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FUNGI ASSOCIATED WITH OVER-WINTERING TRICOLORED BATS, *PERIMYOTIS SUBFLAVUS*, IN A WHITE-NOSE SYNDROME REGION OF EASTERN CANADA

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Abstract: The tricolored bat (*Perimyotis subflavus*) is threatened by white-nose syndrome (WNS), a fungal disease caused by *Pseudogymnoascus destructans* (*Pd*) and was recently ranked as endangered under the Canadian Species-at-Risk Act. There have been few prior studies on the fungi associated with over-wintering bats. Such information is important in assessing overall fungal diversity within the cave habitat, in determining the ecological role that bats may play as dispersers of fungi, and in the identification of fungal species potentially antagonistic to *Pd*. We swabbed twenty-two *P. subflavus* over-wintering in caves and mines in New Brunswick, Canada, in 2012 and 2013. This produced 408 isolates comprising 60 taxa in 49 fungal genera with an average of 10.2 $\pm$ 3.9SD fungal taxa recorded per bat. We found fungal assemblages on *P. subflavus* (post-WNS) very similar to those we cultured previously from *Myotis spp*. (pre-WNS) at the same sites. We suggest that the variation in fungal assemblages observed from site-to-site on hibernating *P. subflavus* is largely due to environmental and ecological characteristics of individual caves, rather than the presence of *Pd* or roosting habits.

INTRODUCTION

The tricolored bat (*Perimyotis subflavus*) is considered one of the most common and widely distributed species of bats in eastern North America (Briggler and Prather, 2003). However, the species is threatened by white-nose syndrome (WNS), a disease of hibernating bats caused by the fungus *Pseudogymnoascus destructans* (*Pd*) that was first observed in 2006 in Albany, New York (Lorch et al., 2011). *Perimyotis subflavus* has suffered mortality of up to 100% in multiple caves, with an average of 76% mortality in the northeastern-state hibernacula surveyed (Turner et al., 2011). Cumulative declines for the species in overall regional abundance from its peak levels to 2011 have been estimated at 34% (Ingersoll et al., 2013).

In Canada *P. subflavus* occurs in southern Ontario, Quebec, New Brunswick, and Nova Scotia (van Zyll de Jong, 1985), the northern limit of the species range where it is considered rare to uncommon (Hitchcock, 1965; Forbes et al., 2010; Mainguy et al., 2011). The arrival of WNS has placed the Canadian population of *P. subflavus* at particular risk, and as of December 2014, the species has been ranked as endangered under the Canadian Species-at-Risk Act.

There have been few studies of fungi associated with over-wintering bats, but with the advent of WNS there has been increasing interest in bat- and cave-associated fungi (Johnson et al., 2013; Lorch et al., 2013a; Vanderwolf et al., 2013). Such information is important in assessing overall fungal diversity within the cave habitat, in determining the ecological role that bats may play as dispersers of fungi, and in the identification of fungal species potentially antagonistic to *Pd*. Our earlier study in Maritime Canada, carried out prior to *Pd* arrival, determined that assemblages of ectomycota cultured from over-wintering *Myotis lucifugus* and *M. septentrionalis* (hereafter *Myotis* spp.) were relatively diverse (>100 species) (Vanderwolf et al., 2013). *Myotis* spp. are widespread in eastern Canada with hundreds to thousands of individuals over-wintering together in hibernacula, while *Perimyotis subflavus* are relatively rare (<10 individuals/hibernaculum) and usually roost singly (Vanderwolf et al., 2012). It has been suggested that roosting alone may slow the transmission of *Pd* (Langwig et al., 2012). If roosting habits affect the diversity of fungi on hibernating bats, we hypothesize that the fungal assemblage on *P. subflavus* may differ from *Myotis* spp. within the same hibernaculum. Here we report on an investigation of the fungi associated with over-wintering *P. subflavus* carried out in 2012–2013 in a WNS-positive region of eastern Canada. Our sample interval is especially noteworthy because it straddles the period from the first detection of *Pd* on *P. subflavus* in Canada in 2011 to the apparent extirpation of *P. subflavus* from hibernacula in New Brunswick due to WNS.

