An exploration of the fungal assemblage in each life history stage of the butterfly, *Lycaeides melissa* (Lycaenidae), as well as its host plant *Astragalus canadensis* (Fabaceae)

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ABSTRACT

Understanding the ecological role of symbiotic microbes is an area of interest. With regard to mediating plant-insect interactions, it has become apparent that symbiotic microbes are of fundamental importance. However, elucidation of the roles of symbiotic microbes in these systems has been hampered by a lack of knowledge regarding microbial community structure; with fungi, in particular, being understudied components of symbiont assemblages. Here, we present one of the first studies to characterize fungi associated with each life history stage of a lepidopteran herbivore (egg, larva, pupa, and adult) and the leaves of its host plant. We use a culture-independent approach and explore the possibility that plant-associated fungi may colonize butterflies. We recovered a distinct fungal assemblage from each butterfly life history stage and from host plant leaves. Interestingly, the most diverse fungal assemblage characterized was from eggs of wild-caught females. We recovered plant-associated fungi from all butterfly life history stages, which raises the possibility that butterflies could disperse fungi between plants. Additionally, we observed a marked reduction in fungal diversity during pupation. As one of the first studies examining the fungal microbiome of a holometabolous insect throughout its life history, our results highlight the diversity of fungi associated with insect herbivores and suggest possibilities for further work examining plant-insect-fungal interactions.

INTRODUCTION

A growing appreciation for the pervasive influence of microbes on ecological interactions is part of the zeitgeist within the science of ecology decades (Frago et al. 2012, Engel and Moran 2013, Christian et al. 2015). This interest has been driven by confirmation that the majority of eukaryotic organisms host numerous, microbial taxa within their tissues (Zilber-Rosenberg and Rosenberg 2008, Rodriguez et al. 2009, Gibson and Hunter 2010, Huffnagle and Noverr 2013, McFall-Ngai et al. 2013). We refer to these microbial denizens as endosymbionts, and limit our definition to microbes that live for at least a portion of their life history wholly within host tissues, either intra- or intercellularly, or as gut microbes. While studies of bacterial endosymbionts are comparatively well represented in the literature (Ryan et al. 2008, Gibson and Hunter 2010, Douglas 2015, Flórez et al. 2015), it is clear that fungi are also ubiquitous endosymbionts of both plants and animals (Rodriguez et al. 2009, Gibson and Hunter 2010, Porras-Alfaro and Bayman 2011).

For instance, endosymbiotic fungi of plants are a hyper-diverse, polyphyletic group with members colonizing every plant species, and plant-tissue type so far examined (Arnold and Lutzoni 2007, Rodriguez et al. 2009). These fungi are termed endophytes, and by definition live for at least a portion of their life asymptotically within plant tissues (Carroll 1988, Rodriguez et al. 2009). Endophytic mycobiota may include latent saprotrophs (e.g. Promputtha et al. 2010, Mayerhofer et al. 2015) and latent pathogens (e.g. Slippers and Wingfield 2007, Yan et al. 2015) as well as numerous fungi with no known ecological role (Arnold 2007). Endosymbiotic fungi are ubiquitous among arthropods (Buchner 1965, Vega and Dowd 2005, Gibson and Hunter 2010) though they have received

limited study. Most known fungal endosymbionts of arthropods colonize the gut (e.g. Suh et al. 2005, Nguyen et al. 2007, Kaltenpoth and Steiger 2014), but fungi may occur elsewhere in the hemocoel as well (Vega and Dowd 2005).

