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REVIEW OF THE CURRENT STATUS AND FUTURE PERSPECTIVES ON *PSEUDOGYMNOASCUS DESTRUCTANS* STUDIES WITH REFERENCE TO SPECIES FINDINGS IN BULGARIA

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Abstract. Emerging infectious diseases are a well-known threat to the wildlife and require complex research. There is a rapidly accumulating knowledge on the infectious disease of bats, named firstly White Nose Syndrome (WNS) and afterwards – White Nose Disease (WND), and its causative agent – the pathogenic fungus *Pseudogymnoascus destructans.* Although mass mortality of bats, known since a decade, is currently restricted to North America, the pathogen is of global concern as a potential threat to other hibernating bat populations. Therefore five years after the first comprehensive synthesis on the fungal ecology and relevant knowledge gaps (FOLEY ET AL. 2011), we decided to summarize the published information on the pathogen morphology, reproduction, ecological requirements, geographic distribution and systematic position. In addition, the present review compiles the available data on the affected bat species, mechanisms of WND, on the host response and on the effective treatment strategies with possible methods for *fighting* the pathogen to reduce the mortality in affected regions as well. Special attention is paid to the finding of the fungus in Bulgarian caves.

Key words: bats, caves, *Geomyces destructans*, geomycosis, nature conservation, White Nose Disease, White Nose Syndrom

Introduction

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Emerging infectious diseases are a well-known menace to the wildlife, often causing mass mortalities of different organisms, threatening them with extinction (e.g. DASZAK ET AL. 2000; DE CASTRO & BOLKER 2004; HOYT ET AL. 2015 and citations there-in). The parasitological threats to biodiversity conservation have been defined also as a pathogen pollution (CUNNINGHAM ET AL. 2003). Among them is the White Nose Disease (WND), named after the White-Nose syndrome (WNS). It was first reported in 2006 and since then was continuously emerging (e.g. BLEHERT ET AL. 2009; TURNER AND REEDER 2009; FRICK ET AL. 2010; CAVEN ET AL. 2012 among the many others). It affected solely hibernating bat species and lead to regional population bats collapses with extensive local extinctions in North America (PIKULLA ET AL. 2012; FRICK ET AL. 2015). There it had been documented in 26 states of U.S.A. and 5 Canadian provinces and caused the death of around 6 millions individuals (U.S. FISH AND WILDLIFE SERVICE 2015; FRICK ET AL. 2016). This zoonosis is comparable with the chytridiomycosis in amphibians, Colony Collapse Disorder in bees, and Snake Fungal Disease in snakes, and is probably the most large-scale extinction of mammals in modern history (CRYAN ET AL. 2010; HOYT ET AL. 2015). Its causative agent is the psychrophilic ascomycetous fungus *Pseudogymnoascus destructans* (Blehert et Gargas) Minnis et D. L. Lindner (Syn. *Geomyces destructans* Blehert et Gargas). Later on *P. destructans* was found in Europe and Asia, but with apparently little or no mortality among the bats from these regions and this lead to suggestions on the fungus origin from these areas and its long co-evolution with the bats there (e.g. PUECHMAILLE ET AL. 2011C; ZUKAL ET AL. 2016). In addition to the unprecedented numbers of sick and killed animals (90-100% of the populations in some areas of North America), it was registered that the bats affected by WNS act strangely during cold winter months, including flying outside during the day and clustering near the entrances of caves and other hibernation areas (COLEMAN 2014). Recent evaluation of the ecosystem services provided by bats have revealed that many species offer unique and large-scale monetary benefits to agricultural industry (e.g. through pollination, controlling of pest insect populations in subtropical coffee and cacao plantations) and we have just started to understand their ecological role in natural ecosystems (e.g. top-down regulators of insect populations in forest habitats - for details see VOIGT & KINGSTON 2016). In the same time bats are extremely vulnerable to anthropogenic impact, especially nowadays, in the changing world of the Anthropocene (OP. CIT.). Therefore their conservation is of key importance for the environment and indirectly for the human society. Logically, the significance of the "novel fatal infectious disease of hibernating bats" provoked strong interest to the fungal pathogen and its effects, first generalized by FOLEY ET AL. (2011) with outlining of the relevant knowledge gaps. Many of these gaps have been fulfilled through the research carried during the last years, but meanwhile still other questions remained and also new questions raised. Thus, according to COHN (2012) bats and WNS continued to remain a conundrum. Therefore we decided to summarise the available information on *P. destructans*, on the mechanisms of disease and the host-pathogen interactions, including the host response, on effective treatment strategies and on the possible methods for *fighting* the pathogen with emphasis on the newest investigations. Special part of the review is targeted on the species findings in Bulgaria.

1. The White Nose Syndrome (WNS), the White Nose Disease (WND) and the *geomycosis:* Historical notes, spread of the infection, terminology, affected bat species, descriptions of symptoms and causative fungal agent

The **White Nose Syndrome (WNS)** was first documented on a photograph, taken on 16th of February 2006 in Howe's cave, New York (TURNER AND REEDER 2009; GARGAS ET AL. 2009) and named after the white fussy growth on bat wings, ears and muzzle (VEILLEUX 2008; REEDER AND TURNER 2008; TURNER AND REEDER 2009; BLEHERT ETAL. 2009). It was also associated with unusual winter activity of bats and mass mortality in New York state (VEILLEUX 2008) and later on in North-eastern United States and South-eastern Canada, where it has led to severe population declines (BAT CONSERVATION INTERNATIONAL: HTTP://WWW.BATCON.ORG/, 2015). The according disease was defined later by the presence of cupping erosions on the skin caused by infection by *P. destructans*, which is determined by histopathological examination (METEYER ET AL. 2009), although the name WNS was still used in the paper. Therefore, following FRICK ET AL. (2016) it has to be stressed that term WNS was originally used to describe the symptoms associated with bats in the field (visible fungal growth on skin surfaces, depletion of fat reserves, altered torpor patterns and aberrant winter behaviour) and had an original definiton of syndrome (e.g. VEILLEUX 2008; REEDER AND TURNER 2008; TURNER AND REEDER 2009) before the disease was fully characterized as a pathogenic cutaneous infection of skin tissues. A lot of confusion arose around application of the term WNS for infections occurring in Europe since they were pathologically similar to those in North America but did not include mass mortality or unusual winter behaviour (PUECHMAILLE ET AL. 2011C). Then CHATURVEDI & CHATURVEDI (2011) stated that WNS was neither an exclusive presentation nor an all-encompassing description of *P. destructans* infections in bats. They insisted that continued use of this terminology to describe bat disease carried the risk of undue focus on one symptom of what was likely to be a complex host– pathogen interaction. Therefore both authors, following the conventional way of formation of mycological and veterinary terms, proposed to use the term ***geomycosis*** **(**from the fungal first name *Geomyces* and suffix *–cosis* [Gr.] used for a disease, morbid state) instead of WNS. However, in their proposal, the term *geomycosis* was adopted to describe infections caused by two different psychrophillic pathogens from the gender *Geomyces* Traeen - *G. destructans* and *G. pannorum* (Link) Sigler et J. W. Carmich.. The last is a rare pathogen which causes skin and nail infections in humans, and bone infections in dogs (for details and citations see CHATURVEDI & CHATURVEDI 2011). Practically, it could be applied to any other *Geomyces* pathogen find in future. The name *geomycosis* was used by some authors (e.g. PUECHMAILLE ET AL. 2011B; PIKULA ET AL. 2012), but obviously in order to avoid new misinterpretations caused by this broader term and to reflect the taxonomic renaming of *G. destructans* in *Pseudogymnoascus destructans*, a new term - **White Nose Disease (WND)** - was coined as a synonym of White Nose Syndrome (WNS) by PAIVA-CARDOSO ET AL. (2014). This led to a certain new confusion among researchers regarding the distinction between the WNS and the WND and, most probably, could be overcome by wider acceptance of the term WND (PAIVA-CARDOSO ET AL. 2014; FRICK ET AL. 2016). However, we have to mention that despite its original definition as a syndrome, the term WNS is still routinely used to refer the cutaneous infection caused by *P. destructans*. The terminological discussions on the appropriate usage of both terms from medical point of view, in which

generally there is a difference between *syndrome* and *disease*, is out of the scope of the present review. Therefore, below both terms WNS and WND will be used in the way in which they were originally applied by the cited authors.

