Grazing of Ectomycorrhizae by Microarthropods

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Abstract

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Microarthropods, including Collembola and mites, are a highly diverse and abundant group of soil animals. Previous information on interactions between microarthropods and ectomycorrhizal fungi has been mainly based on mycelium grown in pure culture. Collembola feeding preferences were assessed by offering naturally occurring ECM to the Collembola species *Folsomia candida*. Some ECM types, including *Tricholoma aestuans, Piloderma bicolor* and *Suillus spraguei*, were grazed. *Cenococcum geophilum* and *Lactarius vinaceorufescens* were unpalatable. However, fungi forming unpalatable ECM were grazed when not in symbiosis with the host root. To determine levels of ECM grazing occurring in the field, gut contents of a species of *Folsomia* and a species of Phthiracaridae were assessed molecularly. No ECM fungi were detected. Although the degree to which soil microarthropods graze ECM in the field is still unknown, this work demonstrates that there is a high degree of variation in palatability among ECM formed by different fungal species.

Keywords: Microarthropod grazing, Fungal preference, Antifeedants

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Chapter 1

Introduction

Soils are full of life and are one of the most species-rich ecosystems (Wall et al. 2005), supporting diverse communities of both fauna and microorganisms. This includes the fungi, a highly diverse group of organisms with an estimated $1.5x10^6$ fungal species – although only 80,000 to 120,000 species are described (Hawksworth and Rossman 1997; Hawksworth 2001; Kirk et al. 2001).

With regard to fauna, it is estimated that arthropods account for 0.2 Gt C in soil (Bar-On et al. 2018), but an estimated 95% of soil dwelling species are still not described (Wall et al. 2005). One of these soil dwelling groups, the Collembola, are a focus of this study. Collembola are fungivorous soil microarthropods (Shaw 1998; Bardgett et al. 1993; Hopkin 1997; Fountain and Hopkin 2005; Jørgensen et al. 2005; Kanters et al. 2015). They are one of the most abundant groups of arthropods and can reach densities of up to 120,000 per square meter of soil (5cm depth) (Ponge et al. 1997; Fountain and Hopkin 2005). Collembola are commonly called springtails, due to their characteristic furca, a flexible appendage used to evade predators by jumping (Hopkin 1997), Although the furca is the most well-known and defining feature of Collembola, some species that live deeper in soil (euedaphic) have a reduced furca or no furca at all, making movement though tightly packed soil easier (Hopkin 1997). *Folsomia candida* is a Collembola species that is widely used in laboratory experiments due to its wide range and ease of culture. *F. candida* is considered a "tramp species" because it has been spread throughout the world through soil and plant pots making its exact origin almost impossible to determine (Hopkin 1997).

Borkott and Insam (1990) performed a study on *F. candida* to test the chitinolytic abilities of the bacteria in their gut content. They found symbiotic bacteria in their guts that break down chitin, the main component of fungal cell walls, indicating that fungi are an important part of their diet. Collembola grazing on fungi, including the mycorrhizal fungi that form symbiotic relationships with plants, influences the shape of fungal communities in soil (Klironomos et al. 1992). It is estimated that 86% of terrestrial plant species have a relationship with symbiotic mycorrhizal fungi and benefit from this symbiosis (Brundett 2002), and mycorrhizal fungi make up 57% of forest soil fungal biomass (Nilsson et al. 2005).

Collembola feed on both arbuscular mycorrhizal fungi (AM) and ectomycorrhizal (ECM) fungi (Shaw 1998; Gagne 2000; Scheu and Folger 2004; Ngosong et al. 2014; Kanters et al. 2015; Anslan et al. 2016). Although AM fungi are not considered to be a preferred food source for Collembola (Klironomos et al. 1996; 1999), AM grazing by Collembola can still cause the connection between plants and their mycorrhizal symbiont to be damaged, which can, at high Collembolan densities, reduce plant growth (Warnock et al. 1982; Harris and Boerner 1990; Ek et al. 1994).

AM fungal structures are contained inside of plant roots, with only the extraradical hyphae available for microarthropod grazing, while the mantle of ECM fungi is located on the outside of the root, making them more available and vulnerable to microarthropod grazing. The present study focuses on the relationship between ECM fungi (ECMf) as a food source for microarthropods.

There are an estimated 20,000 - 25,000 species of ECMf (Rinaldi et al. 2008; Brundrett 2002; Roy-Bolduc et al. 2016), forming symbiotic relationships with the roots

of woody plants (Fogel and Hunt 1983). The roots and fungal tissues combine at the cellular level to create the mycorrhiza where an exchange of nutrients and carbon takes place (Smith & Read 2008). ECMf are integral in seedling survival, growth, and establishment in several different forest ecosystems (Smith and Read, 2008; Sebastiana et al. 2017), especially conifer forests, where they can account for 47 to 84% of soil biomass (Baath et al. 2004). Some ECMf will form symbioses with a large variety of hosts, or in some cases with multiple species of the same genus (Dickie 2007).

The importance of Collembola in the breakdown of ECM is not well known, but there is evidence to suggest that they do find ECMf palatable (Ponge 2000; Anslan et al. 2016). Grazing of the fungal mantle on the outside of the root tip could cause damage to the fungi and inhibit their mutualistic function (Martin and Plassard 2001). Some ECM contain suspected antifeedant mechanisms that are assumed to have developed to deter microarthropod grazing (Böllmann et al. 2010). However, there have only been a handful of studies that have examined the grazing of Collembola on ECMf in association with roots (Ek et al. 1994; Setälä 1995; Kaneda and Kaneko 2004; Kanters et al. 2015), so this is clearly an area that requires more research.

Another type of soil microarthropod investigated during this study were the Oribatid mites (Acari, Oribatida). In one study, Oribatid mites are considered "choosy generalist" feeders because when presented with a wide variety of food choices they prefer darkly pigmented fungi, but when limited choices are available they will eat what is available (Schneider and Maraun 2005). Some authors have studied the overall population of Oribatid mites in soil before and after tree girdling (Remen et al. 2008; Remen et al. 2010), and Schneider et al. (2005) have examined Oribatid mite feeding on

ECMf. However, mites were fed ECMf grown in pure culture, which can influence results because of the high nutrient content of hyphae when grown on rich media. There is also considerable structural difference between an ECM mantle surrounding a fine root and mycelium from pure culture.

The present study investigates soil microarthropod feeding on ECMf in two ways; in the lab and through the analysis of the guts of field collected fauna. By taking this approach, the hope was to demonstrate the way that microarthropods feed when presented with limited choices (Chapter 2), compared to how they feed when in the *Pinus* stand without this limitation (Chapter 3). It was hypothesized that soil microarthropods would feed on ECM that were presented to them in an experimental setting, and that they would prefer some fungi over others. It was also hypothesized that ECMf fungi would be found in the guts of both Collembola and Oribatid mites. Through the research conducted on the soil microarthropods and fungi in this thesis, the hope is to shed some light on what is really going on below the soil surface.

Objectives and Approach

For Chapter 2, the palatability of five different ECM types was tested. The ECM species associated with *Pinus* were collected from forest soil, and were fed to Collembola separately, and as a choice. It was hypothesized that Collembola would find certain ECM types unpalatable due to suspected antifeedant mechanisms present. Experiments in which the basal hyphae from sporocarps and the ECM of each fungal species were presented (either separately or as a choice) were conducted to determine if there was any difference between in the grazing of colonized root tips and soil hyphae.

The aim of Chapter 3 was to determine which fungal species are found in the gut contents of both field-collected Collembola and Oribatid mites, given all the food choice of a natural soil. It was hypothesized that both Collembola and Oribatid mite gut contents would contain at least some ECMf due to the high ECMf hyphal biomass estimated for forest soils.

Chapter 2

Ectomycorrhizae as a food source for Collembola *(Folsomia candida)*

Abstract

Collembola are microarthropods that are ubiquitous in soils throughout the world. They are generalist feeders but prefer fungi as a food source. This study focuses on Collembola and their relationship with one group of fungi, the ectomycorrhizal (ECM) fungi, which form a symbiotic relationship with the roots of woody plants. The decomposition of ectomycorrhizae in soil is not well understood, and it was hypothesized that Collembola could play a role in this breakdown. To test this, *Folsomia candida* (a model Collembolan) was used in various feeding experiments to determine the relative palatability of five ECM species to Collembola. The food choices were presented on their own, and also paired against one another. The palatability of the fungi as ECM was also compared to basal hyphae from conspecific sporocarps. Further experiments were also conducted to specifically investigate the palatability of the darkly pigmented *Cenococcum geophilum* to Collembola, as Collembola generally would not graze this ECM type, even though Collembola supposedly prefer melanized fungi. A clear hierarchy of preference of some ECM types over others was observed when presented separately and as a choice. Collembola readily grazed *Cenococcum geophilum* mycelium when grown in pure culture, but not as ECM root tips.

Introduction

Collembola are soil dwelling hexapods, thought to be one of the oldest and most plentiful groups of arthropods (Fountain and Hopkin 2005). Collembola range in size from 1 to 5 mm (Hopkin 1997; Bellinger et al. 2014), and are classified as microarthropod, a grouping that also includes Acari and enchytraeid worms (Bradford et al. 2002). The defining feature of Collembola is the furca, colloquially known as the springtail (Hopkin 1997), an appendage used to evade predators. There are approximately 8,500 described species of Collembola found throughout the world (Barjadze et al. 2016), but there may be as many as 50,000 species (Cicconardi et al. 2013). Skidmore (1995)

identified 412 species of Collembola in Canada. One square meter of soil can support up to 120,000 Collembola (5 cm depth) (Ponge et al. 1997; Fountain and Hopkin 2005).

Although Collembola are generalist feeders and will ingest plant litter (Endlweber et al. 2009), nematodes (Chamberlain et al. 2006), animal waste and bacteria (Hopkin 1997), it is generally accepted that they feed mainly on fungi (Bardgett et al. 1993; Hopkin 1997; Ponge et al 2000; Jørgensen et al. 2005). Some Collembola have symbiotic chitinolytic bacterial gut symbionts that aid in the utilization of chitin as a nutrient source (Borkott and Insam 1990), indicating the importance of fungi in their diet. Collembola have a positive impact on plant diversity (Sabais et al. 2011) and play an important role in shaping fungal communities (Klironomos et al. 1992). Collembola and other soil fauna increase the decomposition rate of plant derived C through feeding (Bonkowski et al. 2000; Bradford et al. 2007).

Collembola feed on mycorrhizal fungi, which form symbiotic relationships with plants. The relationship between the ectomycorrhizal fungus and the tree is mutualistic, with the fungi receiving photosynthetic carbon and in return aiding the plant with access to nutrients and with water uptake. At high Collembola densities, grazing can damage the hyphal connections, which can be detrimental to plant growth (Warnock et al. 1982; Harris and Boerner 1990; Ek et al. 1994).

Collembola feed on both endomycorrhizal and ectomycorrhizal (ECM) fungi (Shaw 1998; Gagne 2000; Scheu and Folger 2004; Ngosong et al. 2014; Kanters et al. 2015; Anslan et al. 2016). In conifer forests, ECM hyphae may represent between 47 to 84% of the soil's biomass (Baath et al. 2004). ECM fungi form a mantle of fungal tissue and networks of hyphae, representing a highly accessible food source for Collembola. As

with ectomycorrhizal hyphae, Collembola grazing on ECM mantles would have a detrimental effect on the plant host, due to the nutrient transport and storage that the mantle provides (Martin and Plassard 2001).

There have been very few studies in which Collembola have been fed ECM fungi in symbiosis with roots (Ek et al. 1994; Setälä 1995; Kaneda and Kaneko 2004; Kanters et al. 2015). Instead, most researchers utilize pure fungal cultures, the nutrient content of which is dependent on the media used. Nevertheless, some of these studies still showed that Collembola exhibited preferences among ECM fungal species (Schultz 1991; Hoil et al. 1994; Kanters et al. 2015). Another drawback of using pure fungal cultures of ECM fungi in feeding studies is that they lack the unique structures that arise when ECM fungi are in a symbiotic relationship with plant roots. Since trees transfer a significant portion of their fixed carbon to ECM fungi, and ECM decomposition rates vary across fungal species (Kiode et al. 2009; Kiode et al. 2011), it is important to understand the mechanisms involved in ECM decomposition. Collembola, along with microbial decomposition, are likely a major factor in the breakdown of ECM.

Some groups of ECM fungi appear to be relatively unpalatable to Collembola, including members of the *Russulaceae* (*Russula* and *Lactarius*) and possibly *Piloderma spp*. Many species of *Russulaceae* produce secondary metabolites known as sesquiterpenes (Sterner et al. 1985), and some *Piloderma sp*. produce calcium oxalate crystals on their hyphae. Both features are thought to act as antifeedant mechanisms that are believed to deter grazing by soil fauna (Böllmann et al. 2010). However, secondary metabolite production can vary based on the species of fungi and the soil conditions, and animals react to them in different ways, so there is a possibility that certain species of

Collembola may not be affected by these compounds. Collembola and other soil organisms generally prefer to feed on melanized fungi (Maraun et al. 2003; Bollmann et al. 2010). This is of interest, as the ECM formed by *Cenococcum geophilum* are highly melanized but remain in soil for years after its symbiotic partner expires (Koide & Malcolm 2009; Fernandez et al. 2013). This anomaly is explored in detail in the current work.