To date, endosymbiotic fungal taxa that are passed down trans-generationally (vertically-transmitted) have garnered the most attention. These fungi often have dramatic consequences for host ecology and evolution (Clay and Schardl 2002, Friesen et al. 2011, Gibson and Hunter 2010). For instance, maternally transmitted, yeast-like symbionts of several death-watch beetles and planthoppers synthesize sterols and provide detoxification services beneficial for their hosts (Dowd and Shen 1990, Nasir and Noda 2003, Noda and Koizumi 2003). Similarly, vertical transmission of clavicipitaceous *Epichloë* (anamorphs: *Neotophyidium*) fungi in various perennial grasses leads to systemic fungal colonization of host tissues which has far-reaching consequences (Clay and Schardl 2002), including affecting arthropod diversity on hosts (Rudgers and Clay 2007, 2008, Jani et al. 2010) and host competitive ability (Clay and Holah 1999). In recent years, vertical transmission of fungal endophytes has been documented in many non-graminoid plant taxa (Ralphs et al. 2011, Hodgson et al. 2014, Tello et al. 2014, also see Quesada-Moraga et al. 2014). However, vertical transmission of fungi is thought to be the exception, not the norm, for both plants and animals; rather, horizontal-transmission of symbionts is thought to be a more common life history strategy (Rodriguez et al. 2009, Gibson and Hunter 2010).

Little is known regarding the biology, ecology, and distribution of horizontally transmitted fungal symbionts, either of plants or animals (Arnold 2007, Gibson and Hunter 2010, Friesen et al. 2011, Christian et al. 2015). In plants, these fungi are not passed down from parent to offspring, but instead appear to rely on environmental transmission among

hosts via rainwater (Guyot et al. 2005), air currents (Kaneko and Kaneko 2004) or leaf litter (Herre et al. 2007). In contrast to the systemic growth of the seed-borne endophytes discussed above, horizontally-transmitted endophytes may occupy only a small portion of, in some cases only a few square millimeters (Lodge et al. 1996, Yan et al. 2015).

Endophyte ecology is a nascent field, but several ecological roles have been assigned to these fungi, including protecting the host from pathogens (e.g. Herre et al. 2007), perhaps by influencing the response of the plant defense system (Meija et al. 2014). The ecological roles of horizontally-transmitted fungal endosymbionts of arthropods remains dramatically understudied, though some evidence suggests that these fungi may provide important nutritional benefits to their hosts (reviewed in Vega and Dowd 2005).

There is much interest in understanding how both vertically and horizontally microbes might mediate plant-insect interactions, and the impacts to the microbial communities of hosts (see reviews by Hartley and Gange 2009, Frago et al. 2012, Hansen and Moran 2014, Zhu et al. 2014). For instance, it has been hypothesized that plants facilitate transmission of fungi between arthropod hosts (Vega and Dowd 2005, Vega 2008). This hypothesis is supported by observations of plant-associated fungi being ingested by insect herbivores (Monk and Samuels 1990, Devarajan and Suryanarayanan 2006) or becoming attached to passing arthropods (Malloch and Blackwell 1992). Interestingly, in a few instances, insect pathogens living as endophytes have been documented (reviewed in Vega 2008, Quesada-Moraga et al. 2014). For instance, *Beauveria bassiana* (Hypocreales: Clavicipitaceae), a known entomopathogen, has been found in diverse plants from maize, to pine and fir trees (Lewis and Cossentine 1986, Arnold and Lewis 2005, reviewed in Quesada-Moraga et al. 2014). In fascinating work, Behie et al

(2012, 2014) showed that plants infected with *B. bassiana* or various *Metarhizium* taxa were more productive by virtue of recovering nitrogen from herbivorous insects killed by these fungi. Conversely, many plant-associated fungi, including important pathogens, are known to be dispersed by arthropods (e.g. Jennersten 1988, Webber and Gibbs 1989, Kluth et al. 2002, Sugiura and Masuya 2010, Hofstetter and Moser 2014). Given the examples mentioned here, and the specialized nature of plant-insect herbivore relationships (Forister et al. 2015) it seems plausible that some endosymbiotic fungi may have evolved to take advantage of host plant interactions with insects, yet this possibility remains to be explored for all but a fraction of fungal taxa.