The Little brown bat (*Myotis lucifugus*) is the most **affected species** by WND and WNS. Although a few examples of its summer colonies persisting in pockets around the affected areas have been documented (e.g. DOBONY ET AL. 2011; COLEMAN & REIHARD 2014), its numbers decreased by 90-91% in 5 states (TURNER ET AL. 2011; COLEMAN & REICHARD 2015) and, in case that no action is taken, a local extinction of the species by 2020 is predicted (FRICK ET AL. 2010). Great risk of extinction at a global scale is faced also by Northern long-eared bat *Myotis septentrionalis* (LANGWID ET AL. 2012), which since 2015 is enlisted as a federally threatened species by the U.S. Fish and Wildlife Service. However, no connection between colony size and disease impact has been observed (FRICK ET AL. 2015), probably because the initial mortality of the species due to WND was higher in larger colonies (LANGWIG ET AL. 2012). Except these two species, according to THOGMARTIN ET AL. (2013), COLEMAN (2014) and COLEMAN & REICHARD (2014) five more cave hibernating bats, including two endangered (EN) species have been confirmed with WNS: *Eptesicus fuscus, Myotis leibii, Myotis grisescens* (EN)**,** *Myotis sodalis* (EN) and *Perimyotis subflavus.*

However, the presence of *P. destructans* or skin infection by the pathogen not obligatory coincides with lethality. It was proved that mortality rates differ by species even in America (TURNER ET AL. 2011) and that European and some Palearctic Asian populations have not been affected to mass mortality (e.g. PUECHMAILLE ET AL. 2011C; ZUKAL ET AL. 2016 and citations there-in). According to COLEMAN (2014) and COLEMAN & REICHARD (2014) bat species on which *P. destructans* has been detected with no confirmation of disease, were as follows: *Lasiurus borealis, Myotis austroriparius, Lasionycteris noctivagans, Corynorhinus rafinesquii, Corynorhinus townsendii virginianus* (EN**)** and one federally listed species was found in the affected area that have not yet been confirmed with WNS or fungal infection: *Corynorhinus townsendii ingens* (EN). Even earlier, during spring of 2010, DNA of *P. destructans* was detected in three additional species of hibernating bats (*Myotis austroriparius, Myotis grisescens, Myotis velifer*) west of the Appalachian Region (e.g. Missouri and Oklahoma), yet mortality was not observed (USGS 2010- cit. acc. to FLORY ET AL. 2012). However, it has to be underlined that in some cases observations of fungal presence without lethality or additional clinical signs of disease (e.g. bats flying during daytime in winter – for details see the text below) may simply reflect detection of the disease in its earliest stages (FLORY ET AL. 2012). Non-lethal WND is reported for the European species *Myotis myotis* (PIKULA ET AL. 2012), *Myotis daubentonii, Myotis bechsteinii, Myotis nattereri, Myotis brandtti, Myotis emarginatus, Myotis dasygneme, Eptesicus nilssonii, Barbastella barbastellus, Plecotus auritus* and *Rhinolophus hipposideros* (ZUKAL ET AL. 2014). Infections with *P. destructans* in Europe without evidence for mortality were reported also by BÜRGER ET AL. 2013 for *Myotis myotis, M. oxygnathus* and for *Myotis blythii* by PAIVA-CARDOSO ET AL. (2014). In addition to WNS documentation, the presence of *P. destructans* in North America was detected by swab sampling and quantitative PCR methods (MULLER ET AL. 2013) in *Myotis austroriparius, Corynorhinus townsendii virginianus, Corynorhinus rafinesquii* and *Lasionycteris noctivagans* (BERNARD ET AL. 2015), and in Europe –

in *Myotis mystacinus* (MARTINKOVA ET AL. 2010) and *Myotis blythii* (Syn. *M. oxygnathus* - WIBBELT ET AL. 2010). One additional species, *M. escalerei*/sp. A is classified as Gd-suspect via photographic documentation (PUECHMAILLE ET AL. 2011 C). Recently, the WND causative agent was found in North-eastern China (HOYT ET AL. 2016) in 6 more species of bats: *Myotis macrodactylus, Myotis chinensis, Murina ussuriensis, Myotis petax, Myotis leucogaster* and *Rhinolophus ferrumequinum* without causing mortality. With the increase of the scope of the investigated regions and studies on *P. destructans* by different methods, the species is detected in new areas and the list of affected or associated with the fungus species is increasing and is periodically updated at the Bat Conservation International website http://www.batcon.org. The geographic distribution of *P. destructans* and the reasons for lack of mass mortalities in the Palearctic are discussed below in the text and in $3.

Recently there is no doubt that the WND **causative agent is the fungus *Pseudogymnoascus destructans*** (e.g. BLEHERT ET AL. 2009; METEYER ET AL. 2009; CHATURVEDI ET AL. 2010; CRYAN ET AL. 2010; LORCH ET AL. 2011; WARNECKE ET AL. 2012; ZHANG ET AL. 2014). Bats dying of WNS had no consistent significant pathologic changes in their internal organs (WIBBELT ET AL. 2013). The *Pseudogymnoascus destructans* infection (**Pd infection** hereafter) of bat wings, which represent the biggest surface of exposed skin in the body, is presumed to be a primary cause of WNS and subsequent mortality (CRYAN ET AL. 2010; FLORY ET AL. 2012; KNUDSEN ET AL. (2013). Unlike other fungal skin pathogens in endothermic animals, it invades deeply the host skin in addition to the skin superficial infections (METEYER ET AL. 2009). The hyphae of *P. destructans* are visible as a white cotton-like growth on the bat muzzle, wings and ears (e.g. BLEHERT ET AL. 2009), where they penetrate hair follicles and the associated sebacious and apocrine glands. The Pd infection ranges from cup-like intraepidermal colonies with erosions to severe ulceration of the affected skin and deep invasion by fungal hyphae into the underlying dermal connective tissue (e.g. METEYER ET AL. 2009; PIKULA ET AL. 2012; WIBBELT ET AL. 2013). According to FRICK ET AL. (2016) the damage of the muzzles is less important than deep damage of the bat wings. The last leads to severe physiological disorders most notably related to the homoestasis (electrolytic and water balance) and thermoregulation with subsequent behavioral changes during hibernation (e.g. BLEHERT ET AL. 2009; BOYLES & WILLIS 2010; CASTLE & CRYAN 2010; CRYAN ET AL. 2010; LORCH ET AL. 2011; WILLIS ET AL. 2011; FLORY ET AL. 2012; BEN-HAMO ET AL. 2013; KNUDSEN ET AL. 2013; WARNECKE ET AL. 2013; VERANT ET AL. 2014). Neither behavioural (choosing roosts with high air humidity, licking condensed water from the fur, seeking warm conditions and⁄or insect prey to offset metabolic costs of remaining euthermic, etc.) nor physiological adaptations (e.g. metabolic warming of the bodies to euthermic conditions of ca. 35 ^{o}C by arousing from hibernation) are able to compensate fully the resulting bat dehydaration to which animals are especially sensitive during hibernation, when all the vital functions are minimized (e.g. BOYLES & WILLIS 2010; DOBONY ET AL. 2011; METEYER ET AL. 2011; STORM & BOYLES 2011; FLORY ET AL. 2012; BROWNLEE-BOUBOULIS & REEDER 2013). In addition, according to the summary in the WNS News in The Underground Movement (ANONYMOUS 2014) it is possible to suggest that arousing is provoked also by: 1) skin irritation and, once awakened, bats should groom in attempt to clear the fungus from the affected skin; or 2) motivation of bats to leave the hibernaculum in an adaptive response to limit the spread

