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Higher antibody titres against *Pseudogymnoascus destructans* are associated with less white-nose syndrome skin lesions in Palearctic bats

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Introduction: Serological tests can be used to test whether an animal has been exposed to an infectious agent, and whether its immune system has recognized and produced antibodies against it. Paired samples taken several weeks apart then document an ongoing infection and/or seroconversion.

Methods: In the absence of a commercial kit, we developed an indirect enzyme-linked immunosorbent assay (ELISA) to detect the fungus-specific antibodies for *Pseudogymnoascus destructans*, the agent of white-nose syndrome in bats.

Results and Discussion: Samples collected from European *Myotis myotis* (n=35) and Asian *Myotis dasycneme* (n=11) in their hibernacula at the end of the hibernation period displayed 100% seroprevalence of antibodies against *P. destructans*, demonstrating a high rate of exposure. Our results showed that the higher the titre of antibodies against *P. destructans*, the lower the infection intensity, suggesting that a degree of protection is provided by this arm of adaptive immunity in Palearctic bats. Moreover, *P. destructans* infection appears to be a seasonally self-limiting disease of Palearctic bats showing seroconversion as the WNS skin lesions heal in the early post-hibernation period.

KEYWORDS

emerging wildlife infection, adaptive antifungal immunity, disease severity, indirect ELISA, antibody prevalence, *Myotis* bat species

1 Introduction

Novel and emerging wildlife infections that threaten biodiversity, domestic animals and/or humans are of great interest to researchers seeking to gain insights into host-pathogen interactions (1). In such cases, risks of infection are driven by multiple factors, including host-pathogen co-evolution and dynamics, life history traits, community

structure of reservoir hosts, transmission rate and dispersal of the agent and environmental change (2, 3). Understanding the immune responses of bats, which are recognized as reservoir hosts of zoonotic agents, has recently become a critical issue to identify mechanisms that allow pathogen circulation and emergence of severe infections (4). However, the majority of articles on this subject concentrate on viruses of bats and mention the extraordinary ability of chiropterans to cope with RNA viral infections (5–11); consequently, much less is known about bat immunity against non-viral pathogens and/or pathogens that cause clinically manifesting diseases in bats (6).

The need for addressing gaps in our knowledge of bat immunity has also been highlighted by conservation concerns associated with the emergence of white-nose syndrome (WNS), a major threat to naïve bat species in North America (12–14). The causative fungal agent of WNS, *Pseudogymnoascus destructans*, has proven to be devastating for hibernating insectivorous bats in the Nearctic; however, it appears to be tolerated by Palearctic bats (15), with only sporadic cases of WNS-associated fatality being documented in Europe (16, 17), despite hyperendemic (i.e., highly prevalent and persistent) exposure to this psychrophilic skin invasive pathogen in contaminated hibernacula. The high prevalence and infection intensity (measured as fungal load and number of skin lesions) of WNS without mass mortality suggests an equilibrium in host-pathogen interactions between Palearctic bats and *P. destructans* (15). Exceptional infection tolerance in bats is thought to be due to a balance between protective and pathologic immune responses mediated through pro- and anti-inflammatory cytokines (4, 6, 18, 19).

Exposure of a bat to *P. destructans* may result in an invasive skin infection, with extensive damage to its flight membranes (16, 20, 21) and severe disruption of the effective skin barrier function explaining the pathophysiology of the disease (22–25), resulting in altered torpor patterns, increased arousal frequency, premature depletion of fat reserves and dehydration during hibernation (26). Enzymes secreted by *P. destructans*, e.g., destructin-1 peptidase, subtilisin-like serine peptidase and lipases, enable the fungus to invade and digest cutaneous tissues (27, 28). On histopathology of the flight membrane, specifically distinctive lesions include both cupping erosions and/or full-thickness invasion, where the fungus breaches the skin basement membrane (16, 20, 21), the WNS skin lesions being loaded with hyperaccumulated riboflavin, a secondary fungal metabolite emitting an intensive orange-yellow fluorescence following excitation with 366-385 nm ultraviolet (UV) light (29). This fluorescence can be used as a non-lethal diagnostic method to identify bats showing WNS skin lesions in their UV-trans-illuminated wing membranes (30).

Analysis of blood parameters in greater mouse-eared bats (*Myotis myotis*) has revealed a threshold of ca. 300 skin lesions on both wings induced by *P. destructans*, combined with suboptimal hibernation conditions, that distinguish healthy hibernating bats from those with disruption of body homeostasis (31). Pathophysiological effects of WNS in European bats tend to manifest as a mild metabolic acidosis, decreased blood glucose and peripheral blood eosinophilia. Bats displaying blood homeostasis disruption had a lower body mass index (BMI) and hibernated with a 2°C lower body surface temperature (31).