Through this study, I examined feeding preferences of Collembola across different ECM fungal species; something that has not been widely studied. ECM from various fungal species were assessed for palatability to *Folsomia candida*. Some types of ECM with suspected antifeedant mechanisms were expected to not be palatable. Experiments in which the basal hyphae from sporocarps and the ECM of each fungal species were presented were conducted to determine if there was any difference between the grazing of colonized root tips and soil hyphae. It was expected that the soil hyphae would be grazed more readily that the ECM of the same species due to the hyphae lacing antifeedant strategies. A comparison of feeding preference of highly melanized fungi was also performed and I hypothesized that *Cenococcum geophilum* ECM decompose relatively slowly because they are unpalatable to Collembola.

Methods

Site description

Collection of ectomycorrhizal fungi - both ECM and sporocarps - took place in a forest that is adjacent to the Blue Mountain-Birch Cove Lakes Wilderness area (44^{o}41' 26.67" N, 63^{o}41' 38.58.58" W, 71 M elevation) (Figure 2-1), near Kearney Lake and the

Maskwa Aquatic Club, in Halifax, Nova Scotia. Daily average temperatures have a range of -4.1°C in January to 19.1°C in August. The total annual precipitation for the area is 1468.1 mm (Environment Canada Climate Normals 2010). The soil profile for the area is a Lithic Humo-Ferric Podzol. The average organic horizon temperatures were 7.8^{o}C (November) and 16.6^{o}C (July). The average moistures were 35.8% (November) and 27.9% (July) (LeFait et al. 2019). The average pH was 6.2 (LeFait et al. 2019). The forest is dominated by mature *Pinus strobus* (white pine), but also contains *Picea rubra* (red spruce) and hardwood trees, including *Acer rubrum* (red maple).

Figure 2-1: An aerial photo showing the area where soil samples were collected for this project. The red flag shows the center of the field site (Google Map data 2019).

Collection of ECM

Three 10 cm^2 random samples of forest floor organic horizon (average 5 cm depth) were collected for morphological assessment of the proportions of ECM

morphotypes on the site. Samples were taken along a transect with a 2.5 cm soil corer (down to mineral soil) to identify distinctive ECM types that could be readily re-collected. Twelve haphazard samples were taken along a 130 m transect to collect ECM for use in feeding trials. The samples were assessed to determine the ECM morphotypes present at the site, and to determine which types would be suitable to use in grazing preference experiments. The rationale for this was to gain a general idea of what types of ECM were present on the site and to ensure that they could be located again as needed. This initial survey took place in September of 2015, and samples were collected as needed throughout the duration of the project.

Morphological identification of ECM

Pine ECM were chosen for this project because of their larger size compared to other ECM root tips, as well as their unique bifurcation, simplifying identification of the host tree (Figure 2-2). The 12 random samples were rinsed through a sieve to remove all the soil, and the roots were viewed with a dissecting microscope (Nikon model SMZ800, Nikon Inc., Tokyo, Japan) in distilled water. Five distinct ECM types were identified morphologically (Agerer 1987-1996) and chosen for use in feeding trial experiments. These ECM types were selected because they represented a wide range of ECM fungal groups and morphotypes. Fruiting bodies, collected for basal hyphae, were identified morphologically.

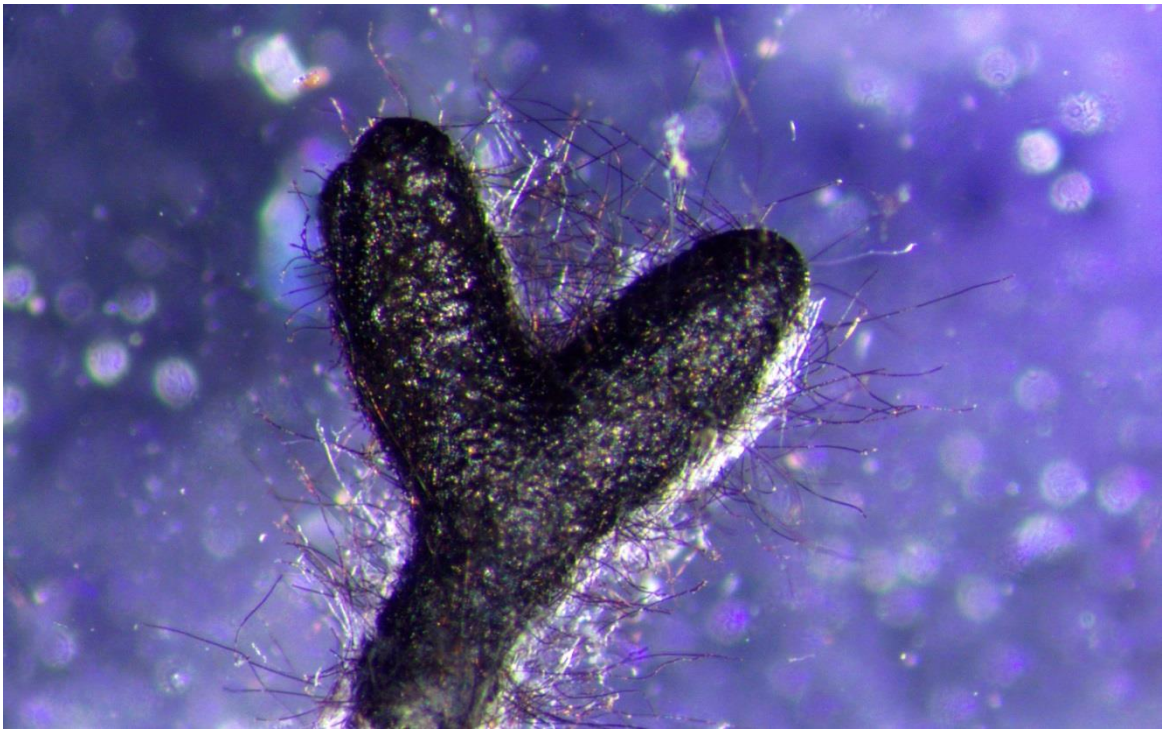

Figure 2-2: A *Cenococcum geophilum* root tip exhibiting the unique bifurcation of pine mycorrhizae.

Molecular identification of ECM

ECM types with distinctive morphologies were selected for identification by DNA sequencing. ECM tips from each of the five morphotypes that were collected from the study site were placed in a root washer to remove any debris and to ensure that there would be no contamination from other fungi. Root washers were constructed from a centrifuge tube with a piece of fine mesh covering each end. The tube is clamped to a ring stand and tap water is run through in order to clean the root tips. DNA was extracted from the ECM using the DNeasy mini plant kit (Qiagen, Hilden, Germany).

PCR reactions with a volume of 50 µl were performed on a Veriti 96 Well Thermocycler (Applied Biosystems, Foster City, CA). Each reaction included 25 µl GoTaq® Green Master Mix (Promega, Madison, WI), 5 µl each of the primers ITS1f (Gardes and Bruns 1993) and NL6C2 (Kernaghan et al. 2017) (IDT, Coralville, IA; each

at 0.2 μM final concentration) and 15 μl of DNA (diluted 1:10). PCR parameters were: 94°C for 85 s, followed by 35 cycles of 95°C for 35 s, 55°C for 60 s, and 72°C for 45 s, with elongation at of 72°C for 10 min. Products were then run on 1.5% agarose gels with 1X sodium-borate buffer and EZ-vision gel dye (Amresco®, Solon, OH). An EC 105 electrophoresis power supply (E-C Apparatus Corporation, USA) was used to run the gels. Gels were imaged at 365 nm wavelength using an AlphaImager HP gel imaging system (ProteinSimple, San Jose, CA).

PCR products and primers were sent to McGill University and Genome Quebec Innovation Centre for sequencing using the primers ITS1 and ITS4 (White et al. 1990). Resulting sequences were aligned using Sequencher 5 software and trimmed at the primer sites using BioEdit software. Consensus sequences were identified by using BLAST against the NCBI and Unite (Kõljalg et al. 2005) databases.

Grazing Preference Experiments

To test the preference of Collembola grazing on various ECM types, experimental arenas to hold feeding trials were built (Figure 2-3). Petri dishes (100 x 15 mm) (VWR 25384-302 Petri Dish, Polystyrene, VWR®, Radnor, Pennsylvania) were filled 3.5 mm deep with an activated charcoal-plaster of Paris mix (4 parts charcoal, 8 parts plaster of Paris, 5.5 parts water) (Gist et al. 1974). The mixture was then allowed to dry and harden for one week. After the mixture had set, the dishes were re-wetted using distilled water, and the excess charcoal was rinsed from the surface. A piece of Pellon® 830 Easy Pattern interfacing (70% polyester, 30% rayon) was placed over top of the surface, and a ring of plastic and hot glue were used to seal the edges of the Pellon to the dish. A 2 cm^2 piece of

1 mm graph paper was covered with tape and placed in each dish under the various fungi used in experiments.

A culture of *Folsomia candida,* a model Collembolan species, was acquired from a commercial distributor (Understory Enterprises, Chatham, Ontario), for use in grazing experiments. The supplier reared *Folsomia candida* on a potting soil mixture, so cultures were transferred and maintained in Mason jars containing the same charcoal/plaster mix described above. This was done to ensure that *Folsomia candida* did not eat the potting soil and so that they would be acclimated to the charcoal/plaster mix used in feeding trial arenas. The Collembola were fed baker's yeast and watered weekly to ensure that desiccation did not occur. Before use in experiments, Collembola were starved for 48 hours to ensure they were driven to eat the food sources presented to them (Shaw 1998). For each feeding trial, ten Collembola were added to each arena, and the dishes were sealed with parafilm. Petri dishes were kept in a dark area, at 25°C, and were assessed visually after one week for moisture levels, and if needed water was added to the Petri dish. Experiments were conducted for a two week period.

There were two experimental set-ups, single ECM choice and dual ECM choice. In experiments where a single ECM type was presented to Collembola, a 3 mm^2 area of fresh fungal material was placed on graph paper in the center of the Petri dish. Three replicate arenas were prepared for each trial (1 arena per Petri dish), for a total of 15 arenas. In experiments with two different ECM types, ECM covering 2 mm^2 areas were placed on graph paper 4.5 cm apart. All combinations of ECM were prepared on 3 replicate Petri dishes (Figure 2-3) (30 in total). The single choice experiments simply demonstrate that *Folsomia candida* will graze on particular ECM types if necessary, but

the dual choice experiments highlight the phenomenon of actual preference for one ECM type over another. Fecal pellets were used as the response variable because they were clearly visible and easy to measure (Figure 2-9). In both single and dual choice experiments, the fecal pellets of Collembola that were on the 2 cm^2 graph paper were photographed with a dissecting microscope and counted using ImageJ 1.52h software.

To ensure that the fecal pellets of *Folsomia candida* were located near to the food source that they were grazing on, a separate experiment was conducted. *Folsomia candida* were fed *Piloderma bicolor* ECM that was either dyed blue with food coloring (McCormick & Company, Inc.) or not dyed (natural yellow colour), using the method described above for dual choice experiments. Nine replicate plates were prepared, and fecal pellets were analyzed after two weeks to determine the location of pellets in relation to the color of the food source.

A comparison of grazing of ECM with basal hyphae of conspecific fruiting bodies was also conducted. A 2 mm^2 section of graph paper was filled with each ECM type or the basal hyphae of their conspecific fruiting body and placed 4.5 cm apart. Experiments were set up within 24 hours of specimen collection to ensure freshness. As with earlier feeding trials described, after two weeks pictures were taken, and fecal pellets were counted. Experiments were also conducted using intact *Suillus sparguei* tuberculate ECM versus tubercules of the same species with the fungal peridium removed. *Cenococcum geophilum* was omitted from this experiment as it does not form fruiting bodies.

Experiments on Melanized Fungi

Cenococcum geophilum ECM from *Pinus strobus* was tested against various forms of *Cenococcum*, as it appeared that Collembola did not graze readily on *Cenococcum geophilum*. This included: immature *Cenococcum geophilum* ECM on *Pinus strobus*, *Cenococcum geophilum* ECM on *Picea rubra*, fungal mantles separated from *Cenococcum geophilum* ECM and thoroughly macerated in liquid nitrogen, and *Cenococcum geophilum* mycelium from broth culture at two nitrogen levels in basal media comprised of 10 g glucose, 1 g magnesium sulfate ($MgSO_4$ 7H2O), 2 g potassium phosphate ($KH2PO_4$) and 1 ml micronutrient solution (ZnSO4, H3BO3 CuSO4) per liter. Nitrogen was supplied as ammonium sulfate ($(NH_4)2SO_4$), at either C:N 10:1 or C:N 100:1. *Pinus* ECM formed by *Tomentella* sp. was also included in this experiment, as it is another example of a melanized ECM fungus.

Table 2-1: Fungal pairing combinations for dual choice experiment.

