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Evidence of 'sickness behaviour' in bats with white-nose syndrome

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Abstract

Many animals change behaviour in response to pathogenic infections. White-nose syndrome (WNS) is a fungal skin disease causing rapid declines of North American bats. Infection with *Pseudogymnoascus destructans* causes hibernating bats to arouse from torpor too often, potentially causing starvation. Mechanisms underlying increased arousals are not understood but fungal invasion of the wings could trigger thirst to relieve fluid loss or grooming to relieve skin irritation. Alternatively, bats might exhibit 'sickness behaviour', a suite of responses to infection that save energy. We quantified behaviours of healthy and experimentally inoculated little brown bats (*Myotis lucifugus*) that could reflect active (i.e., drinking, grooming) or inactive (i.e., sickness behaviour) responses to infection. Infected bats groomed less and were less likely to visit their water dish compared to controls. These results are consistent with research suggesting that *P. destructans* causes sickness behaviour which could help bats compensate for energetic costs associated with infection.

Keywords

little brown bat, *Myotis lucifugus*, fungal pathogen, wildlife disease, *Pseudogymnoascus destructans*.

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1. Introduction

Parasites and pathogens can influence host behaviour in a range of ways (Poulin, 1995; Weary et al., 2009). Considerable recent attention has been paid to manipulation of host behaviour by pathogens to enhance transmission (e.g., Adamo et al., 2014; Toscano et al., 2014) but some behavioural changes are adaptive for the host and can enhance host survival or reduce transmission to genetic relatives (Hart, 1988; Bos et al., 2012). Post-infection behavioural changes can also help reveal aspects of disease pathology and predict impacts of disease on populations (Hart, 1988; Adelman & Martin, 2009).

One category of adaptive responses to infection is collectively referred to as 'sickness behaviour' (Hart, 1988). Sickness behaviour represents a suite of responses, mediated by pro-inflammatory cytokines released by leukocytes, that may enhance survival by reducing energetic demand following infection (Hart, 1988; Dantzer, 1998; Grossberg et al., 2011). For example, male song sparrows (*Melospiza melodia morphna*) reduce investment in territoriality, to decrease behaviours that do not directly benefit immediate survival, after experimental infection with bacterial lipopolysaccharide (Owen-Ashley & Wingfield, 2006; Weary et al., 2009).

Sickness behaviour has potential to improve survival following infection, but active and energetically costly behavioural responses could also be beneficial. Behavioural fever is one such response that is often associated with sickness behaviour in ectotherms. For example, desert locusts (*Schistocerca gregaria*) experimentally infected with the fungus *Metarzhium anisopliae* preferentially select warmer microhabitats than uninfected individuals (Elliot et al., 2002). In addition to fever, energetically costly grooming behaviour could be a beneficial response to some infections, particularly those caused by ectoparasites. The damselfly *Enalagma erbium* increased grooming as mite parasitism increased and grooming successfully reduced parasite intensity (Léonard et al., 1999). Impala (*Aepyceros melampus*) similarly increased grooming with increasing tick (*Boophilus decoloratus*) density (Mooring et al., 1996). Grooming might also be effective against some microparasite infections, particularly cutaneous fungal pathogens, but active behavioural responses to microparasites have received much less attention than responses to ectoparasites.

White-nose syndrome (WNS) is a recently emerged disease devastating North American populations of hibernating bats (Langwig et al., 2012; Frick et al., 2015). WNS is caused by cutaneous infection with the cold-tolerant,

invasive fungus *Pseudogymnoascus destructans* (Gargas et al., 2009; Lorch et al., 2011; Warnecke et al., 2012). This disease has been confirmed in seven species of North American bats (Frick et al., 2015), three of which (*Myotis lucifugus*, *M. septentrionalis*, *Perimyotis subflavus*) are now listed as endangered under the Species at Risk Act in Canada (Canadian Wildlife Service, 2014) with *M. septentrionalis* listed as threatened under the Endangered Species Act in the United States (U.S. Fish and Wildlife Service, 2015). At least six of these species, including *M. lucifugus*, were likely increasing in abundance prior to the emergence of WNS due in part to conservation efforts (Langwig et al., 2012).

WNS kills bats by disrupting hibernation energetics. Infection with *P. destructans* causes an increase in energy expenditure during hibernation (Verant et al., 2014), in part because it increases the frequency of arousals to normothermic body temperature (T_b; Reeder et al., 2012; Warnecke et al., 2012). Mechanisms underlying this increased energy expenditure are not fully understood but a leading hypothesis suggests that invasion of highly vascularized wing membranes by fungal hyphae increases fluid and evaporative water loss causing dehydration, which is known to trigger arousal in hibernators (Thomas & Geiser, 1997; Cryan et al., 2010, 2013; Willis et al., 2011). This suggests that bats might increase certain active behaviours as a response to infection, such as grooming to reduce fungal growth, or drinking to compensate for fluid loss. On the other hand, if WNS leads to increased energy expenditure infected bats might benefit from expressing sickness behaviour, including reducing overall activity. Self-isolation, as a component of sickness behaviour, could also help reduce energetic costs by reducing the potential for bats in torpor to be disturbed by conspecifics in the midst of arousals (Wilcox et al., 2014; Turner et al., 2015) or reduce an individual's chance of multiple exposures to *P. destructans*, and multiple points of infection on the wings, which might increase disease severity (Frick et al., 2015; Willis, 2015).

Behaviour of bats with WNS has been examined in two previous experiments with captive bats, with some conflicting results. Brownlee-Bouboulis & Reeder (2013) and Wilcox et al. (2014) both analysed a range of behaviours during arousals, comparing drinking, grooming, and overall activity. Neither study found an effect of infection on drinking behaviour despite the compelling physiological evidence for dehydration in WNS-affected bats (Cryan et al., 2010, 2013; Willis et al., 2011; Warnecke et al., 2013; Verant

et al., 2014). Brownlee-Bouboulis & Reeder (2013) observed a higher frequency of grooming and overall activity of infected bats whereas Wilcox et al. (2014) observed no difference in grooming but found that infected bats were less active overall, and clustered dramatically less compared to controls. Reduced clustering has also been observed for free-ranging bats with WNS (Langwig et al., 2012) and combined with reduced activity could reflect sickness behaviour.