Note, however, there is still some discrepancy about the presence and/or absence of a systemic response to WNS infection in European bats. While it has been detected at both the organismal (31) and transcriptional (32) levels, it has not been recognized in the plasma proteomic profile (33). Likewise, a study of adaptive immunity in two European bat species detected no antibodies against *P. destructans* in winter, and only low titres in spring, concluding that antibody-mediated immunity cannot explain the survival of European bats infected with the WNS fungus (34). These differences in findings on European bat responses may or may not be due to differences in WNS status and the severity of infection in individuals selected for the studies.

In response to these conflicting findings, the objective of the present study was to develop an enzyme-linked immunosorbent assay (ELISA) for measuring antibody response to *P. destructans* infection based on antigens produced by pathogenic fungal strain isolates from North America, Europe and Asia. We predicted that Palearctic bats would show differences in antibody prevalence and titres against *P. destructans* in relation to species, their age and infection severity at the time of examination. We then tested surviving bats in the early post-hibernation period to assess whether there was a rise in antifungal antibody titre of WNS infection.

2 Materials and methods

2.1 Ethics statement

Bats were sampled in the field in accordance with Czech Law No. 114/1992 on Nature and Landscape Protection, based on permits 1662/MK/2012S/00775/MK/2012, 866/JS/2012 and 00356/KK/2008/AOPK issued by the Czech Agency for Nature Conservation and Landscape Protection. Sampling in caves in the Ural Mountains (Russia) was approved by the Institute of Plant and Animal Ecology, Ural Division of the Russian Academy of Sciences (No. 16353–2115/325), and the Tyumen State University (No. 06/162). Experimental procedures were approved by the Ethical Committee of the Czech Academy of Sciences (No. 169/2011). All authors were authorized to handle free-living bats under Czech Certificate of Competency No. CZ01341 (§17, Act No. 246/1992).

2.2 Sample collection and bat examination

We sampled a total of 46 bats, comprising 35 greater mouse-eared bats (*Myotis myotis*) from the Czech Republic and 11 pond bats (*Myotis dasycneme*) from Russia. Sampling was performed at two bat hibernation sites in the Czech Republic (the Šimon and Juda mines in the Jeseníky Mountains and the Mořina Quarry in Bohemia) in 2018 (April) and at three hibernacula in the Russian Ural Mountains (Arakaevskaja, Komsomolskaja and Partizanskaja caves) in 2017 (April). The sex of each bat was determined and the age estimated based on epiphyseal ossification of the thoracic limb fingers and tooth abrasion (35). We also measured the forearm length using callipers and body mass using a portable top-loading balance. BMI was determined as a bat's body mass (g) divided by

the left forearm length in mm (36). To examine the status and intensity of *P. destructans* infection, we took a swab of each bat's wing surface (FLOQ Swabs, Copan Flock Technologies s.r.l, Italy) for later examination in the laboratory, where presence of the fungus was tested using polymerase chain reaction (PCR) and WNS skin lesions were manually enumerated from photographs of both wings taken over a 368 nm ultra-violet (UV) lamp using the individual object counting tool of ImageJ, as described elsewhere (30, 31). A wing membrane biopsy targeting fluorescing lesions with a 4 mm sterile punch (Kruuse, Denmark) was collected from each bat under UV guidance to check for invasive fungal growth distinctive for WNS on histopathology (16).

To measure titres of antibodies against *P. destructans*, samples of blood were collected from bats ca. 60 minutes after capture, this providing the re-warming period necessary for efficient blood flow from punctured veins under field conditions of late hibernation. The procedure of blood collection comprised skin surface disinfection with an alcohol pad, the spreading of a small drop of heparin over the intended skin puncture site and puncturing of the uropatagial vessel with a sterile needle to obtain a 100µl blood sample using a heparinised pipette tip. The puncture site was then sealed using a drop of surgical absorbable tissue glue (Surgibond, SMI AG, Belgium) to stop further bleeding. All bats were handled gently and, prior to release at the hibernaculum, provided orally with 5% glucose and physiological saline solution for rapid replenishment of energy and fluids. The whole blood samples were centrifuged 15 min at 1500g to separate the plasma, which was then stored at -80°C until further use in antibody measurements.

Eleven adult *M. myotis* bats (three female, eight male) from the Šimon and Juda hibernation site were kept in captivity for four weeks to obtain paired blood samples in order to test for a rise in the antifungal antibody titre of WNS infection surviving bats in the natural early post-hibernation period (i.e., in April to May). These bats were housed at a temperature of 21°C in an indoor flight chamber with soft mesh on the inner walls, cloth layers to provide roosting and hiding places and humidifiers to maintain humidity between 60 and 70%. The bats were exposed to a natural day and night cycle, provided with a water dish for drinking and encouraged and taught to self-feed on mealworms and crickets.

