LETTER

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Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome

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White-nose syndrome (WNS) has caused recent catastrophic declines among multiple species of bats in eastern North America[1,2]. The disease's name derives from a visually apparent white growth of the newly discovered fungus *Geomyces destructans* on the skin (including the muzzle) of hibernating bats[1,3]. Colonization of skin by this fungus is associated with characteristic cutaneous lesions that are the only consistent pathological finding related to WNS[4]. However, the role of *G. destructans* in WNS remains controversial because evidence to implicate the fungus as the primary cause of this disease is lacking. The debate is fuelled, in part, by the assumption that fungal infections in mammals are most commonly associated with immune system dysfunction[5–7]. Additionally, the recent discovery that *G. destructans* commonly colonizes the skin of bats of Europe, where no unusual bat mortality events have been reported[8–10], has generated further speculation that the fungus is an opportunistic pathogen and that other unidentified factors are the primary cause of WNS[11,12]. Here we demonstrate that exposure of healthy little brown bats (*Myotis lucifugus*) to pure cultures of *G. destructans* causes WNS. Live *G. destructans* was subsequently cultured from diseased bats, successfully fulfilling established criteria for the determination of *G. destructans* as a primary pathogen[13]. We also confirmed that WNS can be transmitted from infected bats to healthy bats through direct contact. Our results provide the first direct evidence that *G. destructans* is the causal agent of WNS and that the recent emergence of WNS in North America may represent translocation of the fungus to a region with a naive population of animals[8]. Demonstration of causality is an instrumental step in elucidating the pathogenesis[14] and epidemiology[15] of WNS and in guiding management actions to preserve bat populations against the novel threat posed by this devastating infectious disease.

To test the ability of *G. destructans* to act as a primary pathogen, we housed healthy little brown bats (*Myotis lucifugus*; $n = 29$) in the laboratory under hibernation conditions and treated them with conidia of *G. destructans* harvested from pure culture. Histological examination of treated bats that died during the course of the experiment showed that lesions diagnostic for WNS were apparent by 83 days after treatment. All treated bats were positive for WNS by histology when the trial was terminated at 102 days after treatment. In contrast, at the end of the experiment, all bats from the negative control group (bats treated identically but not exposed to conidia of *G. destructans*; $n = 34$) were negative for WNS by histology.

We also investigated the potential for WNS to be transmitted from infected to healthy animals by co-housing hibernating bats naturally infected with WNS (collected from an affected hibernaculum and showing clinical signs of the disease; $n = 25$) with healthy bats (contact exposure group; $n = 18$). Eighty-nine per cent of bats in the contact exposure group developed WNS lesions by day 102, demonstrating for the first time that WNS is transmissible. This has important epidemiological and disease management implications, because many of the bat species most commonly impacted by WNS often form tight, occasionally mixed-species clusters during hibernation, facilitating the transfer of fungus among individuals and species. In addition, bat species affected by WNS engage in 'swarming' behaviour at hibernacula just before hibernation. During this time, there is much direct contact between individuals as they participate in a promiscuous mating system[16]. Furthermore, individual bats have been documented to move long distances between hibernacula during this period[17], which may, in part, facilitate the spread of WNS across the landscape.

To determine if WNS could be spread between bats through the air, healthy bats ($n = 36$) were placed in mesh cages in close proximity to (separated by 1.3 cm), but not in direct contact with, the positive control and treated groups. After a period of 102 days, none of the animals exposed to possible airborne conidia from bats with WNS showed histopathological evidence of infection. This may be due to an inability of *G. destructans* conidia to travel through air at levels sufficient to establish infections in neighbouring individuals over the experimental interval or could reflect that conditions within the incubators (for example, airflow patterns and/or static charges) were not conducive to airborne transfer of conidia.

