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Surveillance of *Geomyces destructans* in Swedish Bats and Bat Hibernacula

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ABSTRACT

White-Nose Syndrome, WNS, is an emerging fungal disease in the Northern American bat population causing mass mortality in infected hibernacula. *Geomyces destructans* is a newly discovered psychrophilic fungus causing WNS but the pathogenesis and epidemiology of the disease is not yet fully clarified. *Geomyces destructans* has been found in Europe but has not been a cause of mass mortality there. This study is the first investigation done to see if *G. destructans* is present in Sweden. Nine hibernacula were visited in early 2011. In the hibernacula 277 bats were visually inspected and 135 samples from the environment were analyzed by fungal cultures. In the summer of 2011 another 90 bats caught in mist nets for surveillance of European Bat Lyssavirus, EBLV, were also sampled from facial fur for mycology cultures. All culture results in this limited initial study were negative for *G. destructans* and no visible changes indicating WNS were seen. The widespread distribution of *G. destructans* in Europe and the absence of bat mortalities in Europe suggests that even if *G. destructans* was to be found in Sweden it would presently not be considered to be an immediate threat to Swedish bat populations. Annual bat inventories are an important tool for early detection of increased mortality and morbidity within a population.

SAMMANFATTNING

White-Nose Syndrome, WNS, vitnossjuka på svenska, är en ny sjukdom som snabbt sprider sig i den nordamerikanska fladdermuspopulationen. Sjukdomen orsakas av en svamporganism, *Geomyces destructans*. Den snabba spridningen och den höga mortaliteten på smittade övervintringsplatser kan leda till att fladdermuspopulationer på regional nivå kan försvinna inom en snar framtid. *Geomyces destructans* har påvisats på flera håll i Europa men ger då inte upphov till någon massdödlighet. Denna studie är den första undersökningen av förekomsten av *G. destructans* i Sverige. Genom okulär inspektion och miljöprover undersöktes förekomst av *G. destructans* på nio övervintringsplatser i mellersta och södra Sverige mellan januari och mars 2011. Totalt inspekterades 277 fladdermöss under vintern, och 135 miljöprover togs för svampodling. En undersökning genomfördes också i juli 2011 i samband med övervakningen av European Bat Lyssa virus, EBLV i den svenska fladdermuspopulationen, då ytterligare 90 fladdermöss inspekterades och provtogs för svampodling. *Geomyces destructans* kunde inte påvisas hos övervintrande fladdermöss eller i miljöproverna. Inga positiva prover hittades heller från sommarprovtagningen i denna inledande studie. Den omfattande spridningen i Europa och avsaknaden av dödlighet på kontinenten tyder på att även om *G. destructans* skulle påvisas i Sverige skulle det troligen inte vara ett direkt hot mot den svenska fladdermuspopulationen. En årlig inventering är ett viktigt redskap för att tidigt upptäcka en ökad morbiditet och mortalitet i en population.

INTRODUCTION

Background

White-Nose Syndrome, WNS, is an emerging disease in the North American bat population. The disease has since it was first discovered in the state of New York in February 2006 (Blehert *et al.*, 2009) killed more than 5.7 million bats (U.S. Fish and Wildlife Service, 2012). It has spread more than 1 200 kilometers (Frick *et al.*, 2010) over the continent and is now found in 19 states in USA (Bat Conservation International, 2011) and four Canadian provinces (Puechmaille *et al.*, 2011a). White-Nose Syndrome is associated with a characteristic growth of powdery fungus on muzzle, ears and/or wings of affected bats which also has given the disease its name (Blehert *et al.*, 2009). Fungal growth on wings can appear as an opaque white layer of varying density and distribution (Meteyer *et al.*, 2009). Bats affected by WNS generally have depleted fat reserves and they die of starvation (Blehert *et al.*, 2009; Meteyer *et al.*, 2009; Courtin *et al.*, 2010). Infected hibernacula have shown mortality rates of up to 99%, with a median of 73% (Frick *et al.*, 2010).



Fig 1. Hibernating little brown bats in New York cave.

Photo: Al Hicks, New York Department of Environmental Conservation.

Etiology

The pathogenesis behind White-Nose Syndrome is not yet fully understood. The cause of the disease is found to be a newly discovered psychrophilic fungus, *Geomyces destructans*, a very slow growing fungus with an optimal growth rate between 7 and 14°C, and with no growth at temperatures above 24°C (Gargas *et al.*, 2009). Microscopically, predominantly curved conidia are characteristic for *Geomyces*. *Geomyces spp.* have previously not been associated with disease, with a few exceptions of *Geomyces pannorum* var. *pannorum* which on rare occasions has been associated with skin lesions in humans and animals with underlying immunosuppressive disease (Gianni *et al.*, 2003; Christen-Zaech *et al.*, 2008).

Pathogenesis

Geomyces destructans invades the epidermis and underlying connective tissue of the wing membranes, pinnae and muzzles of affected bats, inducing erosions. (Meteyer *et al.*, 2009; Courtin *et al.*, 2010). Fungal hyphae are also found in hair follicles, sebaceous and apocrine glands of the epidermis. In hibernating bats the infiltration of hyphae and involvement of underlying tissue does not provoke an inflammatory response with infiltration of inflammatory cells in the connective tissue. In contrast, bats with WNS caught outside of hibernacula (winter hibernation sites) *G. destructans* infection and visibly damaged wings is associated with a suppurative inflammatory response (Meteyer *et al.*, 2009; Courtin *et al.*, 2010; Meteyer *et al.*, 2011). In some bats there is secondary bacterial infection that results in extensive suppurative inflammation and necrosis. Internal organs are all free of gross lesions and there has not been any indication of major organ failure.