2.3 ELISA for measuring antibodies against *Pseudogymnoascus destructans*

Six pathogenic strain isolates of *P. destructans* from little brown bats (*Myotis lucifugus*), *M. myotis*, common long-eared bats (*Plecotus auritus*) and *M. dasycneme* infected in North America, Europe and Asia, listed in (29) as 20631-21[T], CCF3941, CCF3943, CCF4103, CCF4987 and CCF4986 with ITS rDNA accession numbers EU884921, HM584956, HM584957, LN852366, LN852358 and LN852359, respectively, were used to develop an ELISA for detection of antifungal antibodies in cooperation with the Biovendor Research and Diagnostic Product Department (Brno, Czech Republic). The fungal strains were cultivated on sterile Sabouraud dextrose agar plates wrapped in Parafilm (Fisher Scientific, USA) after inoculation at 10°C in darkness. After eight

weeks of colony growth, phosphate buffered saline (PBS; Sigma-Aldrich, Germany) was used to harvest the fungal conidia. Next, the number of *P. destructans* fungal elements was determined in a Bürker counting chamber (Fisher Scientific, USA), inactivated with formaldehyde (Roth, Germany), sonicated in a Sonorex Super RK 156 BH sonic bath (Bandelin, Germany) and then used to coat 96-well microtitration plates (Nunc Inc., Denmark) in 0.1M carbonate buffer (pH 9.6; Merck Sigma-Aldrich, Germany). An equal coating ratio of all six *P. destructans* pathogenic strains was used in each well. The carbonate buffer used for microtitration plate coating contained 50 000 conidia per 1mL. Each well was treated with a volume of 100µL of the coating solution. After 48h of coating and immobilization at 4–6°C, the plates were washed and blocked to suppress non-specific binding and stabilized in sucrose solution (Sigma-Aldrich) for 1h before drying, sealing under vacuum in the presence of silica desiccant (Roth, Germany) and storing at 2–8°C until use.

Prior to antibody measurement, bat sera were PBS diluted 1:100, then placed into the testing wells and incubated in the plates for 1h at 25°C, after which a two-step protocol was used for detection of anti-*P. destructans* specific antibodies. The first step included addition of biotinylated monoclonal anti-bat antibody (Goat anti-Bat IgG Heavy and Light Chain Antibody Biotinylated, Bethyl Laboratories, USA) and incubation for 1h. This was followed by addition of streptavidine-poly HRP (horseradish peroxidase) conjugate (Mir Biotech s.r.o., Czech Republic) and 1h of incubation. In each case, a Multi Bio 3D Mini Shaker (Biosan, Latvia) set at 300rpm was used during the period of incubation. The microtitration plates were washed four times with PBS containing 0.05% of Tween-20 (Sigma-Aldrich) in-between the incubation steps. A color reaction was developed through addition of a reagent peroxide and TMB substrate (3,3′,5,5′-tetramethylbenzidine; Sigma-Aldrich) and stopped with 1M sulphuric acid (Sigma-Aldrich). IgG (0.01% solution) in Tris bovine serum albumin (BSA) buffer (Sigma-Aldrich) was used as a blank, and plasma samples collected from 12 noctule bats (*Nyctalus noctula*) in a non-related study (37) served as a negative control, this species being a non-cave hibernator that is not exposed to the *P. destructans* fungus. The intensity of *P. destructans* specific antibody binding was detected through duplicate dual wavelength measurements on an Elx808 ELISA microplate Reader (BioTek, USA), with 630nm absorbance serving for subtraction of optical non-homogeneity and 450nm for measuring the specific signal, the absorbance values being measured in duplicates and multiplied by the plasma dilution factor (x100) to obtain antifungal antibody titres. Paired blood samples of *M. myotis* bats, positive for WNS skin lesions and showing a rise in the antifungal antibody titre after healing from the WNS infection in the early post-hibernation period, were considered as positive controls.

2.4 Statistical analysis

All statistical analysis was undertaken using the TIBCO Statistica® software package v.14.0.0 (TIBCO Software Inc., USA). Normal distribution of variables was tested using the Shapiro-Wilk test. As WNS UV lesion counts and antibody titres were not

normally distributed, these variables were log transformed (log n +0.5 and log n, respectively), re-checked for normality and used for all statistical analyses. Differences between means of variables (WNS UV lesion count, antibody titre and relative increase in antibody titre) were tested using the t-test for independent and/or paired samples. As there were no differences in BMI values between female and male and adult and subadult *M. myotis* (t = -0.154, p = 0.879 and t = 0.011, p = 0.991, respectively), the data were pooled for subsequent analyses. The effect of WNS UV lesion count on antibody titre was tested using univariate general linear model (simple regression) separately for each bat species. The Pearson correlation was used as a measure of linear correlation of variables.