The fungal skin lesions that developed in treated and contact-exposed animals were indistinguishable from those that occurred in the positive control bats (Fig. 1). Additionally, the prevalence of infection was similar between the two groups (Table 1), indicating that the treated group did not develop disease from exposure to an excessively high dose of conidia. Similar disease pathology between groups also indicates that the contact-exposed bats did not develop WNS through exposure to an agent other than *G. destructans*. Histological examination of hearts, intestines, livers, lungs and kidneys from a subset of animals (positive control group $n = 5$, negative control group $n = 3$, treated group $n = 10$, contact exposure group $n = 5$) did not reveal any tissue damage or other signs of infectious processes that might have predisposed the animals to skin infection by *G. destructans*. Furthermore, live *G. destructans* was cultured from the skin of bats confirmed to have WNS lesions. Development of lesions diagnostic for WNS in the absence of other signs of disease provides the first experimental evidence that *G. destructans* is a primary pathogen and causes WNS in healthy bats.

The large-scale mortality seen in wild bat populations with WNS was not observed in the treated or contact exposure groups. Although all of the positive control animals died before the termination of the trial, survivorship ($P = 0.72$) and body mass index (BMI; $P = 0.96$) of the remaining groups did not significantly differ from the negative control group (Fig. 2a). The lack of WNS-related mortality in the treated and contact exposure groups is best explained by the short period of time these groups were exposed to *G. destructans*. On the basis of an analysis of wild bats submitted to the US Geological

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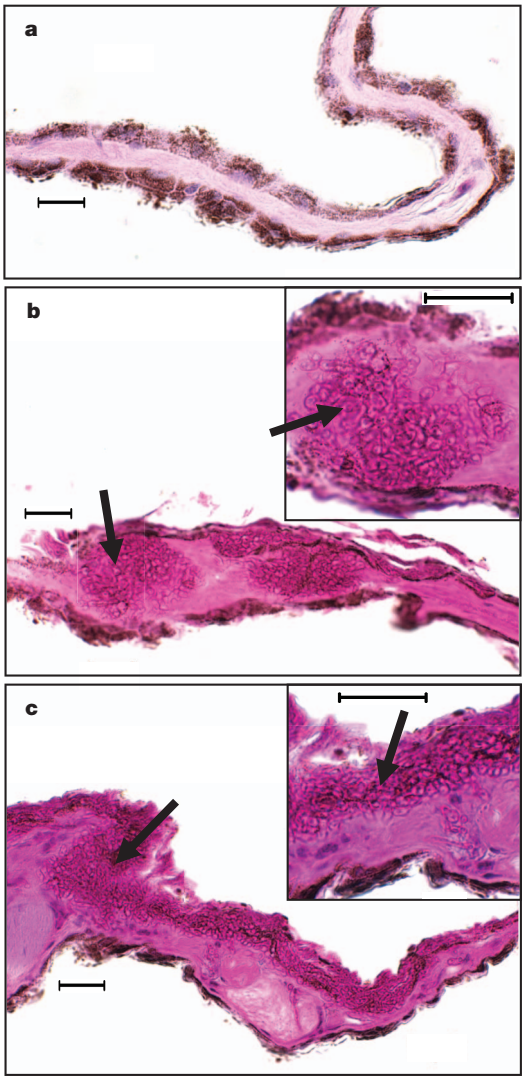

Figure 1 | Histological sections of representative wing membranes (periodic acid-Schiff stain). **a**, Normal wing membrane of a healthy bat from the negative control group showing no signs of fungal growth. **b**, **c**, WNS lesions, including invasion of the underlying connective tissue by fungal hyphae (arrows), are visible in sections from a bat with WNS from the positive control group (**b**) and a bat from the treated group that developed WNS after experimental exposure to *G. destructans* (**c**). Insets are higher magnification images and scale bars indicate 20 μm.

Survey (USGS)–National Wildlife Health Center (NWHC) for diagnostic testing (January 2008 to June 2011), WNS lesions have seasonally first been detected during autumn (late September), just before the start of long-term hibernation; major mortality events caused by WNS have seasonally not been observed among wild bats until the end of January (Fig. 2b). These data indicate that mortality

Table 1 | Development of WNS in experimentally infected bats

Treatment group	Number with WNS lesions present	Number with no WNS lesions	Total	Per cent infected
Negative control	0	34	34	0
Treated	29	0	29	100
Contact exposure	16	2	18	89
Airborne exposure	0	36	36	0
Positive control	25	0	25	100

The data show prevalence of WNS-associated fungal infections established in groups of healthy little brown bats inoculated with conidia of *G. destructans* from pure culture or exposed to bats known to have WNS (positive control group). Infection status was determined by histological examination of the wing.

