ENTOMOPATHOGENIC FUNGI OF THE WINTER TICK IN MOOSE WALLOWS: A POSSIBLE BIO-CONTROL FOR ADULT MOOSE?

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ABSTRACT: Soil fungi were cultured from 24 wallows and proximal control sites in Maine and New Hampshire, USA during the autumn moose (*Alces alces*) breeding season of 2016 to investigate the presence of soil fungi pathogenic to winter tick larvae (*Dermacentor albipictus)*. Twenty genera of fungi were isolated, and all are considered common in a forested ecosystem. The predominant genera isolated in wallows were pathogenic to winter tick larvae and included *Aspergillus* spp. (in particular *A. flavus*), *Beauveria bassiana*, *Mortierella* spp., *Mucor* spp., *Paecilomyces* spp., *Penicillium* spp., and *Trichoderma* spp. Wallow soils had specific characteristics and differed from proximal control sites by having: 1) lower fungal diversity, 2) a higher frequency of primary colonizers including *Mortierella* spp., *Mucor* spp., *Penicillium* spp., and *Trichoderma* spp., and 3) a more variable total amount of fungi indicative of changing (disturbed) soil conditions. We conclude that wallows are sites of soil disturbance that concentrate fungi known to be pathogenic to larval winter ticks. Fungi acquired by breeding moose using wallows might subsequently act as an on-host mechanism of tick control.

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INTRODUCTION

The breeding season and wallowing behavior of moose (*Alces alces*) coincide directly with the questing season of larval winter ticks (*Dermacentor albipictus*) as they seek a moose host (Samuel 2004). As a one-host species, the winter tick is unique in that it remains and feeds on the same moose for its 3 parasitic life stages: larva, nymph, and adult. Excessive blood loss and other complicating factors associated with heavy infestations of > 30,000 ticks cause periodic, high mortality of calf moose in early spring when adult winter ticks take a blood meal. For example, average annual mortality of radio-collared calf moose with high tick abundance was >70% in New Hampshire and Maine in 3 consecutive winters in 2014-2016 (Jones et al. 2017).

Wallowing is believed to reduce external parasites in several programmed grooming animals including bison (*Bison bison*), deer (*Odocoileus virginianus*), and pigs (*Sus* spp.) (Espmark and Langvatn 1979, McMillan et al. 2000, Bracke 2011). The mechanism would largely seem mechanical via rubbing, abrading, and damaging parasites while an animal rolls vigorously in the soil. In addition to possibly reducing tick infestations, wallowing behavior of moose may have the added benefit of exposing larvae to pathogenic soil fungi.

Soil contains an abundance of spore-producing ascomycetes that function as agents of decay and include a group of fungi that are the greatest cause of tick mortality in nature (Fernandes et al. 2012, Cafarchia et al. 2015). Infection of ticks occurs by spores, which upon contact, germinate and produce hyphae that penetrate the tick via mouth, anus, glandular openings, and soft membranes between leg segments, killing it in the process. *Beauveria* and *Metarhizium* spp. are the most noteworthy entomopathogenic fungi used in biological control of ticks (Samish et al. 2004, Fernandes et al. 2012). Indeed, *Beauveria bassiana*, *B. caledonica*, and *Metarhizium anisopliae* (now *M. robertsii*) are known pathogens of winter tick larvae (Yoder et al. 2017b). Depending upon their relative concentration, these fungi may possibly serve as natural regulators of winter tick abundance on moose. The purpose of this study was to identify whether entomopathogenic fungi are present in wallow soil, and to test the hypothesis that fungi isolated from wallow soils may be infective to winter tick larvae.

MATERIALS AND METHODS

All laboratory materials and instruments were obtained sterile from the manufacturer (Fisher Scientific, Pittsburgh, Pennsylvania, USA) or were sterilized by autoclave (121 °C, 19 psi, 15 min), Bunsen burner flame, or 95% ethanol; all methods followed standard aseptic techniques. Powder-free gloves (Microlex Co., Reno, Nevada, USA) were worn in the field and laboratory. All work in the laboratory was performed in a vertical laminar flow hood (Cole-Palmer, Vernon Hills, Illinois, USA) sterilized daily. Methods for isolating and identifying the fungi, as well as testing pathogenicity to larval ticks, conformed with standard practice (Tuininga et al. 2009, Suleiman et al. 2013, Cafarchia et al. 2015, Yoder et al. 2017a, b).

Soil collection

Soil samples were collected from wallows and proximate control sites located in typical moose habitat (forest description in Jones et al. 2017) in northern New Hampshire and central Maine, USA from 2 October – 1 November 2016. The wallows (n = 24) were located in 3 distinct geographic locations: 1) Mount Katahdin, Maine (Katahdin, 2-8 October, n = 4); Greenville, Maine (Greenville, 5 October – 1 November, n = 13); and Milan, New Hampshire (Milan, 7-17 October, n = 7). The Universal Transverse Mercator (UTM) coordinates of the wallow locations are held by P. J. Pekins (University of New Hampshire, Durham, New Hampshire) and S. McLellan (Maine Department of Inland Fisheries & Wildlife, Greenville, Maine). Samples were collected during the moose breeding period (generally the month of October) coinciding with when wallows were active and winter tick larvae were questing. Samples were scooped from surface soil (~8-10 cm depth) with 50 mL polypropylene centrifuge tubes and stored in Whirl-Pak bags (Nasco, Salida, California, USA). The samples were held at approximately 15 °C in a 5 L cooler containing cold packs (Koolit; FDC Packaging, Medfield, Massachusetts, USA) for transport to the laboratory where they were stored at 4 °C (frost-free refrigerator; Fisher) and processed within 24-36 h.