3 Results

PCR examination confirmed *P. destructans* infection in 100% of *M. myotis* and *M. dasycneme* bats and histopathological findings matched WNS diagnostic criteria in all *M. myotis* bats examined. WNS prevalence in *M. dasycneme* bats was 81.82% based on UV skin lesion detection and histopathology (Table 1). Severity of infection ranged between 99 and 2949 skin lesions in Czech *M. myotis* bats and from 0 to 625 skin lesions in Russian *M. dasycneme* bats. All bats of both species were seropositive for anti-*P. destructans* antibodies (Table 1).

While no significant difference was observed between bat species in their antibody titres (t = 0.413, p = 0.681), they differed significantly in WNS UV lesion counts (t = 5.897, p < 0.001; Figure 1). There were no differences in antibody titres even when just the subset of females was compared (t = 0.925, p = 0.370 and t = 2.705, p = 0.016, respectively), with female *M. dasycneme* having significantly fewer skin lesions (mean = 115) than *M. myotis* (mean = 676). A significant relationship between WNS UV lesion count and antibody titre was only confirmed for *M. myotis* (F = 8.512, p = 0.006) with a significant negative correlation (r = -0.453, p = 0.006; Figure 2). After dividing the *M. myotis* data into two groups based on the threshold of 300 skin lesions on both wings (the point at which WNS affects the bat's health status),

significantly lower antibody titres (t = -2.847, p = 0.008) were observed in the > 300 lesion group (Figure 3).

Comparison of paired titres for antifungal antibodies measured in the same *M. myotis* individuals at the end of the hibernation period and after four weeks of euthermy in captivity revealed a significant positive rise in all individuals, except for three bats showing a minor titre decrease of up to 30%. A total of 64% of bats showed a 2- to 6-fold seroconversion, with the relative increase in antibody titres being negatively, though non-significantly, correlated with titre values obtained during the first measurement (r = -0.590, p = 0.056; Figure 4). Adult bats exhibited an average 2.87-fold seroconversion of antibody titres during the four-week period. While there was no difference in the relative increase between adult females and males, subadult females displayed a significantly higher relative increase in antibody titres than subadult males (t = -5.568, p = 0.031).

4 Discussion

4.1 *P. destructans* infection status at the study sites

Our study combined detection of *P. destructans* infection in Palearctic bats using standard procedures for non-lethal examination, such as PCR (38), wing membrane trans-illumination with a modified Wood's lamp (30) and histopathology of skin lesions collected under UV guidance (16, 20) with a newly developed ELISA for measuring antibodies against *P. destructans*. Data gathered in the present study correspond with earlier reports on bat species-specific prevalence, with levels ranging from 64 to 100% (15, 39). The knowledge gained on infection status and severity in this study can be seen as a major advance. On the other hand, it is our opinion that the unknown *P. destructans* infection status of bats collected from Germany and Finland by Johnson et al. (34), along with their relatively small sample size, may limit the validity of their results and conclusions drawn as regards European bats.

TABLE 1 Characteristics of bats examined for antibodies against *Pseudogymnoascus destructans*.

Bat species	*Myotis myotis*				*Myotis dasycneme*
Age	Adult n=27		Subadult n=8		Adult n=11
Gender	Female n=5	Male n=22	Female n=1	Male n=7	Female n=11
Body mass [g]	19 – 28	20.0 – 26.5	18.5	19 – 27	13.5 – 19
Antebrachium length [mm]	60.0 – 64.4	56.7 – 64.4	61.6	56.3 – 65.2	45.9 – 48.5
Body mass index	0.31 – 0.47	0.33 – 0.44	0.3	0.32 – 0.44	0.28 – 0.41
WNS UV lesions	139 – 454	99 – 2949	2318	143 – 2462	0 – 625
Antibody titre I	0.113 – 0.658	0.047 – 1.223	0.151	0.151 – 0.551	0.086 – 0.648
Antibody titre II	0.370 – 1.904	0.151 – 0.58	0.316	0.191 – 0.541	N.A.