METHODS

The number of *Perimyotis subflavus* over-wintering in caves and mines in New Brunswick, Canada, were recorded during regular surveys as described in Vanderwolf et al. (2012). Data on physical characteristics of study sites, including location, length, and temperatures, can be found in Vanderwolf et al. (2012). Where *P. subflavus* was present,

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numbers at hibernacula ranged from one to seven per site. With the exception of a single *P. subflavus* removed from each site for WNS confirmation by histology and sequencing at the Canadian Wildlife Health Cooperative, live bats were assessed in the field for the presence of characteristic *Pseudogymnoascus destructans* fungal growth by visual inspection of exposed skin surfaces only. However, lack of visible *Pd* growth does not equate to the absence of WNS (Verant et al., 2014). Since all *P. subflavus* observed in New Brunswick roosted singly, generally 1 to 2 meters from the hibernaculum floor, we had access to all *P. subflavus* observed. While our assessment method minimized disturbance to hibernating bats, it prevented determination of sex.

Perimyotis subflavus individuals were swabbed for fungi February–March 2012 and March–April 2013 using methods identical to those reported in Vanderwolf et al. (2013). All *P. subflavus* encountered during these two hibernation periods were sampled (n=22). None of the fifteen *P. subflavus* sampled in 2012 showed visible signs of *Pd* growth, while four of the six *P. subflavus* sampled in 2013 had *Pd* growth based on visual inspection. Swabs were taken with a sterile, dry, cotton-tipped applicator from the dorsal fur or skin of live bats; the term skin meaning one or more of the face, ears, patagium, or uropatagium, with sampling dependent on which skin surfaces were accessible. Bats were swabbed while they were roosting and were not removed from cave walls. Swabs were cultured on either dextrose-peptone-yeast extract (DPYA) agar (Papavizas and Davey, 1959) or Sabouraud-Dextrose (SAB) agar, both of which contained the antibiotics chlortetracycline and streptomycin. Four swabs were taken using all combinations of fur or skin on either SAB or DPYA from each *P. subflavus*, except where swabbing was terminated with one and three swabs for two bats that awoke during swabbing. A new applicator was used for each swab. After swabbing, the applicator was immediately streaked across an agar surface in a petri plate. Dilution streaks were completed in the hibernaculum within 3 h of the initial streak, after which plates were sealed in situ with parafilm (Pechiney Plastic Packaging, Chicago, IL).

Samples were incubated, inverted, in the dark at 7 °C in a low temperature incubator to approximate the hibernaculum environment and target fungi adapted to cave microclimates. The average winter temperature in the dark zone of New Brunswick hibernacula is 5.1 $\pm$ 1.1 °C, with winter defined as 1 November–30 April (Vanderwolf et al., 2012). Samples were monitored over four months until no new cultures had appeared for three weeks on a plate or the plate had become overgrown with hyphae. Once fungi began growing on the plates, each distinct colony was subcultured to a new plate. DPYA without oxgall and sodium propionate was used for maintaining pure cultures.

Identifications were carried out by comparing the micro- and macromorphological characteristics of the microfungi to those traits appearing in the taxonomic literature and compendia (Domsch et al., 2007; Seifert et al., 2011). We also had access to reference collections of cultures from *Myotis* spp. identified previously using a mix of sequencing and morphological features (Vanderwolf et al., 2013). Permanent cultures of fungi reported here are vouchered in the University of Alberta Microfungus Collection and Herbarium (UAMH 11335, 11725, 11730, 11731), and desiccant-dried samples are housed in the New Brunswick Museum (NBM# F–04824–04839, 04841–04843, 04871–04882, 04916–04941, 04949–04951, 04961). After testing for normality, a 2-sample t-test was used to compare the number of fungal taxa on individual *P. subflavus* that did and did not culture positive for *Pd*. Since the data were not normally distributed, a Mann-Whitney test was used to compare the number of fungal isolates recovered from fur versus skin and DPYA versus SAB. Minitab statistical software was used for all tests.

We followed the protocol of the United States Fish and Wildlife Service for minimizing the spread of WNS during all visits to caves (revised decontamination protocol: June 25, 2012. Available online http://www.whitenosesyndrome.org/resource/revised-decontamination-protocol-june-25-2012). Necessary permits were obtained from the New Brunswick Department of Natural Resources.