Here, we characterize the fungal assemblage in each life history stage of an insect herbivore, and the foliar tissue of its host plant, to understand potential links between the plant and insect mycobiota. We take a culture-independent, next-generation sequencing approach to describe the fungal assemblage in the butterfly *Lycaeides melissa* (Lycaenidae) and its host legume, *Astragalus canadensis* (Fabaceae). *L. melissa* is widespread throughout the arid west and feeds on several legumes, including members of the genera *Astragalus*, *Lupinus*, and *Medicago*. A preferred host of *L. melissa* is *A. canadensis* (Forister et al. 2013), which is widespread throughout western North America. We use this system to ask 1) how similar are the fungal assemblages in host plant foliar tissue and an associated insect herbivore? 2) how does the fungal assemblage in *L. melissa* change during butterfly ontogeny? 3) can we ascribe a putative ecological role to fungi found in either *L. melissa* or *A. canadensis*? Specifically, might *L. melissa* harbor any known plant pathogens, or conversely, does *A. canadensis* harbor any known insect pathogens? Our goal with this study was to use a high-power molecular approach to assay fungal diversity across life

history stages of an insect herbivore, as a first step towards understanding the natural history of plant-fungal-herbivore interactions in this system.

METHODS

Sample collection and butterfly rearing

Female adult *L. melissa* were captured near Washoe Lake in northwestern Nevada in July 2014. Washoe Lake is a shallow, seasonal lake with a large-population of *A. canadensis* on its shoreline. Females were allowed to oviposit on *A. canadensis* (also taken from Washoe lake) in arenas consisting of plastic cups covered in fine mesh (as per Forister et al. 2013). Arenas were stocked with multiple female *L. melissa* and kept outside under ambient conditions. The mesh was periodically sprayed with water and Gatorade (a for nectar). Host plant tissue used in arenas was collected from the field at the same time as adult females. After , eggs were removed from leaves and placed under a heat lamp until hatching. A subset of these eggs (n = 40) were combined and frozen prior to DNA extraction. Upon hatching, first instar larvae were placed individually in small etri dishes along with a leaf from *A. canadensis* (again taken from Washoe Lake). Throughout larval development, leaves were replaced as needed. Larval development occurred over a period of approximately weeks. Petri dishes were kept under heat lamps on a lab bench and cleaned periodically to remove frass. At each life history stage (larvae, pupae, and adults), twelve individuals were haphazardly selected and frozen (-20° C) in preparation for DNA extraction. Frozen larvae included representatives from the 2nd, 3rd, and 4th instars (4 individuals from each instar) to encompass potential differences in fungal communities accrued during larval development. Adults selected for sequencing were lab-reared. Foliar tissue of *A. canadensis* was collected from the same plants used to feed larvae. Tissue was

collected at the beginning of the rearing experiment and frozen (-20° C) until DNA extraction. Leaflets were removed from the rachis prior to sequencing.

Sample preparation and sequencing

All samples were surface-sterilized prior to DNA extraction (after Arnold et al. 2007 because we were primarily interested in fungi occurring endosymbiotically in samples. Samples were agitated for 30 sec in 95% ethanol, followed by agitation for 2 min in 10% bleach, another 2 min in 70% ethanol, and a final rinse in deionized water. Due to their fragility, eggs were placed on filter paper in a vacuum funnel and serially rinsed with these solutions in the order presented. To determine the of this sterilization technique, we performed a culturing experiment using both sterilized, and unsterilized eggs and adults. Eggs were obtained by allowing female butterflies collected near Silver Lake, NV to oviposit onto *A. canadensis* (also taken from Silver lake). Adults were collected near Verdi, NV. Sterilized (n = 24) and unsterilized (n = 24) eggs were placed onto potato dextrose agar (PDA) plates (three eggs per plate). PDA was also used to culture fungi from adult butterflies (n = 7 sterilized butterflies; n = 7 unsterilized butterflies). The underwings of each butterfly were touched to opposite sides of a plate, and the legs touched to the center of the plate. All plates were incubated at 45° C in the dark. Plates were checked for fungal growth daily.