Myotis myotis and *Myotis dasycneme* bats were sampled in the Czech Republic and Russia in 2017 and 2018. Quantitative characteristics are given as minimum and maximum values. WNS UV lesions, white-nose syndrome skin lesions in both wing membranes, identified and enumerated using trans-illumination with 366-385 nm ultraviolet light; Antibody titre I, values based on blood samples collected in the field towards the end of the hibernation period (April); Antibody titre II, values based on blood samples of WNS infection-surviving bats (n=11) after four weeks of captivity in the early post-hibernation period; N.A., not available.
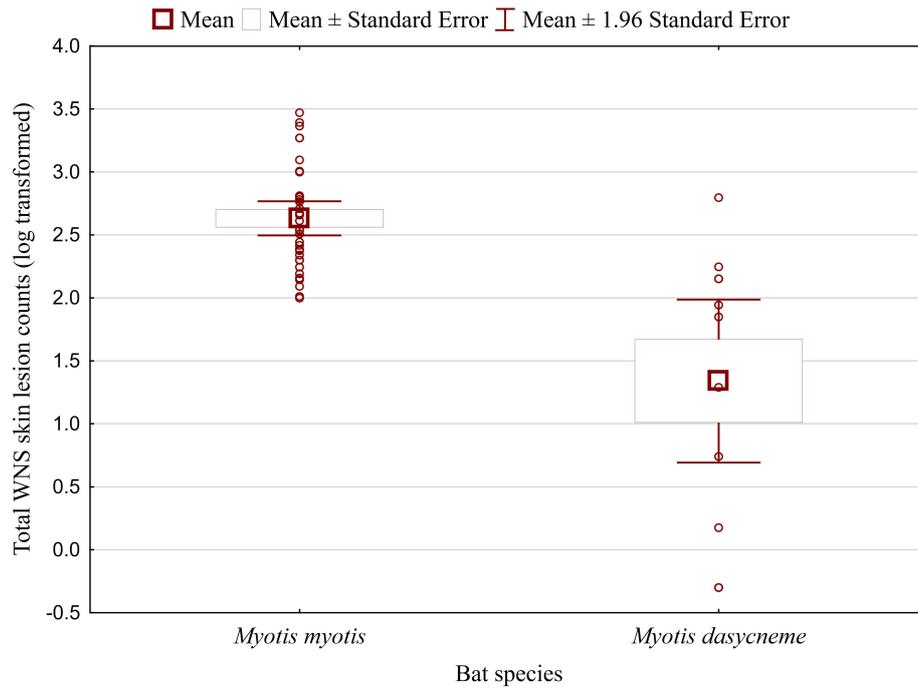
FIGURE 1
WNS UV lesion counts on both wings of *Myotis myotis* and *Myotis dasycneme* bats sampled in the Czech Republic and Russia in 2017 and 2018. WNS UV lesions = white-nose syndrome skin lesions identified and enumerated using trans-illumination with 366-385 nm ultraviolet light. *Myotis myotis* and *Myotis dasycneme* bats differed significantly in WNS UV lesion counts (t = 5.897, p < 0.001).
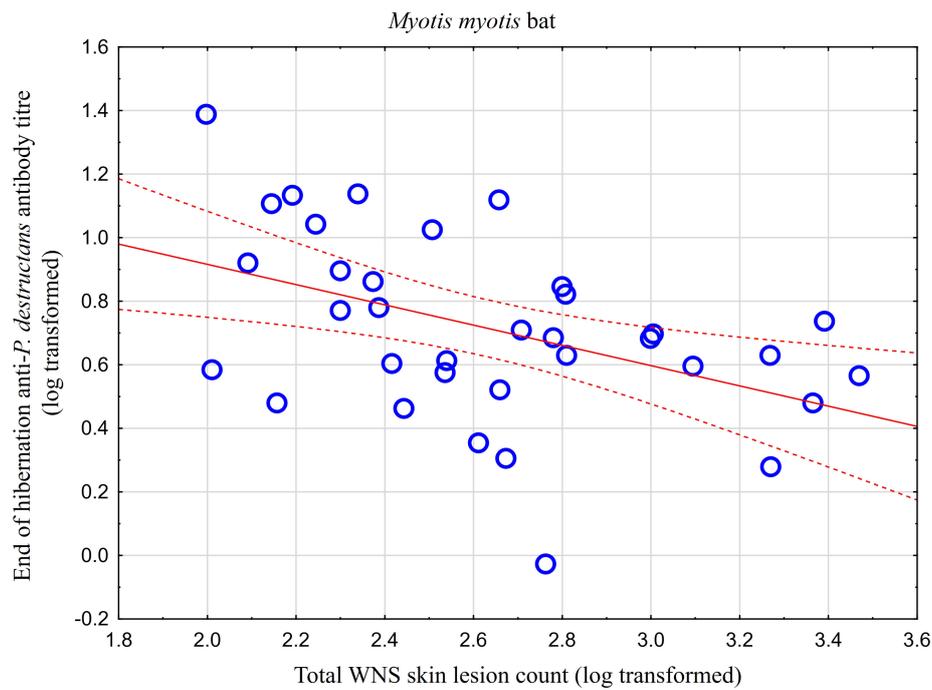