Eight soil samples were taken (filling 50 mL tubes) from each wallow site: 4 randomly collected from within the wallow (designated "wallow"), and 4 control samples collected in opposite directions 1–2 m from the edge of the wallow (designated "proximate"). These proximal samples were assumed close enough to the wallow to be representative of the same site location.

An estimate of soil pH was measured by placing 20 g of soil into 40 mL of distilled, deionized (DI) water. The soil samples (5 g from each of the 4 tubes per site) were

weighed to 0.1 g with a Light Balance (Mettler Toledo, Columbus, Ohio, USA). The soil-water mixture was stirred periodically for 30 min (Kalra 1995), and pH was measured (Oakton pH meter, Vernon Hills, Illinois, USA) at 1, 2, and 3 h; data were combined (mean ± SE). The pH of the fungal agar growth media was adjusted to approximate that of the wallow soil sample.

Fungus isolation and identification

A 1 g sample from each soil sample (equalized to dry weight) was weighed to 0.01 g using a microbalance (Ventron Co., Cerritos, California, USA) according to standard practice (Kumpula et al. 2000, Brown 2007, Shubina et al. 2013). This sample was placed into a 9.0 mL DI water blank mixed with a vortex (Scientific Industries, Bohemia, New York, USA), and used as the stock solution for serial dilution with DI water (Brown 2007). An optimum dilution was used for all subsequent platings based on the standard counting plate method of 30 – 300 colonies per plate (Brown 2007). Aliquots of 0.1 and 1.0 mL were each plated using calibrated glass micropipettes. The DI water was used as a control to gauge the extent of contamination during the experimental manipulations. Potato dextrose agar (PDA + lactic acid to pH 5.5 + 0.05% chloramphenicol) in 100 × 15 mm Petri plates was used as the growth media for plating. Selection of this mildly acidic PDA was based on the soil pH values (see RESULTS); mildly acidic PDA supplemented with chloramphenicol as a bacterial growth regulator is a common agar for enumerating soil fungi (Brown 2007). Incubation was at 25 ± 0.5 °C in darkness in a programmable incubator.

Colonies were counted after 7 days with an automatic colony counter (Bantex Co., Burlingame, California, USA). Each individual fungal colony was considered an isolate. A 1 cm^3 block from the advancing edge of the fungus mycelium was removed using a scalpel under a stereoscopic microscope at 40X for subculturing, and each isolate was then purified by 3 successive rounds of subculturing hyphal tips.

Fungal identification was based on macroscopic colony characteristics, and microscopic spore and phialide characteristics under oil emmersion at 1000x (Barnett and Hunter 2003). Further confirmation of identification was done by comparison with previously identified, authentic cultures (nucITS identification) from 1) the University of Alberta Microfungus Collection and Herbarium (UAMH) Centre for Global Microfungal Biodiversity (Toronto, Ontario, Canada), 2) the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF), United States Department of Agriculture - Agricultural Research Service (USDA-ARS; Ithaca, New York, USA), and 3) the University of Cincinnati Microfungus Collection, Department of Biological Sciences, University of Cincinnati (Cincinnati, Ohio, USA).

We analyzed 3, 1-g samples (dry weight equivalent) from each tube of soil at the wallow; that is, 4 tubes of soil within the wallow and 4 tubes of soil that were proximal (control). Data from the 2 groups of 4 tubes at each wallow site were pooled and represented as a mean ± SE. Data were compared using an analysis of covariance (ANCOVA; $P = 0.05$; JMP, SAS Institute, Cary, North Carolina, USA). Fungus diversity was measured with the reciprocal Simpson diversity index (1/D; Simpson 1949) by treating all members of the same genus as a category. The total amount of fungus from the enumeration was expressed as colony forming units (CFU), and values were compared using a least significant difference (LSD) test.

Fungus inoculum

Spores from 1-month-old cultures of individual fungal isolates were scraped from

the plates with a scalpel and placed into a 1 mL phosphate buffered saline (PBS, pH 7.5) + 0.05% Tween 20 (emulsifier) to form an aqueous inoculum, 1 inoculum for each isolate. Isolates of the same genus were pooled to reflect the diversity (mixture of strains) of fungi that ticks would be exposed to naturally from soil. The spore count of the pooled inoculum was adjusted to 1.6×10^8 spores/mL using a hemocytometer (AO Spencer Bright-Line hemocytometer, St. Louis, Missouri, USA); the control inoculum was PBS + 0.05% Tween. Topical application of this concentration of spores/tick conforms to standard practice in pathogenicity tests (Tuininga et al. 2009, Suleiman et al. 2013, Cafarchia et al. 2015, Yoder et al. 2017a, b). Three different inocula representing a particular treatment group were prepared such that treatments to ticks were not made from a single tube of spores. Only fungal isolates cultured from the wallow samples were used for the pathogenicity experiments. Presumably, adult moose would acquire these fungi on their fur during wallowing, and these fungi would have the greatest likelihood of making contact with ticks on the moose.

Lethal testing

Larvae are one of two stages potentially exposed to fungi via wallowing (the other being nymphs; Addison and McLaughlin 1988), but the only stage available for testing. Larvae were obtained from hatched egg masses that had been laid by 42 blood-engorged females of winter ticks collected from 3 dead calf moose in Milan and Berlin, New Hampshire (P. Pekins holds permits). The fed females were transported to the laboratory in Whirl-Pak bags in 5 L coolers containing cold packs (Koolit). In the laboratory, a single, fed, female tick was placed into a mesh-covered, 50 mL polypropylene tube in an incubator held at 93% RH (Winston and Bates 1960) in a 3000 mL desiccator at 25 ± 0.5 °C at 10 h L:14 h D. After the female laid eggs, the egg mass was removed, placed into a clean 50 mL polypropylene tube and kept at 93% RH, 25 °C, and 10 h L:14 h D for hatching. The females and eggs were not surface sterilized because they were stored in sterile tubes for oviposition and hatching. Any differences in mortality would be the result of fungus treatment because the larvae originated from the same batch of ticks. Keys were used to confirm the identity of adult ticks as *D. albipictus* (Lindquist et al. 2016). The larvae were ~4 months of age when exposed to treatment with spore inocula to match the age at which they quest for a host.