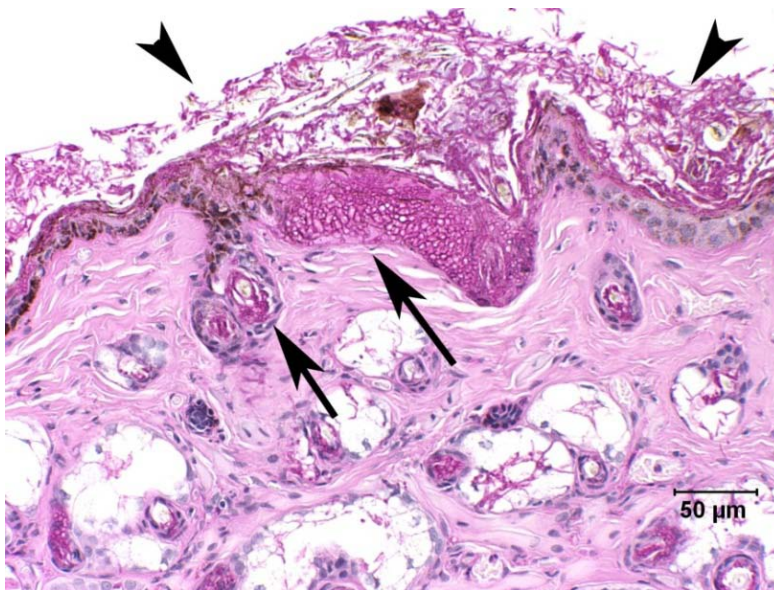


Fig 2. Muzzle of hibernating little brown bat. Arrows point to invasion through epidermis and into hair follicles beneath the skin surface. Arrowheads point to fungal hyphae colonizing the surface of the skin. Photo: C.U. Meteyer USGS NWHC

The association between the cutaneous lesions and death has not been completely understood, but it is believed that infected bats are disturbed from hibernation, consume energy and die from starvation (Blehert *et al.*, 2009). Extensive wing damage can also disrupt the homeostasis of bats leading to dehydration (Cryan *et al.*, 2010). This is supported by both Meteyer *et al.* (2011) and Fuller *et al.* (2011) who have shown that naturally infected bats given supportive treatment can improve and recover from White-Nose Syndrome.

White-Nose Syndrome in North America

Geomyces destructans has been found to infect bats of more than nine different species in Northern America (Bat Conservation International, 2011). Of these nine species six have developed White-Nose Syndrome (Puechmaill, 2011a). The biggest loss in numbers has been recorded for the little brown bat, *Myotis lucifugus*, with estimates of over a million dead individuals (Frick *et al.*, 2010). Other species that are affected includes the already endangered Indiana bat, *Myotis sodalis*, and the northern long eared bat, *Myotis septentrionalis* (Dzal *et al.*, 2011). Bats are important parts of the ecosystem and a possible local extinction of bats can have tremendous ecological and economic effects (Boyles *et al.*, 2011).

The European situation

After the first cases of WNS were reported from North America, investigations have been done to assess the situation in Europe. Surveillance studies of *Geomyces destructans* have identified the fungus in fifteen European countries since 2008, with positive bats or environmental samples in France (Puechmaill *et al.*, 2010), Hungary, Germany, Switzerland (Wibbelt *et al.*, 2010), The Czech Republic, Slovakia (Martinkova *et al.*, 2010), Austria, Belgium, Denmark, Estonia, The Netherlands, Poland, Romania, Turkey and Ukraine (Puechmaill *et al.*, 2011b). The presence of positive isolates of *G. destructans* in Europe has not been associated with mass mortality of bats, as in North America. The fungus is widespread over the European continent and photographic evidence from the 1980s and forward suggests that it has been present on the continent for at least some decades (Wibbelt *et al.*, 2010; Martinkova *et al.*, 2010). Infected bats followed over time have been able to remove the white fungal growth seen and stayed healthy and well-nourished (Wibbelt *et al.*, 2010). In Europe, only bats belonging to *Myotis* sp. have been found to be affected; *Myotis myotis*, *M. dasycneme*, *M. daubentonii*, *M. brandtii*, *M. blythii*, *M. mystacinus*, *M. natterei* and *M. beechsteinii* (Puechmaill *et al.*, 2010; Wibbelt *et al.*, 2010; Martinkova *et al.*, 2010).

Sweden

The aim of this study was to see if *G. destructans*, the cause of WNS, is present in the Swedish bat population and in Swedish bat hibernacula. This has previously not been investigated. During the winter we looked for signs of *G. destructans* growth on hibernating bats and also examined the possibility to identify presence of *G. destructans* in the environment using environmental sampling of hibernacula. In the summer, surveillance was conducted by sampling mist net caught bats to see if the fungus was present in bat fur and skin during the active season of the bats.

MATERIAL AND METHODS

Winter sampling

Nine Swedish hibernacula were visited between the 29th of January and 26th of March 2011. The hibernacula were selected out of location, density of bats, previous knowledge about the local hibernating population and importance for the Swedish bat population in general. The survey was conducted in late winter prior to the bats leaving their hibernacula in the spring, which is the time when the highest incidence of fungal growth on bats from *Geomyces destructans* is found in Europe (Puechmaille *et al.*, 2011b).