FIGURE 2
Relationship between WNS UV lesion counts and antibody titres measured in blood samples collected towards the end of the hibernation period (April). Linear regression with 95% confidence intervals. A significant relationship was only confirmed for *M. myotis* (F = 8.512, p = 0.006) with a significant negative correlation (r = -0.453, p = 0.006). Higher antibody titres against *Pseudogymnoascus destructans* were associated with less white-nose syndrome skin lesions.
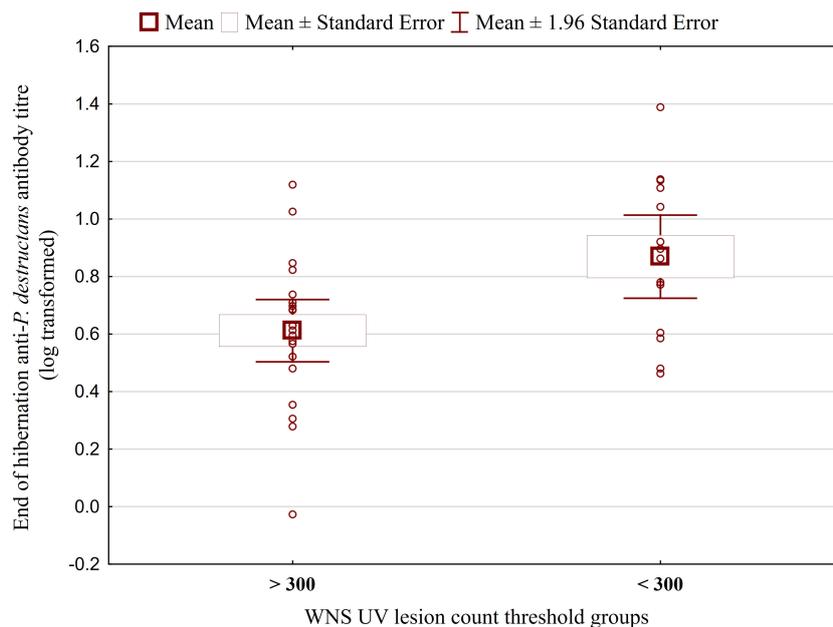
FIGURE 3
Antibody titres against *Pseudogymnoascus destructans* in *Myotis myotis* bats grouped based on a threshold of 300 WNS skin lesions on both wings. After dividing the *M. myotis* data into two groups based on the threshold of 300 skin lesions on both wings (the point at which WNS affects the bat's health status), significantly lower antibody titres (t = -2.847, p = 0.008) were observed in the > 300 lesion group. For bats > 300 n=21, for bats < 300 n=14.

4.2 Bats produce antibodies against *P. destructans*

Contrary to the findings of Johnson et al. (34), the results of the present study indicate that European *M. myotis* bats respond to *P. destructans* infection by producing antibodies. Antibody findings in both European *M. myotis* bats and Russian *M. dasycneme* bats may be similar to naturally infected *M. lucifugus* bats in North America showing the highest titres in regions with longer histories of WNS (34). Even subadult *M. myotis* individuals tested seropositive, indicating that they responded to the fungal infection during their first hibernation winter. Lorch et al. (40) showed experimentally that clinical signs and skin infection with lesions diagnostic of WNS develop within 83 to 102 days of hibernation and the disease peaks at the end of hibernation period (41). The time-course of antibody response to *P. destructans* infection in bats is rather unknown. However, for example in human histoplasmosis, antifungal antibodies generally take up to eight weeks to reach detectable levels (42). As the bat winter hibernation period lasts from five to six months (43, 44), this provides ample time for disease progression and an immune system response. Clearly, detection of seropositive bats does not mean that antibodies will be present indefinitely; however, it is still unclear whether the antibody titres against *P. destructans* remain to the next hibernation winter, and what antibody levels are associated with the threshold of protection. Normally, one might assume that antibodies in Palearctic bats would decline in the absence of further fungal challenge in summer and, indeed, Johnson et al. (34) documented higher anti-*P. destructans* titres in spring-sampled North American bats than in

summer. On the other hand, the high prevalence of antibodies in Palearctic bat populations may reflect either a high rate of infection or a low rate of antibody loss, with repeated challenges every winter. Likewise, there have been no studies on antibody titres in lactating females and potential antibody transfer in the colostrum, and little is known about factors that stimulate seroconversion in some individuals and seroreversion in others. One may hypothesize that both infection intensity and severity (16) and bat health status (31) are correlates of the immune response. Interestingly, two *M. dasycneme* bats in this study displayed antibodies despite being negative on UV skin lesion detection and histopathology, though both bats subsequently tested positive for the *P. destructans* fungus on PCR examination, suggesting that their antibodies developed rather in response to infection during the previous winter hibernation.