Equipment limitations, and the types of behaviours that were scored, could have influenced results of both these studies. To avoid disturbing hibernating bats, both Brownlee-Bouboulis & Reeder (2013) and Wilcox et al. (2014) used infrared (IR) security cameras to record behaviour within the artificial hibernacula that housed bats in the lab. Drinking was quantified based on the presence of an individual at a water dish (Brownlee-Bouboulis & Reeder, 2013; Wilcox et al., 2014) but this may have underestimated drinking if bats were exploiting condensation on cage surfaces. Free-ranging little brown bats select hibernacula with high humidity (Wilder et al., 2011) and could drink from condensation in these humid sites (Davis, 1970) but both behavioural studies used relatively low-resolution cameras, which may not have allowed detection of this less obvious form of drinking. In addition, neither study determined where bats focused their grooming effort. If bats were grooming in an attempt to relieve discomfort from infection, they might have focused on flight membranes where fungal growth is most pronounced, as opposed to furred areas of the body, which are typically uninfected. Thus, both studies may have missed patterns of grooming and drinking indicative of an adaptive response to WNS.

Our objective was to use more detailed behavioural observations to better understand mechanisms underlying increased arousal frequency of bats with WNS, and to resolve discrepancies between the only two behavioural studies that have been conducted so far. We used higher resolution IR cameras than those used by Brownlee-Bouboulis & Reeder (2013) and Wilcox et al. (2014) to test two competing hypotheses about behavioural consequences of WNS. What we term the 'active mitigation hypothesis' predicts that bats inoculated with *P. destructans* should increase drinking during arousals to offset physiological dehydration, and spend more time grooming their flight membranes (compared to furred areas of the body) potentially to slow fungal growth and transmission. Alternatively, the 'sickness behaviour hypothesis' predicts that infected bats should drink and groom less than controls and exhibit reduced overall activity, presumably to reduce energetic costs.

2. Methods

2.1. Inoculation and housing

All procedures were approved by the University of Saskatchewan Committee on Animal Use and Supply and conducted under a Manitoba Conservation Wildlife Scientific Permit. Healthy male little brown bats were captured from a WNS-negative hibernaculum approximately 75 km east of The Pas, MB, Canada (53.825°N, 101.253°W) in November 2011. Bats were removed from the walls of the hibernaculum by hand and transferred to cloth bags, which were suspended within a plug-in cooler. The cooler was maintained at approximately 7°C and high relative humidity to encourage bats to use torpor during transport. Bats were transported first by helicopter from their capture site to The Pas (about 30 min) and then approximately 480 km by car to the animal holding facility at the Western College of Veterinary Medicine at the University of Saskatchewan. Bats were then randomly assigned to either a sham-inoculated control group or an experimentally inoculated group ($N = 10$ per group). Inoculation was conducted inside a biosafety cabinet and all inoculation procedures followed Warnecke et al. (2012). Bats were only handled once during the inoculation process. Body mass and forearm length were obtained for each individual and an individually numbered aluminium bat-band (Porzana, Icklesham, UK) was attached to the forearm of each bat. Each bat was also outfitted with a temperature datalogger (DS1922L-F5 Thermochron iButton; Maxim, Sunnyvale, CA, USA, modified according to Lovegrove (2009) and Reeder et al. (2012)) between the shoulders. A small (<1 cm^2) patch of fur was trimmed and dataloggers were affixed using non-toxic latex-based skin adhesive (Osto-Bond, Montreal, QC, Canada). Each datalogger was marked with a unique alphanumeric symbol to enable individual identification in the IR video recordings. Bats in the treatment group were then inoculated by pipetting a 20 μl solution of *P. destructans* conidia (500 000/μl) suspended in phosphate buffered saline and a detergent, Tween-20 (PBS-Tween-20), onto each wing. Controls were sham-inoculated with a solution containing only PBS-Tween-20 and no conidia.

Inoculated and control bats were housed in separate mesh cages (Reptarium, Apogee, Dallas, TX, USA) within temperature- and humidity-controlled environmental chambers (Model 6040-1; Caron, Mariette, OH, USA) that maintained conditions similar to natural hibernacula (i.e., $>97\%$

relative humidity and 7°C), with no food but water ad libitum. We housed both inoculated and control cages in the same environmental chamber to minimize potential chamber effects as previous studies have demonstrated that *P. destructans* is transmitted by contact, and not airborne exposure in the laboratory (Lorch et al., 2011). A motion-activated IR camera (Model HT650IRVFHQS; Speco Technologies, Amityville, NY, USA) was suspended from the top of each cage, and active bats triggered recording during arousals (Warnecke et al., 2012; Wilcox et al., 2014). Bats remained in the hibernacula until the experiment was terminated, after which they were humanely euthanized under isoflurane anaesthesia. We confirmed infection using post mortem histopathology, qPCR, UV fluorescence, and wing necrosis (Warnecke et al., 2013; McGuire et al., 2016).

2.2. Video analysis

We analysed behaviour during bats' first (29–56 days since infection) and last (67–105 days since infection) arousals to assess behavioural effects of WNS during both early- and late-stage disease. For both first and last arousals we analysed behaviours during what we defined as the activity period — the time from when a bat first exhibited activity that triggered the camera until the activity stopped. For last arousals we were also able to define an arousal period based on skin temperature (T_{sk}) recorded by the dataloggers; we programmed dataloggers to begin recording T_{sk} after 64 days of hibernation to ensure they would have sufficient memory capacity remaining to record T_{sk} during advanced WNS. Therefore, we were not able to record T_{sk} during each individual's first arousals. For last arousals, T_{sk} was recorded every 10 min and we followed Jonasson & Willis (2012) and Warnecke et al. (2012) to delineate the start and end of each arousal. Increases in T_{sk} from steady-state torpor at arousal onset, and the sharp decline back to steady-state torpor at the end of arousals, were obvious in the T_{sk} data. We defined arousals as periods when T_{sk} was higher than 15°C.