of infection (reflecting either the movement of infected bats away from healthy ones or the movement of healthy bats away from infected ones). It has to be stressed that due to more frequent disrupting of the torpor WNS-infected bats may roost closer to the cave entrance than uninfected bats and roosting in clusters may reduce evaporating loss during torpor (OP. CIT.). Nevertheless that behavioral changes are an important part of the bat response to the pathogen infection, yet they are not fully understood. One of the reasons for this lies in the difficulties of observation of free-ranging bats in nature. Therefore it is not clear whether the behavioral changes detected so far represent adaptive or maldaptive responses (OP. CIT.). Continuous infrared videography in laboratory conditions allowed WILCOX ET AL. (2014) to make observations on the behavior of infected *Myotis lucifigus* that would have been impossible in the field and to obtain some rather unexpected results: 1) infected bats did not demonstrate an increase in grooming behavior compared to uninfected controls; 2) infected bats did not visit a water source in the enclosure more often than uninfected controls; 3) activity levels in infected bats were similar to those of uninfected controls in terms of latency to onset and frequency of activity; however, infected bats were active for less time than uninfected controls; 4) reduced rates of clustering were observed in infected bats compared to uninfected controls, with fewer bats in clusters and more bats roosting alone. These results, inspite of the need to be interpreted with caution due to some unpredictable differences in laboratory and wild conditions, provide additional insight into the mechanisms of disease responses. Up to know there is accumulated clear evidence that the Pd infection is connected with considerable fitness reduction and hypotonic dehydration and it has been suggested that infected bats were more often forced to interrupt their torpor to drink and to activate their immune system, which finally depletes their fat stores and causes death because of starvation and weakness (e.g. FOLEY ET AL. 2010; REEDER ET AL. 2012; WARNECKE ET AL. 2012; CRYAN ET AL. 2013; LANGWIG ET AL.2015A). The multiple early arousals in mid winter and outdoor day flights are generally considered as a typical, but abnormal hibernation behaviour related with Pd infection and mass bat mortalities. In a few cases only, bats with Pd infection were capable to survive by arousing from hibernation (FLORY ET AL. 2012).

The lethal outcome can be enhanced or caused also by the chronic respiratory acidosis (MOORE ET AL. 2013), oxidative stress (MOORE ET AL. 2013) and some immune system malfunctions (LEIBUNDGUT-LANDMANN ET AL. 2012). Paradoxically, all the adaptations that allow bats to conserve energy and survive the adverse winter conditions (such as decreased body temperature and roosting in big groups) also provide perfect conditions for the growth of the pathogen due to its specific ecological requirements (for details see $3).

In this context, it is very important to understand the mechanisms underlying the ability of European bats to survive the infection. After the first genetic confirmation of the presence of *P. destructans* in Europe by PUECHMAILLE ET AL. (2010), based on the 2009 samples from hibernating *M. myotis* in France, during the last few years it became clear that *P. destructans* is widely distributed all over the Old continent without causing mass morbidity or mortality. Up to now the fungus has been confirmed in Austria, Belgium, the Czech Republic, Croatia, Denmark, Estonia, France, Germany, Hungary, Luxemburg, the Netherlands, Poland, Portugal, Romania, Russia, Slovakia, Switzerland, Turkey (European part), Ukraine and the United Kingdom (e.g. KUBÁTOVÁ A ET AL. 2011; GEBHARDT 2010; MARTÍNKOVÁ ET AL. 2010; WIBBELT ET AL. 2010;

PUECHMAILLE ET AL. 2010, 2011A-C; ŠIMONOVIKOVA ET AL. 2011; MESTDAGH ET AL. 2012; PIKULA ET AL. 2012; SACHANOWICZ ET AL. 2014; BÜRGER ET AL. 2013; PAIVA-CARDOSO ET AL. 2014; PAVLINIĆ ET AL. 2014; FRICK ET AL. 2016; ZUKAL ET AL. 2016) and recently was documented in Bulgaria (see details below in §6). So far, the species had not been recorded from Italy, Slovenia and Sweden (VOYRON ET AL. 2010; NILSSON 2012; MULEC ET AL. 2013).

Two different hypothesis explained the disparity of mortality between North America and Europe: 1) the European fungus may be less virulent or European bats may have evolved immunity to it (WIBBELT ET AL. 2010; PUECHMAILLE ET AL. 2011C); 2) differences in winter environmental conditions outside hibernacula in both continents (e.g. sustained subfreezing temperatures) were accepted as important co-factor for WNS virulescense and disease mortality (FLORY ET AL. 2012). Although at first it was thought that infection with *P. destructans* in Europe was restricted to superficial skin layers only (WIBBELT ET AL. 2013), the later electron microscopic studies of bat wings revealed the same cup-like erosions characteristic of WNS on both sides of the Atlantic (BANDOUCHOVA ET AL. 2015). The last data, based on studies of bats from the Czech Republic (individuals from 6 species, PCR-positive for *P. destructans*), were the first which confirm the presence of severe WNS lessions aside of North America. The authors suggested that the European bats may be only tolerant but not resistant to the fungus and that the inter-continental differences in the outcome of WNS in bats in terms of morbidity/mortality may not be due to differences in the pathogen itself. Earlier it had been already proved that the experimental infections with European isolates of *P. destructans* cause mortality in American bat species (LORCH ET AL. 2011). The new data obtained by ZUKAL ET AL. (2016) provided evidence for both endemicity and tolerance to this persistent virulent fungus in the Palearctic, suggesting that host-pathogen interaction equilibrium had been established**.** After it became clear that the differences in bat response to the fungus were not due to differences in the pathogens, it is possible to suppose with a high probablility that the differences in the bat response to the fungus are mostly due to the factors such as environmental conditions in the roost, the bat or the cave microbiome, or species specific physiological reactions.

In attempt to clarify the exact **mechanisms of WND pathogenesis**, O'DONOGHUE ET AL. (2015) conducted a thorough research on the fungus secretome and recorded 3 serine endopeptidases, 2 serine carboxipeptidases, an aspartyl endopeptidase and lipolytic enzymes such as lipases and phospholipases. The serine endopeptidases isolated were named Destructin 1, 2, and 3 respectively. Out of these Destructin 1 has the highest activity and is able to degrade β-sheets of collagen in contrast to collagenases which aim at the α-spirals. It shows homology with enzymes produced by the nematophitic fungi *Dactylellina varietas* Yan Li, K.D. Hyde & K.Q. Zhang and *Arthrobotrys conoides* Drechsler for degradation of nematode cuticle (YANG ET AL. 2007A,B), as well as with the endopeptidase isolated from *Engyodontium album* (Limber) De Hoog, better known as Proteinase K, and some peptidases from entomophilous fungi. *P. destructans* carboxipeptidases are similar to carboxipeptidases in *Saccharomyces cerevisiae* Meyen ex E. C. Hansen and *Aspergillus niger* Van Tieghem, and the aspartyl endopeptidase has a homolog in *Candida albicans* (C. P. Robin) Berkhout, where it serves for adhesion to the cells of the epithelium, degradation of host proteins, penetrating the mucose layer, and evading host immune response (NAGLICK ET AL. 2004). In many dermatophytic fungi serine, as well as

aspartyl endopeptidases, are also able to degrade keratin (SANTOS & BRAGA-SILVA 2013). Subtilisin serine protease is recently identified in *Batrachochytrium dendrobatidis* Longcore, Pessier et D. K. Nichols, where it degrades antimicrobal peptides on frog skin. Potentially applicable inhibitors of Destructin action are PMSF, antipain, and chemostatin, the last reducing collagen degradation by 77%.

2. *Pseudogymnoascus destructans* morphology, reproduction and systematic position

Morphological features of the pathogen **mycelium** are quite clear. On Saboraud Dextrose Agar (SDA) colonies are white at the margin and with central sterile white overgrowth (GARGAS ET AL. 2009). Conidial masses at colony centers are grey to grey-green and the colony reverse is uncoulored on Corn Meal Agar (CMA), and drab to hair brown on Sabouraud agar (GARGAS ET AL. 2009). Colonies on Malt Extract Agar (MEA) are initially white, but after spore production and aging they quickly darkened from the center to a dull gray, often showing a faint green hue (PUECHMAILLE ET AL. 2010). Similar characteristics are given also by MARTÍNKOVÁ ET AL. (2010) and ŠIMONOVIČOVÁ ET AL. (2011).