RESULTS

WNS was first observed in New Brunswick in Berryton Cave in March 2011, at which time a single dead *Perimyotis subflavus* was collected among thousands of dead *Myotis* spp. This bat was subsequently confirmed *Pseudogymnoascus destructans* and WNS-positive, the first such detection for *P. subflavus* in Canada (S. McBurney, Canadian Wildlife Health Cooperative, pers. comm.). Thereafter, we did not encounter *P. subflavus* with visible *Pd* growth until December 2012, although *P. subflavus* were observed roosting near *Myotis* spp. with visible *Pd* growth during this interval. *P. subflavus* has not been observed at any of our study sites since December 2013.

Pd was cultured from three *P. subflavus* in February–March 2012, though visible fungal growth was not seen on *P. subflavus* until 2013. *Pd* was cultured from 100% of *P. subflavus* with visible *Pd* growth (n=4) and from 27.8% of *P. subflavus* without visible *Pd* growth (5 of 18 bats). However, failure to culture *Pd* from a bat does not demonstrate that *Pd* was absent. Sixteen of the eighteen *P. subflavus* sampled without visible *Pd* growth, including the five that cultured *Pd*-positive, were located in hibernacula with *Myotis* spp. that had visible *Pd* growth. *Pd* was isolated at similar frequencies from fur (n=10) and skin (n=13) swabs and on DPYA (n=13) and SAB (n=10) media.

Fungi were successfully cultured from all bats and from 73 of 79 swabs (92.4%), producing 408 isolates. An average of 10.2 $\pm$ 3.9SD fungal taxa were recorded per bat (n=22, range 2–22; Table 1). *Perimyotis subflavus* that cultured

positive for *Pd* had 12.1 ± 4.3SD fungal taxa per bat (n=9 bats) compared with 8.9 ± 3.2SD for *P. subflavus* that cultured negative (n=13 bats). There was no significant difference in the number of fungal taxa cultured between *Pd*-negative and *Pd*-positive bats ($T_{1,13}$= –1.89, P=0.082).

During this study sixty taxa in forty-nine fungal genera were isolated, plus nine sterile fungal morphs (Table 1). Twenty six (43.3%) fungal taxa were found on only a single *P. subflavus*. The most common taxa isolated were *Leuconeurospora* spp. (detected on 86.4% of the twenty-two bats sampled), *Cephalotrichum stemonitis* (68.2%), *Humicola* cf. UAMH 11595 (63.6%), *Pseudogymnoascus pannorum sensu lato* (63.6%), *Penicillium* spp. (54.5%), *Wardomyces* spp. (54.5%), *Trichosporon* spp. (50.0%), and *Pseudogymnoascus destructans* (40.9%). The number of isolates recovered for each fungal taxon was not significantly different between fur and skin swabs ($W_{1,60}$=3999, P=0.194) or DPYA and SAB media ($W_{1,60}$=3878.5, P=0.503), although fur and DPYA tended to yield greater fungal diversity (182 isolates on fur, 157 on skin; 176 on DPYA, and 163 on SAB).

Discussion

The diversity of fungi isolated from *P. subflavus* is similar to that isolated from *Myotis* spp. during previous investigations at the same sites (Vanderwolf et al., 2013). In 2010 (pre-WNS), fifty-two fungal taxa in thirty-eight genera were isolated from *Myotis* spp. (n=20) in Markhamville Mine and Glebe Mine, the principal sites that we found *P. subflavus* selected for over-wintering. In comparison, fifty-five fungal taxa in forty-two genera were isolated from *P. subflavus* (n=19) at these two sites post-WNS. Six of the eight most common fungal taxa isolated from *P. subflavus* post-WNS, as well as many of the rarer fungi, were identical to those cultured from *Myotis* spp. pre-WNS at these two sites. The number of fungal taxa isolated per bat was also similar. In 2010, an average of 8.3 ± 3.7 and 8.5 ± 1.7 fungal taxa per *Myotis* spp. were isolated from Glebe Mine (n=10 bats) and Markhamville Mine (n=10 bats) respectively (Vanderwolf et al., 2013). This compares to an average of 10.2 ± 2.2 and 11.2 ± 4.7 fungal taxa per *P. subflavus* isolated from Glebe Mine (n=9 bats) and Markhamville Mine (n=10 bats) respectively when combining 2012 and 2013 data. The average number of fungal taxa per bat is not significantly different between *Myotis* spp. and *P. subflavus* either in Glebe Mine ($T_{1,14}$=1.38, P=0.188) or Markhamville Mine ($T_{1,11}$=1.72, P=0.114).