We used objective criteria to quantify specific behaviours in video recordings (Table 1, Wilcox et al., 2014). Visits to the water dish were obvious but differentiating grooming behaviours was occasionally difficult because bats hanging upside down could obstruct our view of their behaviour. Therefore, we divided grooming into three categories: Grooming of the flight membranes, grooming of the fur or body, and indeterminate grooming during which it was clear bats were grooming based on head or body movements

Table 1.
Behavioural definitions used to differentiate drinking and grooming behaviours of little brown bats (*Myotis lucifugus*) experimentally inoculated with *Pseudogymnoascus destructans* or sham-inoculated.

Behaviour	Definition
Grooming of the flight membranes	Contact between mouth and wing/tail membrane. Mouth visibly stretching the membrane as it grooms. Wing extended and head tucked out of view but clearly under the wing and moving from grooming effort. Contact between hind foot and wing.
Grooming of the body	Contact between the mouth, hind foot, or thumb with furred areas.
Indeterminate grooming	Biting, or hind foot obviously scratching, but the target of grooming not clear.
Drinking from condensation	Drinking or licking of condensation on sides of the cage, indicated by movement of the head across the sides of the cage and rapid opening and closing of mouth, with the tongue often visible.
Drinking from the water dish	Head over the water dish on the bottom of the cage.

but the observer could not tell what parts of the body were being groomed. Indeterminate grooming was included to calculate total grooming time.

A single observer recorded behavioural observations for all first arousals and a second observer recorded behavioural observations for all last arousals. We recorded the latency, frequency, and duration of drinking from condensation, approaching the water dish, total grooming, grooming the wings, and grooming the fur. We defined latency as the time between the start of an active period and the start of a particular behaviour. We defined frequency as the number of discrete behaviours of the same type per active period. Discrete behavioural bouts were classified as such if they were separated from subsequent behavioural events by at least one second, with the exception of condensation drinking. Bats often paused while drinking condensation from the substrate to swallow, so we classified condensation drinking bouts as discrete if they were separated by more than 30 s.

2.3. Statistics

All statistical calculations were performed in R (version 3.1.2; R Core Team, 2014). Results are reported as mean ± SEM unless otherwise stated. We used Fisher's exact tests to compare the proportion of bats from each group

that visited the water dish and drank from condensation more than once during their active periods. We used a series of generalized linear models (GLMs) to assess the effect of inoculation on latency, frequency, and duration to drink condensation, approach the water dish, groom overall, groom the wing and tail membranes, and groom the fur. To account for variation in the time available for bats to perform different behaviours, we included an offset parameter as the log-transformed duration of the activity period in our models. We included activity period as an offset parameter rather than a covariate because this variable differed dramatically between the inoculated and control groups (see results), so treating it as a covariate would have biased the predictive ability of our models (Verboom & Huitema, 1997; Toelch et al., 2006; Zuur et al., 2009). We specified a negative binomial distribution for our GLMs to account for overdispersion. We used analyses of deviance, and χ^2 values to assess model significance (see results) because the difference in deviance between our models and null models followed a chi-squared distribution (Zuur et al., 2009). We used Welch's two-sample t-tests to compare duration of arousals, duration of activity periods, and day of last arousal between groups.

3. Results

All inoculated bats were confirmed as infected with *P. destructans* based on our diagnostic metrics, and no bats from the control group were infected (McGuire et al., 2016). The high-resolution IR cameras allowed us to quantify a range of behaviours, including drinking from condensation, and distinguish grooming of the flight membranes from furred areas of the body. For first arousals, we scored 10.4 h of arousals for nine control bats (two of which exhibited very long torpor bouts and did not arouse again before the experiment was terminated) and 9.4 h for nine experimentally inoculated bats. For last arousals, observers scored 12.8 h of arousals for eight control bats and 9.4 h for 10 inoculated bats. One bat from each of the control and treatment groups did not have their first arousal before some individuals in their cage had their last arousals. Therefore, we excluded these bats from the early-stage analysis but included their last arousal in the late-stage analysis.

We did not identify any bats from either treatment group drinking from condensation during the first arousal but 75% (6/8) of control bats, and 20% (2/10) of infected bats drank condensation during their last arousal

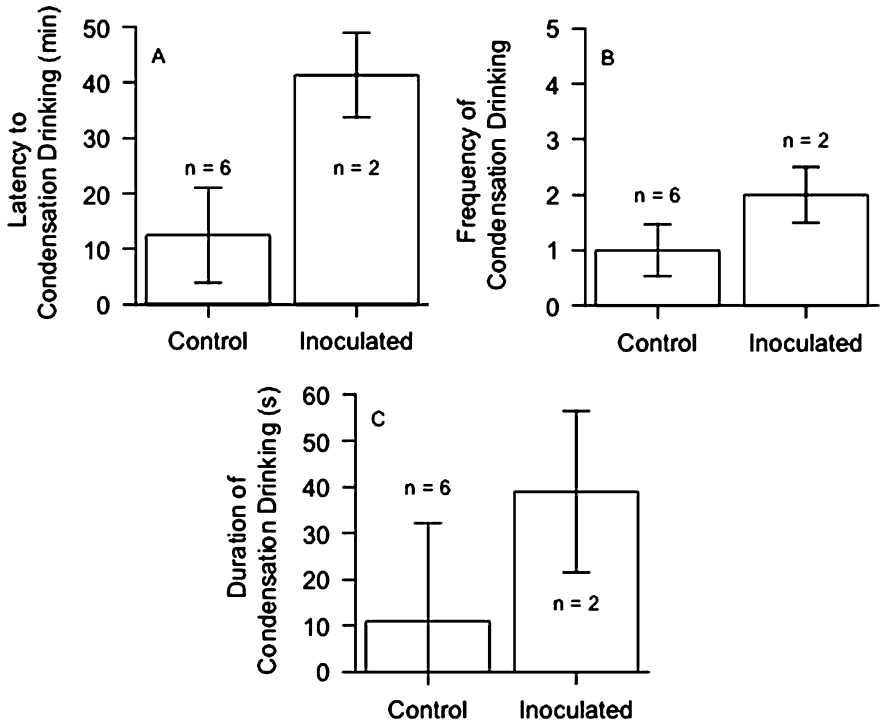

Figure 1. Mean latency (A), frequency (B) and duration (C) of drinking from condensation by little brown bats (*Myotis lucifugus*) inoculated with *Pseudogymnoascus destructans* (inoculated) or sham-inoculated (control) during their last arousal from hibernation before the end of the experiment.