An aspirator was used to handle larvae, one larva at a time. The aspirator was made by attaching a mesh-covered pipette tip to the end of a piece of Tygon tubing. Larvae were selected randomly from 42 tubes of hatched egg masses; thus, all larvae did not originate from the same egg mass for a particular treatment group. Prior to experimentation, larvae were examined at 40x using a stereoscopic microscope and checked for their ability to self-right and crawl 5 body lengths (health threshold); only healthy larvae were used in the experiment.

Larvae were treated in groups of 10 with 1 mL of a particular spore inoculum, or PBS + 0.05% Tween control, in a 1.5 mL microcentrifuge tube. The tube containing ticks was gently agitated for 2 min after which the contents were poured on a piece of filter paper (No. 3, Whatman, Hillsboro, Oregon, USA). Individual larvae that could self-right and crawl 5 body lengths were collected off the filter paper, and each was placed into a clean, 1.5 mL mesh-covered microcentrifuge tube (1 larva per tube). Larvae were stored at 80% RH (Winston and Bates 1960), 25 °C, and 10h:14h L:D cycle in a sealed glass desiccator placed in an incubator. Larvae were

examined daily at 40-45X using stereoscopic microscopy and the number of dead larvae was counted 10 days post-treatment; indication of death included lack of movement, curled legs, and deflated opisthosoma. Each spore inoculum experiment was replicated 10 times on 10 tubes of larvae (n = 100 larvae for each treatment). Data are expressed as the mean ± SE. An Abbott correction and logit-transformation, and ANCOVA for number of survivors ($P = 0.05$; JMP, SAS Institute, Cary, North Carolina, USA) were used to compare mortality between treatment and control groups. Larvae were exposed to those fungi isolated most frequently from the wallow soil samples.

Reisolation of fungi

Dead larvae were rinsed in a solution of DI water:absolute ethanol:5.25% NaOCl (18:1:1 v/v/v) for 1 min, followed by 2, 1-min rinses in DI water. This treatment in mild bleach solution was to externally remove fungi from the tick's body surface. After the final rinse, each larva was sectioned in half with a scalpel, and each portion was embedded separately into PDA growth medium in a Petri plate and incubated at 25 °C in darkness. Plates were examined daily at 45X for the appearance of hyphae emerging from within the tick's internal body contents. A 1 cm^3 block of agar containing the hyphal tip was removed from the plate for subculturing on a fresh plate of PDA (25 °C, darkness). Three additional subcultures were performed to purify the fungus for identification. The fungus that was isolated internally from within the larval tissues was compared to the fungus that was used to prepare the treatment inoculum as confirmation of infection (Koch's postulates). All dead larvae were analyzed for internal fungi within a particular treatment group and separated into statistical replicates. The number of dead larvae that tested positive for the treatment fungus was expressed as the mean ± SE and compared using an analysis of covariance (ANCOVA - Tukey's Test; significance at $P < 0.05$).

RESULTS

Sampling locations

Soil pH of samples from wallow and proximal samples ranged, respectively, from 5.6 – 6.3 and 5.1 – 5.8 at Katahdin, 5.0 – 6.4 and 5.4 – 6.0 at Greenville, and 5.8 – 6.6 and 5.1 – 6.0 at Milan. To match these soil conditions, we used slightly acidified potato dextrose agar (PDA + lactic acid to pH 5.5) as the agar growth medium. Preliminary observations indicated that wallow sample cultures contained a larger number of bacterial colonies than the proximal samples. Bacteria appeared prior to the appearance of the majority of fungal colonies that were discerned by the presence of hyphae, a mycelium, and capacity to produce spores. Accordingly, the media were supplemented with 0.05% chloramphenicol to limit bacterial growth.

Mycoflora profile

Tables 1-3 list the genera that were isolated. Water-treated controls showed 1 *Trichoderma* spp. colony/45 plates. Large variability existed in the amount of fungi (CFU/g dry soil) between wallow and proximal samples. The fungal diversity index was lower in all 4 wallow (versus proximal) samples at Katahdin, in 9 of 13 wallow samples at Greenville, and 5 of 7 wallow samples at Milan (Tables 1-3). There were 1-3 samples at each geographic location that had either much higher or lower (orders of magnitude) relative amounts of fungi (Fig. 1).

Aspergillus flavus was present at each geographic location, with 75% abundance at Katahdin, 62% at Greenville, and 29% at Milan (Tables 1-3). Abundance of *A. fumigatus* was 50% Katahdin, 38% at Greenville, and 14% at Milan. There was a lower

Table 1. Percent occurrence of fungus genera in soil from moose wallows and adjacent control sites from Katahdin, Maine, USA.

Fungi	% mean # isolates							
	K1		K2		K3		K4	
	P	W	P	W	P	W	P	W
Composition (± SE ≤ 3.2)								
Absidia spp.	11	0	14	0	0	0	0	0
Alternaria spp.	0	0	0	0	0	0	2	0
Aspergillus spp.	0	0	14	0	0	0	0	0
Aspergillus flavus	0	16	0	0	8	0	7	0
Aspergillus fumigatus	0	0	0	0	8	0	2	0
Beauveria bassiana	0	0	14	8	0	0	4	0
Cladosporium spp.	11	0	0	0	15	11	4	0
Fusarium spp.	0	16	0	0	8	0	7	11
Mortierella spp.	11	0	14	25	23	22	20	11
Mucor spp.	34	16	14	33	8	22	13	22
Paecilomyces spp.	0	0	0	0	0	11	9	0
Penicillium spp.	11	0	0	8	23	11	16	43
Scopulariopsis spp.	11	0	0	8	8	0	4	0
Trichoderma spp.	11	48	29	17	0	22	11	11
Simpson's index, 1/D	5.5	3.1*	5.6	4.5*	6.7	5.7*	9.0	3.7*
SE	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.1

Isolate, 1 cm^3 block of an individual fungus colony on a culture plate. P = proximal soil sample; and W = wallow soil sample. * denotes a significant difference between the proximal-wallow pair at the geographic site.