DNA from *A. canadensis* foliar tissue (~100mg per individual) was extracted using the DNeasy Plant Mini Kit (Qiagen). *L. melissa* tissue samples were extracted using the DNeasy Blood and Tissue Kit (Qiagen). Eggs were combined prior to DNA extraction, but for other life-history stages each individual was extracted separately (e.g. each larva was extracted separately). Extracted DNA was pooled by sample type, vortexed, and two

aliquots from each pool were sent to the Research and Testing Laboratory (Lubbock, TX) for library preparation and sequencing. Forward and reverse fusion primers were used for library preparation. These fusion primers consisted of an Illumina adapter, an 8-10 base pair (bp) barcode, and a primer pad, as well as the forward or reverse primer. The ITS 1 and ITS 2 loci were targeted for amplification using the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3') (White et al. 1990). PCR reactions were carried out in 25 μ l total volume using 1 μ l template DNA, 1 μ l of each 5M primer, and with Qiagen HotStar Master Mix (Qiagen). PCR conditions were 95° C for 5 min, followed by 35 cycles of 94° C for 30 sec, 54° C for 40s, and 72° C for 1 min, elongation at 72° C for 10 min, and a final hold at 4° C. Amplicons were pooled and a two-round purification step performed using Agencourt AMPure XP SPRI magnetic beads (Beckman Coulter Inc.). Paired-end (2x300) sequencing was conducted using the Illumina MiSeq platform.

Bioinformatics

Our sequence processing pipeline was adapted from Balint et al. (2014). Reads were first sorted by quality and those sequences with an Illumina phred score of less than 20, or an overall quality score of less than 26, were omitted. Reads shorter than 150 bp were also omitted. All reads were truncated to 280 bp due to quality decline near the ends of the reads. Because of the short read length inherent to the Illumina platform and the primer pairs chosen, the reads originating from the forward and reverse primers did not overlap and therefore could not be as is typically performed for paired-end sequence data. Each single-end read was therefore analyzed separately. Sequencing of a mock community by Nguyen et al. (2014) showed that analyzing single-end reads separately improved OTU

recovery as compared with analysis of paired-end reads. After quality-control analyses, sequences were dereplicated (duplicated sequences removed) and singleton reads removed. ITS sequences were extracted from the pool of reads using ITSx (Bengtsson-Palme et al. 2013). This step is necessary to ensure removal of conserved portions of the small rRNA subunit gene that can bias clustering. ITS sequences extracted were of variable length. The USEARCH (Edgar 2010, 2013) clustering algorithm is very sensitive to such length polymorphisms as they may result in terminal gaps post-sequence alignment. Consequently, all ITS sequences were padded with ambiguous base calls ("N") using a custom PERL script so that all sequences were 280bp long prior to clustering. Clustering of sequences into operational taxonomic units (OTU) was performed with the USEARCH software. Reads were clustered using a 97% similarity threshold and checked for chimeras with USEARCH. Resulting sequences were blasted (Camacho et al. 2009) against the NCBI nucleotide database (accessed: March 2, 2015). OTUs were parsed by taxonomy using MEGAN (Huson 2007). Parameters used for the least common ancestor algorithm utilized by MEGAN were a minimum bit score of 170, and no minimum read complexity. Only those sequences <3% different from the best read (in terms of BLAST matches) were utilized for taxonomic assignment. MEGAN sorts through BLAST output to determine the most conserved taxonomic level across matched database accessions. Consequently, if multiple accessions are matched, then the taxonomic status of an OTU as suggested by MEGAN may be poorly resolved. Therefore, OTUs deemed of particular interest due to their occurrence across samples were BLASTed via the NCBI web interface (Johnson et al. 2008), and taxonomic status manually assigned (based on match quality).

The similarity of fungal assemblage among samples was explored via non-metric multidimensional scaling (NMDS) using the isoMDS function in the R package MASS (Venables and Ripley 2002, R Core Team 2015). Prior to ordination, fungal community data was represented in matrix form, where rows corresponded with samples, and columns corresponded with OTUs. Cells were filled with the number of reads for a given OTU in that sample. This matrix was converted to a distance matrix using a Bray-Curtis distance measure and ordinated via NMDS. Samples were only ordinated in two dimensions because examination of stress values showed no benefit from ordinating in additional dimensions.