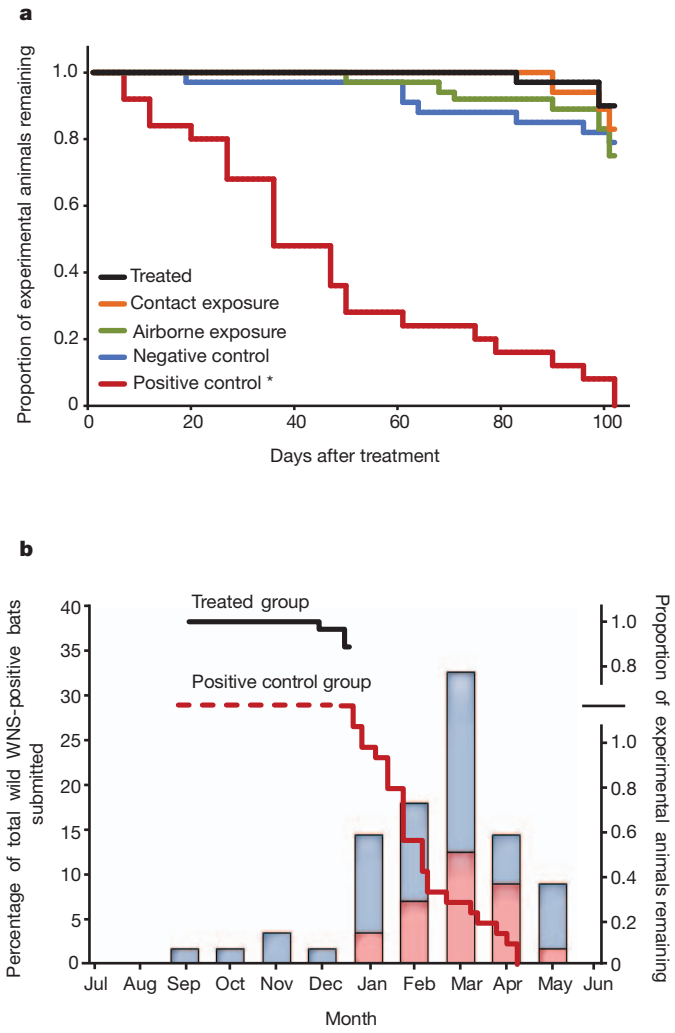

Figure 2 | Survival curves. **a**, Survival curves for the treated ($n = 29$), contact exposure ($n = 18$), airborne exposure ($n = 36$), negative control ($n = 34$) and positive control ($n = 25$) groups. Bats in the positive control group, which consisted of animals naturally infected with WNS at the time they were collected, exhibited significantly decreased survival (asterisk) relative to the other groups ($P < 0.001$). Survival among bats of the remaining groups did not differ significantly from one another ($P = 0.72$). **b**, Percentage of bats submitted by month (January 2008 to June 2011) to the USGS–National Wildlife Health Center that tested positive for WNS ($n = 54$ submission events). The blue bars represent submissions that were not associated with major mortality events; the red bars depict submissions associated with high mortality. Annually, WNS-associated mortality events are first observed in January; the number of submissions involving mortality events for a given month peaks in March. Assuming the positive control bats were first exposed to *G. destructans* in late September, mortality due to WNS did not occur in the laboratory until approximately 120 days after exposure, consistent with what is observed in free-ranging wild bats (the dotted line represents the exposure period in the wild before the animals were collected for this study). The duration of this infection trial (102 days) was insufficient to observe WNS-associated mortality in the treated and contact exposure groups (the treated group mortality curve is shifted such that duration of exposure corresponds to that of the positive control group; contact and airborne exposure group mortality curves are not shown).

from WNS does not manifest until approximately 120 days after bats enter hibernation and assume a cold physiological state conducive to proliferation of *G. destructans*; mortality subsequently peaks about 180 days after bats first enter hibernacula (in the month of March). Assuming that initial exposure of positive-control bats to the fungus occurred in late September, these animals survived about 110 to 205 days after exposure, with approximately 50% having died by the

150-day mark. The treated and contact-exposure bats were only exposed to *G. destructans* for 102 days. Thus, the experiment was terminated before the disease had progressed to the degree that mortality would be expected among treated and contact-exposed animals.