Lactarius vinaceorufescens and *Suillus spraguei*
Lactarius vinaceorufescens and *Piloderma bicolor*
Lactarius vinaceorufescens and *Tricholoma aestuans*
Lactarius vinaceorufescens and *Cenococcum geophilum*
Suillus spraguei and *Piloderma bicolor*
Suillus spraguei and *Tricholoma aestuans*
Suillus spraguei and *Cenococcum geophilum*
Piloderma bicolor and *Tricholoma aestuans*
Piloderma bicolor and *Cenococcum geophilum*
Tricholoma aestuans and *Cenococcum geophilum*

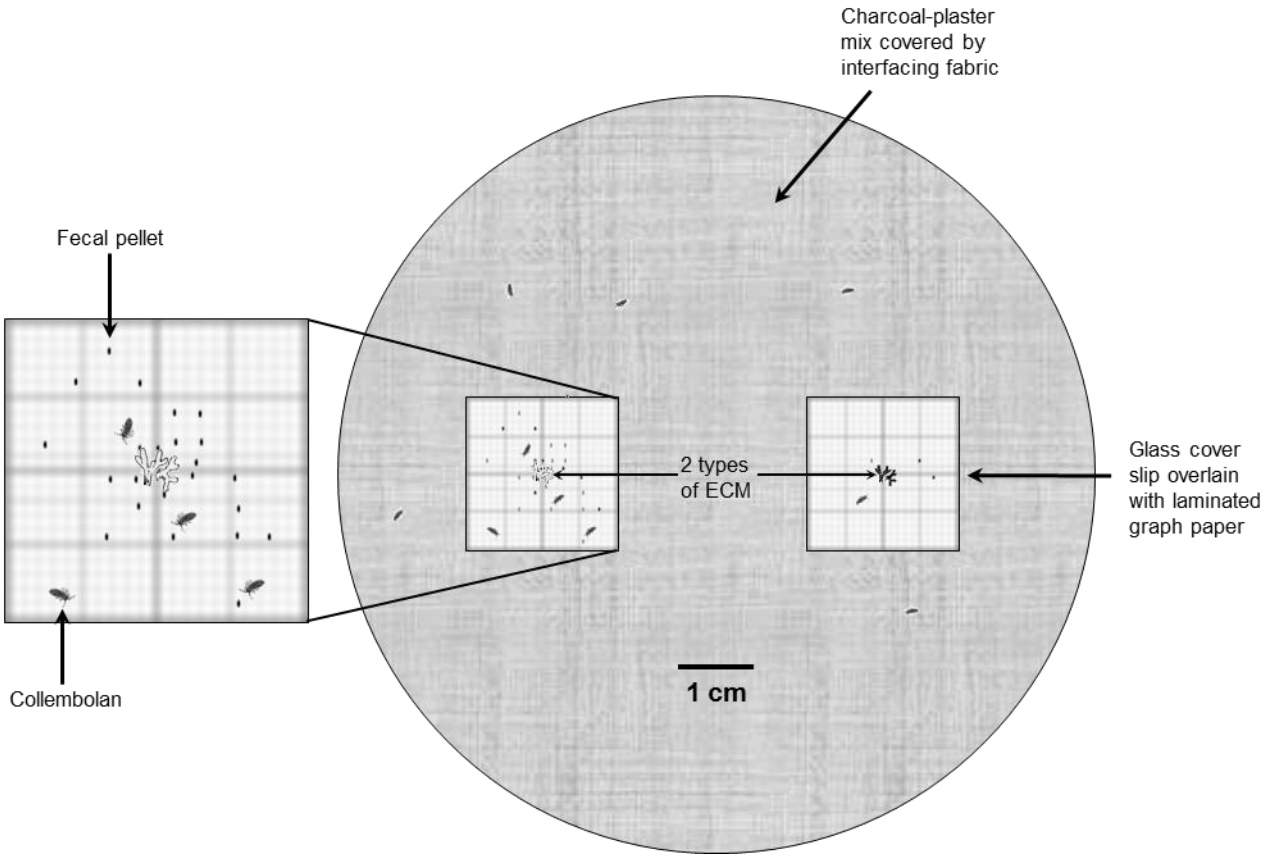

Figure 2-3: A diagram showing the set-up of the feeding trail arena. This is a dual choice set up. A single choice arena would have the cover slip in the center of the plate.

Analysis of Grazing Preference

For single choice experiments, data were analyzed by a one-way ANOVA followed by Tukey HSD to determine if there were any significant differences between the fungi offered as a single choice. For experiments with two choices, paired t-tests were performed to determine if there were significant differences between the two choices offered. The cases that were statistically significantly different were ranked to show a hierarchy of preference. All data were first normalized with a square root transformation and analyzed with GraphPad Prism 8 software.

Results

The sampled ECM community was dominated by Russulaceae (*Russula* and *Lactarius*) (56% of ECM) and *C. geophilum* (32% of ECM). Four other unidentified types at 8.5%, 2%, 1% and 0.5% made up the remainder of the ECM found. Russulaceae and *C. geophilum* were distributed evenly throughout the site, while other ECM types such as *Piloderma bicolor, Tomentella* sp. and *Tricholoma aestuans* were found in only one or two small isolated locations. *Suillus spraguei* ECM (tuberculate) were common but in localized patches. Table 1 shows the closest database match to each ECM used in the feeding trial experiments.

The fecal pellet location experiment in which *Piloderma bicolor* was used either in its natural hue, or dyed, revealed that there was very little crossover in the location of fecal pellets. Less than 1.5% of fecal pellets from either tip were near the opposite choice, which shows that Collembola deposit their fecal pellets near (within ~5 mm) to the food source that they are grazing on.

The feeding trials in which there was only one ECM choice presented to *F. candida* clearly showed that some types of ECM were preferred over others (ANOVA, $p < 0.001$). The preference hierarchy with only one ECM choice per plate was *Suillus spraguei* > *Tricholoma aestuans* = *Piloderma bicolor* > *Lactarius vinaceorufescens* = *Cenococcum geophilum* (Figure 2-4).

Preferences were also demonstrated when *F. candida* was presented with two choices. The preference hierarchy with two ECM choices per plate is as follows: *S. spraguei* = *T. aestuans* = *P. bicolor* > *L. vinaceorufescens* = *C. geophilum* (Figure 2-5).

In the experiment in which the tuberculate ECM of *S. spraguei* was presented to *F. candida* with the peridium intact or removed, there was no significant difference in the fecal pellet analysis between the two choices.

Grazing on ECM compared to basal hyphae from sporocarps of the same species gave variable results. Fecal pellets assessed were several times higher for the basal hyphae of *L. vinaceorufescens* than from its ECM in experiments involving a choice between basal hyphae and ECM ($p = 0.0243$, 2-7) and with a single fungal type per arena ($p = 0.0186$, Figure 2-6). *P. bicolor* hyphae had more fecal pellets than on ECM ($p = 0.0116$) when presented independently, but there was no statistical difference when a choice between the ECM and hyphae was offered ($p=0.1540$). For both *T. aestuans* and *S. spraguei*, there were no significant differences between the number of fecal pellets produced on basal hyphae and ECM when presented separately or as a choice.

When melanized fungi were assessed for their palatability to *F. candida*, various results were observed (Figure 2-8). *Cenococcum geophilum* presented to *F. candida* grown in culture at both high and low C:N ratios were statistically higher than all other melanized fungal types tested, including all *Cenococcum geophilum* collected from nature in all forms: on pine, on spruce, immature, macerated, and sclerotia. Cultures of *Cenococcum geophilum* were also grazed at higher levels than the melanized ECM of *Tomentella*, and *Tomentella* ECM was grazed at a significantly higher rate than that of *Cenococcum geophilum* ECM on pine. There was no statistically significant difference between *Cenococcum geophilum* collected from nature in all forms (on pine, on spruce, immature, macerated, and sclerotia), but the pine ECM was eaten at a slightly lower rate.

Table 2-2. Identities, closest database matches, and accession numbers sequences of ectomycorrhizae and Collembola used in feeding trials.

Identity	Closest Database Match	Coverage	Similarity	GenBank Accession #
Ectomycorrhizae (ITS rDNA)				
Cenococcum geophilum	*Cenococcum geophilum* strain CC042r (LC095050)	100%	99%	MH809945
Lactarius vinaceorufescens	*Lactarius vinaceorufescens* voucher JN2007-018 (KF241542)	97%	99%	MH809946
Piloderma bicolor	*Piloderma bicolor* voucher UC2023238 (KP814514)	97%	100%	MH809947
Suillus spraguei	*Suillus pictus** isolate AFTOL-ID 717 (AY854069)	98%	99%	MH809948
Tomentella sp.	Uncultured *Thelephoraceae* clone 34D (KP403037)	100%	99%	MH809949
Tricholoma aestuans	*Tricholoma aestuans* genomic DNA MC94008 (LT000007)	91%	98%	MH809950

**Suillus pictus is a synonym of S. spraguei*

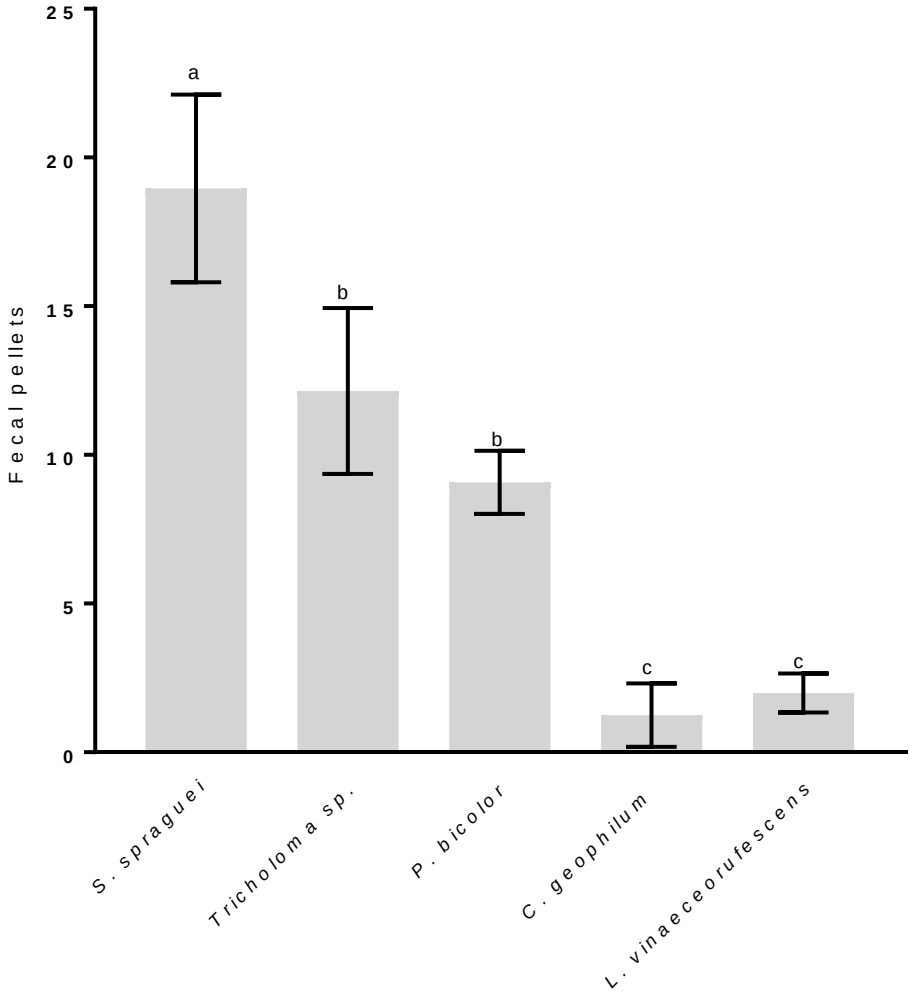

Figure 2-4: Number of fecal pellets produced after two weeks during feeding trials with *Folsomia candida* and five different ECM types. Error bars represent standard error of the mean. A single mycorrhizal type was provided for each trial. Mycorrhizal types with different letters are significantly different (Tukey Test). Bars represent the average of six replicates.

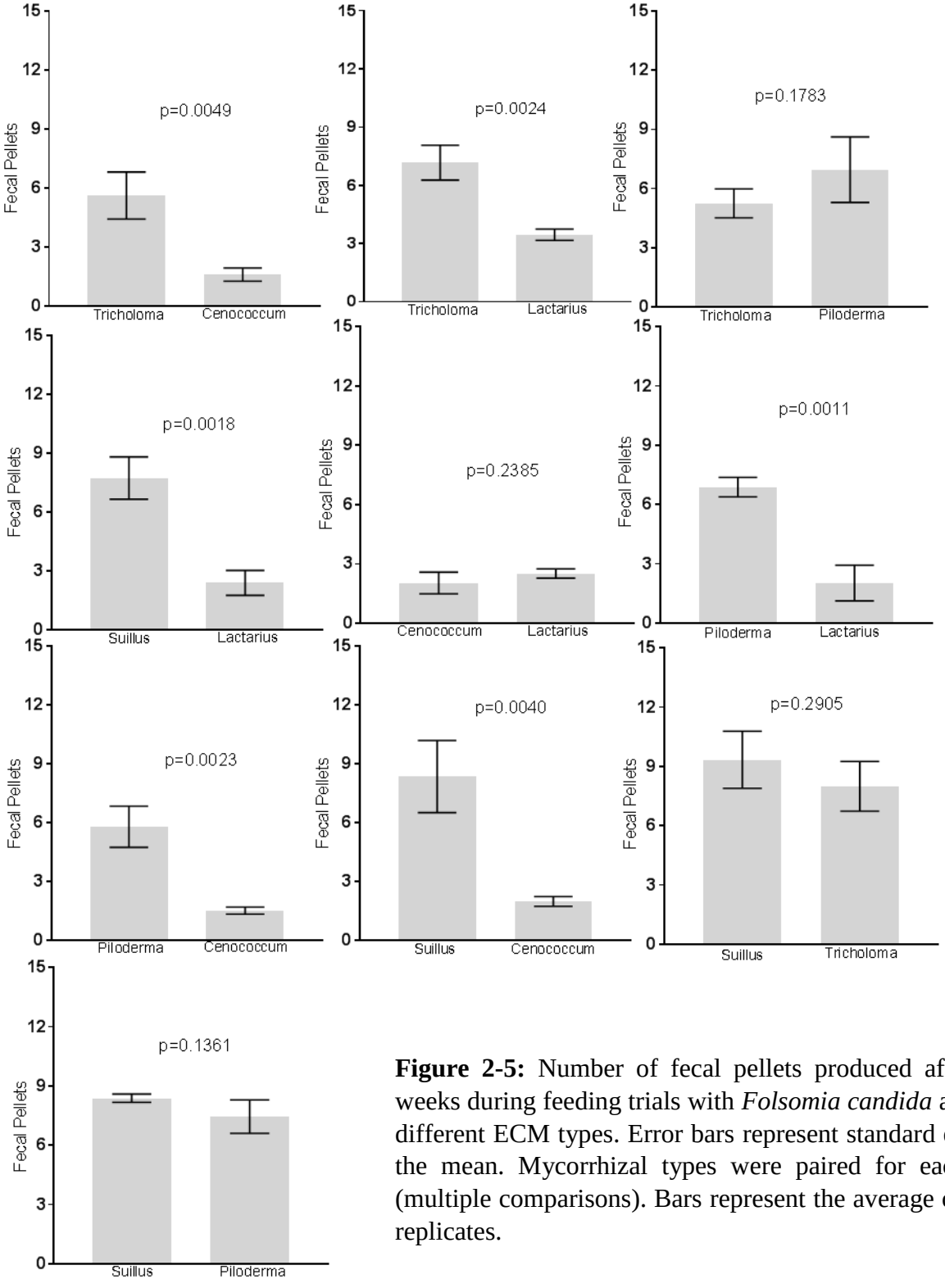

Figure 2-5: Number of fecal pellets produced after two weeks during feeding trials with *Folsomia candida* and five different ECM types. Error bars represent standard error of the mean. Mycorrhizal types were paired for each trial (multiple comparisons). Bars represent the average of three replicates.