The most characteristic feature of the fungus is the morphology of the anamorph and in particular of the asexual reproduction spores - conidiospores. On CMA they are 5–12 × 2.0–3.5 μm, tapering basally to 1.5–2.0 μm and apically to 0.5–1.5 μm, truncate with prominent scars at one or both ends, smooth and lightly pigmented; predominantly curved, sometimes oval, obovoid, or cymbiform, moderately thick-walled at maturity and readily seceding, borne singly at the tips, on the sides, or in short chains on verticillately branched conidiophores (GARGAS ET AL. 2009). Conidia on MEA are hyaline, irregularly curved, broadly crescent-shaped (typically 6–8 μm long and 3–4 μm wide), and narrowed at each end, one of which was broadly truncate, often showing an annular frill (PUECHMAILLE ET AL. 2010). The size of conidiospores according to ŠIMONOVIČOVÁ ET AL. (2011) is 4.6–6.0 x 1.5–3.1 μm and they are formed in short chains on branched erect conidiophores. Details on the fungal conidiophores are provided by GARGAS ET AL. (2009). There is only one species, known that to be macroscopically similar to *P. destructans,* which can be confused with it when growing on bats: *Trichophyton redellii* Minnis, Lorch, D.L. Lindner et Blehert (LORCH ET AL. 2015). This species, described from Wisconsin, Indiana and Texas, seems to be native to the U.S.A. and as far as we know, does not cause any harm to the bats. The two species can be distinguished when the bats are illuminated from above with UV light (infections caused by *T. redellii* were not observed to produce an orange-yellow fluorescence when exposed to ultraviolet light as has been reported for *P. destructans* infections by TURNER ET AL. 2014, LORCH ET AL. 2015), by histological examination (only *P. destructans* penetrates deep in the derma and forms cup-like erosions, whereas with infections of *T. redellii,* the fungal colonization pattern often has an active edge with a central zone of clearing, similar to what is observed in classic human ringworm infection - LORCH ET AL. 2015) or by observations of spores under the microscope (conidiospores of *T. redellii* are radially symmetric, obovate to pyriform, attached laterally to the sides or ends of hyphae and are sessile or on very short pedicels, while in *P. destructans* conidia are distinctive asymmetrically curved, or crescent-shaped, borne at the ends of verticillately branched conidiophores - GARGAS ET AL. 2009, LORCH ET AL. 2015).

According to our best knowledge, so far the **teliomorph and sexual process of *P. destructans* remains cryptic** (for details see the text below) but PALMER ET AL. (2014) suggested that the sexual recombination may allow the pathogen to adapt to its environment and hosts, despite its slow growth.

An intriguing and at the same time controversial question is that of *P. destructans* **actual systematics position** and evolutionary origin. The fungus was first described in 2009 after being isolated from infected bats of the species *M. lucifugus* and *M. septentrionalis* (GARGAS ET AL. 2009). According to the phylogenetic tree built on the bases of small subunit (SSU) and internal transcribed spacer (ITS), the newly described fungus was placed in the ascomycetous genus *Geomyces*. There its closest relatives were *Geomyces pannorum* and *Pseudogymnoascus roseus* Raillo, according to the SSU analysis and *Pseudogymnoascus verrucosus* Rice et Currah, according to the ITS analysis, comparative sequences being searched through BLAST in GenBank.

The species epitheton *destructans* was given because of the devastating effect the fungus had on bat populations. As a consequence, the main attention was turned towards studies of the fungus in the bat hibernacula and this led to the documentation of many *Geomyces* "species". For example, JOHNSON ET AL. (2013) obtained eleven *Geomyces* isolates spread in seven clades and LORCH ET AL. (2013a) also recorded a significant diversity of *Geomyces* isolates in 24 soil samples from bat hibernacula based on sequencing of ribosomal RNA regions (ITS and PIS - partial intergenic spacer), in both studies the alignment of *Geomyces* was based on maximum-likelihood phylogenetic analysis. A special note has to be made that the samples in the last study were the same as those used for molecular analysis by LINDNER ET AL. (2011), in which many *Geomyces* isolates, including non-pathogenic to bats, were found.

The lack of a modern taxonomic evaluation and of a phylogenetic framework of the group motivated MINNIS & LINDNER (2013) to apply a larger number of molecular markers and to revise the place of *Geomyces destructans* and its relatives in the Tree of Life. By sequencing and analysing the ITS region, large subunit (LSU), rDNA, MCM7, RPB2, and TEF1 from a diverse array of *Geomyces* and allies, MINNIS & LINDNER (2013) came to the conclusion that the fungus should be placed in the genus *Pseudogymnoascus* Raillo with the members of the *Pseudogymnoascus roseus* species complex as its closest relatives. True *Geomyces* species were defined as the members of the basal lineage based on phylogenetic placement of the type species, *Geomyces auratus* Traaen. However, the obtained results should be interpreted with caution because all the species used in the analyses originated from the U.S.A., where the pathogen was just recently introduced (LEOPARDI ET AL. 2015). Therefore the demonstrated position of the WND causative agent may be biased by the lack of data from both Asia and Europe, where it originally evolved (e.g. ZUKAL ET AL. 2016) and further changes in its classification may be expected.

Sexual reproduction in *P. destructans* is not yet observed and therefore it has to be stressed that the position of the species among Ascomycota is due only to the molecular data and therefore it is positioned ***Incertae sedis* in Dothideomycetes of Ascomycota**. The traditional mycological classification approach would require to keep it among the mitosporic fungi untill the observation of the sexual process and its relevant structures. In spite of the lack of direct observation of the sexual reproduction, it has to be outlined that PALMER ET AL. (2014) discovered and molecular characterized heterothallic mating system in fungal isolates from the

Czech Republic. In the opinion of the authors, the coexistence of two mating types of *P. destructans* suggested the presence of mating populations in Europe. So far, fungal populations in North America are thought to be clonal, but the potential for sexual recombination indicates that continued vigilance is needed (OP.CIT.). Further work is needed to find and characterize the sexual cycle of *P. destructans* regarding both theoretical and practical needs.

3. *Pseudogymnoascus destructans* ecology, transporting vectors and distribution

Ecologically, *Pseudogymnoascus destructans* is considered to be a **psychrophilic species** with a growth temperature ranging from 3 to 20°C and no growth occurring at 24°C or higher (e.g. JOHNSON ET AL. 2013). The optimal growth has been pointed to be between 5 and 10°C (BLEHERT ET al. 2009), between 8 and 14°C (LANGWIG ET AL. 2012), between 10 and 14°C (VERANT ET AL. 2012), or between 12.5–15.8 ^{o}C (GARGAS ET AL. 2009; TURNER ET AL. 2011). This "cold-loving" peculiarity is quite important for the fungus because it is similar to the temperature which can be found in cavernous humid hibernacula (e.g. caves, adits, cellars, old mines) of many bat species (e.g. WEBB ET AL. 1996; FLORY ET AL. 2012; BÜRGER ET AL. 2013) and therefore to the temperature of the bats in torpor (e.g. BOYLE & WILLIS 2010; HOYT ET AL. 2015) .

In addition to the cold preferences, or perhaps in relation with it, is the typical for the species **slow growth.** According to GARGAS ET AL. (2009) colonies on CMA and SDA after 16 days reach diameter 1.0 mm at 3°C, 5 mm at 7°C, 8 mm at 14°C. No fungal growth has been observed at 24°C (GARGAS ET AL. 2009).

An alarming fact regarding *P. destructans* ecology is its **ability to survive and to grow not only on bats, but also in cave environment even in the absence of the host** (LORCH ET AL. 2013a, b), which points on the ability of the infected caves to serve as pathogen reservoirs (RAUDABAGH & MILLER 2013). The ability to survive long in the absence of the host was experimentally proved in the lab by HOYT ET AL. (2014). This **potential facultativity of *P. destructans* as a pathogen**, greatly increases the risk of further WNS distribution and prevents bats to recolonize a site once after the pathogen has arrived (OP.CIT.). The ability of fungal pathogens like *P. destructans* to persist outside their host, likely increases their impact on populations and increases the risk of extinction (FISHER ET AL. 2012; HOYT ET AL. 2014).