Table 2. Percent occurrence of fungus genera in soil from moose wallows and adjacent control sites from Greenville, Maine, USA.

Fungi	% mean # isolates													
	G1		G2		G3		G4		G5		G6		G7	
	P	W	P	W	P	W	P	W	P	W	P	W	P	W
Composition (± SE ≤ 3.6)														
Absidia spp.	7	8	17	0	17	13	0	0	2	3	0	13	5	4
Acremonium spp.	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Alternaria spp.	4	0	0	0	0	0	7	0	0	6	10	0	3	9
Aspergillus spp.	7	4	0	14	0	13	0	0	0	0	10	0	0	9
Aspergillus flavus	2	4	0	0	0	0	7	0	4	3	0	0	5	0
Aspergillus fumigatus	4	0	0	0	0	0	0	0	4	0	0	0	3	0
Aspergillus niger	0	0	0	0	0	0	0	0	0	3	0	0	0	0
Beauveria bassiana	4	0	17	0	0	0	0	0	2	5	0	0	3	0
Cladosporium spp.	7	13	0	0	0	0	0	0	4	3	10	0	3	9

Table 2 Continued ...

Table 2. continued

Fungi	% mean # isolates													
	G1		G2		G3		G4		G5		G6		G7	
	P	W	P	W	P	W	P	W	P	W	P	W	P	W
Epicoccum spp.	2	0	0	0	17	0	0	0	4	0	0	0	0	4
Fusarium spp.	0	0	0	0	0	13	10	0	6	8	20	0	8	4
Mortierella spp.	16	4	33	14	0	13	19	14	14	21	10	13	19	9
Mucor spp.	9	33	17	29	17	25	10	29	10	10	10	25	5	13
Paecilomyces spp.	0	4	0	0	0	0	16	0	12	0	0	0	11	4
Penicillium spp.	13	17	17	14	0	0	16	14	8	19	20	13	11	13
Rhizopus spp.	7	0	0	0	0	0	0	0	0	0	0	0	0	0
Scopulariopsis spp.	0	0	0	0	0	0	0	0	2	2	0	0	5	0
Trichoderma spp.	13	13	0	29	33	25	16	43	16	21	10	38	16	22
Umbelopsis spp.	4	0	0	0	17	0	3	0	8	0	0	0	5	0
Simpson's index, 1/D	11.2	5.7*	4.7	4.6	4.7	5.6*	8.0	3.3*	11.3	7.6*	7.6	4.2*	10.5	8.9*
SE	0.2	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.2

Fungi	% mean # isolates											
	G8		G9		G10		G11		G12		G13	
	P	W	P	W	P	W	P	W	P	W	P	W
Composition (± SE ≤ 3.1)												
Absidia spp.	0	3	0	5	5	2	3	15	0	14	0	9
Acremonium spp.	0	0	0	0	0	0	0	0	0	0	4	0
Alternaria spp.	3	0	0	3	0	0	3	0	0	0	0	0
Aspergillus spp.	3	0	5	0	16	7	6	0	0	14	7	0
Aspergillus flavus	5	7	0	3	0	0	0	0	6	0	2	0
Aspergillus fumigatus	0	0	0	0	0	0	6	0	6	0	0	0
Aspergillus niger	0	0	5	0	0	0	0	0	0	0	0	0
Beauveria bassiana	3	3	0	8	0	0	3	0	0	0	2	4
Cladosporium spp.	11	7	0	5	0	9	19	23	6	29	15	4
Fusarium spp.	11	14	0	0	11	11	0	4	0	0	2	0
Mortierella spp.	5	17	19	10	16	7	13	15	25	14	13	22
Mucor spp.	11	14	10	18	16	17	6	23	13	0	11	9
Mycelia sterilia	3	0	0	0	0	0	0	0	0	0	0	0
Nigrospora spp.	3	0	0	3	5	0	0	0	0	0	0	0
Paecilomyces spp.	8	0	14	13	0	0	3	4	0	0	7	4
Penicillium spp.	8	17	19	13	16	19	10	12	13	14	22	22
Rhizopus spp.	0	0	0	0	0	0	3	0	13	0	0	0
Scopulariopsis spp.	5	0	0	0	5	0	6	0	0	0	0	0
Trichoderma spp.	16	21	24	18	11	17	10	8	19	14	15	26
Umbelopsis spp.	5	0	5	5	0	11	6	0	0	0	0	0

Table 2 Continued ...

Table 2. continued

Fungi	% mean # isolates											
	G8		G9		G10		G11		G12		G13	
	P	W	P	W	P	W	P	W	P	W	P	W
Verticillium spp.	3	0	0	0	0	0	0	0	0	0	0	0
Simpson's index, 1/D	13.2	7.4*	6.4	9.3*	8.1	7.8	10.9	6.5*	6.7	5.6*	7.9	5.6*
SE	0.2	0.1	0.2	0.1	0.2	0.2	0.1	0.2	0.1	0.1	0.1	0.2

Isolate, 1 cm^3 block of an individual fungus colony on a culture plate. P = proximal soil sample and W = wallow soil sample. * denotes a significant difference between the proximal-wallow pair at the geographic site.