RESULTS

A total of 187,564 reads were generated by sequencing; 187,458 of the reads were retained after quality filtering. Of those reads, were identified as fungal in origin (Table 1). Fungal reads were obtained from every sample sequenced, however relatively few reads were obtained from larval and pupal samples as compared with either foliar tissue, or tissue from other life history stages. Results from culturing confirmed the success of our surface sterilization technique in reducing or eliminating fungal presence. Six out of seven cultures from unsterilized adults showed fungal growth after d, while three out of seven cultures from sterilized adults showed growth. Butterflies were touched to each agar plate in three places; for unsterilized adults 62% of these spotting locations showed fungal growth, while for sterilized adults 33% of spotting locations showed growth. Out of all eggs plated ($n = 48$), fungal growth (an unidentified fungus) was only observed on two unsterilized eggs after a week incubation period, no fungal growth was observed from sterilized eggs.

The majority of OTUs assigned belonged to the Ascomycota, though a number of Basidiomycota taxa were also present (Fig. 1, Table 2). Identification beyond phylum proved difficult for many OTUs, however a number of OTUs were assigned to classes. Of the Ascomycota classes recovered, the Dothideomycetes was well represented in both foliar tissue, and butterfly eggs. The Sordariomycetes was also well represented in egg samples. Additional classes recovered included the Saccharomycetes, Arthoniomycetes, Leotiomycetes, and Eurotiomycetes. Of the Basidiomycota recovered, the Exobasidiomycetes, Tremellomycetes, Agaricomycetes, and Microbotryomycetes were represented.

Fungal sequences were assigned to 63 unique OTUs (Table 2.). OTU richness was greatest in egg and foliar tissue samples (>30 OTUs each), and lowest in pupal samples (3 OTUs) (Fig. 2, Table 1.). Overall, fungal assemblages differed markedly between each *L. melissa* life history stage, and also between life history stages and *A. canadensis* foliar tissue (Fig. 2, Fig. 3). Eggs and *A. canadensis* foliar tissue had the most OTUs in common (14 OTUs, Fig. 4). However, the fungal assemblages recovered from pupal and larval tissues were proportionally most similar to those of leaves: 2 of the 3 OTUs within pupae were also recovered from foliar tissue, and 8 of the 12 OTUs found in larvae were also in leaves. Relatively few OTUs found in leaves were also found in adult butterflies (4 OTUs). The majority of OTUs assigned uniquely to a single tissue type were sequenced from either eggs, or foliar tissue, though several OTUs were only recovered from other life history stages (Table 2). OTUs uniquely found in eggs were assigned variously to the Tremellomycetes, Exobasidiomycetes, Agaricomycetes, Eurotiomycetes, Arthoniomycetes, Dothideomycetes, and Sordariomycetes. OTUs unique to *A. canadensis* foliar tissue were assigned to the

Saccharomycetes, Arthoniomycetes, Dothideomycetes, Leotiomyces, and Sordariomycetes. Very few OTUs were found only in larvae (3 OTUs); most OTUs recovered from larvae were also found in either eggs, or foliar tissue. Of these larval OTUs, all were members of the Ascomycota, but only one OTU could be assigned a class (Sordariomycetes). Only three OTUs were recovered from pupal sequences (this despite the fact that the highest DNA concentrations obtained during extraction were from pupae). Two of these OTUs were assigned to *Malassezia restricta* (OTUs: 13, 14; Basidiomycota: Exobasidiomycetes: Malasseziales), while the third OTU could not be assigned taxonomic status beyond the Ascomycota. Of the 11 OTUs recovered from adult butterflies, 6 were unique to those samples. These OTUs included members of the Pezizomycotina, including representatives of the Sordariomycetes. Of Basidiomycota unique to adult samples, all were assigned to the Agaricomycetes.