All bats present in the hibernacula were visually inspected for fungal growth on muzzle, ears and/or wings while resting. Flashlight and binoculars were used for the inspection. If no indication of fungal growth was present on visible parts of the bats, they were left untouched and undisturbed. To avoid handling and disturbing the bats, the wing membranes could not be examined. All bats were identified to species with help from bat biologists and the regular bat inventory personnel in the different locations.

Outdoor and hibernaculum inside temperatures and humidity was noted on all locations. In big hibernacula, or if the nature of the location was such that differences in temperature and humidity could be expected, several measurements were taken as sampling was carried out and an average was calculated. Four different measuring instruments were used on the different locations.

The environment

Environmental samples were taken from the walls of the hibernaculum as close to the bats as possible, within 10 cm of the bat. Because of *Myotis* sp. in North America as well as in Europe are more prone to be infected (Puechmaille *et al.*, 2011b; G. Wibbelt, personal communication), a higher frequency of sampling was done close to *Myotis* bats. In addition, samples were taken from white discoloration on the walls which could be fungal growth. Sampling from the environment was done with environmental sampling Sabouraud dextrose, SAD-agar, plates (5cm in diameter, SVA substrate department, Uppsala, Sweden). During the first five visits sampling was done both with SAD-agar with as well as without chloramphenicol antibiotics to see if there were any noticeable differences in growths between the two substrate types. Both types of plates were pressed against the wall close to each other and close to the bat. For the next four visits we only used environmental sampling SAD- agar plates with chloramphenicol for the sampling of the environment. The samples were brought chilled to the Swedish national veterinary institute, SVA, for culturing within 48 hours of sampling. On a few locations with bats situated far inside blast holes, swab samples were taken from the wall surface in front of the muzzle and transferred to SAD-agar for culturing.



Fig 3. Inventory, with ocular inspection of bats and environmental sampling for *Geomyces destructans* in Sala Silvergruva. Photo: Stina Nilsson.

Bat sampling

If fungal growth was present on a bat or suspicion of *G. destructans* had arisen, sampling with adhesive tape and direct investigation with light microscopy of tape samples would be done as well as sampling with sterile cotton swabs of affected site. Swabs from suspected fungal growth would be transported chilled to SVA for culture on Sabaroud dextrose, SAD-agar, similar to the environmental samples.

Dead bats found in visited hibernacula were brought to SVA for further examination and fungal culturing.

Culturing

Sabouraud dextrose agar plates were inoculated in 8°C until overgrowth under daily observations for up to six weeks. Identification by macroscopic appearance as well as direct microscopy of the fungus was carried out once between day 7 and 14, and a second time if needed. Microscopic identification was done by lifting colony material from the agar with adhesive tape to glass slides and stained with cotton blue. Presence of any slow growing colonies, 5 mm in diameter after 16 days in 7°C, consistent with the description of *G. destructans* published by Gargas *et al.* (2009), described as marginally white colonies with sterile overgrowth centrally and with conidial masses in the center, colored powdery gray to gray-green, were to be transferred to a new SAD agar with chloramphenicol for refinement to reduce overgrowth from more fast growing fungi. Under the microscope *Geomyces* conidia are recognized as predominantly

curved, sometimes oval, egg- or boat-shaped (Gargas *et al.*, 2009). Verification of suspected *Geomyces destructans* was to be done by PCR and DNA sequencing.

Sampling locations

Örsundet, N 59° E 18°, an old military fortification bunker blasted into the mountain.

Fredriksborg, N 59° E 18°, a brick fortress with bats hibernating in the ceiling where the mortar has vanished and left holes.

Oskar-Fredriks Borg, N 59° E 18°, an old military fortification blasted in to the mountain on different levels with tunnels.

Sala silvergruva, N 59° E 16°, reaches more than 300 meters down into the ground and is made up of over 20km of paths and rooms on several different levels and containing of various sunken shaft. Inventory was carried out on the 40, 55, 60, 75 and 85 meter levels.

Taberg, N 57° E 14°, an old mine made up of several systems and is the home of one of Sweden's largest population of hibernating bats with annual inventory counts of between 200 and 300 individuals. A portion of the system was visited in this study.

Kleva, N 57° E 15°, a mine made up of a centrally situated shaft surrounded by several smaller tunnels expanding from it in a sunlight pattern.



*Fig 4. Tykarpsgrötan, Skåne county, a closed down old underground limestone quarry. It is used by bats for hibernation including the rare *Myotis bechsteini*. In the picture two *Myotis sp.* are hanging in the roof. Photo: Stina Nilsson*

Tykarpsgrottan, N 56° E 13°, an underground limestone quarry extending over 20 000 km² about ten meters below ground level. Only a small part of the mine system was visited and investigated.

Bergensgrottan, N 56° E 13°, made out of limestone. Much smaller than its neighbor Tykarpsgrottan but share the same environmental conditions.

Champinjongrottan, N 56° E 13°, made up of two small rooms less than 100 m² in total, a few meters below ground level.