World-wide known is the great spectrum of enzymes in saprotrophic fungi and therefore it has to be expected that diverse enzymes should be found in *P. destructans* in case it is capable of **saprotrophic activity**. Even before the findings of LORCH ET AL. (2013A, B) and HOYT ET AL. (2014), it was shown *in vitro* that the fungus was able to produce b-glucosidase, N-acetyl-b-glucosaminidase, acid and alkaline phosphatases, esterase/esterase lipase/ lipase, leucine and vailine arylamidase, naphthol-AS-B1-phophohydrolase, various proteinases (albumin/casein/gelatin), and urease, while no enzymatic activity had been indicated for cystine arylamidase, a-chymotrypsin, alpha/beta galactosidase, trypsin, bglucoronidase, a-fucosidase, and a-mannosidase (CHATUVERDI ET AL. 2010). Some of these enzymes (urease, proteinase /aspartyl/ and superoxide dismutase) exist in other pathogenic fungi (BROCK 2008; CASADEVALL ET AL. 2003) and are considered dual virulence factors (SMYTH ET AL. 2013).

RAUDABAGH & MILLER (2013) examined six isolates from four Eastern and Midwestern states and demonstrated that the fungus is alkalitolerant, able of nitrogen utilization, and is capable of saprobically utilizing many complex carbon-containing cave substrates. They demonstrated that all six isolates were capable of growth and sporulation on dead fish, insect, and mushroom tissues. Regarding details of this study it has to be stressed that in neutral to alkaline environments, nitrate, nitrite, ammonium, and amino acids sources are all sufficient for good growth of the fungus, while uric acid is a potential nitrogen resource under alkaline conditions. Importantly, *P. destructans* demonstrated urease activity which had been proposed as a dual use virulence factor in the pathogenesis of *Cryptococcus neoformans* (San Felice) Vuill. and other pathogenic fungi by CASADEVALL ET AL. (2003) and HUNG ET AL. (2007). The results of RAUDABAGH & MILLER (2013) suggest that regardless of whether *P. destructans* is keratinophilic or keratinolytic, it is capable of generating a microenvironment in which keratinaceous substrates found in caves and cave soils (such as bird feathers and mammal hair and skin, incl. bat skin) are more susceptible to degradation and can serve as an important resource for *P. destructans*. The same authors showed that similar to keratinaceous substrates, chitinaceous substrates are important resources for the fungus. It cannot degrade chitin but rather utilizes other nutritional components found in chitinaceous substrates (e.g. proteins and lipids). SMYTH ET AL. (2013) demonstrated that *P. destructans* could penetrate dead moss cell walls. Taking this into account, RAUDABAGH & MILLER (2013) proved that the fungus could produce b-glucosidase and therefore, most probably, it could degrade cellulosic substrates. However, they stated that although cellulosic substrates could be potential substrates for *P. destructans*, they are not suitable for long-term colonization in caves or portions of caves that have frequent moisture fluctuations.

REYNOLDS & BARTON (2014) also compared the saprotrophic activity of the pathogen and other closely related species from soil and showed that all the enzymes required for saprotrophic growth (especially in peculiar cave conditions where light and substrate resources are limited) are present in *P. destructans*. Among them are the cellulases and lipases which decompose plant debris, chitinases which degrade dead insect bodies and ureases which are very useful for utilizing nitrogen from bat urine or guano. The obtained data characterize *P. destructans* as a generalist decomposer and suggest that it may evolved not from dermatophytic, but from saprotrophic cave fungi. The reduced activity of the enzymes described in comparison to the enzymes of obligate saproprophs, was taken by the authors as a sign for reduced selective pressure on the ability to use decaying matter as a primary source of food, associated with its long evolution as a pathogen. REYNOLDS & BARTON (2014) proved low chemolitic activity and explained it as an aid in survival on the nutrient-limited surface of the bat wing membrane. Doubtless, these statements could be proved by further genetic studies which could also potentially provide a molecular clock for the timing of the movement of *P. destructans* out of the soil/cave sediment environment into its host (OP. CIT.).

Through controlled experiments, it was determined that WNS is spread by direct contact with its causative fungus (LORCH et al. 2011). Most evidence show that **the main vector of the disease are the animals themselves** (for example, new WNS sites are situated along bat migration routes - REYNOLDS AND BARTON 2013), but the facultative pathogen character of the fungus suggests that the **infection via contact with**

contaminated substrate is surely possible. According to LANGWIG ET AL. (2015B), when *P. destructans* is introduced to a new site, it is found only in close proximity to bats during the first year, but the next season it is already found in half of the environmental samples even far from the animals. The second year is also characterized by an elevated risk of mortality as bats get infected right after they enter the winter roost. In attempt to estimate the risk of extinction for whole colonies, REYNOLDS ET AL. (2015) used a mathematical model of *P. destructans* distribution considering the quantity of organic carbon in the soil, the length of the hibernation season and the availability of substances that inhibit the growth of the fungus. The results showed that *P. destructans* was most abundant in substrates rich in organics, especially cellulose, although growth was possible even in a silicate sand with a very low organic content. Especially alarming is the fact that according to the model, if no inhibitors are present, the pathogen can be found in the environment a 100 years after its introduction even in the absence of bats, and if inhibition is taken into account it can survive in substrates with high organic content. However, the main factor on which bat survival depends, however, is the length of the hibernating season – according to the model, period of 120 days seems to the critical, although other authors point out 102 days (LORCH ET AL. 2011). A question remains if a minimal amount of *P. destructans* persists in the organisms of bats that survived the infection during the summer.

Another possible vector for the fungal mycelia and spores are the wing mites from the family Spinturnicidae, which are ectoparasites of hibernating bats (LU

AN ET AL. 2016). Doubtless further research will confirm or reject this hypothesis, which points that in addition to the transport of fungal propagules, mites may facilitate entry of the fungal hyphae through the epidermis of bats via injuries caused by their bites. These injuries could explain previous findings of virulent skin infections by BANDOUCHOVA ET AL. (2015) when no signs of fungus keratinolytic activity were observed in the stratum corneum of bats under ultramicroscopy. The trnmission of the fungus by parasites logically explains also the earlier observations of higher infections in bats, which overwinter in closer clusters (e.g. ZUKAL ET AL. 2014).

The **macroecological interactions between bats and the fungus** were invesyigated by FRICK ET AL. (2015). Using data from 1118 winter roosts of 16 bats species on both continents collected for the past 30 years, the authors show that bat population density in Europe is similar to that in America after the introduction of *P. destructans*, suggesting that the fungus could potentially be an important factor that shaped the biogeography of bats in Europe.

The **origin of *P. destructans*** was long debatable. Some years ago FLORY ET AL. (2012) still pointed out two possible, but controversial opinions, based on te patterns of WNS spread: that the fungus may be an exotic-invasive species that was recently introduced to the United States (WIBBELT ET AL. 2010) or that the fungus is native and only recently became pathogenic to bats (PUECHMAILLE ET AL. 2011C). Recently it is strongly believed that introduction from Europe and not evolution *in situ* led to the appearance of *P. desctructans* in America (e.g. WARNECKE ET AL. 2012; LEOPARDI ET AL. 2015). LEOPARDI ET AL. (2015) sequenced and compared 8 genomic loci of fungal isolates from both continents and showed that while European isolates are highly polymorphic (8 different haplotypes), there is almost no variation among North American isolates.

Moreover, the haplotype that is most common in Germany, France, Belgium and Luxembourg is 100% identical to the one from the U.S.A. and Canada which points at Western European populations of *P. destructans* as the source of the American isolate. KHANKET (2014) found that the population in Canada had the same genotype as those from the US and there was also evidence of minor genetic variation in three Canadian isolates. All these results agree with the photographic data on the presence of white fungal growth on bats in Europe much before WND was known, as well as with the fact that the fungus is not associated with mass bat mortality in the Old Continent (PUECHMAILLE ET AL. 2011B, C; WIBBELT ET AL. 2013). Newest investigations by ZUKAL ET AL. (2016) support this opinion and even spread the area of the Palearctic origin of *P. destructans* to the Asian territory and authors claimed the endemicity of the species.

Taking into account that bat movements across the Atlantic are rare events on geological times, it is possible to suggest with a high probability that the pathogen was firstly introduced in New York state by a human, most probably a tourist, caver or researcher that visited caves in Europe prior to visiting Howe's cave in New York (PUECHMAILLE ET AL. 2011C; LEOPARDI ET AL. 2015). All this once more emphasises the need of strict control on the transport of biological materials between continents and of high hygiene culture for cavers and tourists visiting relatively isolated ecosystems.