($p = 0.054$). There was also no effect of infection on whether or not bats approached the water dish during their first arousal, with 44% (4/9) of control bats and 55% (5/9) of infected bats approaching the dish at least once ($p = 1.0$). However, during the last arousal, more control bats approached the water dish than infected bats, with 100% (8/8) of control bats and 40% (4/10) of infected bats approaching the dish at least once ($p = 0.01$).

Infection had no effect on the latency to drink condensation at least once during the last arousal ($\chi^2 = 0.03$, df $= 1$, $p = 0.86$; Figure 1A) and no effect on the frequency ($\chi^2 = -1.78$, df $= 1$, $p = 1.0$; Figure 1B) or duration of bouts of drinking from condensation (Figure 1C). Infection had no effect on the latency to approach the water dish at least once during either first ($\chi^2 = 0.28$, df $= 1$, $p = 0.6$; Figure 2A) or last arousals ($\chi^2 = 2.87$, df $= 1$, $p = 0.09$; Figure 2B). There was also no effect of infection on the frequency

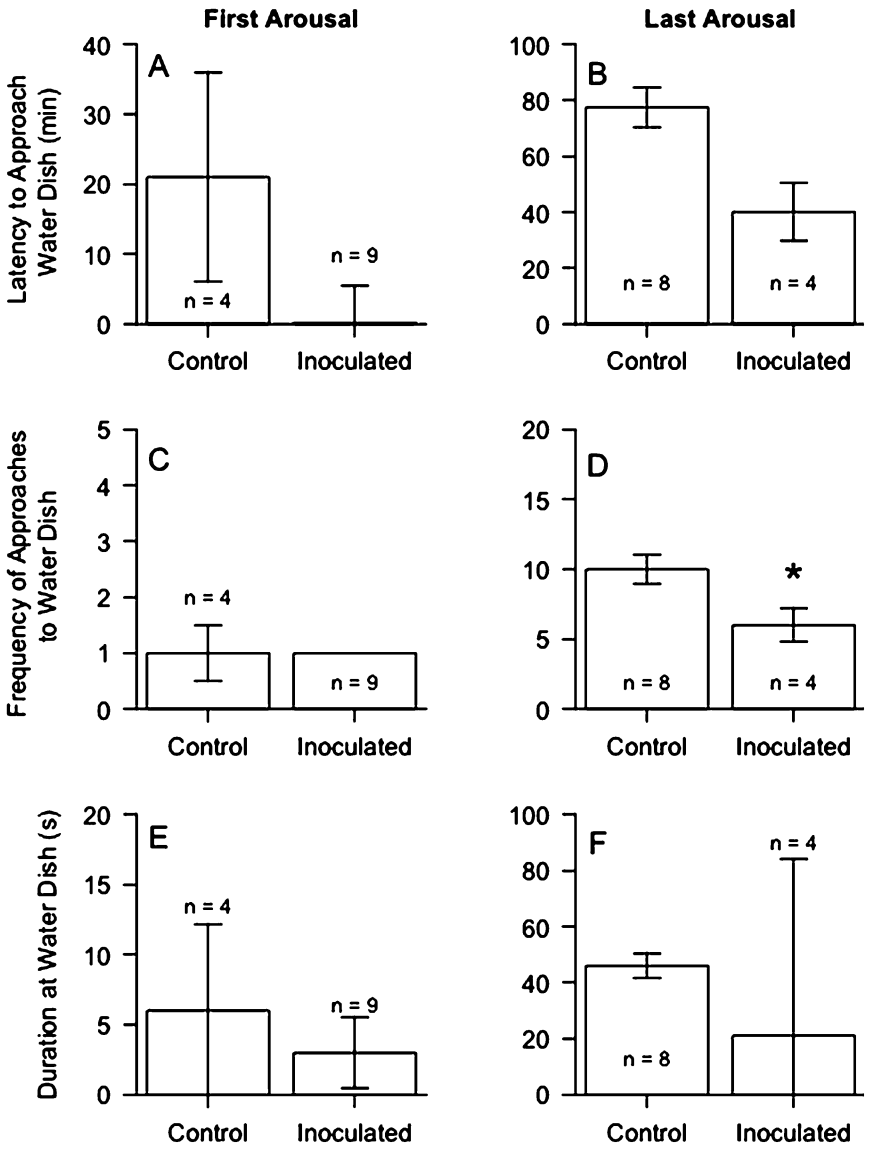

Figure 2. Mean latency to approach the water dish within an artificial hibernaculum during the first (A) and last arousal (B), frequency of approaches to the water dish during the first (C) and last arousal (D), and duration spent at the water dish during the first (E) and last arousal from hibernation before the end of the experiment (F) by little brown bats (*Myotis lucifugus*) inoculated with *Pseudogymnoascus destructans* (inoculated) or sham-inoculated (control). Error bars represent standard error, and an asterisk indicates significance ($p < 0.05$).

of approaches to the water dish during first arousals ($\chi^2 = -0.42$, df = 1, $p = 1.0$; Figure 2C), but control bats approached the water dish more than infected bats during last arousals ($\chi^2 = 4.28$, df = 1, $p = 0.04$; Figure 2D). There was no effect of infection on the duration spent at the water dish during first ($\chi^2 = 1.4$, df = 1, $p = 0.23$; Figure 2E) or last arousals ($\chi^2 = 3.62$, df = 1, $p = 0.06$; Figure 2F).