Table 3. Percent occurrence of fungus genera in soil from moose wallows and adjacent control sites from Milan, New Hampshire, USA.

Fungi	% mean # isolates													
	M1		M2		M3		M4		M5		M6		M7	
	P	W	P	W	P	W	P	W	P	W	P	W	P	W
Composition (± SE ≤ 3.3)														
Absidia spp.	2	0	3	0	5	11	0	0	3	2	9	5	0	10
Alternaria spp.	0	0	0	0	0	0	0	0	0	6	8	0	0	0
Aspergillus spp.	0	0	11	0	5	9	13	0	6	0	9	3	0	0
Aspergillus flavus	0	0	0	0	2	0	0	0	6	0	0	0	0	0
Aspergillus fumigatus	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Beauveria bassiana	0	0	0	0	6	0	0	0	0	0	4	8	0	0
Cladosporium spp.	10	9	6	6	7	2	13	0	11	0	8	10	0	0
Epicoccum spp.	2	0	0	0	0	0	0	9	11	6	0	0	0	10
Fusarium spp.	12	1	4	0	7	7	0	0	6	6	0	0	0	0
Geotrichum spp.	0	0	0	0	0	0	0	0	3	0	0	8	0	0
Mortierella spp.	21	19	19	56	17	13	0	36	8	23	13	8	17	20
Mucor spp.	10	42	19	11	7	18	25	9	8	17	6	18	17	10
Nigrospora spp.	2	0	0	0	0	2	0	0	0	0	9	0	0	0
Paecilomyces spp.	0	0	3	0	5	0	0	0	3	0	0	0	0	0
Penicillium spp.	21	13	13	11	12	18	40	18	17	17	11	18	33	20
Rhizopus spp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Scopulariopsis spp.	0	0	3	0	7	2	0	9	3	0	0	3	0	0
Trichoderma spp.	14	13	12	17	13	13	13	18	14	23	15	18	17	30
Umbelopsis spp.	5	3	6	0	7	0	0	0	3	0	6	0	17	0
Verticillium spp.	0	0	3	0	0	0	0	0	0	0	2	0	0	0
Simpson's index, 1/D	7.1	4.0*	8.9	2.8*	11.5	7.2*	4.1	4.6	11.4	6.0*	11.3	8.5*	4.7	5.2
SE	0.1	0.1	0.2	0.2	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.1

Isolate, 1 cm^3 block of an individual fungus colony on a culture plate. P = proximal soil sample; and W = wallow soil sample. * denotes a significant difference between the proximal-wallow pair at the geographic site.

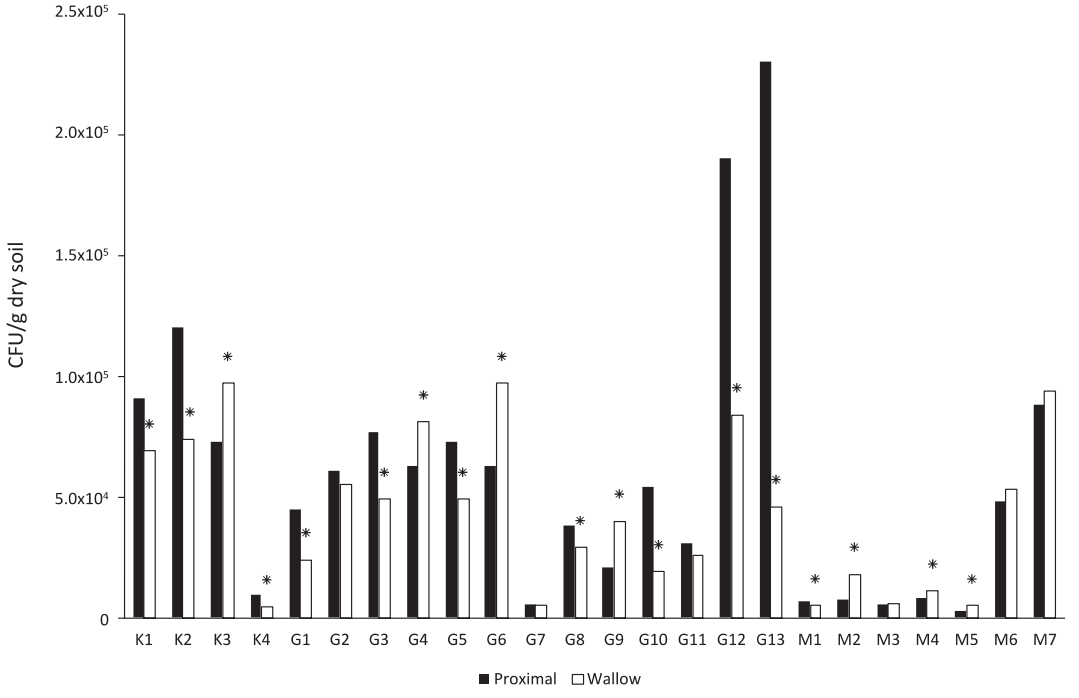

Fig. 1. Amount of fungi (colony forming unit, CFU/g dry soil) in soil samples collected in Katahdin (K) and Greenville (G), Maine, and Milan (M), New Hampshire, USA. The * above a bar denotes a significant difference ($P < 0.05$) between proximal and wallow soil samples at that geographic site. Note the variation in fungus amounts: sometimes proximal > wallow, proximal < wallow, and proximal = wallow.

presence of *A*. *niger*: 0% at Katahdin, 15% at Greenville, and 0% at Milan. Occurrence of *B*. *bassiana* was 50% at Katahdin, 62% at Greenville, and 29% at Milan. Species detectability and abundance was higher in proximal than wallow samples, except for *A*. *niger* at Greenville.