Malassezia restricta was the only fungal OTU recovered from all life history stages and from foliar tissue. Three additional OTUs (OTUs 2, 29, and 59) were recovered from every sample, excluding pupal samples. Two of these OTUs were members of the Basidiomycota and assigned to the Sporidiobolales (class Microbotryomycetes). Manually BLASTing sequences associated with these OTUs left the second OTU still unresolved, while OTUs 29 and 59 matched the anamorph/teleomorph pair of *Rhodotorula* and *Rhodospiridium* respectively (100% match).

In most cases, few of the OTUs recovered could be assigned to ecologically relevant functional classes. However, several OTUs were assigned to genera that include known plant pathogens: OTU 28 and 57 were assigned to the genus *Myrothecium* (Hypocreales),

OTU 34 to *Fusarium* (Nectriaceae), and OTU 58 to the genus *Alternaria* (Pleosporales).

Only OTU 33 was assigned to a genus of known insect pathogens, *Torrubiella* (Cordycipitaceae).

DISCUSSION

Here, to our knowledge, we present the first sequencing of the fungal microbiome of all life history stages of a butterfly and its host plant using next-generation sequencing technology. Ten fungal classes were represented by 63 OTUs recovered from all samples sequenced (Table 2.). Fungal assemblages in each life history stage, and in foliar tissue, were quite different from one another (Fig. 3). Interestingly, the most diverse fungal assemblage recovered was from eggs, including many OTUs not recovered from other life stages or from leaves (Fig. 2). While we did surface sterilize eggs, due to their fragility we needed to utilize less violent agitation than for our other samples. Therefore, it is possible that fungi from ovipositing adults or from the host plant may have adhered to the outside of the eggs, and passed through the sterilization process unharmed. If true, this could be responsible for the elevated fungal richness found in egg samples. However, the results of our culturing experiment seem to counter this explanation, as we observed very little fungal growth from unsterilized eggs, and no growth at all from sterilized eggs.

Several fungi were found in all life history stages of *L. melissa*, save the pupal stage, as well as *A. canadensis* leaves (OTUs 2, 29, and 59). These OTUs were assigned to the genera *Rhodotorula* and *Rhodosporidium* (anamorph and teleomorph respectively). These genera have been isolated from many orders of insects, including Hemipterans, Dermapterans, Dipterans, and Hymenopterans (Zacchi et al. 2002) as well as numerous plants (Andrews and Harris 2000, Lachance et al. 2001). It is unclear from our results if

these fungi colonize butterflies during a single event and then persist throughout butterfly ontogeny, or if repeated colonization events occurred. The latter hypothesis seems more likely given the lack of *Rhodotorula/Rhodospiridium* in pupal samples. Since these fungi were recovered from *A. canadensis* foliar tissue and butterflies were kept in the same etri dishes throughout development, these fungi may have repeatedly colonized *L. melissa* over the course of the experiment; for instance during larval feeding, during initial oviposition, and post-eclosion. Regardless of colonization mechanism, the presence of these fungi in foliar tissue and in multiple life-history stages, along with the diversity of fungi recovered from eggs, suggests that butterflies at least have the potential to act as dispersal vectors for plant-associated fungi.

Interestingly, we discovered a marked reduction in fungal reads and OTUs obtained from pupal DNA. This parallels a recent finding by Hammer et al. (2014) who showed a reduction in bacterial community richness during pupation of the *Heliconius erato* butterfly. hypothesized that excretion of ingested bacteria by caterpillars prior to pupation may be the cause of this pattern. We have noticed that *L. melissa* caterpillars tend to avoid eating for prior to pupation, in which time fungi obtained from leaves could be excreted, thus leading to the reduction in fungal richness observed in pupae. Alternatively, in other insects an upregulation of the immune response during pupal diapause has been described, which may have consequences for microbial persistence and growth within the pupa (Lee et al. 2002, Hao et al. 2012). When taken together, our results, along with those of Hammer et al. (2014), suggest a constriction of microbial richness, both fungal and bacterial, during the pupal stage of holometabolous insects.