Infection had no effect on the latency to groom during first ($\chi^2 = 0.69$, df = 1, $p = 0.41$; Figure 3A) or last arousals ($\chi^2 = 0.1$, df = 1, $p = 0.75$; Figure 3B). However, in general, control bats groomed more frequently than inoculated bats during both first ($\chi^2 = 15.83$, df = 1, $p < 0.001$; Figure 3C) and last arousals ($\chi^2 = 20.85$, df = 1, $p < 0.001$; Figure 3D). Control bats also spent more time grooming than inoculated bats during first ($\chi^2 = 12.56$, df = 1, $p < 0.001$; Figure 3E) and last arousals ($\chi^2 = 9.63$, df = 1, $p = 0.002$; Figure 3F). There was no effect of infection on the latency to groom fur during first ($\chi^2 = 0.29$, df = 1, $p = 0.59$; Figure 4A) or last arousals ($\chi^2 = 0.03$, df = 1, $p = 0.87$; Figure 4B) but control bats groomed their fur more frequently than inoculated bats during both first ($\chi^2 = 8.61$, df = 1, $p = 0.003$; Figure 4C) and last arousals ($\chi^2 = 14.91$, df = 1, $p < 0.001$; Figure 4D). Control bats also groomed their fur for longer than inoculated bats during their first arousal ($\chi^2 = 10.77$, df = 1, $p = 0.001$; Figure 4E) and there was a similar trend for last arousals but it was not statistically significant ($\chi^2 = 3.61$, df = 1, $p = 0.06$; Figure 4F). Infection had no effect on the latency to groom flight membranes during first ($\chi^2 = 3.8$, df = 1, $p = 0.051$; Figure 5A) or last arousals ($\chi^2 = 0.1$, df = 1, $p = 0.75$; Figure 5B) but, as for grooming of the fur, control bats groomed their flight membranes more frequently than inoculated bats during both the first arousal ($\chi^2 = 16.71$, df = 1, $p < 0.001$; Figure 5C) and last arousal ($\chi^2 = 23.07$, df = 1, $p < 0.001$; Figure 5D). Control bats also spent more time grooming their flight membranes than inoculated bats during both first ($\chi^2 = 8.74$, df = 1, $p = 0.003$; Figure 5E) and last arousals ($\chi^2 = 12.82$, df = 1, $p < 0.001$; Figure 5F).

We were not able to quantify the duration of arousals based on T_{sk} for first arousals (see methods). For last arousals, duration ranged from 30 to 120 (84 $\pm$ 15.4) min for the control group and 45 to 104 (64.8 $\pm$ 10.6) min for the inoculated group but there was no statistical difference ($t = 1.0$, df = 7.4, $p = 0.34$). In terms of the active period (i.e., the time between the first sustained movement by an active bat that triggered the video recorder and the

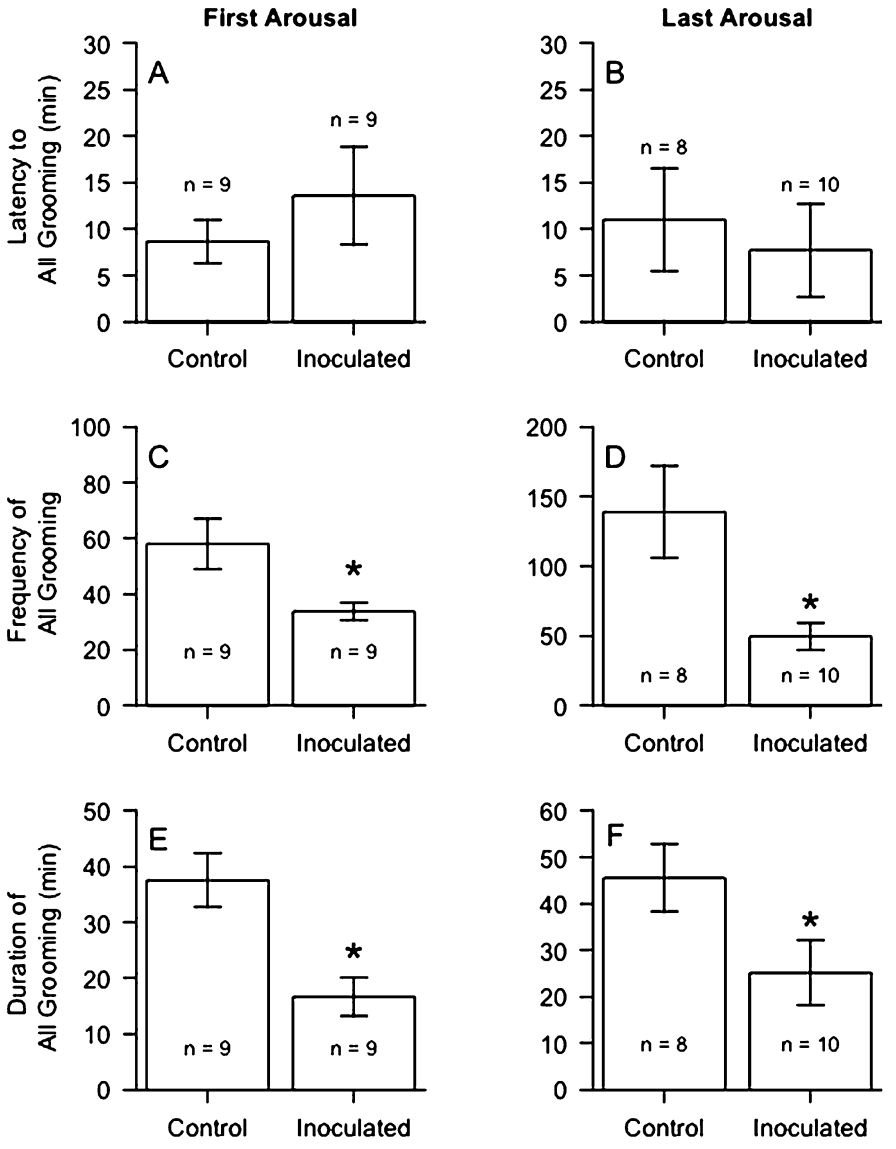

Figure 3. Mean latency to groom anywhere on the body during the first (A) and last arousal (B), frequency of grooming during the first (C) and last arousal (D), and duration spent grooming during the first (E) and last arousal from hibernation before the end of the experiment (F) by little brown bats (*Myotis lucifugus*) inoculated with *Pseudogymnoascus destructans* (inoculated) or sham-inoculated (control). Error bars represent standard error, and asterisks denote significance ($p < 0.05$).