Tick mortality

The most common fungi at each wallow (n = 24) were tested for pathogenicity against winter tick larvae. Higher mortality ($P < 0.05$) compared to the PBS + Tween-treated control (5-16% mortality) occurred consistently for 8 genera that were isolated at all 3 geographic locations (*Aspergillus* spp., *Beauveria bassiana*, *Fusarium* spp., *Mortierella* spp., *Mucor* spp., *Paecilomyces* spp., *Penicillium* spp., and *Trichoderma* spp.). The mean proportion killed by these genera ranged from ~30-95%, with *Beauveria bassiana*, *Mortierella* spp., *Mucor* spp., and *Paecilomyces* spp. the most lethal (>50%; Tables 4-6). These 4 genera were also recovered from larval cadavers at a rate higher than controls. *Trichoderma* spp. was recovered from cadavers for 20 of 24 wallows, and *Penicillium* spp. at 18 of 22 wallows; both were less lethal (typically <50%) than the other 4 genera (Tables 4-6). *Aspergillus flavus* was the sole *Aspergillus* isolate at 4 wallows and occurred within a test mixture of *Aspergillus* at another. It induced mortality (23-78%) and was recovered from larval cadavers at these specific wallows; otherwise, it did not cause significant larval mortality. Mortality associated with *Fusarium* spp. was mixed and recovery

Table 4. Mortality of healthy larvae of *Dermacentor albipictus* after 10 days exposed to spores (1.6×10^8 spores/larva) from wallow soil fungi from Katahdin, Maine, USA.

Treatment	# of larvae 10 days post-treatment				
	Control	K1	K2	K3	K4
Dead/100 larvae	11 ± 2.8				
% positive for fungus	27 ± 1.2				
Aspergillus spp.					
Dead/100		76 ± 2.7*	-	-	-
% positive for fungus		82 ± 1.4*	-	-	-
Beauveria bassiana					
Dead/100		-	86 ± 4.1*	-	-
% positive for fungus		-	93 ± 2.7*	-	-
Cladosporium spp.					
Dead/100		-	-	6 ± 0.7	-
% positive for fungus		-	-	0	-
Fusarium spp.					
Dead/100		11 ± 0.5	-	-	16 ± 1.6*
% positive for fungus		9 ± 0.3	-	-	19 ± 2.1
Mortierella spp.					
Dead/100		-	67 ± 3.1*	89 ± 2.4*	66 ± 2.4*
% positive for fungus		-	93 ± 2.0*	95 ± 1.9*	86 ± 1.9*
Mucor spp					
Dead/100		58 ± 4.0*	36 ± 3.4*	73 ± 2.2*	84 ± 3.7*
% positive for fungus		86 ± 3.2*	61 ± 1.7*	90 ± 1.1*	95 ± 2.9*
Paecilomyces spp					
Dead/100		-	-	86 ± 3.7*	-
% positive for fungus		-	-	85 ± 2.4*	-
Penicillium spp.					
Dead/100		-	65 ± 2.0*	21 ± 2.9*	41 ± 3.6*
% positive for fungus		-	49 ± 1.8*	33 ± 2.1	66 ± 3.2*
Trichoderma spp.					
Dead/100		63 ± 2.4*	44 ± 3.1*	71 ± 4.5*	39 ± 2.3*
% positive for fungus		78 ± 2.8*	68 ± 1.9*	58 ± 2.2*	79 ± 3.1*

Control, PBS + 0.05% Tween. - indicates not determined, isolate not present in wallow soil. Data are the mean ± SE. * indicates significant greater difference from respective control ($P < 0.05$).

from larval cadavers was irregular. Isolates of *Cladosporium* spp. and *Umbelopsis* spp. did not cause larval mortality.

DISCUSSION

The 8 genera of fungi found pathogenic against winter tick larvae have been previously identified as entomopathogenic against ticks (Chandler et al. 2000, Samish et al. 2004, Greengarten et al. 2011; *Trichoderma* spp. listed as its teleomorph *Hypocrea* in Greengarten et al. 2011; Yoder et al. 2017a). When artificially infected by topical application, each of these fungi was

Table 5. Mortality of healthy larvae of *Dermacentor albipictus* after 10 days exposed to spores (1.6×10^8 spores/larva) from wallow soil fungi from Greenville, Maine, USA.

Treatment	# of larvae 10 days post-treatment						
	G1	G2	G3	G4	G5	G6	G7
Aspergillus spp.							
Dead/100	81 ± 3.2*	42 ± 4.3*	21 ± 1.7*	-	68 ± 2.1*	-	28 ± 3.1*
% positive for fungus	91 ± 2.2*	21 ± 2.8	38 ± 2.4	-	94 ± 1.3*	-	21 ± 2.1
Beauveria bassiana							
Dead/100	-	-	-	-	91 ± 2.7*	-	-
% positive for fungus	-	-	-	-	97 ± 2.1*	-	-
Cladosporium spp.							
Dead/100	11 ± 0.6	-	-	-	7 ± 0.9	-	5 ± 1.4
% positive for fungus	18 ± 1.1	-	-	-	14 ± 0.8	-	0
Fusarium spp.							
Dead/100	-	-	16 ± 2.5*	-	20 ± 1.9*	-	11 ± 2.0
% positive for fungus	-	-	25 ± 1.5	-	40 ± 2.2*	-	27 ± 1.8
Mortierella spp.							
Dead/100	43 ± 2.8*	71 ± 3.3*	78 ± 3.1*	61 ± 3.3*	39 ± 2.2*	42 ± 1.2*	72 ± 2.8*
% positive for fungus	84 ± 1.7*	76 ± 2.0*	90 ± 2.2*	82 ± 2.4*	77 ± 1.7*	90 ± 2.5*	88 ± 3.1*
Mucor spp.							
Dead/100	60 ± 2.9*	54 ± 3.1*	56 ± 1.9*	46 ± 3.1*	52 ± 1.9*	71 ± 1.3*	60 ± 3.4*
% positive for fungus	82 ± 3.1*	91 ± 2.7*	82 ± 2.3*	70 ± 2.6*	85 ± 1.4*	83 ± 1.4*	68 ± 2.1*
Paecilomyces spp.							
Dead/100	57 ± 2.8*	-	-	-	-	-	61 ± 2.9*
% positive for fungus	65 ± 1.4*	-	-	-	-	-	90 ± 2.1*
Penicillium spp.							
Dead/100	21 ± 2.7*	49 ± 3.5*	-	24 ± 2.0*	47 ± 2.9*	31 ± 2.2*	45 ± 3.3*
% positive for fungus	38 ± 2.1	63 ± 2.4*	-	71 ± 1.7*	83 ± 1.9*	55 ± 2.3*	64 ± 3.1*
Trichoderma spp.							
Dead/100	29 ± 3.4*	43 ± 2.6*	19 ± 3.1*	34 ± 2.6*	41 ± 3.1*	22 ± 3.0*	42 ± 2.4*
% positive for fungus	58 ± 4.2*	70 ± 3.1*	37 ± 2.1	76 ± 3.1*	85 ± 2.2*	32 ± 3.0	79 ± 1.3*