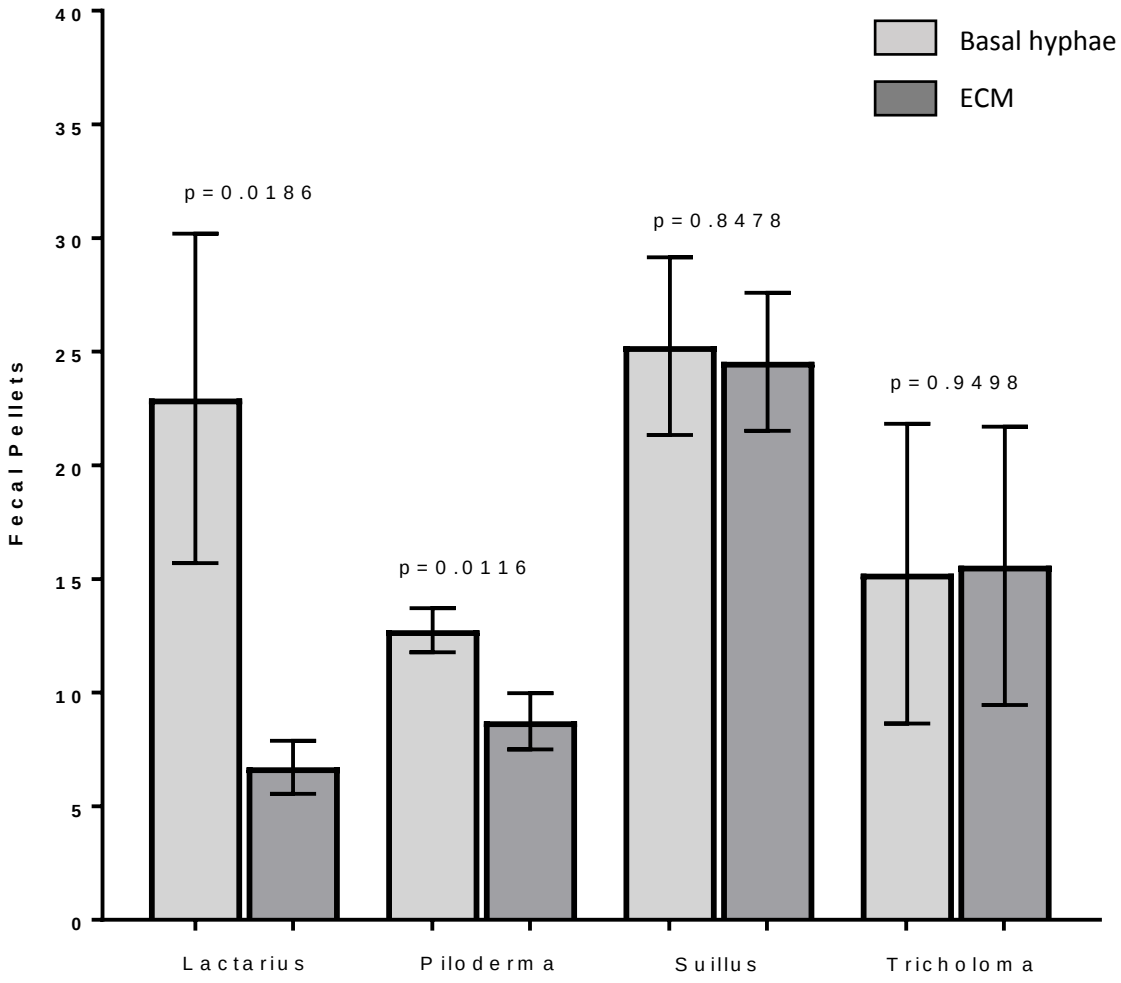

Figure 2- 6: A comparison of basal hyphae and ECM presented on their own to *F. candida*. Error bars represent standard error of the mean. Fecal pellets produced when mycorrhizal types were alone (multiple comparisons). Bars represent the average of three replicates.

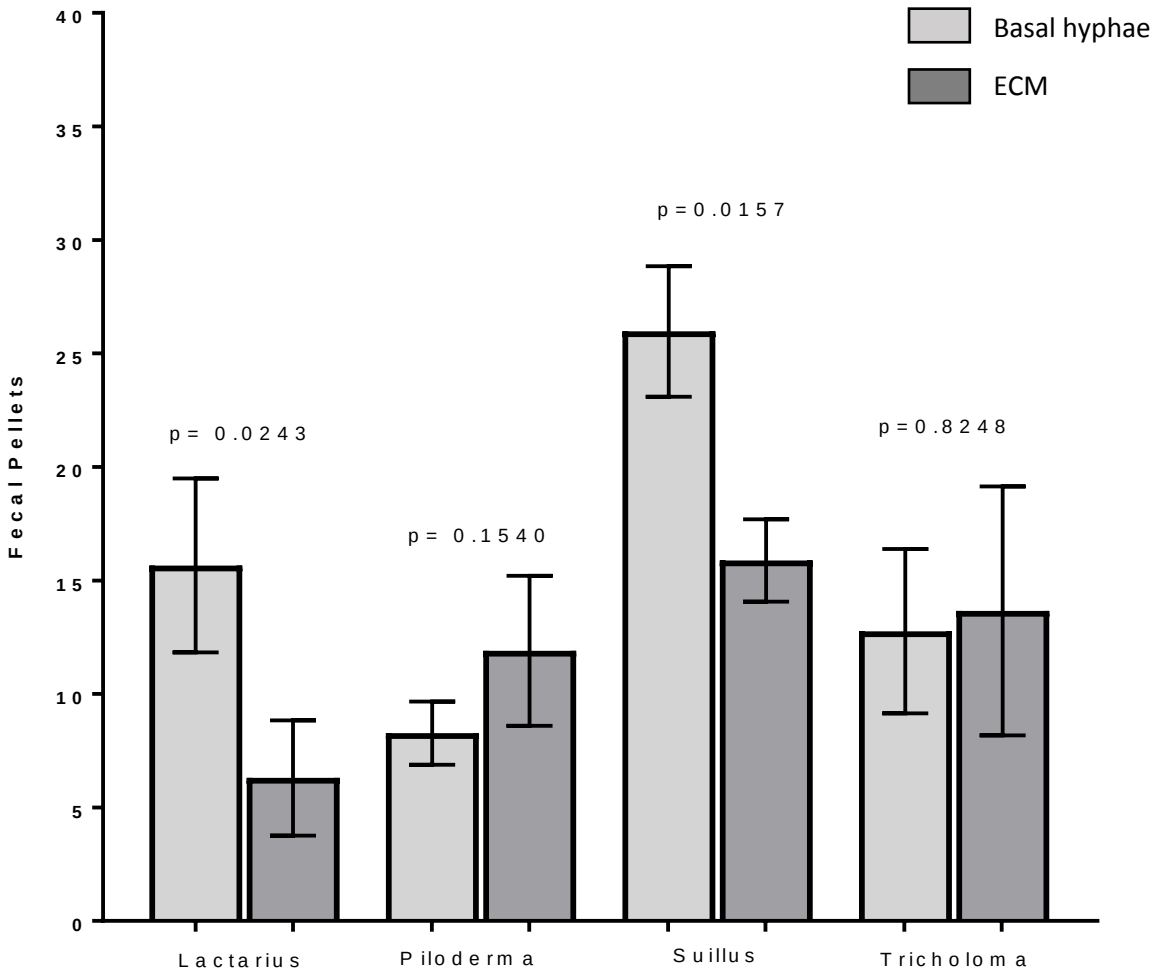

Figure 2-7: A comparison of basal hyphae and ECM presented as a choice to *F. candida*. Error bars represent standard error of the mean. Fecal pellets produced when mycorrhizal types were paired (multiple comparisons). Bars represent the average of three replicates.

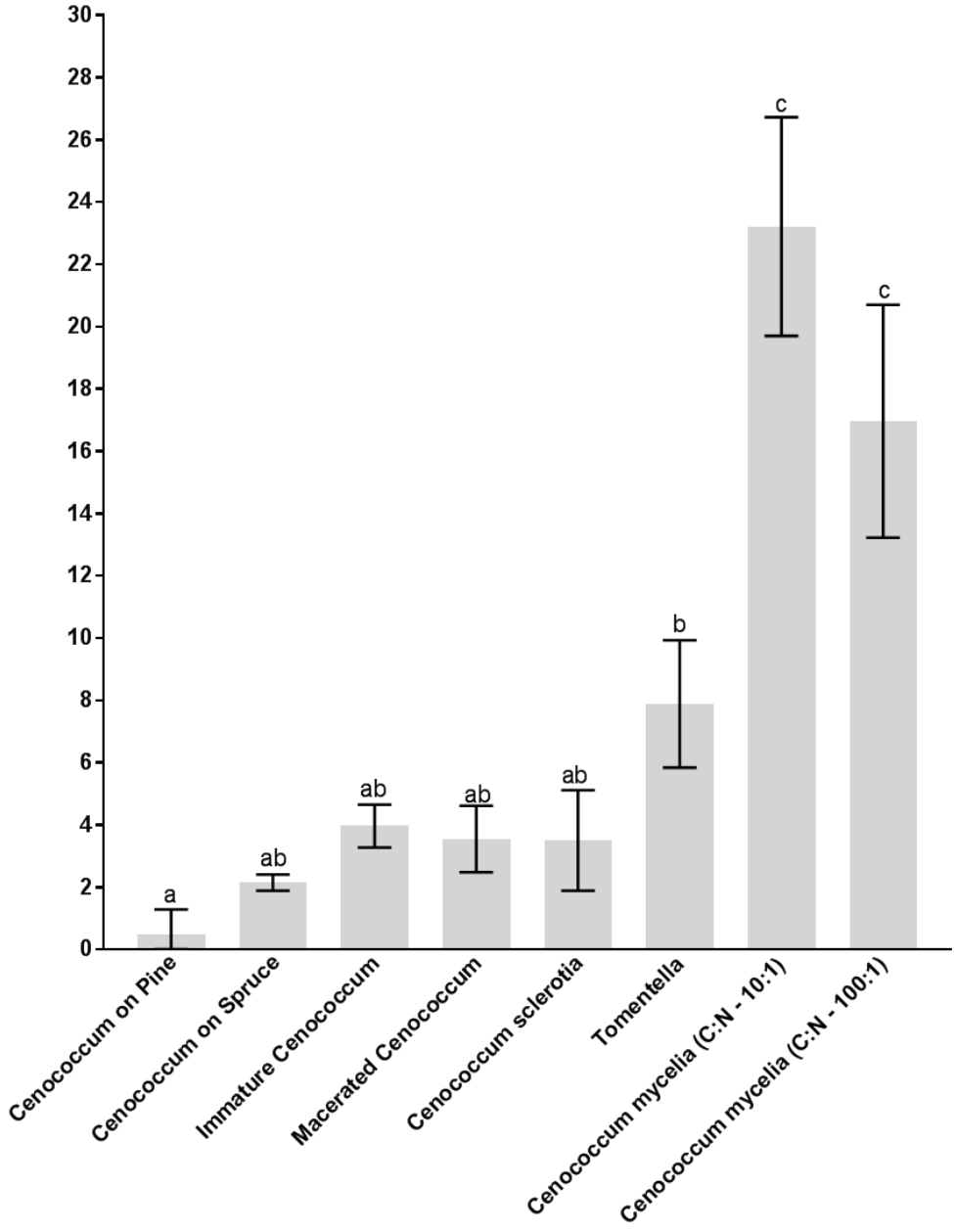

Figure 2-8: Comparison of palatability of melanized fungal material to *F. candida* based on number of fecal pellets produced after two weeks. Food types were presented on their own. Each bar represents an average of three replicate feeding trials. Values with the same superscript letters are not significantly different. For macerated *Cenococcum*, ECM mantle tissue was separated from root tissue and macerated in liquid nitrogen.