4.3 Fungal immunogenicity

The design of the ELISA kit used in this study was based on fungal conidia triggering stronger immunogenic responses than mycelial components (45). The immune system of *P. destructans*-infected bats is, however, mainly exposed to fungal hyphae invading deeper skin layers and secondary metabolites produced by the fungus. Conidia predominate on the skin surface because they are produced by aerial hyphae that colonize skin surface, meaning that the immune system is less exposed to conidia (16). As little is known about differences in the immunogenicity of pathogenic *P. destructans* strains isolated from bats over extensive geographic
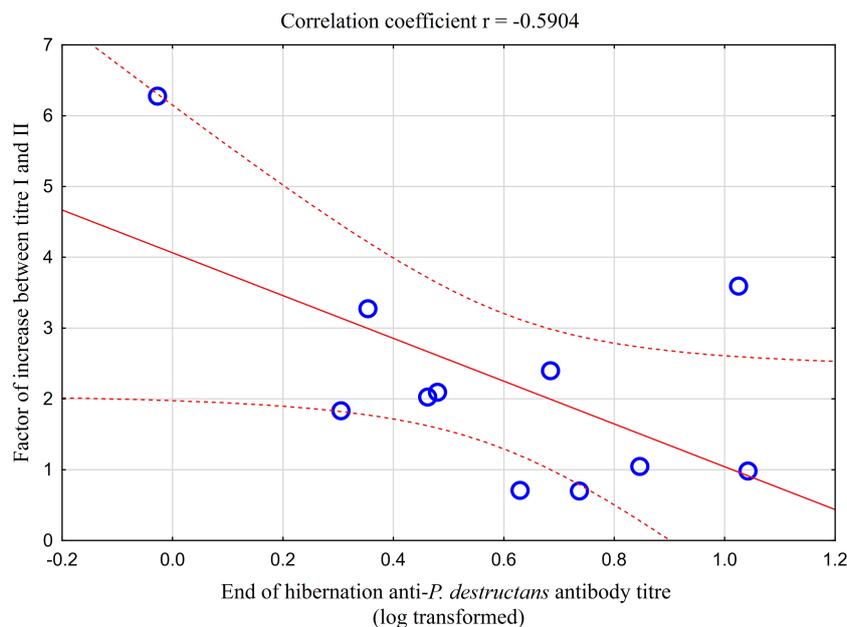
FIGURE 4
Relationship between relative increase in antibody titres in captive bats after four weeks of euthermy (titre II) and antifungal antibodies measured in the same individuals at the end of the hibernation period (titre I). Linear regression with 95% confidence intervals. The relative increase in antibody titres was negatively, though non-significantly, correlated with titre values obtained during the first measurement (r = -0.590, p = 0.056).

areas of the Palearctic and Nearctic regions, our ELISA kit was produced using antigens of six pathogenic strains to cover the available genetic variability of the fungus, including its mating types (15, 29). This is a similar strategy to that used in the study of Brennan et al. (46), who also tested multiple *P. destructans* strains to determine ELISA specificity. Brennan et al. (46), using immunofluorescence antibody testing, also demonstrated that various *Pseudogymnoascus* species share a conserved immunoreactive antigen, while Carvalho et al. (47) showed that the immunogenic stimuli of fungi depend on the ability of the innate immune system to sense pattern recognition receptors. Interestingly, this suggests that the immunogenic antigens of *P. destructans* that stimulate antifungal immunity, i.e., calnexin and destructin-1, could be used to prepare a vaccine promoting protection against WNS (48).

4.4 Immune suppression and immune response to fungal infection in bats

Seasonality is a driver of the annual cycle of temperate insectivorous bat species (49), and governs the year-round dynamics of most physiological functions, including the immune response (50). In winter, hibernating bats are subject to physiological extremes as they go through cycles of prolonged torpor with shorter periods of arousal (51–53). During winter hibernation torpor, bats lower their body temperature close to the ambient temperature, which tends to range between 0 and 12°C in hibernacula of temperate vespertilionids and rhinolophids (54, 55). As body temperature is an important factor modulating bat

immune defense mechanisms (34, 37, 56–61), they may face reduced innate and adaptive functions in winter (57–59, 62). While immune suppression enhances fungal pathogenesis, fungal infections such as WNS will threaten such immune-compromised hosts (47, 63, 64).

Our results indicate that immune suppression of Palearctic bats during winter hibernation allows invasive infection of living skin tissues by the fungal agent, manifesting as distinctive WNS lesions. At the same time, the degree of immune suppression does not prevent production of antibodies against the invading agent. The immune system becomes more effective in the production of antifungal antibodies in the post-hibernation period, as documented by the comparison of paired titres measured in the same *M. myotis* bat individuals at the end of the hibernation period and after four weeks of natural euthermy.