Our work demonstrates experimental infection of little brown bats by *G. destructans* with subsequent development of WNS in the absence of underlying health conditions. It follows that the recent widespread detection of *G. destructans* in Europe without apparent detriment to bat populations indicates that the fungus may be endemic to that region where it co-evolved with continental bat species[8,10]. In North America, the data indicate that WNS originated at a single site[1,18] with high tourist traffic, consistent with the introduction of an exotic species[19]. Thus, the pathological effects caused by *G. destructans* in North American bats may reflect exposure of a naive host population to a novel pathogen. Future studies are needed to investigate the origin of *G. destructans* in North America and to elucidate differences in physiology and behaviour between North American and European bats that might account for disparate disease outcomes observed among the two continents.

Fungal pathogens have the unique capacity to drive host populations to extinction because of their ability to survive in host-free environments[5]. Given the high mortality rate and speed at which WNS has spread, the disease has the potential to decimate North American bat populations and cause species extinctions[20] similar to those documented for amphibians affected by chytridiomycosis[21]. Advancement of WNS research and management has been limited by uncertainty over the causative agent of this disease. With the causative agent now conclusively identified through fulfilment of Koch's postulates, future research efforts can focus on mitigating the effects of WNS before hibernating bat populations suffer losses beyond the point of recovery.

METHODS SUMMARY

Little brown bats (*Myotis lucifugus*) naturally infected with WNS (positive control group; $n = 25$) were collected from a hibernaculum in New York. Healthy (based upon body condition and histopathology findings) little brown bats were collected from a hibernaculum in Wisconsin outside of the known range of WNS. Healthy bats were divided into four groups: negative control ($n = 34$), treated ($n = 29$), contact exposure ($n = 18$) and airborne exposure ($n = 36$). Conidia of *G. destructans* (5×10^5 conidia suspended in 20 µl of phosphate buffered saline solution containing 0.5% Tween 20 (PBST)) were applied to one of the wings of bats in the treated group, and an additional 5×10^5 conidia were applied to the fur between the eye and ear. Negative control bats were treated identically with PBST lacking conidia. Animals were maintained in mesh enclosures (Supplementary Fig. 1) under conditions approximating bat hibernacula for 102 days. The experimental end point was set to correspond with the timing by which wild bats naturally emerge from hibernation. Infection status was determined by histological examination of the muzzle and skin from each wing[4], and *G. destructans* was re-isolated from wing skin as previously described[22]. The identity of fungal isolates resembling *G. destructans* was confirmed by PCR amplification/double-stranded sequence analysis of the rRNA gene internal transcribed spacer[23].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions D.S.B., A.C.H., P.M.C. and D.M.R. designed the study. A.C.H., J.T.H.C. and D.N.R. collected wild animals for the study. J.M.L., D.S.B., C.U.M., M.J.B., J.G.B., A.C.H., A.E.B., J.T.H.C., D.N.R. and D.M.R. performed the experiment and/or assisted to collect samples upon completion. C.U.M. and M.J.B. read and interpreted histopathology. J.M.L. and J.G.B. analysed the experimental data. A.E.B. compiled data on wild bats submitted to the NWHC for diagnostic testing. J.M.L. wrote the manuscript and all co-authors provided input. D.S.B. supervised data analyses and edited the manuscript.