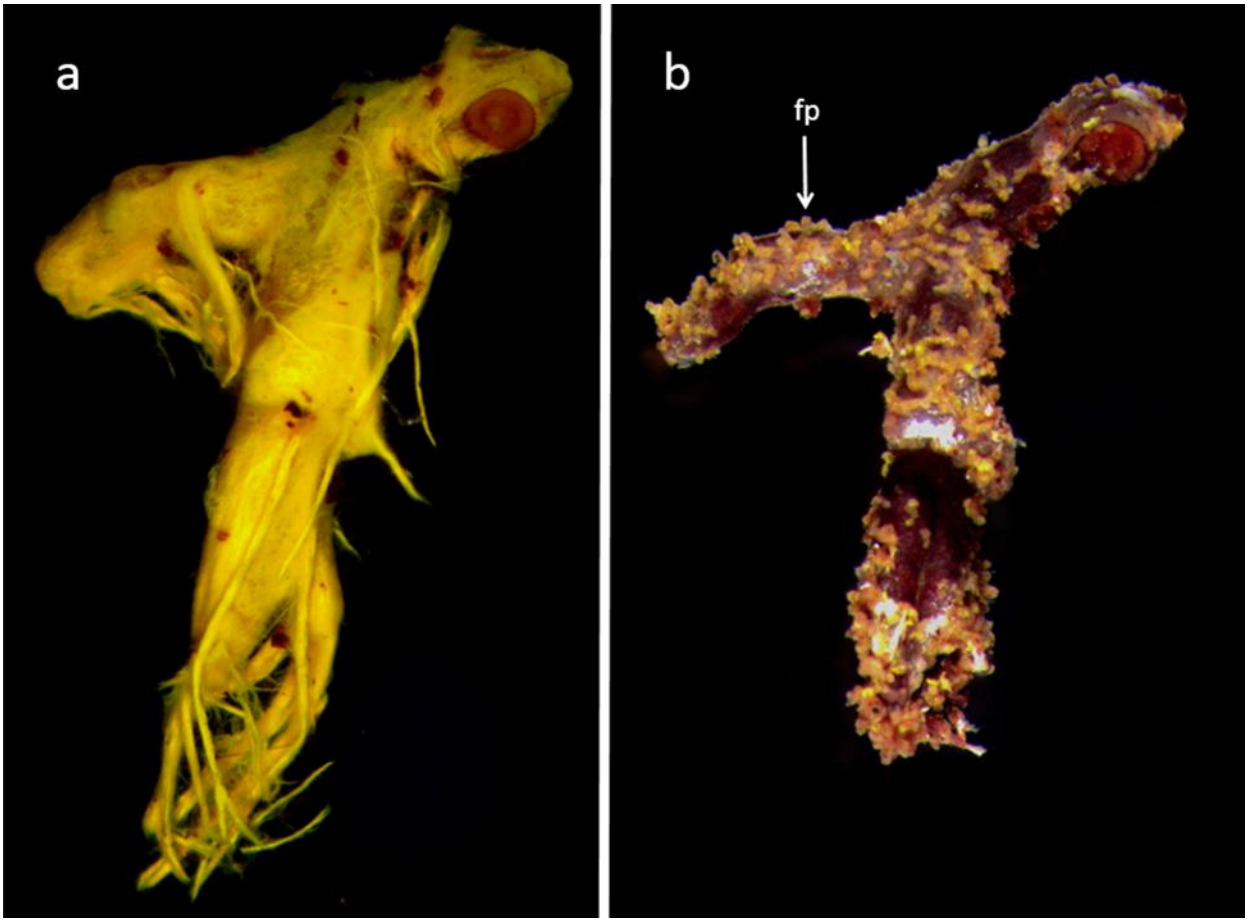

Figure 2-9: An example of a *Piloderma bicolor* ECM root tip used in a feeding trial experiment showing the root tip a) before and b) after feeding has taken place (fp= fecal pellet).

Discussion

ECM Palatability

The most palatable ECMf, *S. spraguei,* was the only ECM type in the feeding trails that forms tuberculate ECM. Tuberculate ECM are unique structures, in that the ectomycorrhizal root tips are located within a fungal peridium, resulting in a low oxygen environment in which nitrogen fixation can occur (Paul et al. 2007; Paul et al. 2012). Collembola grazing on this type of ECM could therefore cause damage to the host plant not only through severing of exploratory hyphae, but also by compromising the nitrogen

fixing capabilities of the tubercle. It is possible that *S. spraguei* was the most preferred ECM because it represented the highest quality food source presented (Booth and Anderson 1979; Usher et al. 1982), although Wallander et al. (1997) found no difference between the protein content of tuberculate ECM and other ECM types. *S. spraguei* may have been preferred as it does not possess any obvious microarthropod antifeedant mechanisms. *S. spraguei* is also the only fungus offered that produces safe edible sporocarps for human consumption (Bessette et al. 2016).

The second most palatable ECMf was *T. aestuans*, the sporocarps of which carry the common name of "the Acrid Knight", due to their extremely acrid taste to humans. This is likely due to the same foul-smelling bitter indole derivatives found in close relatives of *T. aestuans* (Garlaschelli et al. 1994). Although the bitterness of *T. aestuans* would seem to be a potent antifeedant mechanism, it did not seem to have a negative effect on *F.candida*, as it was readily consumed.

The ECMf *P. bicolor* was the third most palatable. Calcium oxalate crystals are often found encrusting the hyphae of *P. bicolor* (Arocena et al. 2001), and Böllmann et al. (2010) proposed that these might act as a deterrent to microarthropod grazing. However, *F. candida* readily consumed *P. bicolor* ECM in the present study. This might be explained by the fact that our local soils are calcium deficient (Lawrence et al. 2015), which may reduce the abundance of calcium oxalate crystals formed. Indeed, crystals were not seen in H_2O squash mounts of *P. bicolor* ECM from the study site.

L. vinaceorufescens was one of the least preferred types in the ECM in the hierarchy of ECMf palatability. *L. vinaceorufescens* and other members of the Russulaceae produce peppery tasting, toxic sesquiterpene dialdehydes derived from their

milky latex as defense mechanisms (Sterner et al. 1985; Stadler and Sterner 1998; Clericuzio et al. 2008; Malagòn et al. 2014). This antifeedant appears to have had a deterrent effect on Collembola because *L. vinaceorufescens* was minimally grazed even when it was the only food source available.

Cenococcum geophilim was also among the least preferred ECM types and was rarely grazed even when it was the only food choice available to *F. candida*. *Cenococcum geophilum* is one of the most widespread ECM fungi (Trappe 1962) and comprised 32% of the ECM at the research site. Although it degrades slowly due to the fact that the high melanin content of its hyphae inhibits microbial activity (Fernandez et al. 2013), it does not appear to possess any specific microarthropod antifeedant mechanism, as microarthropods are generally thought to prefer darkly pigmented fungi as a food source (Maraun et al. 2003). These factors led to the decision to look more closely at *C. geophilum* as a food source for Collembola.

As all of the ECM root tips fed to *F. candida* in the single and dual choice experiments were associated with *Pinus,* it was important to also determine if the symbiotic host played a role in the palatability of *C. geophilum* ECM. *Folsomia candida* did not graze *Picea - C. geophilum* ECM any more than those of *Pinus,*, indicating that species of conifer host does not play a major role in the palatability of *C. geophilum*. Macerated *C. geophilum* ECM mantle was also not palatable to *F. candida*, indicating that a chemical, rather than physical, antifeedant mechanisms was protecting *C. geophilum* ECM. Another interesting result was that immature *C. geophilum* was grazed at a higher rate than mature *C. geophilum,* which could indicate a build-up of an antifeedant chemical over time. Other authors have found *C. geophilum* hyphae to be

palatable to Collembola when grown in pure culture (Böllmann et al. 2010), or possibly as soil mycelium (Ponge et al. 1997). The current results also show that this is not merely due to the artificially high nitrogen content of nutrient media. This is yet more evidence that *C. geophilum* ECM may possess a chemical antifeedant mechanism present when in association with a symbiotic partner (Duhamel et al. 2013). The observed aversion of *F. candida* to *C. geophilum* ECM might therefore be an additional factor, in combination with the microbial inhibition from melanization (Fernandez 2013), explaining the slow turnover rate of *C. geophilum.*

Basal Hyphae

Although these experiments gave variable results, all fungal species were palatable as basal hyphae, while unpalatability was seen only in ECM (*L. vinaceorufescens* and *C. geophilum* ECM). *C. geophilum* does not produce sporocarps, but the mycelium was palatable in pure culture. This would seem to be further evidence for the presence of antifeedant mechanisms in some ECM types that, whether produced by the host plant or by the fungi, are only produced when the two are in symbiosis.

Conclusion and Future Directions

The Russulaceae represent one of the most diverse groups of ECMf with an estimated 2000 species (Looney et al. 2018), and were dominant at the research site. Another ECMf that comprised a large portion of the community was *C. geophilum,* which has a wide habitat range (Trappe 1964) and is considered to be one of the dominant ECM species in temperate forests (Trappe 1964; Molina and Trappe 1982). As

these two ECM types were also the least palatable to Collembola, it seems that local ECMf species composition may have a large effect on the grazing habits of Collembola and other microarthropods, which may in turn influence the relationship between the plant and its mycorrhizal symbiont (Warnock et al. 1982; Harris and Boerner 1990; Klironomos et al. 1992; Ek et al. 1994; Bradford et al. 2007).

This study has demonstrated that *F. candida* finds certain ECM types palatable but not others. Since some ECMf have developed mechanisms to deter microarthropod grazing, one might infer that these adaptations have arisen in response to soil faunal grazing pressure. Furthermore, the sheer abundance of Collembola in forest soils is enough that even if ECM are not the first food choice, there should still be enough ECM grazing to have an impact.

This study presented only a small number of ECMf species to Collembola. In the future, it would be interesting to test a wider range of ECMf for their palatability. Also, this study included only one Collembola species - *F. candida* - as a model organism to test the palatability of ECMf. Future experiments should be conducted using more species of Collembola to see if there are microarthropod species dependent differences in ECM grazing. Research should also be conducted into the palatability of certain ECMf in association with a wider variety of host species, as this would provide more evidence regarding the presence of chemical deterrents that might only be produced when certain fungi are in symbiosis with particular plants.

Chapter 3

Fungi in gut contents of field collected microarthropods

Abstract

The gut content of microarthropods were assessed for the presence of ECMf. Collembola and Oribatid mites are known fungivores. Oribatid mites and Collembola were extracted from pine forest soils, and the most common of each were identified morphologically and by COX I sequencing. Mites were determined to be a species from the family Phthiracaridae and Collembola were *Folsomia* sp. The fungal communities within the guts of these microarthropods were then characterized by constructing and sequencing ITS clone libraries. Fungal diversity was very low within both the mites and the Collembola, and no ectomycorrhizal fungi (ECMf) were detected. The Chaetosphaeriaceae and the yeast *Tuenomyces kruisii* were detected in both microarthropods, and *Penicillium spinulosum* and an unidentified fungal endophyte were found only in the mites. The microarthropods assessed for gut fungi tend to inhabit the upper layers of the forest litter and may not routinely come into direct contact with ECM roots, explaining the lack of ECMf species.

Introduction

Ectomycorrhizal fungi (ECMf) are important symbiotic partners with the roots of woody plants (Fogel and Hunt 1983), facilitating plant establishment, growth and survival by trading water and soil nutrients for photosynthetically fixed carbon (Smith and Read, 2008; Sebastiana et al. 2017). There are estimated to be between 20,000 - 25,000 species of ECMf (Rinaldi et al. 2008; Brundrett 2009; Roy-Bolduc et al. 2016) and ECMf mycelium can account for up to 84% of soil biomass in forests which was assessed with fungal DNA (Baath et al. 2004).

Although there is an abundance of information on the initiation and development of ECMf, there is a lack of knowledge on their breakdown, which releases carbon and

nitrogen back into soil (Bååth 2004). Clearly ECMf are important in forest soils, but their ultimate fate is not well understood.

Many types of microarthropods are fungivores, including Collembola and Oribatid mites (Bardgett et al. 1993; Hopkin 1997; Ponge et al 2000; Jørgensen et al. 2005). Schneider et al. (2005) demonstrated that Oribatid mites will eat ECMf when grown in pure culture, but to the best of my knowledge, no studies have been conducted on Oribatid mite grazing of ECMf in the field. In one study, Oribatid mites are considered to be "choosy generalists", in that they will eat whichever fungi are available when options are limited, but tend to prefer darkly pigmented (melanized) fungi when given a choice (Schneider and Maraun 2005).

Collembola are generalist feeders that prefer fungi (Shaw 1998; Bardgett et al. 1993; Hopkin 1997; Fountain and Hopkin 2005; Jørgensen et al. 2005; Kanters et al. 2015). Collembola feed on both ECMf and the endomycorrhizal (arbuscular mycorrhizal) fungi of non-woody plants (Shaw 1998; Gagne 2000; Scheu and Folger 2004; Ngosong et al. 2014; Kanters et al. 2015; Anslan et al. 2016). Unlike mites, there has been a fair amount of research conducted on Collembola and ECMf, with numerous studies offering ECMf grown in pure culture, and some studies on grazing of ECM when in symbiosis with roots (Ek et al. 1994; Setälä 1995; Kaneda and Kaneko 2004; Kanters et al. 2015). Anslan et al. (2016) detected ECMf in the gut contents of epidaphic (above ground) Collembola, and suggested that at least some of this was the result of Collembolan feeding on sporocarps. It is important to consider that Collembola as well as Oribatid mites are generalist feeders, and will eat a wide variety of fungi, not just mycorrhizal fungi.

The previous chapter examined variation in ECMf palatability to cultured Collembola *invitro*, while this chapter deals with the grazing of microarthropods on ECMf in the field. The aim was to determine which fungal species are found in the gut contents of both field-collected Collembola and Oribatid mites, given all the food choices of a natural soil. It was hypothesized that both Collembola and Oribatid mite gut contents would contain at least some ECMf due to the high ECMf hyphal biomass estimated for forest soils.