The only fungal OTU obtained from all butterfly life history stages and from foliar tissue, was identified as *Malassezia restricta*. Fungi from the genus *Malassezia* appear to colonize a variety of ecological niches and have been recovered from a diversity of substrates, including: ocean sediments, the skin of humans and many other animals, Antarctic soils, and the guts of marine invertebrates (Amend 2014). Environmental RNA sequencing of deep-sea sediments found sequences likely from *Malassezia* spp. (Edgcomb et al. 2011). Since RNA degrades rapidly, this finding suggests that these fungi may actually occur in deep-sea sediments, and by extension, their presence in other unexpected locations may not simply be the result of contamination.

Though we did recover a potential insect pathogen from adult butterflies (*Torribiella* sp., OTU 33) and several potential plant pathogens from eggs and leaves (OTUs 28, 34, 57, 58), unfortunately, we were unable to assign most OTUs a taxonomic status highly resolved enough to ascribe putative ecological function. However, many OTUs recovered were placed within the Sordariomycetes and Dothideomycetes, classes known to contain numerous endophytic fungi (Rodriguez et al. 2009). None of the Dothideomycetes recovered were assigned to the vertically-transmitted, fungal genus *Undifilum* found within other *Astragalus* species (Cook et al. 2011, Baucom et al. 2013). We suggest that the poor taxonomic resolution we achieved may be evidence of the uniqueness of the fungi recovered in our samples.

Conclusion

Here we present one of the first studies examining the fungal microbiome of a holometabolous insect throughout its life history. We found a different fungal assemblage associated with each life history stage. Fungal diversity recovered from eggs of wild-caught

females was much higher than that observed in other life-history stages. We also observed a marked decline in fungal richness during pupation. Several fungi found on foliar tissue were also recovered from adult butterflies, which when taken together with the diversity of fungi recovered from eggs, suggests that butterflies have the potential to disperse fungi between plants. Our work adds to the existing literature suggesting that insects plant-associated fungi, and points to the need for further work examining the ecological role of endosymbiotic fungi of both plants and insects.

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Table 1. Summary of data obtained through sequencing of the butterfly *Lycaeides melissa* at all life history stages, and of its host *Astragalus canadensis*. Extracted DNA from twelve surface-sterilized individuals was pooled, and two aliquots sequenced, for each life history stage. Foliar tissue from twelve plants was similarly extracted, pooled, and sequenced. Totals shown represent pooled sequences from both analyzed aliquots. Reads shown are the number of high-quality, single-end reads remaining after quality filtering (including both forward and reverse reads, see main text for details). All OTUs were assigned at 97% sequence similarity match and singletons excluded from OTU counts.

Sample	Reads	Fungal reads	Fungal OTUs
<i>L. melissa</i>			
Adults	34,814	2,054	11
Pupae	5,274	223	3
Larvae	3,542	574	12
Eggs	33,740	7,289	35
<i>A. canadensis</i>			

Leaves	110,088	50,623	31
Totals	187,458	60,763	63

Table 2. Fungal OTUs in each life history stage of *Lycaeides melissa* (Lycaenidae), and in foliar tissue of its host plant *Astragalus canadensis* (Fabaceae). Sequences were clustered into OTUs using a $\geq 97\%$ similarity threshold using USEARCH. Presence in a sample is denoted in the first column through the use of the following abbreviations: adult (A), pupa (Pu), larva (L), egg (E), and host plant (H). Taxonomic status of each OTU was determined through parsing BLAST output using MEGAN, or by manually reviewing output of web BLAST searches.