Perimyotis subflavus characteristically roost alone during hibernation, although clusters of two to four have been reported (Briggler and Prather, 2003; Vincent and Whitaker, 2007). It has been suggested that this roosting habit may slow the transmission of WNS and may help explain why *P. subflavus* has lower mortality rates from WNS than *M. lucifugus*, which often roost together (Turner et al., 2011; Ingersoll et al., 2013). In New Brunswick, *P. subflavus* did show a delay in the development of visible *Pd* growth and ensuing mortality relative to *Myotis* spp. (Vanderwolf et al., unpublished). However, once *Pd* becomes widespread on cave walls and in cave sediment (Lorch et al., 2013b;), substrate to bat transmission may negate any protection that roosting alone provides.

Although the transmission of *Pd* may be density-dependent, at least initially (Langwig et al., 2012), this does not appear to be the case with other fungi found on bats. We found over-wintering *P. subflavus* harbor a similar diversity of fungi compared to colonial *Myotis* spp. Aside from *Pd*, *Trichophyton redellii* is the only fungus identified to date that grows on cave-hibernating bats (Lorch et al., 2015). However, McAlpine et al. (2015) have recently reported of unidentified ascomycetes growing on big brown bats (*Eptesicus fuscus*) over-wintering in buildings in New Brunswick. The genus *Trichophyton* appears to be rare on bats in our study area, as we only isolated one *Trichophyton* sp. culture from among eighty hibernating *Myotis* spp. sampled in 2010 (Vanderwolf et al., 2013), and we detected no *Trichophyton* sp. isolates in the current study. Lorch et al. (2015) cultured samples on SAB at 7 °C, so it is likely we would have detected *Trichophyton redellii* if present. It is unknown if the transmission of *Trichophyton redellii* is density-dependent.

As with hibernating *Myotis* spp. (Vanderwolf et al., 2013), over-wintering *P. subflavus* seem to acquire spores from their environment, both prior to and during hibernation. It is likely that bats play a role in the dispersal of fungal spores, although there is virtually no data on this aspect of fungal ecology. Sources of spores within Glebe Mine and Markhamville Mine, the main over-wintering sites for *P. subflavus* in New Brunswick, include old mine timbers and mammal dung, both of which exhibit visible growth of Basidiomycota and Ascomycota. It is likely that fungi such as *Cephalotrichum stemonitis* and *Leuconeurospora polypaeciloides* have some association with mammal dung (unpubl. data). These species are particularly abundant on bats at sites where mammal dung is present (Vanderwolf et al., 2013).

The mean number of fungal taxa per bat in Markhamville Mine was noticeably higher in 2013 (15.3 ± 5.9, n=3) compared to 2012 (9.4 ± 3.0, n=7). The trend is not statistically significant; if it is real, the cause is unclear. Some fungal species in oligotrophic caves will proliferate opportunistically when presented with a food source (Cubbon, 1976). No bat carcasses were observed in the mine 2012–2013, but raccoon (*Procyon lotor*) activity in the mine, as evidenced by the presence of dung, seemed higher in 2013 compared to 2012. Based on visual sightings, the occasional carcass, and dung, raccoons often shelter in Markhamville Mine during the winter, and their droppings support luxuriant fungal growth. This may increase spore density in the mine, potentially leading to an increase in

Table 1. The number of *Perimyotis subflavus* sampled for fungi, the total and mean number of fungal taxa isolated, and the number of individual *P. subflavus* from which specific fungal taxa were cultured in each hibernaculum in each year. Mark= Markhamville Mine.