4. Bat immune and neuroendocrinological response to WNS/WND

The topic of immunity to fungal infections is of interest and understanding the nature and function of the immune response to fungi is an exciting challenge that might set the stage for new approaches to the treatment of fungal diseases, from immunotherapy to vaccines (ROMANI 2011). The past decade has witnessed the development of a wide range of new approaches to elucidate events that occur at the host-fungus interface (OP. CIT.). Hibernation is generally associated with a significant reduction in all metabolic processes and profoundly affected immune system regulation, but little is known on how bat immune system function and vary seasonally (e.g. REEDER & MOORRE 2013 and citations there-in). Therefore it is of particular interest to study the immune response to *P. destructans* in bats. Successful resistance against pathogen invasion involves the coordinated elevation of multiple innate and adaptive immune mechanisms but there is a paucity of information regarding bat immune responses against fungal pathogens in paricular (e.g. MOORE ET AL. 2013; REEDER & MOORE 2013; RAPIN ET AL. 2014 and citations there-in). From one side, dermatophytic fungi are known to activate the innate immune response, which slows down the growth of the pathogen and leads to some tolerance towards it (NETEA ET AL. 2008). On the other hand, most often the infection can be cleared completely after the activation of the adaptive immunity, but it is exactly the adaptive immunity that is most suppressed during hibernation in different from bats mammals (e.g. CAHIL ET AL. 1967; MANIERO 2000; BOUMA ET AL. 2012; SIECKMANN ET AL. 2014). Taking this into account, it might be not surprising that *P. destructans* can overcome host defensive mechanisms. In any case, before providing the recent achievements on the topic, we would like to stress that yet many results are contradictory, some processes respond to Pd infection/WNS to different degrees and even in different directions and therefore underlying mechanisms and their biological meaning are yet to be described (e.g. MOORE ET AL. 2013).

Quite recently FIELD ET AL. (2015) proved that WNS caused significant changes in gene expression in hibernating bats including pathways involved in inflammation, wound healing and metabolism. The comparison of the transcriptome of healthy and Pd infected *M. lucifugus* by these authors shows elevated levels of lectin receptors of C-type such as CLEC4D (MCL), CLEC4E (MINCLE), CLEC7A (dectin-1), CLEC6A (dectin-2) and of Toll-like receptor 9. They are a part of the innate immune response and are typical for the initial stages of other fungal infections (e.g. like those caused by *Candida albicans*). Up-regulated are also the levels of multiple cytokines, including interleukins IL-1β, IL-6, IL-17C, IL-20, IL-23A, IL-24, and G-CSF and chemokines, such as Ccl2 and Ccl20 and G/H synthase 2 (cyclooxygenase-2), that generate eicosanoids and other nociception mediators. However, monocytes, neutrophils, and active T-helper cells, which promote the adaptive immunity, have been not detected, which is on conformity with the results of other histological investigations. The may be due to the shortness of euthermic periods or to the specific inhibition of hemotactic signals provoked by *P. destructans*, which is the case with the amphibian infecting fungus *Batrachochytrium dendrobatidis*. Up-regulation of interleukins 1 and 6, kallikrein-6, katepsin S, and the enzymes cyclooxygenase-2 and phospoholipase A2, which participate in the acute inflammation processes, has mostly negative effects on bats as it increases wing membrane damage and interrupts torpor. Activation of genes from the lipid metabolism is also detrimental as it is associated with faster depletion of fat reserves crucial for surviving the winter. However, no significant levels of antibodies against *P. destructans* have been detected in European bats infected with the pathogen, suggesting that it is not the adaptive immune response that accounts for the differences in WNS survival rate on the two continents and on some remnant American populations, which may be developing resistance to WNS (JOHNSON ET AL. 2015).

A research by MOORE ET AL. (2013) finds elevated levels of circulatng leukocytes in WNS-affected *M. lucifugus* – an attempted defence against *P. destructans*, probably related to documented changes in thermoregulatory behaviors of diseased bats. Although this response is not enough to clear the pathogen, it raises the possibility that some bats may be better equipped to resist infection than others (PUECHMAILLE ET AL. 2011c) with the potential for directional selection and evolution of the immune defence towards the fungus. Earlier studies of Moore et al. (2011) showed that bats affected by WNS experience both relatively elevated and reduced innate immune responses depending on the microbe tested, although the cause of observed immunological changes remains unknown. Additionally, considerable trade-offs may exist between energy conservation and immunological responses. Relationships between immune activity and torpor, including associated energy expenditure, are likely critical components in the development of WNS.

Since the physiological adjustments which influence energy balance and thermoregulation before, during and after hibernation result from precise regulation of neuroendocrine activity, the euroendocrinological research of bats is of great importance in the attempts to improve understanding of mechanisms underlying mortality and test the potential of bat populations to evolve resistance or tolerance in response toWNS (WILLIS & WILCOX 2014). The last authors reviewed the effect of three key hormonal mechanisms – leptin, melatonin and glucocorticoids – in hibernating animals and proposed hypotheses regarding bats WNS-effects on these systems and their evolution. They suggested that bats with the least sensitivity to leptin (a lipostat hormone

associated with metabolism, feeding behaviour and therefore with winter energy balance in bats) could accumulate more mass (larger fat stores) in the fall prior to hibernation and therefore would have a better chance of surviving WNS and reproduce in the following spring compared to other individuals (WILLIS & WILCOX 2014). Thus, the bats characterized by the lowest leptin levels in autumn will exhibit greater survival from WNS and relatively high reproductive rates in spring and it was predicted that fall leptin sensitivity should be lower in post-WNS populations compared to populations that have not yet been affected. In relation to melatonin, as a signal influencing seasonal and diurnal biological rhythms, they predicted that bats affected byWNS may have elevated levels of melatonin during the later stages of infection as they reduce clustering and begin to synchronize arousals with the dark phase, i.e. there should be detectable differences in melatonin secretion and sensitivity between pre- and post-WNS bat populations. In relation to glucocorticoids (GCs - steroid hormones underlying the physiological stress response), WILLIS & WILCOX (2014) predicted that bats with WNS should exhibit increased GCs levels beyond those normally seen in healthy, undisturbed hibernating bats and perhaps similar to bats frequently disturbed by predators (or researchers mimicking predators). Thus at least part of the explanation for increased arousal frequency in bats with WNS reflects a heightened physiological stress response.

GCs might also influence the healing and recovery process for the small proportion of bats that survive WNS, particularly if the disease represents a chronic stressor. Contrary to the logical opinion that if infected animals survive the winter period, the rapidly activated immune system under euthermic conditions will easily fight WNS and eradicate *P. destructans* from the organism, it was shown that the sudden reversal of immune suppression in bats upon the return to euthermia is a great risk for them (METEYER ET AL. 2012). The authors proved that some of the infected individuals, which survived winter with WNS, in spring could manifest immune reconstitution inflammatory syndrome (IRIS) and this possesed a great risk for bats emerging from hibernation (METEYER ET AL. 2012). As their immune function is restored in spring and suddenly encounters the pathogen, beginning to combat the fungal infection, the rapid neutrophilic inflammatory response can, paradoxically, cause severe negative pathology (extensive necrosis and oedema) and likely mortality for some individuals (METEYER ET AL. 2012). As with IRIS in humans, the intensity and extent of tissue infection determines if this exuberant inflammatory response will cause severe tissue damage and death, or will eliminate the pathogen and lead to host recovery.

5. "Fighting" *Pseudogymnoascus destructans* and WNS

Due to the key role that bats play in the ecosystems, strategies for limiting *P. destructans* distribution or decontaminating of the already infected sites in America, are of crucial importance. After accepting of the U.S.A. National WNS Management Plan (COLEMAN ET AL. 2011) designed to organize *fighting* against the disease, various solutions to prevent the WNS epidemic have been offered. Some of them included spreading of vaccines or antibiotics in the roosts, removing infected individuals from the populations, or closing caves and changing their microclimate so that it is no longer optimal for the development of *P. destructans* (e.g. LORCH ET AL. 2012, 2015; CORNELISON ET AL. 2014A). However, these classic disease management practices seem not to

be realistic options for management of disease in the wild bat populations with their peculiar ecology and obviously can affect other cave inhabitants, and provoke other undesirrable changes of the cave ecosystems. Therefore the most promising seems to be the implementing of biological control on the pathogen growth, where a special requirement to the control means is they to have inhibitory activity at low levels. Soils and cave sediments in particular, which host numerous microbes that compete with each other, are likely to offer a ready set of fungicides that wait to be tested.