Methods

Field Collection of Collembola and Oribatid Mites

Collembola and mites were collected from three sites within the pine stand described in Chapter 2. I selected collecting sites that represented distinct habitats within the larger pine stand. Site 1 was chosen because the litter was a mixture of coniferous and deciduous leaves. Site 2 and Site 3 were chosen because they were moss covered and relatively moist (Site 3 was covered in *Sphagnum*). Sites were 30-50 m apart.

Soil samples (approx. 1 kg) were collected from each of the three sites in October 2017 (down to the mineral layer). Two Tullgren funnels (Figure 3-1) were used to extract the microarthropods from each soil sample (Tullgren 1918). Each funnel consisted of an 8.5-inch clamp light with a 40-watt light bulb, a ring stand, a ring clamp, a funnel constructed from a 2 L soda bottle, and wire mesh (approximately 0.5 mm) inside the funnel. A 250 ml Erlenmeyer flask containing a 50% solution of ethanol was placed beneath each funnel for collection.

Funnels were left for four to seven days, depending on how long the soil took to fully dry out. The Erlenmeyer flasks with the extracted microarthropods were then covered with Parafilm and refrigerated. Samples from each site were viewed with a dissecting microscope (Nikon model SMZ800, Nikon Inc., Tokyo, Japan) and sorted based on morphology. There were various species of Collembola and mites present, but only the dominant Collembola and the dominant mite (found at all three sites) were selected for processing (Figures 3-2 and 3-3).

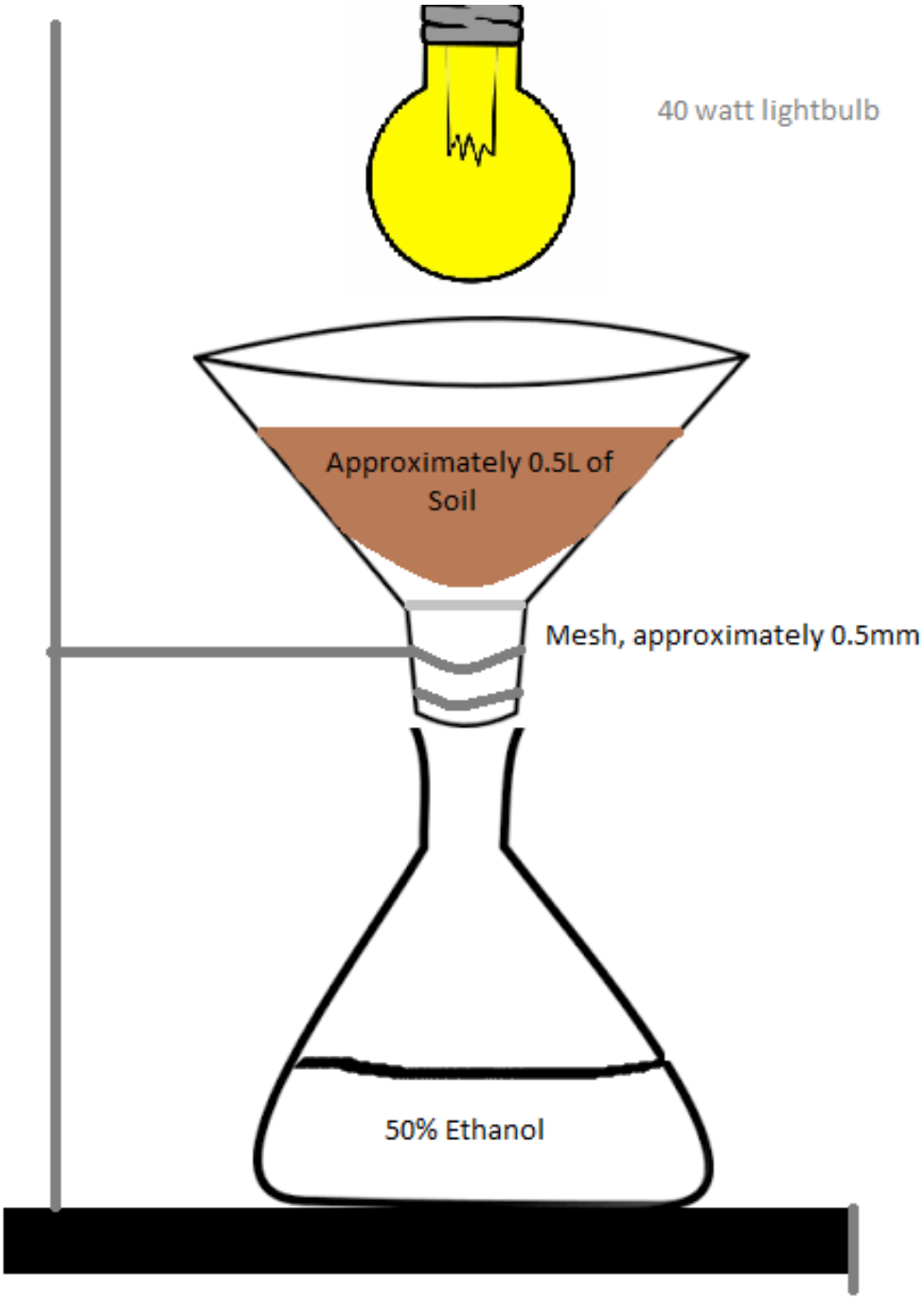

Figure 3-1: The set-up of a typical Tullgren funnel.

Identification of Collembola and Mites

After the Collembola from each of the three sites were sorted into similar morphotypes, the dominant Collembolan were sent to Dr. Jeff Battigelli from the University of Alberta's Department of Renewable Resources for expert identification, and also identified by COX I sequencing as follows.

For each microarthropod group, ten individuals from each site were observed under the dissecting microscope to make sure that no contaminating debris was present. DNA was then extracted from the dominant Collembola from the sites using the DNeasy mini plant kit (Qiagen, Hilden, Germany). The mitochondrial cytochrome c oxidase subunit I (COX I) gene was then amplified with the primers LCO1490 and HC02198 (Folmer et al. 1984). PCR Reactions with a volume of 50 µl were performed on a Veriti 96 Well Thermocycler (Applied Biosystems, Foster City, CA). Each reaction included 25 µl GoTaq® Green Master Mix (Promega, Madison, WI), 5 µl each of the primers, each at 0.2 µM final concentration (IDT, Coralville, IA), and 15 µl of DNA (diluted 1:10). The PCR parameters were: 94°C for 4 minutes, 10 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min and 30 s; then, with 25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C 1 min and 30 s; and a final extension at 72°C for 8 min (Potapov et al. 2010). The PCR products were then sent to McGill University and Genome Quebec Innovation Centre for sequencing using the same primers. The resulting forward and reverse sequences were then used to construct consensus sequences with Sequencher 5 software and trimmed at the primer sites using BioEdit software. Consensus sequences were then identified by comparing them to NCBI database accessions (GenBank) using BLAST. The phylogenetic tree of *Folsomia* species was created with PAUP to show the relationship

between the *Folsomia sp.* used in the gut content experiments and the *Folsomia* species with COX I sequences available on GenBank.

Figure 3-2: An image of the Collembola chosen for gut content sequencing. The species was determined both by a microarthropod expert and by COX I sequencing.

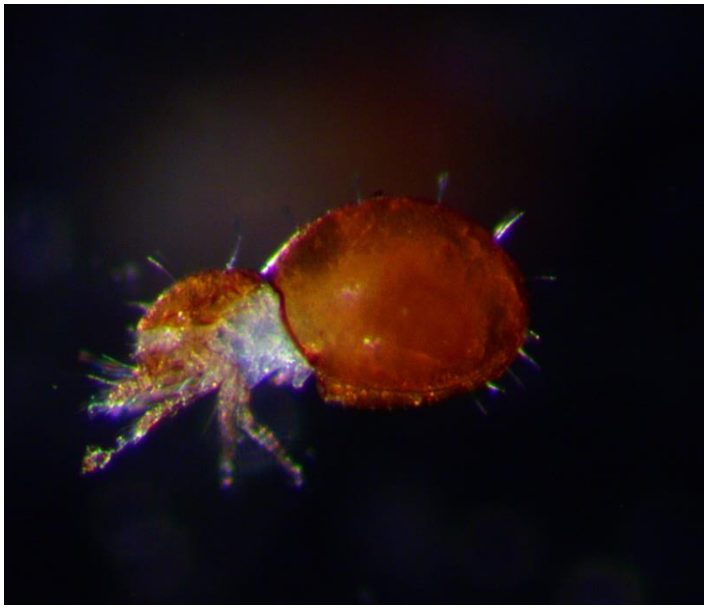

Figure 3-3: An image of the mite chosen for gut content sequencing. The species was determined by morphology and by COX I sequencing.

The mite species selected for gut content analysis was identified using a key from the Canadian Biodiversity Information Facility (http://www.cbif.gc.ca/eng/species-bank/diversity-of-oribatida-in-canada/image-library/?id=1370403266216) and by COX I sequencing. The method used for extraction, PCR, and sequencing was as above, except that five mites were used rather than the ten individuals that were used in Collembola analysis, as the mites were larger.

Extraction and Analysis of Microarthropod Gut Contents

Guts were removed from three sets of 20 Collembola and three sets of ten mites (from the three sites) for analysis of the fungi they contained. Guts were removed using two pairs of Dumont #5 forceps (Fine Science Tools Inc., Vancouver, B.C.). Microarthropods were placed one at a time in a glass Petri dish with distilled water under a dissecting microscope and were held with the forceps on the anterior end and posterior end. The forceps were then used to pull in opposite directions which resulted in the gut sliding out from the inside of the body. A probe was used to remove any excess tissue from the gut and the guts were then placed in 1.5 mL Eppendorf tubes and then stored in the refrigerator until experiments were conducted.

DNA was then extracted from the three sets of Collembola guts and the three sets of mite guts using the DNeasy mini plant kit (Qiagen, Hilden, Germany). PCR Reactions with a volume of 50 µl were performed on a Veriti 96 Well Thermocycler (Applied Biosystems, Foster City, CA). Each reaction included 25 µl GoTaq® Green Master Mix (Promega, Madison, WI), 5 µl each of the primers ITS1f (Gardes and Bruns 1993) and NL6C2 (Kernaghan et al. 2017) (IDT, Coralville, IA), each at 0.2 µM final concentration, and 15 µl of DNA (undiluted). PCR parameters were: 94°C for 4 minutes, 10 cycles of

94°C for 30 s, 45°C for 30 s, and 72°C for 1 min and 30 s; then, with 25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C 1 min and 30 s; and a final extension at 72°C for 8 min. PCR products were then run on 1.5% agarose gels with 1X sodium-borate buffer and EZ-vision gel dye (Amresco®, Solon, OH), using an EC 105 electrophoresis power supply (E-C Apparatus Corporation, USA). Gels were imaged at 365 nm wavelength using an AlphaImager EP gel imaging system (ProteinSimple, San Jose, CA).

Six clone libraries were prepared (one from each microarthropod group collected at each site) in which PCR products were ligated into vector plasmids (pGEM®-T Easy Vector System) (Promega, Madison, WI) according to the manufacturer's instructions. NEB-5-alpha *E.coli* cells (New England Biolabs, Ipswich, MA) were then transformed with the ligated plasmids by heat shock. After incubation for 90 min at 37ºC in SOC media, the transformed cells were plated (two plates per site per microarthropod) onto LB agar media with ampicillin, X-gal and IPTG for blue-white screening and incubated for a further for 24 hours at 37°C in a HeraTherm ICS100 incubator (Thermo Scientific, Waltham, MA). Twenty white colonies from each site were then selected and transferred to 100 µL of sterile water in a 96 well plate using sterile toothpicks.

The fungal ITS region was amplified from *E. coli* cultures using ITS1 and ITS4 primers (Gardes & Bruns 1993). The 25µl reactions contained: 12.5 µl GoTaq® Green Master Mix (Promega, Madison, WI), 2.5 µl ITS1 and 2.5 µl ITS4 (IDT, Coralville, IA) both at final concentrations of 2.5 µM, and 7.5 µl of DNA extract. PCR amplification parameters were: an initial cycle of 10 m at 94°C, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, with a final elongation at of 72°C for 10 min. Products were then viewed on 1.5% agarose gels as described above (Figure 3-4a). The

PCR products from the first four colonies of each clone library were sent to McGill University and Genome Quebec Innovation Centre for sequencing using the primers ITS1 and ITS4. Forward and reverse sequences were then used to construct contig sequences using Sequencher 5 software and trimmed at the primer sites using BioEdit software. Consensus sequences were identified by using BLAST against the NCBI and Unite (Kõljalg et al. 2005) databases.

RFLP (restriction fragment length polymorphism) patterns were then produced for each clone with a distinctive sequence (i.e. each operational taxonomic unit) by cutting the same colony PCR products from the first four clones in each group with Taq$^{\alpha}$I (New England Biolabs, Ipswich, MA)(cuts at T/CGA). RFLP analyses were then performed on the remaining clones and the patterns compared to those from sequenced clones. In all, 15 RFLP patterns were successfully produced from each microarthropod x site combination for a total of 90 patterns (Figure 3-4b).