Summer sampling

During the summer for the last 4 years an active surveillance for European Bat Lyssavirus, EBLV, has been carried out in Sweden by the Swedish national veterinary institute, SVA together with the Swedish biodiversity centre, CBM, the Swedish environmental protection agency and the Swedish Institute for Communicable Disease Control, SMI (National Veterinary Institute, 2012). For this surveillance, bats from Uppland County are mist-net caught by veterinarians and bat biologists from SVA and CBM. The bats are sampled for EBLV and antibodies against EBLV in saliva and blood, respectively. In July 2011, 90 mist-net caught bats were also sampled for *Geomyces destructans*. The sampling for *Geomyces destructans* was noninvasive. With a bat in hand, the muzzle was lightly pressed against a SAD with chloramphenicol culture plate surface at three separate spots, turning the bat so both cheeks and chin were sampled. The plates were incubated in 8°C for over a month under daily observation. Macroscopic and direct microscopy for species identification was carried out once between day 7 and 14, a second reading was done if required for further investigation. Microscopic identification was done in accordance with the method for the environmental samples during the winter surveillance. Colonies with the characteristics for *Geomyces destructans* would be further investigated by PCR and DNA sequencing.

RESULTS

Winter sampling

During the winter sampling nine hibernacula were visited and a total of 277 bats were inspected for signs of fungal growth. In most cases we came close to the bats and were able to do a good visual inspection of the bats. No bat had any kind of visible change that led to sampling with sterile cotton swabs or tape sampling for direct microscopy.



Fig 5. *Myotis* sp. hibernating in Sala silvergruva. Photo: Stina Nilsson.

Bats from eight different species were recognized, among those five different species of *Myotis*; *Myotis daubentonii*, Daubenton's bat (vattenfladdermus), *M. mystacinus*, Whiskered bat, (Mustaschfladdermus), *M. brandtii*, Brandt's bat, (Brandts fladdermus) *M. nattereri*, Natterer's bat (fransfladdermus) and *M. bechsteini*, Bechstein's bat (Bechsteins fladdermus). Other species found were *Eptesicus nilssonii*, northern bat (nordisk fladdermus), *Plecotus auritus*, brown long-eared bat (långörad fladdermus) and *Barbastella barbastellus*, Barbastelle (barbastell). Bats that were located in such a way that identification was impossible due to long distance or due to hidden feet/ears or other characteristics needed for species identification were either noted as to what family they belonged if possible or as species unknown. To differentiate between whiskered bat (*Myotis mystacinus*) and Brandt's bat (*Myotis Brandtii*) the teeth have to be examined, so they were therefore noted together. For 24 bats the species could not be identified (see table 1).

Table 1. Number and species of visually inspected bats during hibernation.

	Örsunda fortet	Fredriks- borgs fästning	Oskar- Fredriks borg	Taberg	Kleva	Sala silvergruva	Tykars- grottan	Bergens- grottan	Champinjon- grottan	TOTAL:
<i>Eptesicus nilssonii</i>	20	1	-	-	5	1	-	-	-	27
<i>Plecotus auritus</i>	2	-	2	7	7	11	-	1	-	30
<i>Barbastella barbastellus</i>	1	-	-	-	-	-	-	-	-	1
<i>Myotis daubentonii</i>	12	1	-	3	20	7	4	4	2	53
<i>Myotis mystacinus/ Myotis brandtii</i>	2	5	7	3	12	45	1	2	11	88
<i>Myotis nattereri</i>	1	6	4	2	8	-	-	3	-	24
<i>Myotis bechsteini</i>	-	-	-	-	-	-	3	1	-	4
<i>Myotis</i> sp.	1	-	-	10	11	2	-	1	1	26
Unknown species	-	9	1	5	3	4	1	1	-	24
TOTAL:	39	22	14	30	66	70	9	13	14	277

In three different locations, Fredriksborgs fästning, Taberg and Sala silvergruva, a total of four dead bats were found, and all were collected for sampling. The carcasses were all too mummified or decomposed for further pathology examination but were swabbed for fungal culture, and all were negative for *Geomyces destructans*.

Environmental samples

In total, 135 environmental samples were taken including 5 swabs transferred to SAD-agar. No sample was culture positive for *Geomyces destructans*. Plates were excluded from further inoculation when overgrown. Other species than suspected *Geomyces* sp. were never identified further than to genus. Fungal growth of some kind was found on all samples. Fungi found were *Mucor* spp., *Chrysosporium* spp., *Cladosporium* spp., *Penicillium* spp. and *Fusarium* spp. In every genus there were several different species present with different macroscopic as well as microscopic characteristics. When small slow growing white colonies were transferred for refinement the following result were colonies of *Chrysosporium* spp., *Cladosporium* spp., *Penicillium* spp. or sterile hyphae. On genus level there were no distinct differences in fungal growth between the different sampling locations. All genera were to be found on all locations. The only noticeable exception was in the three limestone locations in Skåne where an apparently local fungal species was found on several plates. The genus of the fungus could not fully be identified but the characteristics were not similar to *Geomyces destructans*.