Treatment	# of larvae 10 days post-treatment					
	G8	G9	G10	G11	G12	G13
Aspergillus spp.						
Dead/100	73 ± 3.1*	78 ± 2.2*	15 ± 1.8	-	26 ± 3.1*	-
% positive for fungus	84 ± 2.1*	83 ± 1.6*	27 ± 2.0	-	31 ± 2.2	-
Beauveria bassiana						
Dead/100	83 ± 2.1*	91 ± 2.6*	-	-	-	87 ± 3.2*
% positive for fungus	93 ± 1.8*	92 ± 2.5*	-	-	-	84 ± 2.7*
Cladosporium spp.						
Dead/100	14 ± 1.5	12 ± 1.7	16 ± 0.8	6 ± 1.1	10 ± 1.1	5 ± 0.9

Table 5 Continued ...

Table 5. Continued

Treatment	# of larvae 10 days post-treatment					
	G8	G9	G10	G11	G12	G13
% positive for fungus	7 ± 1.8	33 ± 0.7	19 ± 1.1	0	20 ± 1.3	0
Fusarium spp.						
Dead/100	22 ± 3.1*	-	26 ± 3.1*	14 ± 1.9	-	-
% positive for fungus	64 ± 2.3*	-	65 ± 2.4*	36 ± 2.1	-	-
Mortierella spp.						
Dead/100	55 ± 2.4*	66 ± 2.5*	49 ± 2.2*	75 ± 2.7*	81 ± 2.3*	56 ± 3.1*
% positive for fungus	85 ± 3.0*	80 ± 1.2*	82 ± 1.9*	76 ± 1.7*	83 ± 2.7*	88 ± 2.6*
Mucor spp.						
Dead/100	81 ± 2.4*	74 ± 2.2*	59 ± 2.1*	47 ± 3.4*	-	63 ± 2.4*
% positive for fungus	95 ± 2.0*	93 ± 2.0*	93 ± 2.7*	83 ± 3.2*	-	90 ± 2.4*
Paecilomyces spp.						
Dead/100	-	70 ± 2.0*	-	68 ± 3.5*	-	71 ± 3.6*
% positive for fungus	-	81 ± 1.7*	-	88 ± 2.6*	-	73 ± 2.0*
Penicillium spp.						
Dead/100	52 ± 2.4*	26 ± 2.1*	38 ± 2.4*	44 ± 1.9*	51 ± 2.0*	29 ± 2.2*
% positive for fungus	79 ± 2.8*	31 ± 3.0	59 ± 1.9*	75 ± 2.2*	61 ± 2.5*	28 ± 1.9
Trichoderma spp.						
Dead/100	61 ± 3.0*	37 ± 2.1c*	26 ± 2.7*	31 ± 2.8*	46 ± 3.4*	31 ± 3.1*
% positive for fungus	85 ± 2.4*	64 ± 3.2*	31 ± 2.3	77 ± 2.6*	72 ± 2.6*	35 ± 2.1
Umbelopsis spp.						
Dead/100	-	11 ± 1.4	4 ± 0.5	-	-	-
% positive for fungus	-	0	3 ± 0.2	-	-	-

- indicates not determined, isolate not present in wallow soil. Data are the mean ± SE. * indicates significant greater difference from control in Table 4 ($P < 0.05$).

recovered from within the tissues of larval cadavers by internal fungus culture as confirmation of infection.

Changes in moisture level, relative humidity, salts, and extent of aeration quantitatively alter the fungal activity and amount in disturbed soil (Miller and Lodge 2007, Morris et al. 2007). Such changes could be attributed to moose activity: 1) removal of surface habitat by digging that changes the drainage patterns and moisture level within the wallow, 2) concentration and frequency of salts from urination in the wallow, 3) changes in aeration from loosening and compacting soil from the digging, and 4) the overall frequency and intensity of disturbance of the soil. This contrasts to the relatively stable, undisturbed conditions of the proximal soil samples where fungi were typically more abundant and diverse.

Fungi that were not cultured with our methods certainly exist in the soil samples. Whether the amount and composition of soil fungi were measurably influenced by stand type, age, and canopy cover associated with the wallows cannot be answered given our somewhat random approach in locating active wallows. Clearly, moose can

Table 6. Mortality of healthy larvae of *Dermacentor albipictus* after ten days exposed to spores (1.6×10^8 spores/larva) from wallow soil fungi from Milan, New Hampshire, USA.