Fungal Taxa	Mark 2012	Mark 2013	Glebe 2012	Glebe 2013	Harbell 2012	White 2010	Dalling 2013	Total # of Bats
Number of bats sampled	7	3	7	2	1	1	1	22
Number of fungal taxa isolated	34	23	32	12	2	8	13	n/a
Mean number of fungal taxa/ bat ±SD	9.4 ± 3.0	15.3 ± 5.9	10.9 ± 2.0	8.0 ± 1.4	n/a	n/a	n/a	n/a
Ascomycota								
Acremonium sp.	0	0	1	0	1	0	0	2
Arthroderma sp.	0	1	0	0	0	0	0	1
Arthroderma silverae Currah, S.P. Abbott & Sigler	2	0	0	0	0	0	0	2
Arthrographis sp.	1	0	1	0	0	0	0	2
Auxarthron cf. californiense G.F. Orr & Kuehn	1	0	0	0	0	0	0	1
Cephalotrichum stemonitis (Pers.) Link	5	3	6	1	0	0	0	15
Chrysosporium spp.	5	1	0	0	0	0	0	6
Cladosporium spp.	2	0	1	0	0	1	0	4
Clonostachys sp.	0	0	1	0	0	0	0	1
cf. *Cryomyces* sp.	0	1	0	0	0	0	0	1
Diplococcium sp.	1	0	0	0	0	0	0	1
Eremomyces sp.	0	0	1	0	0	0	0	1
Fusarium sp.	0	0	0	0	0	0	1	1
Hormographiella sp.	0	0	0	0	0	0	1	1
Humicola sp.	0	2	0	0	0	0	0	2
Humicola cf. UAMH 11595	3	3	6	1	0	1	0	14
cf. Hyphozyma sp.	0	0	0	0	0	0	1	1
Isaria farinosa (Holmsk.) Fr.	1	0	1	0	0	0	0	2
Leuconeurospora polypaeciloides Malloch, Sigler & Hambleton	3	3	6	2	0	1	1	16
L. capsici (J.F.H. Beyma) Malloch, Sigler & Hambleton	2	0	0	0	0	0	1	3
Mammaria sp.	1	0	0	0	0	0	0	1
Microascus caviariformis Malloch & Hubart	0	0	0	0	0	0	1	1
Myceliophthora sp.	1	2	0	0	0	0	0	3
Myxotrichum sp.	0	0	0	0	0	1	0	1
Oidiodendron truncatum G.L. Barron	0	0	1	0	0	0	0	1
Paecilomyces sp.	1	0	0	0	0	1	0	2
Penicillium spp.	5	3	1	0	0	0	1	10
P. expansum Link	1	0	0	0	0	0	0	1
P. solitum Westling	1	0	0	0	0	0	0	1
Phaeotrichum sp.	0	0	1	0	0	0	0	1
P. hystricinum Cain & M.E. Barr	0	0	5	0	0	0	0	5
Phoma sp.	0	0	1	0	0	0	0	1

Table 1. Continued.

Fungal Taxa	Mark 2012	Mark 2013	Glebe 2012	Glebe 2013	Harbell 2012	White 2010	Dalling 2013	Total # of Bats
Preussia sp.	0	0	4	1	0	0	0	5
P. funiculata (Preuss) Fuckel	0	0	1	0	0	0	0	1
Pseudogymnoascus destructans (Blehert & Gargas) Minnis & D.L. Lindner	1	3	2	2	0	0	1	9
P. pannorum sensu lato (Link) Minnis & D.L. Lindner	6	3	2	1	0	1	1	14
P. roseus Raillo	0	0	0	0	0	0	1	1
Scopulariopsis cf. candida Vuill.	0	1	0	0	0	0	0	1
Scytalidium sp.	0	0	1	0	0	0	0	1
Thelebolus crustaceus (Fuckel) Kimbr.	1	3	0	0	0	0	0	4
Tolypocladium inflatum W. Gams	1	0	0	0	0	0	0	1
Trichoderma sp.	0	2	0	0	0	1	0	3
Trichosporiella sp.	1	3	3	0	0	0	1	8
Thysanophora penicillioides (Roum.) W.B. Kendr.	0	0	0	1	0	0	0	1
Wardomyces sp.	1	1	0	0	0	0	0	2
W. humicola Hennebert & G.L. Barron	1	0	1	0	0	0	0	2
W. inflatus (Marchal) Hennebert	0	0	6	2	0	0	0	8
Zopfiella pleuropora Malloch & Cain	0	0	2	0	0	0	0	2
unidentified ascomycete	1	0	0	0	0	0	0	1
Basidiomycota								
Asterotremella sp.	1	1	1	1	0	0	0	4
Baeospora sp.	0	0	5	0	0	0	0	5
Cystofilobasidium sp.	1	2	1	0	0	0	0	4
Hypholoma sp.	0	0	3	0	0	0	0	3
Trichosporon sp.	3	3	0	1	0	0	0	7
T. dulcitum (Berkhout) Weijman	2	1	1	0	0	0	0	4
unidentified Basidiomycete	1	1	6	1	0	0	0	9
Zygomycota								
Thamnidium elegans Link	0	0	1	0	0	0	0	1
Mortierella sp.	3	0	0	2	0	0	1	6
Mucor sp.	1	3	1	0	0	0	0	5
unidentified yeast	1	0	0	0	0	0	0	1
Sterile	2	1	3	0	1	1	1	9