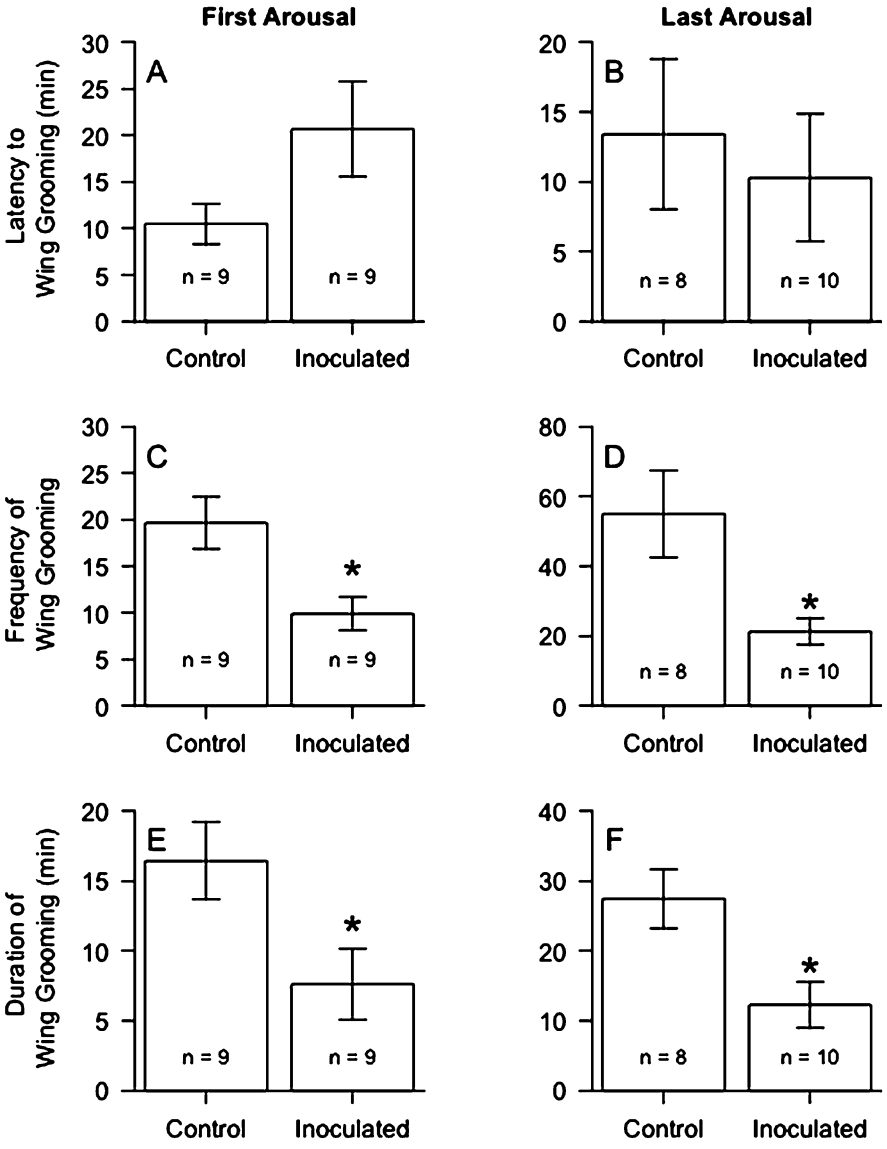

Figure 4. Mean latency to groom the flight membranes during the first (A) and last arousal (B), frequency of grooming the flight membranes during the first (C) and last arousal (D), and duration spent grooming the flight membranes during the first (E) and last arousal from hibernation before the end of the experiment (F) by little brown bats (*Myotis lucifugus*) inoculated with *Pseudogymnoascus destructans* (inoculated) or sham-inoculated (control). Error bars represent standard error, and asterisks denote significance ($p < 0.05$).