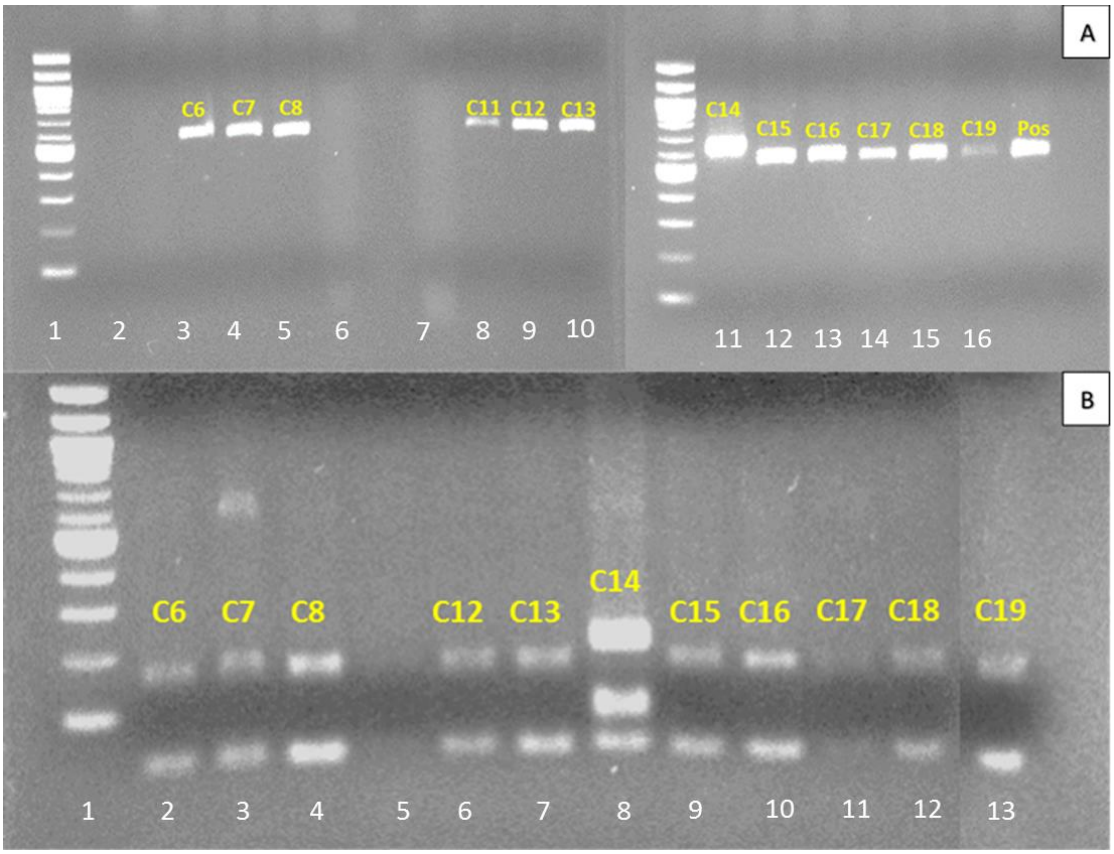

Figure 3-4: Examples of electrophoretic gels produced during the DNA sequence-based identification of Oribatid mite gut fungi with PCR (a) and RFLP (b). (a) PCR products amplified from plasmids that had been ligated with fungal ITS. Lane 1 is a 100 bp size ladder, lane 2 is a negative control. The alphanumeric codes (e.g. C6, C7) signify the clone number from which each PCR product was amplified. b) Example of Restriction Fragment Length Polymorphism (RFLP) analysis of cloned fungal ITS. PCR products were cut with the restriction enzyme Taq$^{\alpha}$I. Two different RFLP patterns are visible. Lane 1 is a 100 bp size ladder.

Ordination Analysis

Relationships between the two microarthropod species at the three sampling sites and their gut fungal communities were explored with a non-metric multiple dimensional scaling (NMDS) ordination performed using the Vegan Package in R (Oksanen et al. 2013) using Bray Curtis distances (default) and 100 iterations to determine significance of the site and the fungi found in the guts of Mites and Collembola.

Results

Identification of Collembola and Mites

COX I sequencing and phylogenic analysis identified the field collected Collembola as *Folsomia* sp. (Figure 3-5). The closest GenBank accession was *Folsomia* sp. MH823679 (99%) from Cape Breton Highlands National Park, Nova Scotia (Table 3-1). Although expert morphological identification placed it close to *Folsomia ozeana*, this species is not known from Eastern Canada, hence the field collected Collembola are referred to here as *Folsomia* sp.

COX I sequencing and phylogenic analysis identified the mites as *Phthiracaridae*, related to *Hoplophthiracus illinoisensis* (Figure 3-6). The closest GenBank accession was Phthiracaridae (HM379318) (99%) from Gros Morne National Park, Newfoundland (Table 3-1). These mites are quite difficult to identify because they have very few morphological features that differentiate them from other members of their family and genus (Parry 1979).

Molecular Analysis of Gut Contents

The fungal richness in the guts of the Oribatid mite species was low, with only four species found: the yeast *Teunomyces kruisii*, a species of Chaetosphaeriaceae, *Penicillium spinulosum*, and an unknown fungal endophyte. The best matching GenBank sequences were EU343840 (100%), HQ207069 (100%), MH861217 (98%), and KF673749 (97%) respectively (Table 1). No ECM species were detected in the gut content of the Oribatid mites tested.

Fungal richness in the guts of *Folsomia sp*. collected from the field was also very low, with only two species detected: the yeast *Teunomyces kruisii* and a species of Chaetosphaeriaceae, matching the NCBI sequences EU343840 (100%) and MH810088 (100%), respectively (Table 3-1). No ECM species were detected within Collembola guts. Example sequences have been deposited in GenBank as MH810087 - MH810088.

Shannon diversity indices averaged across the three sites were similar for both the Collembolan and the mite gut fungi, although diversity was more variable among samples in the mites. Collembola had very low fungal diversity, with the same two fungal species (*Tuenomyces kruisii* and Chaetosphaeriaceae) across all three sampling sites. Mite fungal diversity was more variable, with four species found in their gut contents.

The NMDS ordination indicates that there was a difference between the feeding patterns of the Oribatid mites and Collembola tested (Figure 3-7). Collembola from all three sampling sites were very similar in their fungal diet, with only *Tuenomyces kruisii* and Chaetosphaeriaceae sp. detected in each. However, mite gut fungi varied more from site to site. Mites from site 2 were very similar to the Collembola, while mites from site 1

were characterized by a large proportion of *Penicillium spinulosum*, and mites from site 3 were distinguished by the presence of the unidentified fungal endophyte.

Table 3-1. Identities, closest GenBank matches, and accession numbers for sequences of gut fungi and field collected mites and Collembola.

Identity	Closest Database Match	Coverage	Similarity	GenBank Accession
Fungi from gut content analysis (ITS rDNA)				
Tuenomyces kruisii	*Tuenomyces kruisii* strain MUCL 29848	100%	100%	EU343840
Chaetosphaeriaceae sp.	*Chaetosphaeriaceae sp.* clone Al-WS1	100%	100%	MH810088
Penicillium spinulosum	*Penicillium spinulosum* strain CBS 336.79	98%	99%	MH861217
Fungal endophyte	Fungal endophyte voucher ARIZ:DM0210	97%	96.5%	KF673749
Collembola and Oribatid Mite (Cox I gene)				
Folsomia sp.	*Folsomia sp.* BIOUG13166-C10 (MF610365)	100%	99%	MH823679
Phthiracaridae *sp.*	Phthiracaridae *sp.* MIONT461-09voucher BIOUG<CAN>:09DPMIT-0042 (HM379318.1)	92%	99%	HM379318

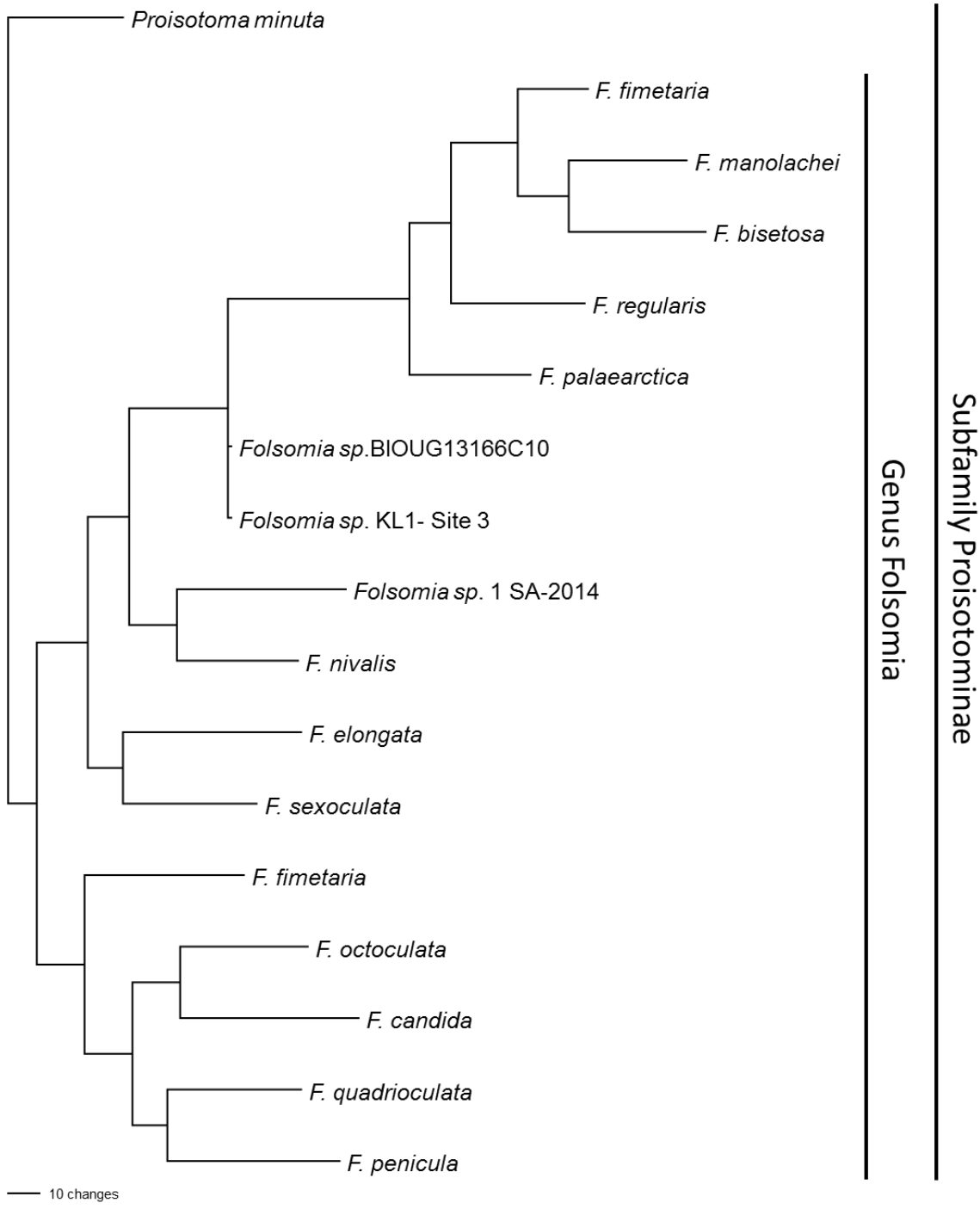

Figure 3-5: A COX I phylogenetic tree comparing the *Folsomia* species used in gut content assessment experiments to other *Folsomia* species. *Folsomia sp.* KL1-Site 3 represents the Collembola species used in gut content experiments.

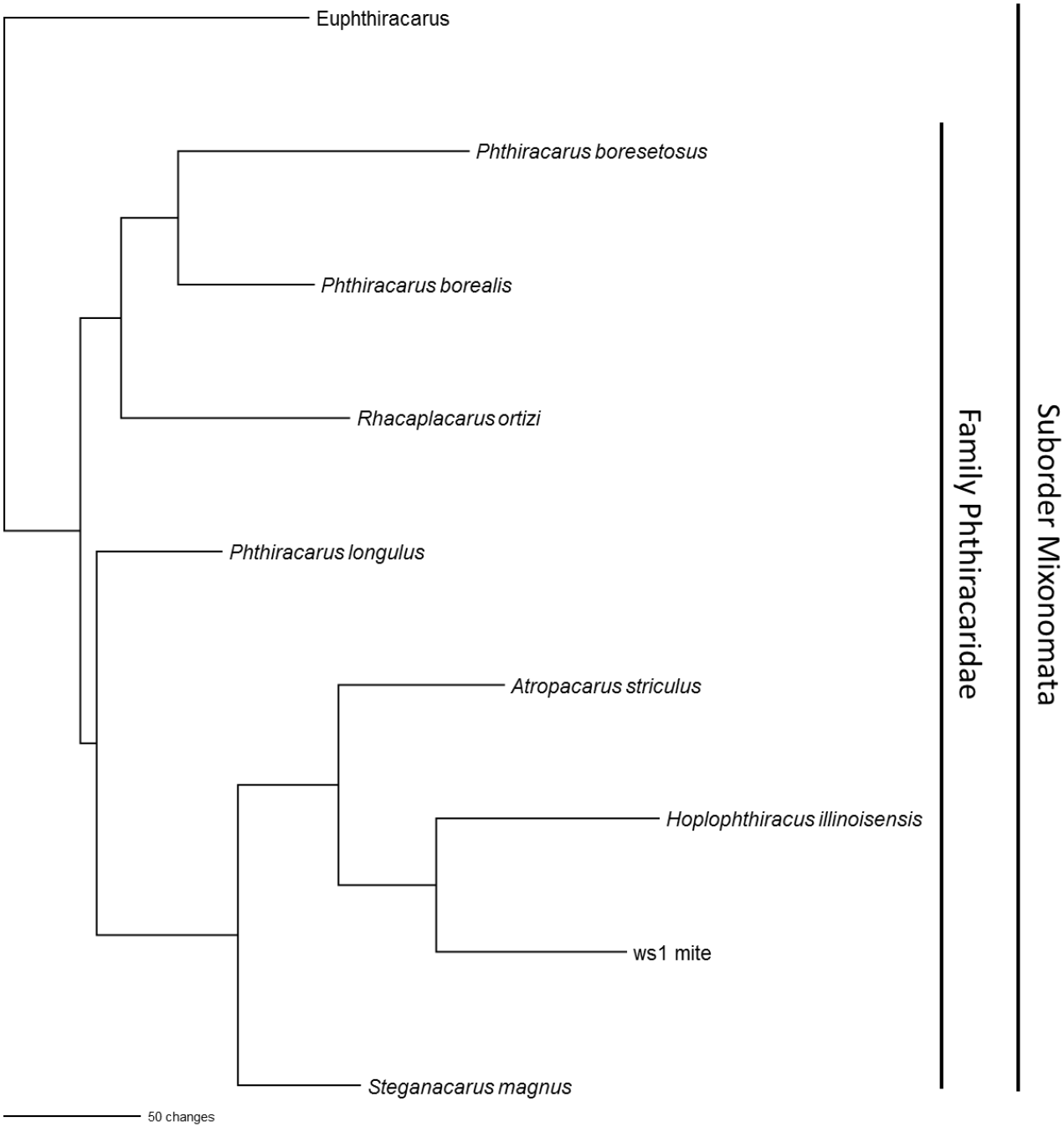

Figure 3-6: A COX I phylogenetic tree comparing the Oribatid mite species used in gut content assessment experiments with other members of the of Phythiracaridea. WS1 mite represents the mite species used in gut content analysis experiments.