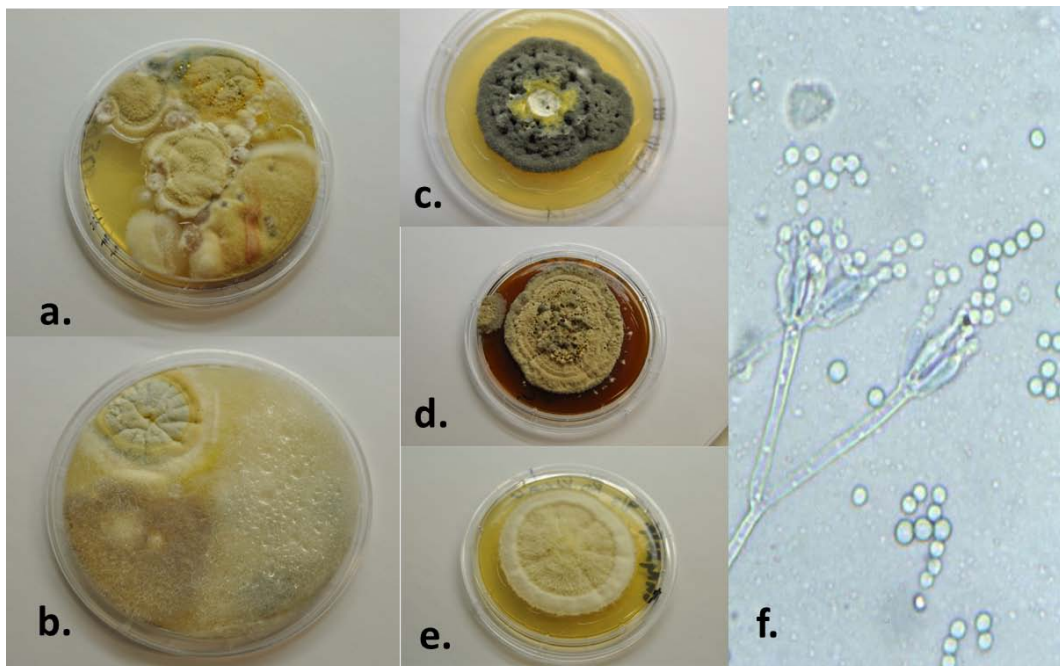


Fig 6. a-b) Typical appearance of plates after inoculation around 1 week in 8°C with various fungi growing at different speed and macroscopic characteristics. c-e) Refinement cultures from plates with slow growing colonies that were saved from overgrowth by faster growing fungi. f) Penicillium sp. as it appears stained with cotton blue during light microscopy. Photo: 6a-e Stina Nilsson; 6f Roland Mattsson.

There was no noticeable difference between the environmental SAD plates with and without chloramphenicol that were both used during the first five samplings.

Temperature and humidity was measured outside as well as on several places within the different locations and the results are presented below in table 2.

Table 2. Environmental conditions, temperature and relative humidity, at sampling locations.

	Outside Temp.(°C)	Outside RH (%)	Max Temp. (°C)	Min Temp. (°C)	Median Temp. (°C)	Max RH (%)	Min RH (%)	Median RH (%)	Number of readings at location
Örsunda fortet	2,0	-	8,0	2,7	3,9	71	53	59	9
Fredriksborgs fästning	2,0	-	4,0	1,9	2,8	53	50	51,5	4
Oskar-Fredriks borg	2,0	-	5,9	4,2	5,0	63	59	61	2
Taberg	-2,2	34	2,0	-3,6	-0,2	42	21	34	11
Kleva	-8,0	50	11,8	2,0	4,0	91	33	51,5	11
Sala silvergruva	5,5	50	11,7	4,3	5,6	83	44	78	16
Tykarps-grottan	6,5	-	11,6	9,7	10,6	74	60	66	14
Bergens-grottan	6,5	-	10,7	9,2	9,7	82	61	80	18
Champinjon-grottan	6,5	-	6,8	6,7	6,8	82	76	79	2

Other findings

Other findings seen on the bats during the visual inspection were parasites. Two different bats were found to have white or red mites around 1 mm long, in the fur around the nose (see fig. 7c). Several bats also had lesions interpreted as old healed injuries on ears and tragus. One bat lacked the entire pinnae of one ear and half of the other. There were no visible signs of active inflammatory reaction and the bat seemed to be in good condition. One bat had an active process (see fig. 7b) with half of the outer ear missing and pus covering the lesion. The affected bat was in good body condition but was very easily awakened. Another bat in Sala silvergruva appeared to be thin, with ragged fur on the hind legs and rump, where the skin was red and appeared irritated (see fig. 7a).



Fig 7 a-c) Bats found with various lesions and parasites during hibernation in late winter 2011. Photo: 7a-b Stina Nilsson; 7c Henryk Hörner.

Summer sampling

Culture plates from the 90 bats sampled by veterinarians and bat biologists from SVA and CBM in the summer of 2011 were all negative for growth of *Geomyces destructans*. There was a big variety of fungi on the plates. Identification further than to genus level was not performed on other than *Geomyces* spp. Fungi found were, from higher to lower prevalence, *Cladosporium* spp., *Scopulariopsis* spp., *Penicillium* spp., *Alternaria* spp., *Chryssosporium* spp., *Absidia* spp., *Fusarium* spp and *Mucor* spp, with *Cladosporium* spp. having the highest prevalence, present on 55 of the 90 plates. *Scopulariopsis* spp. was identified on 20 of the plates. *Absidia* spp. was sampled from two bats on two different locations. There were also sterile hyphae and some unidentifiable fungi present on some of the samples as well as yeast. Eight were negative for growth.

Four of the *Pipistrellus* sp. had furless patches on their backs without skin irritation or signs of dermal infection. The sampling from these bats where not associated with any special findings.

Table 3. Number and species of collected and sampled bats during summer surveillance in July 2011.