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METHODS

Animals. This study was conducted at the NWHC in accordance with Institutional Animal Care and Use Committee Experimental Protocol 081118. WNS-positive little brown bats (*Myotis lucifugus*) (positive control group; $n = 25$) were collected from a hibernaculum in New York in January 2009; only bats showing visible signs of fungal growth on the muzzle and/or wings were collected from the New York site. Healthy (based upon body condition and histopathology findings) little brown bats were collected from a hibernaculum in Wisconsin (approximately 1,000 km distant from the known range of WNS at the time that animals were collected). Bats were transported to the NWHC in coolers at approximately 7 °C.

Experimental infection. Healthy bats were randomly (except for ensuring nearly equal sex ratios) divided into groups: negative control ($n = 34$), treated ($n = 29$), contact exposure ($n = 18$) and airborne exposure ($n = 36$). Negative control, positive control and treated groups were maintained in separate rooms. Animals in the contact exposure group were placed in the same enclosures as the positive control group. Animals in the airborne exposure group were split evenly between separate enclosures, each located 1.3 cm from enclosures housing the positive control and treated groups (Supplementary Fig. 1).

Conidia were harvested from 60-day-old cultures of the type strain of *G. destructans*[3] (American Type Culture Collection number ATCC MYA-4855) by flooding plates with phosphate buffered saline solution containing 0.5% Tween20 (PBST). Conidia were washed, enumerated and re-suspended in PBST. Twenty microlitres of the conidial suspension containing 5×10^5 conidia were pipetted directly onto the dorsal surface of one of the wings of bats in the treated group; an additional aliquot (20 µl) was pipetted onto the fur between the eye and ear. Negative control bats were treated identically with PBST lacking conidia. Bats were housed in mesh enclosures (Reptaria; Apogee) within refrigerators (SRC Refrigeration) under conditions approximating bat hibernacula (complete darkness, approximately 6.5 °C and 82% relative humidity) for 102 days. Termination of the experiment corresponded to the time period during which wild bats begin to emerge from hibernation. Temperatures were recorded daily in each refrigerator to ensure that appropriate hibernation conditions were maintained. The mean temperatures (± standard deviation) for the refrigerators were as follows: negative control group, 6.4 ± 0.8 °C; positive control, airborne exposure (in part) and contact exposure groups, 6.7 ± 0.4 °C; treated and airborne (in part) exposure groups, 6.4 ± 0.8 °C. BMI was calculated by dividing body mass at the time that the bats were euthanized by forearm length. Because animals that died naturally during the trial became desiccated, BMI was only calculated for bats that were euthanized.

Diagnosis of WNS was made through histological examination of the muzzle and a portion of skin from each wing[4]. *G. destructans* was re-isolated in culture from wing skin as described previously[22] and identified by PCR amplification/double-stranded sequence analysis of the rRNA gene internal transcribed spacer[23].

Statistical analyses. Survivorship was compared among groups using the Gehan–Breslow survival test (SigmaPlot 11.0; Systat Software) because this method gives more weight to animals that died naturally during the experiment and less weight to the large number of censored data points (that is, euthanized animals) at the end of the experiment. Pair-wise comparisons were examined with the Holm–Sidak procedure (significance at $P < 0.05$). BMI was compared among groups (negative control group, $n = 27$; treated group, $n = 25$; contact exposure group, $n = 15$; airborne exposure group, $n = 27$) using an analysis of variance test (significance at $P < 0.05$) after confirming that the data met assumptions of normality (Shapiro–Wilk test, $P = 0.07$) and equal variances (Levene median test, $P = 0.87$). One bat from the treated group was excluded from the BMI analysis because its weight was not measured before euthanasia and sample collection. Three bats from the treated group were euthanized 34 days after exposure to assess whether WNS lesions were developing; WNS lesions were not detected in these animals. Because these three animals were prematurely removed from the experiment, they were excluded from further analyses and are not represented in the specified sample sizes.

Equipment and settings. Prepared tissue sections were examined using an Olympus BH-2 upright microscope with SPlan Apo ×40 and ×100 objectives (Olympus Optical). Images were collected in tagged image file format using a digital colour camera (Insight2) and Spot Basic Version 4.0.8 (Diagnostic Instruments).