The volatile organic compounds (VOCs) produced mainly by soil bacteria all over the globe and, most importantly, acting as fungicids even without a direct contact, became a foreground in the research for finding novel treatment options and tools to combat the devastating WND (e.g. KERR ET AL. 1999; ZOU ET AL. 2007; CORNELISON ET AL. 2014A). The last authors tested the efficacy of several VOCs produced by the genera *Pseudomonas* and *Bacillus* with broad spectrum of antifungal activity proved earlier by XU ET AL. (2004) and FERNANDO ET AL. (2005). Laboratory analyses of CORNELISON ET AL. (2014A) showed successful inhibition of *P. destructans* growth by decanal, 2-ethyl-1-hexanol, nonanal, benzothiazole, benzaldehyde and N,N-dimethyloctylamine in concentrations below 1 ppm. In addition to the checking the effects of individual VOCs, the last authors investigated formulations for synergistic effects. Most effective were the synergistic actions of 2-ethyl-1-hexanol combined with benzaldehyde, decanal, N,N-dimethyloctylamine and nonanal respectively, the last pair being able to inhibit the growth of the fungus by 95% for 14 days after inoculation. The long-term eficacy of studied VOCs in low quantities, and their possibilities to be applied directly (without modifications as they have been purchased in pure liquids) in combination with the increased inhibitory effect at low temperatures (ca. 4 ^{o}C) similar to these in hibernacula, proved by the authors, classifies these bacterially derived VOCs as important potential agents of biological control. On the other hand, this study stimulates further research on similar compounds in order to enlarge the potential pool of VOCs, which are able to inhibit the growth of *P. destructans* and thus to disrupt its transmission. Moreover, the stronger effect of the synergistic blends of VOCs mixtures in comparison with pure derivates provides better opportunities for creation of powerful treatment tools and supports the idea of using soil-based fungistasis in fight against Pd infection (OP.CIT.). A problem that still remains to be solved is the spread of these compounds *in vivo*, and the same authors suggest the use of aerosol sprays similar to the commercially available air fresheners after proper scientific assessments and approvals. Neverthelees of the means of application, we should always consider to stuck as close as possible to natural biological processes in attempt to diminish to lowest rates the negative impact on the cave ecosystems that are as unique, as fragile.

The identification of inducible biological agents with contact-independent anti-*P. destruct*ans activity is a milestone in the development of viable biological control options for *in situ* application (CORNELISON ET AL. 2014B). The authors tested the widely distributed bacterium *Rhodococcus rhodococcus*, which is often used for bio-remediation or for slowing down fruit ripening (PIERCE ET AL. 2011). CORNELISON ET AL. (2014B) provided the first example of contact-independent antagonism of this devastating wildlife pathogen obtained after evaluation of various application methods of induced cells of *R. rhodochrous* strain DAP96253 for potential in situ application, including whole-cell application, non-growth fermentation cell-paste, and fixed-cell

catalyst at psychrophillic conditions (at temperatures 15°C, 7°C and 4°C). The non-growth fermentation cell-paste demonstrated persistent inhibitory activity and represented the most promising application method evaluated. It was 100% effective against *P. destructans* for more than 80 days, did not require additional growth media, and did not pose a significant threat to the natural cave ecosystems (OP. CIT.). The first application of the method was in Mark Twain Cave in Hannibal, Missouri (http://www.nature.org/ourinitiatives/regions/northamerica/unitedstates/tennessee/success-in-treating-white-nose-syndrome.xml.): in May 2015, 75 *M. lucifugus* were released back into the wild after successful *R. rhodococcus* treatment and researchers hope that very soon they will be able to apply the method in a much larger scale. Details of this apparently successful trial are yet to be published.

As well as bacteria, fungi can also compete with *P. destructans*. *Candida albicans* secretes trans,transfarnesol (TT-farnesol) – a sesquiterpene and a *quorum-sensing* molecule with antifungal properties (e.g.WEBER ET AL. 2008; SEMIGHINI ET AL. 2006; BRILHANTE ET AL. 2013). TT-farnesol is effective against other important pathogens such as bacterium *Streptococcus mutans* (JEON ET AL. 2011) and therefore it was tested in different concentrations against *P. destructans* by RAUDABAUGH & MILLER (2015). Although it does not actually kill *P. destructans*, even in concentrations that are naturally occurring in the environment, TT-farnesol effectively inhibits fungal growth to permit bat survival till the end of the hibernating season (OP. CIT.).

Another **potential solution for WNS control is given by the microorganism communities inhabiting bat skin.** HOYT ET AL. (2015) tested 133 bacterial morphotypes, all belonging to the genus *Pseudomonas*, isolated from two bat species – *Eptesicus fuscus* and *Myotis lucifugus*. Six of them successfully inhibited *P. destructans* growth in the lab. The advantages of these "treatment tools" in addition to their anti-fungal properties, which make them promising candidates to be possible probiotic protectors of bats against WNS are the ubiquitous character of *Pseudomonas* and its ability to use the mycelial networks of fungal colonies as a mean of transport can be useful for *in situ* implementation (WARMINK et al. 2011). Previous experiments with members of the same genus isolated from the environment have also been successful and proved possibilities to use natural antagonists of *P. destructans* which inhibit its growth and/or limit its effects on bats. FRITZE ET AL. (2012) tested *in vitro* the anti-fungal properties of *Pseudomonas veronii*-like PAZ1, isolated from the mycelium of the causative agent of the European black alder die-off *Phytophthora alni* Brasier et S. A. Kirk. It showed significant inhibition of *P. destructans* on 3 different media, up to complete growth arrest on potato-dextrose agar with yeast extract (mPDA). The authors supposed that the antagonistic effect are due to bacterial peculiar secondary metabolites - cyclolipodepsipeptides, which opens the future possibilities for their isolation and individual testing.

In the present review, we do not discuss the substances from and relations between bacteria and other fungi found in hibernating sites together or near to *P. destructans*, since there is still no knolwedge on the role of these organisms in fungus life and their relationships (e.g. BARLOW ET AL. 2009; AMELON & KNUDSEN 2010; CHATUVERDI ET AL. 2010; CRYAN ET AL. 2010). Studies on the microbiota of bats have focused on gut and fecal microbiota, with little attention given to the external microbiota (e.g. ZANOWIAK ET AL. 1993; MUHLDORFER 2012; PHILLIPS ET AL. 2012). KOOSER ET AL. (2015) showed for first time biogeographic differences in the

abundance and diversity of external bat microbiota. Their study included 202 (62 cave-netted, 140 surfacenetted) bat samples belonging to 13 species of bats from western US uninfected with WNS and the authors managed to identify differences in microbiota diversity among sites, and between cave bats versus surface-netted bats, regardless of sex and species. These results present novel information about the factors that shape external microbiota of bats providing new insights into potential vulnerability of different bat species to WNS. However, still most of the research is turned towards antagonistic bacteria species which can produce active substances inhibiting the fungus (AMELON & KNUDSEN 2010 – cit. acc. to FRITZE ET AL. 2012).

Other decisions for active management of WNS include using artificial roosts that can be cleaned every year in order to exclude the possibility of bats getting infected with *P. destructans* from the environment. Such an experimental hibernacula was built in Tennessee and existing military bunkers have been used as artificial hibernaculum in the northeastern U.S.A., though it is still early to evaluate their effectiveness (FRICK ET AL. 2016). Some authors also suggest supplying hibernating bat with electrolytes, given that their depletion plays an important role in progression of the disease (FRICK ET AL. 2016). Breeding bats in captivity has also been discussed, but in general considered to be too difficult. However, captive colonies could be used at least to provide animals for laboratory experiment, thus reducing researchers' impact on wild populations (FRICK ET AL. 2016).