	Kallerö	Silvhytteå	Vatt- holma	Säva	Tensta	Östra Ekeby	Marie- lund	Funbo kyrka	TOTAL:
<i>Myotis daubentonii</i>	-	10	19	1	6	14	9	3	62
<i>Pipistrellus pygmaeus</i>	12	-	-	-	-	-	-	-	12
<i>Pipistrellus natushii</i>	11	-	-	-	-	-	-	-	11
<i>Eptesicus nilsonii</i>	-	1	1	-	-	3	-	-	5
TOTAL:	23	11	20	1	6	17	9	3	90

DISCUSSION

White-Nose Syndrome is a very important emerging disease substantially affecting the bat population in Northern America and could with time become a major threat for bats worldwide. There is still much information missing in the understanding of this complex disease and its first appearance in 2006. There are no reports of mass mortality in Sweden and with annual inventories being done on different locations over the country such events are expected to have been noted and reported. This pilot study was done to initiate surveillance for presence of *Geomyces destructans* in Sweden. The study also resulted in gathering bat expertise and spreading knowledge about WNS within this network and to the interested public in Sweden.

Further research about *G. destructans* and WNS is of great importance both nationally and internationally. As Puechmaille *et al* (2011a) states it is of great importance that we gather knowledge and cooperate until it is clarified why *G. destructans* in North America cause WNS while European bats seem to stay healthier. Are European bats resistant to the fungus through co-evolution and the American bats immunological naïve or is it a new more virulent strain of *G. destructans* that has mutated and emerged in America in 2006? The answer to this question is of great importance to what precautions and preventative measures need to be taken on national and international level to handle this disease.

The significance of this emerging infectious disease that only affects bats can be questioned, with other pathogens and diseases more easily recognized as having an effect on public health or of economic importance. But a healthy ecosystem is of great importance and bats are an important and underestimated part of it. In a recently published paper Boyles *et al.*, (2011) shows the importance of bats for agriculture and estimates the economic importance of bats to be at least 3.7 billion US dollar a year due to insectivorous bats feeding on insects that are crop and forest pests. Studies done on little brown bat, *Myotis lucifugus*, suggest that a single individual can eat as much as four to eight grams of insects a night during their active part of the year (Anthony and Kunz, 1977). Mortality rates ranging between 30 and 99% (median 73%) in infected hibernacula (Frick *et al.*, 2010) has led to an estimated 7 million dead *M. lucifugus* (U.S Fish and Wildlife Service, 2012). These numbers are extremely concerning and this, once one of Northern Americas most widespread and common bat species, is at high risk of regionally becoming extinct within 16 years if the ongoing negative trend continues (Frick *et al.*, 2010). When a new pathogen emerges there are always some individuals that have some kind of resistance to the disease and survive. Frick *et al* (2010) calculates that there has to be a resistance in the population reducing the annual decline to less than 5% to significantly reduce the risk of local extinction in 100 years.

Swedish situation

The negative results for *Geomyces destructans* found in this study are in par with the absence of reports of fungal growth and mortality in the Swedish bat population. The sample size is too small to state that we are free from *G. destructans*. There are two possible scenarios in Sweden; either *G. destructans* is not present in Sweden or it is present and it has just not been found yet.

The findings of *G. destructans* in our neighboring countries Denmark and Estonia (Puechmaille *et al.*, 2011b) could indicate that we might have *G. destructans* in Swedish hibernacula. If that is the case, the widespread European distribution of *G. destructans* with positive samples from eight different countries and no reports of mass mortality in Europe (Puechmaille *et al.*, 2011b) suggests that even if *G. destructans* would be present in the Swedish bat population it would not be an immediate threat to bats in Sweden. If you consider the number of bats and hibernacula in Sweden and the size of the hibernacula, for example Sala Silvermines over 20 km of paths and rooms, the investigated area is very small. Accessing high sample numbers is one of the difficulties with wildlife surveillance compared to surveillance in domestic animals.

In the scenario of *Geomyces destructans* not being present in Sweden, this can be due to a number of factors. There has been some discussion comparing the size of hibernacula in Europe and North America (Wibbelt *et al.*, 2010) as a factor that may influence the disease through increased disturbance of clustered bats. In North America aggregations of bats of 1 000 to 500 000 animals can be found (Frick *et al.*, 2010) with cluster of bats of up to 300 individuals in a square foot (Fears, 2012) compared to Europe where the hibernacula rarely exceed 1 000 individuals (Wibbelt *et al.*, 2010). In Sweden, the hibernaculum with the highest number of individuals was 300 bats at an inventory in February 2011 (S. Lind, personal communication). The lower bat density also influences the number of possible transmission paths and lowers both the transmission speed and the possible exposure to the pathogen.

Other environmental factors that might influence the presence of *G. destructans* are temperature and relative humidity. To find *G. destructans* the relative humidity need to exceed 80% (G. Wibbelt, personal communication). In the Swedish hibernacula a relative humidity of above 80% was measured on four of the nine locations. Maybe the Swedish hibernacula are too dry or have some other environmental condition that suppresses the growth of *G. destructans*. To further investigate this, geological data should be collected along with longer studies of temperature and relative humidity to get a trend and to investigate if there is any fluctuation with the outdoor conditions and between day and night. Especially in the smaller more open locations. In this study we could see a difference between instruments on the same location this could be an effect of the time for the instrument to tune in and show the right conditions but because of the importance

of not disturbing the bats, the need to move on and not stay at the same place for a longer period made it impossible to stay for a longer period of time.

A colder and dryer climate in combination with a lower population density within the locations is a possible reason to why Swedish bats could be safer than on the continent and why we have not seen or heard of any positive cases of fungal *G. destructans* in the Swedish bat population.