Treatment	# of larvae 10 days post-treatment:						
	M1	M2	M3	M4	M5	M6	M7
Aspergillus spp.							
Dead/100	-	-	23 ± 2.9*	-	-	35 ± 3.4*	-
% positive for fungus	-	-	35 ± 3.2	-	-	63 ± 2.6*	-
Beauveria bassiana							
Dead/100	-	-	-	-	-	91 ± 2.3*	-
% positive for fungus	-	-	-	-	-	80 ± 2.2*	-
Cladosporium spp							
Dead/100	7 ± 0.4	8 ± 0.5	6 ± 1.1	-	-	15 ± 0.9	-
% positive for fungus	0	13 ± 1.2	0	-	-	13 ± 0.4	-
Fusarium spp.							
Dead/100	21 ± 2.0*	-	25 ± 3.4*	-	31 ± 2.2*	-	-
% positive for fungus	19 ± 2.4	-	44 ± 2.8*	-	65 ± 3.1*	-	-
Mortierella spp.							
Dead/100	74 ± 3.1*	79 ± 3.4*	64 ± 2.2*	72 ± 2.4*	58 ± 2.5*	74 ± 2.3*	68 ± 3.3*
% positive for fungus	77 ± 2.9*	77 ± 3.5*	91 ± 3.1*	92 ± 2.1*	84 ± 2.5*	93 ± 2.8*	78 ± 2.6*
Mucor spp.							
Dead/100	81 ± 2.2*	54 ± 3.2*	40 ± 2.8*	66 ± 2.0*	66 ± 2.1*	80 ± 3.1*	59 ± 2.7*
% positive for fungus	93 ± 1.8*	91 ± 2.6*	78 ± 2.6*	86 ± 2.4*	91 ± 3.4*	84 ± 2.2*	85 ± 2.2*
Penicillium spp.							
Dead/100	34 ± 2.5*	45 ± 2.7*	31 ± 3.3*	73 ± 3.1*	38 ± 2.1*	47 ± 2.8*	54 ± 2.9*
% positive for fungus	44 ± 2.2	87 ± 2.4*	26 ± 2.0	90 ± 2.9*	79 ± 2.7*	68 ± 2.4*	84 ± 3.0*
Trichoderma spp.							
Dead/100	57 ± 3.0*	53 ± 2.2*	26 ± 2.8*	33 ± 2.4*	41 ± 1.9*	57 ± 2.5*	53 ± 2.6*
% positive for fungus	81 ± 3.0*	83 ± 1.9d*	73 ± 2.4*	58 ± 2.3*	66 ± 2.2*	68 ± 2.3*	75 ± 2.4*
Umbelopsis spp.							
Dead/100	15 ± 1.2	-	-	-	-	-	-
% positive for fungus	27 ± 1.1	-	-	-	-	-	-

-, not determined, isolate not present in wallow soil. Data are the mean ± SE. *, significant greater difference from control in Table 4 ($P < 0.05$).

acquire fungi at bedding sites, in open, regenerating forest habitats, and in muddy locales – especially on bare soil. Aspects of wallowing behavior and any moose-winter tick-fungal relationship is likely not universal given the wide geographic range, diverse use of habitats and forest types, and variable seasonal and microclimatic ground conditions found throughout North American moose range.

From a fungal ecology perspective, the mycoflora of wallow soil can best be explained relative to soil disturbance, in particular digging and burrowing (Kumpula et al. 2000, Shubina et al. 2013). The proximal soil samples can be considered as undisturbed soils due to the absence of direct digging/activity. The qualitative differences between the wallow and proximal samples presumably reflect the disturbance to wallow

soil that modifies the fungal community structure (criteria from Miller and Lodge 2007, Morris et al. 2007): 1) there was lower fungal diversity in wallow soil than proximal soil, 2) a group of fungi (*Mortierella* spp., *Penicillium* spp., *Mucor* spp., and *Trichoderma* spp.) that function as primary colonizers (i.e., the first to grow on roots) predominates in wallow soil, and 3) the amount of total fungi was highly variable exhibiting no consistent pattern in CFU/g soil between wallow and proximal samples. Qualitatively, each time a moose visits a wallow and disturbs the soil, the fungi subsequently recolonize in response, indicating why *Mortierella* spp., *Penicillium* spp., *Mucor* spp., *Trichoderma* spp., and other genera were isolated most frequently. The extent that male urine alters the soil environment, in positive or negative ways, is not known and invites further inquiry.

Fur, hooves, and horns of animals harbor numerous saprobic fungi acquired from forest soils (Shubina et al. 2013), including many fungi taxa identified here. We conclude that through soil disturbance, wallowing behavior makes spores readily available in infections (*Mortierella* spp., *Penicillium* spp., *Mucor* spp., *Trichoderma* spp.) in wallow soil. We emphasize that some pathogenic genera (i.e., *Aspergillus flavus*, *Beauveria bassiana*, *Paecilomyces* spp., *Scopulariopsis* spp.) were even more abundant in proximal soil samples. Thus, areas outside of wallows are not necessarily less important as potential sources of infection. All of these fungi are heavily spore-producing genera, and our study further identifies that their fungal strains are indeed pathogenic to winter tick larvae, many with a lethal rate >50%. It seems reasonable to suggest that exposure of breeding adult moose to self-made, disturbed soils can provide for a coating of soil on their fur that contains several important groups of entomopathogenic fungi, particularly their spores. As such, we hypothesize that this behavior may provide a form of biological control for adult moose against winter ticks not afforded calf moose, and perhaps contribute to the differentially higher mortality among tick-infested calves (Jones et al. 2017) as compared to tick-infested adult moose. We plan to measure the presence and prevalence of these fungi and tick numbers in reference to sex and age-class of moose harvested in October 2017 to further elucidate possible moose-winter tick-fungal relationships.

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