Note: n/a = not applicable.

fungal assemblage diversity on hibernating bats. In contrast, both *Myotis* spp. and *P. subflavus* in Harbell's Cave yield low diversity of fungi in general, possibly because the passage floor encloses a fast flowing stream that prevents the accumulation of exposed sediment or soil in the cave that might harbor fungi (Vanderwolf et al., 2013). It appears that fungal assemblages on over-wintering bats can vary in response to local factors, both within sites from year-to-year and between sites. These factors include cave morphology, differences in cave fauna, and probably other factors as yet unknown.

The only previous study of fungi associated with *P. subflavus* was conducted by Johnson et al. (2013) in Illinois in April–May 2010 and Indiana in June 2011. Johnson et al. (2013) reported twenty-three fungal genera from *P. subflavus*, with 4.83 ± 2.04SD (n=6 bats) and 7.25 ± 4.57SD (n=4 bats) fungal genera per bat in two WNS-negative Illinois caves, and 2.2 ± 1.64SD fungal genera per bat (n=5 bats) in a single WNS-positive Indiana cave. Although we found a similarly low number of fungal taxa per bat in Harbell's Cave, our low sample size (n=1 bat), and the negative *Pd* status for both the cave and the bat make comparison with the Indiana site investigated by Johnson et al. (2013) inadvisable. However, the fungal taxa isolated from *P. subflavus* by Johnson et al. (2013) include widespread genera, such as *Cladosporium*, *Penicillium*, *Mortierella*, *Mucor*, *Trichosporon*, and *Pseudogymnoascus pannorum* sensu lato, which we also isolated from *Myotis* spp. and *P. subflavus* in New Brunswick (Vanderwolf et al., 2013).

Johnson et al. (2013) attributed the low number of fungal genera on WNS-positive *P. subflavus* in Indiana to the presence of *Pd*. In contrast, we cultured a diverse assemblage of fungi from *Pd*-positive *P. subflavus*. We suggest that since bats in Indiana were captured during flight outside the hibernation period using a harp trap and were subsequently bagged and handled, that this may have influenced the fungal diversity encountered on *P. subflavus* in Indiana, rather than any interactions with *Pd*. It has been our observation that bats will often groom upon waking and prior to flight, which may also remove some fungal spores. Probably more importantly, and as we show above, environmental and ecological characteristics of individual caves may influence the fungal assemblages that can be cultured from hibernating bats at specific hibernacula.

The diversity of cold-tolerant fungi cultured from bats in this study is similar to that found in sediments from other caves in North America (Lorch et al., 2013a; Zhang et al., 2014), further emphasizing that the fungal assemblage on hibernating bats reflects the assemblage found in the surrounding environment. Ascomycota dominate, particularly *Penicillium* spp. and *Pseudogymnoascus pannorum* s.l., and these fungi appear to be adapted to cave conditions. A subgroup of dominant cosmopolitan fungal genera are usually found in studies of cave fungi, accompanied by a diversity of rare fungi (Vanderwolf et al., 2013; Zhang et al., 2014). The high proportions of fungal taxa found singly suggest that the actual diversity of fungi in caves is much higher than detected (present study; Vanderwolf et al., 2013; Zhang et al., 2014). Undoubtedly, additional diversity would be discovered with the use of a greater variety of media.

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