The summer sampling was very interesting, using a method not published earlier. There is a discussion whether fungal spores can be dormant in the fur during the active summer period when the bats live in temperatures where *G. destructans* does not grow. If *G. destructans* can be found in the fur of the bats in the summers this could be an important route of transmission and could be of importance for the transmission from the continent to the Swedish population. There are some bat species that migrate between Sweden and the continent. They are spending the summer months in Sweden and as winter approaches they migrate to warmer areas to extend the active and feeding period, and to hibernate later in the year. If they can carry spores in the fur there is a possibility of them picking up *G. destructans* in the winter hibernation, bringing it to Sweden as they migrate here to feed and live in the summer, infecting Swedish hibernating bats. The method of sampling bats in the summer for *G. destructans* is also an easier way to have an active surveillance for *G. destructans* in Sweden, where it could be carried out as it was done this year by sampling the bats already caught within the EBLV program. It would be very interesting to continue this sampling the upcoming years.

The fungi found in the sampling are to be considered as normal flora in soil and environment. Some of the fungi were very fast growing, e.g. *Mucor* spp. that within days had overgrown the whole plate and suppressed all other fungi. Through refinement cultures slower growing colonies were saved and any *G. destructans* present on the plates would have been identified.

There was some difference in the fungal flora found in the environment in the hibernacula and on the bats in summertime. *Mucor* spp. was far more common in the environment than on the bats in summertime where *Cladosporium* spp. was the most prominent and prevalent fungus. The finding of *Scopulariopsis* spp. and *Absidia* spp. in the fur of the bats is interesting as there are pathogenic species within these genera.

Disease emergence and disease control

The emergence of White-Nose Syndrome in North America in 2006 and the findings of widespread distribution of *G. destructans* without mortality in Europe have led to three dominating theories about this emerging disease.

The most widespread theory is the introduction of *G. destructans* from Europe to North America by a human vector to a naïve population (Puechmaille *et al*, 2010; Wibbelt *et al*, 2010; Puechmaille *et al*, 2011a). The way the disease spread on the North American continent is consistent with models of how an infectious agent introduced to a naïve population would behave. This in combination with the first reports coming from a well visited tourist cave support the theory of the fungus being introduced by a human vector. Photographic evidence of fungal growth on European bats for a few decades and the widespread distribution over the European continent also indicates an introduction from Europe to North America. European bats are thought to have co-evolved with the fungus over a long time and formed a resistance either through immunological competence or through behavioral adaptation.

Another theory is the evolution of a new strain of *G. destructans* in North America (Puechmaille *et al*, 2011a), a more aggressive and virulent strain causing White-Nose Syndrome. DNA analyses between the different continents show that isolates on the both continents are identical on two markers (Puechmaille, 2010). The identical genetic markers suggest there has been an historical exchange between the two continents but the stability of the markers indicate that an exchange can have happened anytime within the last few million years. A more extended DNA analyze needs to be done to reveal if there has been a mutation to a more virulent strain or not (Puechmaille *et al*, 2011a). If this is the case it is of the outer most importance to prevent spreading between the continents and an introduction of this new virulent strain to Europe bats.

A third theory is that *Geomyces destructans* by itself does not cause White-Nose Syndrome (Puechmaille *et al*, 2011a) but some other factor or factors are involved; virus, bacteria or toxic agent, causing immunosuppression and being the underlying cause of WNS. All studies so far have turned out negative regarding other agents being involved and the theory is also contradicted by Lorch *et al*. (2011) who through fulfillment of the criteria for Koch's postulate has determined *Geomyces destructans* as the primary pathogen of WNS.

Understanding how White-Nose Syndrome emerged and the epidemiology behind the disease is important when deciding what measures need to be taken to avoid transmission and management of the disease. If there is a new and more virulent strain that has appeared in the US, then European bats are also at risk. The European situation seems to change as well with Martinkova *et al*. (2010) reporting an increased prevalence of *G. destructans* infection and the first reported cases of WNS in European bats (Pikula *et al*., 2012). Has something changed in the situation of the bats? Human influence with habitat being exploited and animals being stressed causing immunosuppression has been reported many times throughout history with substantial effects to both humans and animals. The whole scenario, whether the disease has been transmitted from Europe or a new

strain has emerged, highlights the importance of good hygiene and biosecurity. When travelling between hibernacula, cleaning of shoes and instruments are crucial to avoid transmission of any kind of pathogens and diseases, not only the ones we are aware of but also those not yet found.

Disease spread

The spread of the fungus between different locations as well as the persistence in the population during the active period of the bats, is not fully understood but a spatial mixing of bats from different hibernacula play a probable role in disease transmission (Frick *et al.*, 2010). Lorch *et al.* (2011) report that disease can be transmitted from bat to bat. Dzal *et al.* (2011) found lowered activity in summer among affected bat species after WNS arrived in an area showing that WNS does not just affect a certain hibernacula but a whole area/ecosystem. The spread of the disease within an ecosystem is rapid, and within two years of WNS arriving in an area, all hibernacula are infected (Frick *et al.*, 2010). In all caves with identified fungal growth but without increased mortality one year, the mortality increased the following year.

Isolation of *G. destructans* from the environment has been done both from a cave wall in Europe (Puechmaille *et al.*, 2011b) and from soil samples in WNS affected hibernacula in North America (Lindner *et al.*, 2010) indicating the environment as a potential reservoir of the disease.