The filamentous growth of fungal pathogens represents a particular challenge for the immune system (65). Neutrophils represent the first line of defense against fungi and are capable of modifying the overall immune response (66). WNS cupping erosions on bat wings, for example, heal through marked neutrophilic inflammation and sequestration of the fungal agent from the skin (16). Hibernating North American *M. lucifugus* bats infected with *P. destructans* express genes associated with local inflammation in the skin (67) and bat populations sampled prior to and after exposure to the WNS agent show a shift in immunogenetic diversity (68). During hibernation, bats may balance their inflammatory response to fungal exposure through energy-saving strategies (69). Regulation of gene expression has also been noted in the fungal agent as a response to the host-pathogen interaction during the course of WNS infection (70). Importantly,

macrophages derived from *M. myotis* have been shown *in vitro* to be able to increase functional performance within minutes of transition from torpor temperatures to euthermic arousal (61).

In practice, however, a combination of cell-mediated and humoral responses is often necessary for clearance of fungal infections (47, 63, 65, 71). Antibodies may then participate in protection against fungal diseases through direct action on fungal cells and cytotoxicity, enhancement of phagocytosis and/or complement activation (65). Antibody-mediated immunity against fungi may even be deleterious (65). In North American *M. lucifugus* bats, for example, a cell-mediated delayed-type hypersensitivity was shown to kill WNS fungus-infected bats weeks after emergence from hibernation (56). Paradoxically, therefore, antifungal immunity can range over a continuum from deficiency to hyperactivity (47). In the present study, WNS infection surviving adult *M. myotis* bats held in captivity in the early post-hibernation period showed no signs of immune-mediated pathology associated with post-emergent healing of wing membrane lesions.

4.5 Can measurements of bat antibody titres against *P. destructans* be used in further studies?

While the direct conservation risks of emerging wildlife infections are clear, the adverse carry-over effects that reduce the success of such conservation efforts are often complex and difficult to document (72). Theory defines carry-over effects in wild animals as non-lethal biological processes that act in one season and influence performance in the next season (73), with carry-over phenomena believed to occur at the individual and/or population or community levels. Studies dealing with carry-over effects measure different indicators of condition and/or health (e.g., body mass, fat mass, pathogen load, blood profile, metabolic rate, immune defense) at the end of one season as a proxy for explaining subsequent variations in fitness, including reproduction and survival.

Very little is known about the health of European *P. destructans*-infected hosts in the period following emergence from hibernation. Upon arousal, early euthermic females may face a trade-off between mounting an immune response and the energetic investment needed to initiate gestation (74, 75). North American bat species recovering from *P. destructans* infection have shown shifts in pregnancy and lactation, suggestive of reproductive fitness consequences (76).

The characteristics of WNS make it an ideal model for examining carry-over effects associated with pathogen pressure (77), as 1) the infection is seasonally limited, 2) all bats roosting in a contaminated hibernaculum are exposed to the pathogen, 3) WNS survivors recover in the post-hibernation period and clear the infection, and 4) WNS pathogen pressure can be quantified, either as fungal load (infection intensity), through image analysis of wing membrane damage and/or through histopathology grading (disease severity) based on non-lethal skin biopsies (16).

Of all the European bat species, *M. myotis* shows the highest prevalence and infection intensity with WNS fungus (15, 16, 21, 31), making it the most suitable model species for studying immune responses and carry-over effects. In the same context, *M. dasycneme* may be used for host-pathogen studies encompassing Palearctic regions outside *M. myotis* distribution (15, 78). An important issue, however, is how to link pathogen pressure during bat hibernation with indicators of condition and health in the post-hibernation period. We argue that comparisons of antibody titres against *P. destructans* infection are a suitable candidate for obtaining such a WNS disease-specific link.

5 Conclusions

The WNS fungus, *P. destructans*, is endemic throughout temperate regions of Europe and Asia, meaning that hibernating insectivorous bats are naturally exposed to this skin-infecting pathogen every winter. Interestingly, reports on histopathology indicate equal findings of focal skin-tissue invasiveness distinctive for WNS lesions in Palearctic and Nearctic bats (15, 16). Here, we show highly prevalent antibodies against *P. destructans* in Palearctic bats, a response pattern indicating an inverse relationship between antibody titre and skin lesion count and an increase in antibody titres in the early post-hibernation period. Further studies will be needed to obtain deeper insights into how both the innate and adaptive arms of immunity contribute to the survival of Palearctic bats exposed to the virulent WNS fungus.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Ethical Committee of the Czech Academy of Sciences. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. JB: Conceptualization, Methodology, Validation, Writing – review & editing. VS: Data curation, Investigation, Methodology, Validation, Writing – review & editing. VP: Investigation, Methodology, Writing – review & editing. JZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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