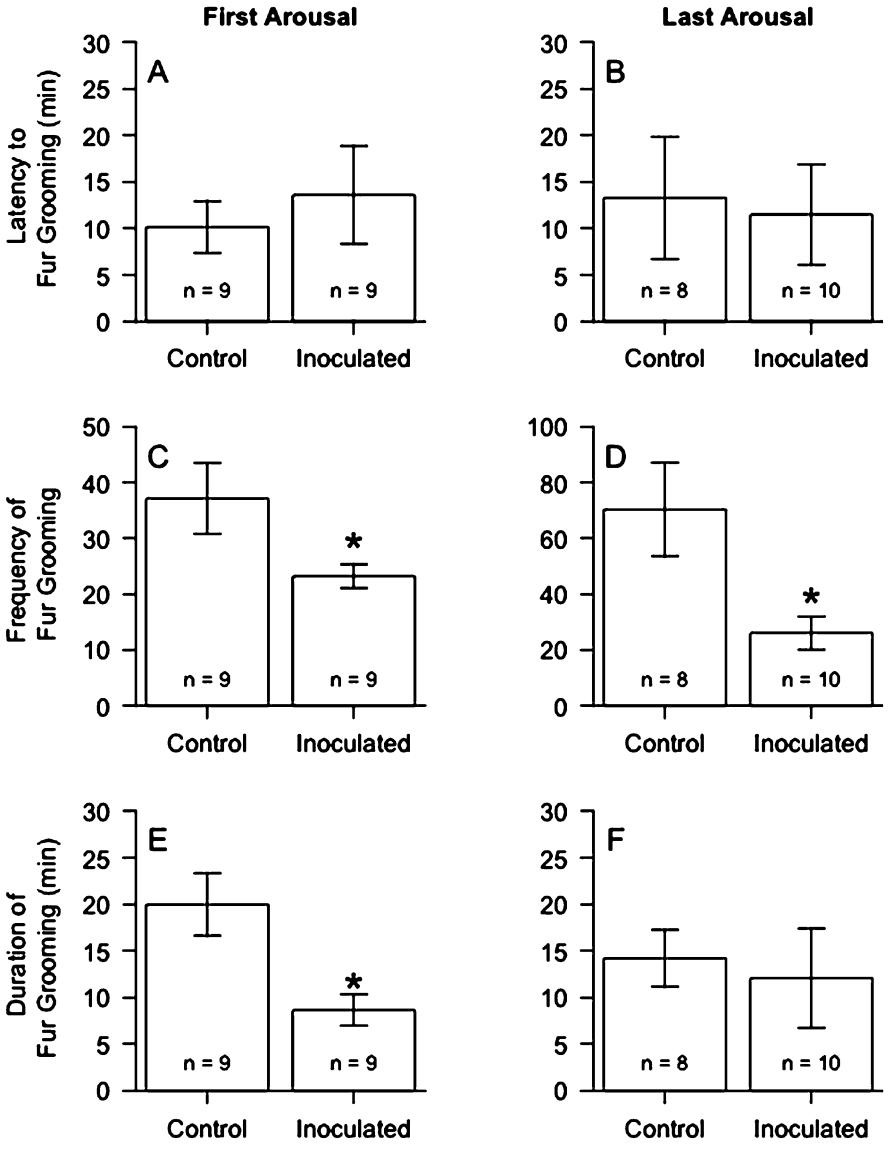

Figure 5. Mean latency to groom the fur during the first (A) and last arousal (B), frequency of grooming the fur during the first (C) and last arousal (D), and duration spent grooming the fur during the first (E) and last arousal from hibernation before the end of the experiment (F) by little brown bats (*Myotis lucifugus*) inoculated with *Pseudogymnoascus destructans* (inoculated) or sham-inoculated (control). Error bars represent standard error, and an asterisk represents significance ($p < 0.05$).

end of the last sustained movement by that individual), there was no difference between control (69.6 ± 6.5 min, range: 48.4–112 min) and inoculated bats (62.4 ± 5.8 min, range 29.5–88 min; $t = 0.8$, df = 15.8, $p = 0.42$) during first arousals. However, active periods during last arousals were more than twice as long for controls (112.7 ± 12.3 min, range: 46.1–143.4 min) compared to inoculated bats (53.8 ± 5.9 min; range: 33.7–74 min; $t = 4.3$, df = 5.8, $p = 0.005$).

There was no difference in the timing of the first arousal after the experiment started between control (52.9 ± 0.1 days post-infection) and inoculated bats (50.1 ± 2.8 days; $t = 1.0$, df = 8.0, $p = 0.35$). Similarly, there was no difference in the timing of the last arousal between control (85.8 ± 6.0 days post-infection) and inoculated bats (97 ± 3.7 days post-infection; $t = -1.6$, df = 12, $p = 0.13$). This suggests that bats were at a similar stage of hibernation when we compared their behaviour for control and treatment groups.

4. Discussion

Our results are consistent with the sickness behaviour hypothesis rather than the active mitigation hypothesis and suggest that bats with WNS may alter behaviour during arousals to reduce energetic costs, rather than increase energetically costly behaviours that might help alleviate negative consequences of infection. Infected bats in our study did not increase drinking behaviour, even when we accounted for the possibility that they might drink from condensation in their hibernacula, and they showed reduced grooming behaviour experience when we distinguished between furred areas and areas more likely to cause fungal irritation. Moreover, despite maintaining high T_b during arousals for just as long as controls, infected individuals triggered the camera for less than half the time as controls during their last arousals, indicating reduced activity overall.

Sickness behaviour is primarily mediated by a suite of cytokines, including interleukin 1β (IL1β) and tumour necrosis factor α (TNF-α), which are released by white blood cells in response to infection with a range of pathogens and parasites (Dantzer, 1998). These cytokines are responsible for the lethargy and depressed activity commonly seen in animals that are fighting infection (Dantzer, 1998; Grossberg et al., 2011). Interestingly, Rapin et al. (2014) detected increased TNF gene expression in *M. lucifugus* infected with *P. destructans*, which is consistent with the sickness behaviour

hypothesis and suggests a mechanism underlying the behavioural patterns we observed.

Infected bats did not groom or drink any sooner than healthy control bats following the start of an activity period (Figures 1–5), which suggests that arousals were not triggered directly by dehydration or skin irritation resulting from infection with *P. destructans*. This further suggests that the characteristic increase in arousal frequency of bats infected with *P. destructans* is triggered by some other consequence of infection. One possibility is that increased energy expenditure during torpor bouts, as observed by Verant et al. (2014), increases the need to eliminate metabolic wastes, triggering more frequent arousals. Other possibilities include increased sensitivity to disturbance by other bats (Turner et al., 2015), altered endocrine function resulting from infection (Willis & Wilcox, 2014), or some combination of all these factors. Surprisingly we still do not understand precisely how WNS kills bats and more research is needed to address this fundamental question.

Adipsia (i.e., the tendency to reduce drinking) and dehydration are common consequences of pathogenic infections for many vertebrates (Hart, 1988). However, it seems unlikely that adipsia is the ultimate cause of physiological dehydration for WNS-affected bats because reduced water consumption on its own leads to hypertonic (i.e., concentrated body fluids), rather than the hypotonic (i.e., reduced plasma electrolyte concentrations) dehydration that is characteristic of infection with *P. destructans* (Cryan et al., 2013; Warnecke et al., 2013). In addition to hypovolemia (i.e., low body fluids associated with dehydration) the fact that bats with WNS exhibit hypotonic dehydration suggests pronounced fluid loss with some replacement of body water (Cryan et al., 2013; Warnecke et al., 2013). One possibility is that in combination with metabolic water production, even low rates of drinking allow bats with WNS to replace lost body water. Additionally, *P. destructans* could preferentially sequester electrolytes (Cryan et al., 2013), which would contribute to the hypotonic dehydration found in previous studies. More work is needed to understand how physiological and behavioural consequences of WNS interact but our results, combined with those of Brownlee-Bouboulis & Reeder (2013) and Wilcox et al. (2014), suggest that bats infected with *P. destructans* do not compensate behaviourally for the physiological dehydration that results from infection.