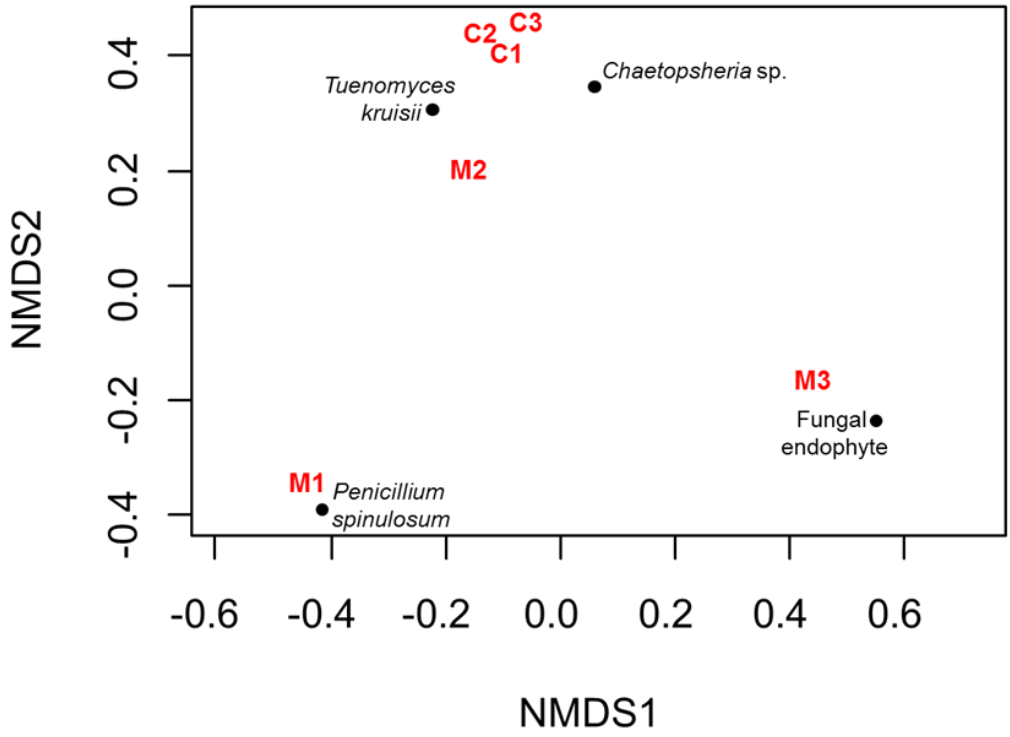

Figure 3-7: Non-metric multidimensional scaling (NMDS) ordination of Collembola and Oribatid mite gut fungi and sampling sites. C1, C2, and C3 represent Collembola samples from site 1, 2, and 3. M1, M2, and M3 represent mite samples taken from site 1, 2, and 3. Stress < 0.0001

Discussion

When the gut contents of field-collected *Folsomia* sp. were analyzed for the presence of fungi, only two fungal species were detected across all three sites, and neither of the species were ectomycorrhizal. While gut contents of field-collected oribatid mites did have more fungal species than *Folsomia* sp., diversity was still low, and mycorrhizal species were also lacking. Sampling location had little influence on the fungi in *Folsomia* guts, but it did play a role in the fungal species composition of the guts of Phthiracaridae species.

The actual fungal diversity of both types of microarthropod guts may have been underestimated due to some methodological constraints. Fungal DNA may have been degraded by microarthropod digestion between feeding and analysis (King et al. 2008). Also, some gut contents might have been expelled during Tullgren funnel extraction. These issues could perhaps be avoided if the microarthropods were harvested by hand and immediately frozen, but this would be difficult and very time consuming (Remen et al. 2010).

The fungal species found in the guts of Collembola were the yeast *Tuenomyces kruisii* (formerly known as *Candida kruisii*) and a species of Chaetosphaeriaceae. These two species were also found in the guts of the mites, as well as *Penicillium spinulosum*, and an unknown fungal endophyte. Chaetosphaeriaceae is an ascomycetous family of saprobes with cosmopolitan distributions (Réblová et al. 1999). They degrade wood and plant litter and have hyaline (non-dematiaceous) hyphae (Cannon and Kirk 2007). Chaetosphaeriaceae was found in the gut contents of all microarthropod samples from all

three sites, and it is reasonable to assume that this fungal species is common in the upper litter layers where both the Oribatid mites and Collembola species sampled likely reside.

The yeast *Tuenomyces kruisii* can be found worldwide, but mainly in association with insects (Kurtzman et at, 2016). The majority of the species of *Tuenomyces*, including *T. kruisii*, have been identified in the gut contents of numerous beetles and other insects (Suh et al. 2006; Sylvester et al. 2015). This likely accounts for the detection of *Tuenomyces kruisii* in the gut contents of both microarthropods at all sites, with the exception of the mite sample from site 2.

Penicillium spinulosum is a mold which is widely found in forest soils throughout the world (De Bellis et al. 2007; Korneikova et al. 2018), and as such it is reasonable to find it in association with the Oribatid mites. *P. spinulosum* was not detected in the gut content of the Collembola from any of the sites. This association would be interesting to explore further, as it suggests differences between Collembola and Orabatid mites with respect to preference for some soil saprobic fungi.

The ascomycetous fungal endophyte was found only in the guts of the mites in the sample from site 3. There is no information available as to the species identification of the fungal endophyte (no similar sequences on GenBank with taxonomic assignments), but it would seem reasonable to assume that the Oribatid mites had fed on plant tissue colonized internally by a symbiotic endophyte.

The lack of any ECMf species in the microarthropod guts is not unexpected, as the importance of microarthropod ECM grazing under field conditions is controversial. Malmström and Persson (2011) investigated the importance of ECMf in Collembolan

diets and found that while Collembola do feed on ECMf, they can survive without access to them as a food source. As well, Potapov and Tiunov (2016) analyzed the C and N isotopic signatures of Collembola and determined that ECMf do not account for a large portion of the Collembolan diet. Next generation sequencing has also been performed on the gut contents of Collembola (Anslan et al. 2016). Although several ECM species were detected by this method, the Collembola analyzed were collected from the forest floor (epidaphic), and likely did not come in direct contact with ECM roots. Instead, these gut ECMf may have been from sporocarps, hyphae or spores.

However, it is clear that microarthropods will graze some types of ECM roots (Chapter 2), but this may go undetected in gut surveys as microarthropod species tend to be specialized to specific soil layers (Berg et al. 1998) and ECM roots mainly form in the lower litter and humus layers (Lindahl et al. 2007). This means that only certain Collembola and mite species would come into contact, and potentially feed on, ECM roots, but they would still have access to the emanating soil hyphae of the ECM fungal species. This idea is supported by Ponge (2000), who microscopically assessed the gut contents of Collembola species inhabiting different soil layers and found that the guts of some species from the lower organic horizon contained mainly ECM and plant material. Members of the genus *Folsomia* and the Oribatid mite family Phthiracaridae are both believed to feed on fungi, detritus and litter in the upper litter layers of forest soils (Berg et al. 1998; Ponge 2000; Chahartaghi et al. 2005; Liu and Zhang 2016). As such, the microarthropods assessed in the current study might not co-habit with ECM roots. In future studies it should be confirmed that the microarthropods assessed do live in the same soil layer as ECM to ensure that they have ample opportunity to graze on ECM in

the field. This could be achieved by using a morphological field guide for Collembola, although a reliable guide regarding North American Collembola was a challenge to find. The reason this was not done was because it was decided that a similar species should be used in the gut-content analysis experiments as was used in the feeding trial experiments.

Another reason for the lack of ECMf in the microarthropod guts may involve the fact that the most common and evenly dispersed ECM types at the research site were found to be unpalatable to *Folsomia candida* (Chapter 2), while the palatable species were much less common and more localized. Therefore, the microarthropods collected in the current study might well have not been in close proximity to palatable ECM. Another factor that may have had an effect on the types of ECM that were located in the gut contents of microarthropods could be seasonal changes. In the future it would be beneficial to collect microarthropods during different times of the year to see if there is a difference in fungi found in the gut contents of microarthropods during different seasons.

Conclusions and Future Directions

Some theories suggest that the overwhelming diversity of soil fauna has led different types to specialize toward particular food choices, especially Oribatid mites (Wallwork 1983; Hubert et al. 2001). Future research should therefore include a wider variety of Collembola and Oribatid mite species to assess for gut ECMf. Larger sample sizes would also show a wider picture of soil faunal food choices in the field, also improved sampling methods such as extracting and freezing organisms at the field site, sampling specific soil horizons, and sampling microarthropods at locations known to support palatable ECM would improve precision regarding microarthropods food choice.

Soil animals are important in initiating the decomposition of plant and fungal materials in soils (Bardgett and Griffiths 1997; Ponge 2000). Although the current analysis of the gut contents of field-collected Oribatid mites and Collembola did not detect any ECMf species, this in no way rules out the possibility that Collembola and Oribatid mite species graze ECM in the field, so long as the microarthropods and ECM inhabit the same soil horizon, and palatable ECM are available.

Chapter 4

Research Synthesis and Concluding Remarks

While it has been demonstrated that certain ECMf types are palatable to the Collembola species *F. candida* (Chapter 2) there were no ECMf detected in the gut contents of the field-collected microarthropods *Folsomia* sp. and Phthiracaridae (Chapter 3). These findings demonstrate that while certain ECM species - *Suillus spraguei, Tricholoma aetsuans,* and *Piloderma bicolor* - are palatable when offered on their own or as a choice, ECMf are likely not the main food source of the common Collembola and mite species inhabiting the pine forest studied. The most widely available and evenly dispersed species of ECMf at the research site *Cenococcum geophilum* was unpalatable to *F. candida.* This begs the question - is this ECM type dominant because it possesses an effective antifeedant mechanism to deter microarthropod grazing? If this is the case, then it follows that this mechanism may have evolved through microarthropod grazing pressure, which would imply that microarthropod grazing would be more ecologically important if the majority of ECM were not protected, and that it may still be an important factor for the more palatable, but less common ECM types.

While *C. geophilum* ECM were unpalatable to Collembola through various experiments Collembola readily consumed *C. geophilum* grown in culture even at low N levels in media. This gives evidence that *C. geophilum* ECM possesses an antifeedant mechanism. When Collembola were fed *C. geophilum* ECM mantles that had been macerated they fed at extremely low levels, this would have led to the assumption that it must be a chemical antifeedant mechanism as opposed to a physical mechanism because

maceration would have removed possible physical barriers. One possibility is that there may be high levels of heavy metals bound to the melanin in *C. geophilum* ECM which has been found in species of *Armillaria* (Rizzo et al. 1992), but more work would need to be done to determine if that is the case with *C. geophilum*.

The species of microarthropods for which the gut contents were assessed are commonly found in the upper layers of soil (Berg et al. 1998; Ponge 2000; Chahartaghi et al. 2005; Liu and Zhang 2016), while ECM roots tend to form in the lower litter and humus layer of soil (Lindahl et al. 2007). This implies that the microarthropods assessed in this study may not generally come into contact with ECM roots. This could explain the absence of ECMf in their gut contents, as ECMf have been found in the guts of field-collected Collembola in other studies (Ponge 2000; Anslan et al. 2016),but the emanating hyphae of ECM would be found throughout soil, so the microarthropods would have access to the fungi in soil.

More research needs to be conducted on a wider variety of microarthropod species in feeding trials as well as field-collected gut analyses to apply this research on a larger scale as evidence suggests that microarthropod species could have specialized diets to overcome competition in soil (Wallwork 1983; Berg et al. 1998; Hubert et al. 2001). This study has demonstrated that ECMf are highly variable with respect to palatability to Collembola, and therefore the local community structure of ECMf is likely to have a large influence on microarthropod grazing pressure and therefore reduction in plant growth through disruption of the plant fungal symbiosis.

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