Further Swedish investigations

If the Swedish surveillance for *Geomyces destructans* is to be continued, the method used needs to be discussed. With the present knowledge of pan-European distribution with apparent resistance to *G. destructans*, at what level should the surveillance be carried out? The methods for detection known today are the growing on culture plates or detection of genetic material through PCR. Environmental sampling of walls as done in this study is probably not a sensitive enough method on its own, but a combination of different analytic methods could improve the sensitivity. *Geomyces destructans* has been grown from a wall where an infected bat had been sitting recently (Puechmaille *et al.*, 2011b) showing it is possible to find and grow the fungus from the environment. The sensitivity of the culturing techniques used for identification on bat wings can be rather low (54%) (Lorch *et al.*, 2010). PCR is a much more sensitive method used both on bat wings (Lorch *et al.*, 2010) and for detection in soil (Lindner *et al.*, 2010). The sensitivity of *G. destructans* detection from bat wings is 96% (Lorch *et al.*, 2010) whereas in soil sampling from infected hibernacula the fungus was found in 3 of the 19 samples in a study by Lindner *et al.*, (2010). If environmental sampling is to be done in the future, PCR would be a more sensitive method and probably the method of choice for screening studies. Difficulties with environmental sampling in Sweden, on top of the limits of culture methods, are the often vast size of the hibernacula and the relatively low bat density within the hibernacula.

Suggestions for a future surveillance for *Geomyces destructans* would be a passive surveillance in combination with reports from bat inventories. Sampling opportunities such as the ongoing surveillance for EBLV can be used as a complement to study bats in different seasons. An early detection of increased morbidity and mortality among Swedish bats could then probably be achieved. If suspected cases of WNS are to be found during this passive surveillance further investigation with culturing combined with PCR-analysis would be a good and relatively sensitive way. But PCR-analysis and culturing on its own as a method for active surveillance of the Swedish situation would not be cost effective.

The disease etiology

Geomyces destructans is established to be the primary cause of White- Nose Syndrome (Lorch *et al.*, 2011) and prior to this emerging disease in bats no *Geomyces* spp. have been associated with disease except for a few reports in immunosuppressed humans and animals (Gianni *et al.*, 2003; Christen-Zaech *et al.*, 2008). In other hibernating mammals there is a down regulation in the immune system of both the innate and the adaptive immune system when the core temperature is lowered during hibernation (Bouma *et al.*, 2010). This down regulation might be an evolutionary adaptation to save energy during an energy deprived state. At hibernation temperatures most pathogens do not thrive, but for an opportunistic psychrophilic fungus like *G. destructans* it can coincide with optimal growth temperatures around 7°C (Gargas *et al.*, 2009). Leukocyte drops of around 90% and delayed immunoglobulin production has been recorded during the temperature drop of hibernation. As the hibernating mammals gone back to their euthermic state again the immune system is normalized (Bouma *et al.*, 2010). This has not been investigated in bats but would fit well with the absence of an inflammatory response seen in hibernating bats with WNS and the severe suppurative inflammatory response in bats with visibly damaged wings due to the disease, when they are caught outside the hibernacula (Meteyer *et al.*, 2009; Courtin *et al.*, 2010; Meteyer *et al.*, 2011).

The cause of death is believed to be that the normal hibernation of infected bats is disturbed, e.g. they sleep shallower and are more easily woken up, leading to increased energy consumption and death from starvation (Blehert *et al.*, 2009). Sightings of bats with abnormal behavior, flying outside of hibernacula in the middle of hibernation period and earlier than usual in spring time as well as day active flying bats, supports this theory (Courtin *et al.*, 2010). Disrupted homeostasis can also lead to bats being awoken due to thirst and consuming energy to restore water homeostasis (Cryan *et al.*, 2010). Studies on recovery from fungal infection also indicate that the cause of death is not the infection of *Geomyces destructans* itself but an effect of the infection disrupting normal behavior or physiology (Meteyer *et al.*, 2011; Fuller *et al.*, 2011). Meteyer *et al.* (2011) showed that naturally infected bats with WNS given supportive treatment consisting of warmth, food and water did improve and did not show any clinical

signs after 70 days and were then all PCR negative for *G. destructans*. Fuller *et al.* (2011) also showed a marked improvement of bats with wing damage in the wild within a relative short time (e.g. two weeks) but didn't follow them to see if they could fully recover.

Bats are sensitive to water loss during hibernation, with water loss through the skin accounting for up to 99%. The wings make up a great proportion of the integumentary area. Loss of normal skin structure due to the fungal infection and impairment of the wing membrane function as a barrier against the surrounding climate can increase the water loss. Wings with widespread lesions are easily torn, appear to lose normal tone, tensile strength and elasticity and can in severe cases adhere to each other. There is also discussion whether there is cumulative damage to the wings over several hibernation periods or if spores can lay dormant until the next hibernation when they grow and result in fully flagrant WNS (Fuller *et al.*, 2011; Meteyer *et al.*, 2011). The difficulty seems to be the ability to survive until the end of the hibernating period without too extensive scarring of the wings to be able to fly, catch prey and avoid being killed by predators (Meteyer *et al.*, 2011).

CONCLUSION

Geomyces destructans has not been found in Sweden, and is probably not extensively spread within the Swedish bat population, although this was only a limited pilot study. No clinical signs of WNS have been seen in Sweden.

Since mass mortality has not been reported among bats in Europe, a finding of *G. destructans*, in Sweden at this time, would probably not be regarded as endangering the Swedish bat population.

The Swedish annual surveillance of European Bat Lyssavirus, EBLV, is one useful opportunity to obtain data for risk assessment of the disease situation among Swedish bats, together with annual inventories as an important tool for early detection of increased mortality and morbidity within the bat population.

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