Infected bats groomed less than healthy controls (Figures 3–5) which is consistent with the relative lack of inflammatory response to *P. destructans*

in the midst of hibernation (e.g., Meteyer et al., 2009). However, as with reduced drinking, reduced grooming could also reflect sickness behaviour. Grooming is energetically costly for bats (e.g., Giorgi et al., 2001) and reduced grooming might help conserve at least some energy reserves (Hart, 1988). In a cold hibernaculum, despite heat production from increased muscle activity, the activity required to groom could increase air movement leading to convective heat loss, while licking the fur or wing membranes could also increase evaporative cooling (Hart, 1988; Ochoa-Acuña & Kunz, 1999).

Crawling or flying is also energetically costly for bats and, when combined with conductive heat loss from climbing across cold surfaces to get to a water source, or drinking cold water (Lotz et al., 2003), its effect on energy stores could be dramatic. Infected bats in our study decreased activity by occasionally rewarming but remaining inactive at the start of an arousal, sometimes for more than 20 min, without triggering our motion-sensitive cameras. Reduced drinking, grooming, and a reduction of overall activity during an arousal could simply reflect lethargy as a consequence of dramatically depleted energy reserves. However, as the pattern of reduced grooming by infected bats also occurred for first arousals when fat stores should still be available, we suggest that the observed reduction in activity is more consistent with sickness behaviour.

In contrast to our results and those of Wilcox et al. (2014), Brownlee-Bouboulis & Reeder (2013) found an increase in the time that WNS-affected bats spent active, largely due to increased grooming. This could reflect differences in experimental design between studies. Brownlee-Bouboulis & Reeder (2013) compared naturally infected bats from a WNS-positive site to healthy controls from a WNS-negative site hundreds of kilometers away. While this is an excellent approach for ensuring natural patterns of infection, because the two groups of bats came from different caves it is not possible to separate effects of WNS status from other potential differences between groups due to genetics or environment. Increased grooming can reflect anxiety and stress in rodents (Katz & Roth, 1979) and little brown bats (Menzies et al., 2013). Anxiety and response to novelty are components of animal personality (Martin & Réale, 2008), which is known to be heritable (Dingemanse et al., 2002) and recent work suggests that genetic structure among hibernating colonies of little brown bats may be significant across the distances between hibernacula sampled by Brownlee-Bouboulis

& Reeder (2013) (e.g., Davy et al., 2015). Thus, it is possible that individual bats captured from one cave could exhibit similar stress-related grooming independent of infection, compared to individuals from another site. Cave temperature, humidity, or pre-hibernation diet could also influence hibernation energetics, wing membrane physiology, or water loss in ways that could have influenced the differences in activity and grooming exhibited by the bats studied by Brownlee-Bouboulis & Reeder (2013).

Although they could not perfectly replicate natural exposure to *P. destructans*, two inoculation experiments (i.e., Wilcox et al., 2014; this study), conducted in different years, controlled for potential underlying differences between infected and treatment groups and detected broadly similar patterns of reduced grooming and reduced overall activity in infected bats. Experimental inoculation could cause unusual behaviour patterns, possibly as a result of more severe infection than what might occur in the wild. The naturally infected bats studied by Brownlee-Bouboulis & Reeder (2013) may not have experienced as high a fungal load as experimentally inoculated bats and, thus, might have exhibited less severe symptoms of WNS. However, in our view this explanation seems unlikely because inoculated bats in our experiment had lower fungal loads and levels of tissue damage compared with bats from Wilcox et al. (2014) (see McGuire et al., 2016) but they still exhibited similar behaviour. Moreover, patterns of torpor-arousal behaviour and clustering by naturally infected bats studied in the wild (e.g., Langwig et al., 2012; Reeder et al., 2012) are similar to those observed for the experimentally inoculated bats we studied in the laboratory (Warnecke et al., 2012; Wilcox et al., 2014). We cannot rule out the possibility that experimental inoculation leads to unusual patterns of infection and behaviour but, given similarities in torpor patterns and physiology between naturally- and experimentally-infected bats, we suggest that activity patterns we observed reflect behaviours likely to be expressed by wild bats infected with *P. destructans*.

In general, our results, combined with those of Wilcox et al. (2014), suggest that bats adjust levels of activity during arousals to save energy. This could be beneficial if it allows some bats with WNS to survive longer during winter on their reduced energy budget. These behaviours could be maladaptive, however, if they reduce potentially adaptive responses such as drinking to offset dehydration that results from infection. Verant et al. (2014) found

that bats with early-stage WNS used more water and fat reserves than controls suggesting that the costs of WNS for bats begin relatively early during hibernation. Our results support this hypothesis, given that infected bats in our study showed differences in behaviour as early as their first arousal after infection.

Our results allow for a better understanding of potentially adaptive responses to WNS and their implications for conservation. Although WNS-affected bats exhibit hypotonic dehydration and electrolyte depletion (Cryan et al., 2013; Warnecke et al., 2013), our findings suggest that supplementing water with electrolytes inside hibernacula would not be beneficial since affected bats have a tendency to reduce their drinking behaviour. We recommend future studies using open-flow respirometry or doubly-labelled water to quantify energetic costs of different behaviours during arousals. This would allow for calculation of an energy-activity budget for infected bats to determine if reduced activity could outweigh potential costs of reduced grooming and drinking, at least for a few individuals. If individual bats with WNS have repeatable differences in the amount that they reduce activity and energy expenditure during arousals, and if this difference is heritable, then this trait could influence survival and increase the potential for an evolutionary response to WNS (Maslo & Fefferman, 2015; Willis, 2015). Thus, conservation strategies that might increase survival and reproduction of bats with this particular WNS-survival trait could help improve the potential of evolutionary rescue and facilitate population recoveries.

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