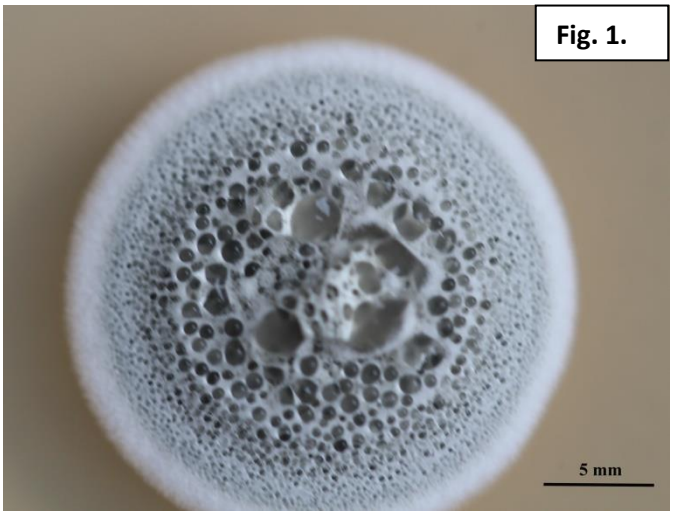
Important impications were provided by JOHNSON ET AL. (2014). They proved that host and environmental characteristics are significant predictors of WNS mortality and outlined environmental and quantitative pathogen characteristics which could be useful in further for pathogen prevention. The authors conducted a captive study of 147 little brown myotis (*Myotis lucifugus*) inoculated with 0, 500, 5 000, 50 000, or 500 000 Pd conidia and hibernated for five months at either 4 or 10 ^{o}C. The results obtained showed that female bats were significantly more likely to survive hibernation, as were bats hibernated at 4 ^{o}C, and bats with greater body condition at the start of hibernation. Although all bats inoculated in this study with *P. destructans* exhibited shorter torpor bouts compared to controls (a WNS characteristic) only the exposure to up to 500 conidia was sufficient to cause a fatal infection. However, JOHNSON ET AL. (2014) pointed the need to quantify dynamics of pathogen exposure in free-ranging bats, as dynamics of WNS produced in captive studies inoculating bats with several hundred thousand conidia may differ from those in the wild. Generally similar are the results of GRIENEISEN ET AL. (2015) from another 2-year captive study of the same species, designed to determine the impact of hibernacula temperature and sex on WNS survivorship in *Myotis lucifigus* that displayed visible fungal infection when collected from affected hibernacula. They demonstrated that colder hibernacula were more favourable for survival, that WNS mortality varied among individuals and that female bats might be more negatively affected by WNS than male bats.

According to the findings summarized above and following ZHANG ET AL. (2014) it is possible to state that all results underscore the need for integrated disease control measures that target both bats and *P. destructans* and that urgent steps are still needed for the mitigation or control of the pathogen to save bats. Currently, many projects are concentrated on different aspects of fighting the disease and positive results are expected. However, before applying whatever strategies for decontamination of already infected sites or healing

already diseased animals, it is of primary importance to eliminate the human factor in transmitting the pathogen. SHELLEY ET AL. (2013) tested various methods of disinfecting cave clothes and equipment. They discovered that washing in temperatures above 50ºC or placing in Lysol solution in concentration 1:64 are 100% effective against *P. destructans* and do not significantly decrease equipment quality. Regarding cave visits for scientific purpose, PUECHMAILLE ET AL. (2011B) pointed out that transport of samples could also be a cross-contamination source as *P. destructans* spores are able to germinate after being stored for 8 days in RNAlater or dry. On the other hand, 70% ethanol can kill the fungus after a minimum of 24 hours, while only 30 minutes are enough for absolute ethanol. That is why, when transporting pathogen samples for DNA research, the use of absolute ethanol is recommended, especially for places where *P. destructans* has not yet been observed.

6. Studies on *Pseudogymnoascus destructans* in Bulgaria and future perspectives

In Bulgaria *P. destructans* was observed on bats by B. Petrov and S. Stoycheva in 2011 and 2012 (B. Petrov, pers. comm.), but was first first discovered microscopically in 2014 by the Bulgarian authors of these review (and D. Zlatanova) in environmental samples from the caves Lednitzata in Rodopi mountains and Raichova Dupka in Central Balkan National Park (TOSHKOVA 2014, ZHELYAZKOVA 2014; TOSHKOVA & ZHELYAZKOVA 2014.) and later its identity was confirmed by DNA analysis in Greifswald, Germany by S. PUECHMAILLE (TOSHKOVA & ZHELYAZKOVA 2014). A few months later the fungus was documented by DNA analysis in the samples from the cave Ivanova Voda, also in Rodopi Mts (PUECHMAILLE, in litt.). The characteristic white fungal growth on the muzzle and the wings of the bats was observed on numerous occasions during the last winter monitoring of bat populations in the country (season 2014-2015) – ZHELYAZKOVA & TOSHKOVA, unpubl. An interesting observation from this year is the



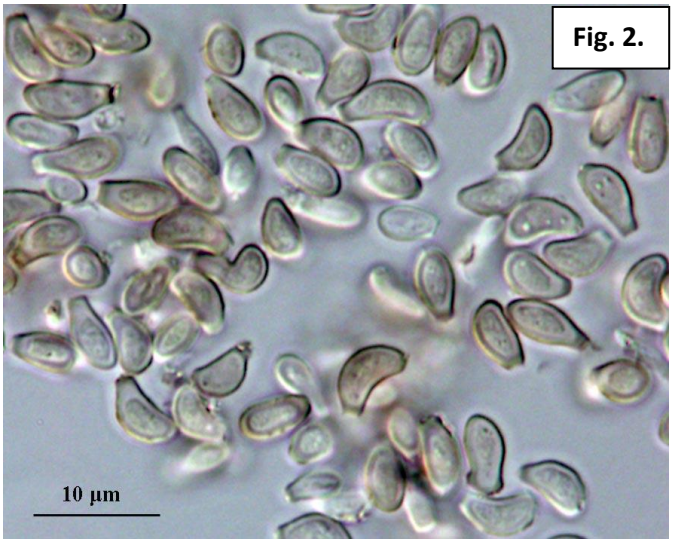


Fig. 1. Colony of *Psedogymnoascus destrutans* on malt extract agar from the cave Raichova Dupka.

Fig. 2. Conidiospores of of *Psedogymnoascus destructans* from the cave Raichova Dupka.

late hibernation of *Myotis myotis/blythii* in the cave Golyamata Balabanova Peshtera in the Western Balkan Mts – in the beginning of June some male bats were still in torpor and all of them had the characteristic for *P. destructans* white growth on the muzzle, ears, and wings. This was observed for the first time in Bulgaria, although other authors have previously reported torpid bats in May and June throughout Europe (PUECHMAILLE ET AL. 2011A).

As Bulgaria is one of the most important countries in Europe regarding bat numbers and species diversity, it is a suitable place for investigations on the biology of *P. destructans* in its native environment and its evolved interactions with its hosts that have led to the *peaceful* co-occurrence of bats and fungi. So far, multiple researches have concentrated on finding the exact processes accounting for the differences between the survival rates in European and American WND-positive bats, but still without any significant results. Pushing this matter further is of great importance for understanding the distribution mechanisms and evolution of wildlife diseases, especially when having in mind the ever increasing traffic of people and products between continents, which inevitably leads to transport of various microorganisms and increases the probability of introduction of new diseases in naïve ecosystems (CUNNINGHAM ET AL. 2003). Up to now, the WNS is devastating for North American bat populations but for the first time it made societies and governments fully aware of the indispensable role these animals play in the ecosystems. WND already greatly influenced conservation planning and population monitoring of temperate bats in North America (FOLEY ET AL. 2011) and mutual efforts of researchers all over the globe already led to some advances in various methods of limiting the mycosis distribution and even curing infected bats and it is possible that some of them soon will be applicable in a large scale. Although in Europe and Asia flying mammals are not directly threatened by WND, it is important to use the disease popularity to promote a responsible attitude towards caves and their inhabitants. At present, many cavers and speleologists do not even wash their equipment between visiting different underground sites, which is not a good strategy for their protection. In order to be effective in fighting the epidemic and to prevent similar cases in the future, a combination of high-tech lab science, regular field monitoring, and education should be used.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article. All authors contributed equally to this paper.

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