Sample	OTU	Num. reads	Phylum	Class	Order	Family	Genus species
E, H	1	88			Unknown fungus		
A, L, E, H	2	162			Unknown fungus		
E	3	12	Basidiomycota	Tremellomycetes	-	-	-
L, H	4	591	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	-
H	5	2219	Ascomycota		Unknown member of Pezizomycotina		
E	6	154	Ascomycota	-	-	-	-
H	7	1247	Ascomycota	Saccharomycetes	Saccharomycetales	-	-
H	8	11733	Ascomycota	-	-	-	-
H	9	176	Ascomycota	-	-	-	-
A	10	140	Ascomycota	-	-	-	-
E, H	11	126	Ascomycota	-	-	-	-
H	12	88	Ascomycota	Arthoniomycetes	Lichenostigmatales	Phaeococcomycetaceae	<i>Phaeococcomyces nigricans</i>
A, Pu, L E, H	13	10384	Basidiomycota	Exobasidiomycetes	Malasseziales	Malassezia	<i>Malassezia restricta</i>

Pu, E, H	14	154	Basidiomycota	Exobasidiomycetes	Malasseziales	Malassezia	<i>Malassezia restricta</i>
A	15	339	Basidiomycota	Agaricomycetes	Polyporales	Ganodermataceae	<i>Ganoderma</i> sp.
E, H	16	424	Basidiomycota	Exobasidiomycetes	Malasseziales	Malassezia	-
E, H	17	136	Basidiomycota	Exobasidiomycetes	Malasseziales	Malassezia	-
H	18	256	Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	<i>Preussia</i> sp.
H	19	59	Ascomycota	Leotiomycetes			
E	20	92	Ascomycota	Eurotiomycetes	Verrucariales	Verrucariaceae	<i>Verrucaria baldensis</i>
E	21	82	Ascomycota	Arthoniomycetes	Lichenostigmatales	Phaeococcomycetaceae	-
E	22	12	Ascomycota	Dothideomycetes	Pleosporales	-	-
L, E	23	95	Basidiomycota	Tremellomycetes	Filobasidiales	-	<i>Cryptococcus</i> sp.
E	24	19	Basidiomycota	Tremellomycetes	Filobasidiales	-	<i>Cryptococcus</i> sp.
E	25	328	Basidiomycota	Exobasidiomycetes	Malasseziales	Malassezia	<i>Malassezia pachydermatis</i>
E	26	168	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	-
L	27	22	Ascomycota		Unknown member of Pezizomycotina		
E	28	96	Ascomycota	Sordariomycetes	Hypocreales	-	<i>Myrothecium</i> sp.
A, L, E, H	29	413	Basidiomycota	Microbotryomycetes	Sporidiobolales	-	-
L, E, H	30	356			Unknown fungus		
E	31	70			Unknown fungus		
E	32	72			Unknown fungus		

A	33	166	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	<i>Torrubiella</i> sp.
E	34	61	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	<i>Fusarium</i> sp.
H	35	137	Ascomycota		Unknown member of Pezizomycotina		
E	36	74	Ascomycota	Dothideomycetes	-	-	-
E	37	59	Basidiomycota	Agaricomycetes	Cantharellales	Botryobasidiaceae	<i>Botryobasidium subcoronatum</i>
E	38	11			Unknown fungus		
H	39	2061	Ascomycota		Unknown member of Pezizomycotina		
A	40	212	Ascomycota		Unknown member of Pezizomycotina		
E	41	52	Ascomycota		Unknown member of Pezizomycotina		
H	42	1209	Ascomycota	Saccharomycetes	Saccharomycetales	-	-
E, H	43	135	Ascomycota	Arthoniomycetes	Lichenostigmatales	Phaeococcomycetaceae	-
H	44	11209	Ascomycota	-	-	-	-
E	45	154	Ascomycota	-	-	-	-
H	46	166	Ascomycota	-	-	-	-
A	47	135	Ascomycota	-	-	-	-
E, H	48	122	Ascomycota	-	-	-	-
E	49	73	Ascomycota	-	-	-	-
A	50	104	Ascomycota	-	-	-	-
H	51	52	Ascomycota	-	-	-	-

Pu	52	14	Ascomycota	-	-	-	-
E	53	146	Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	<i>Preussia</i> sp.
H	54	21	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	-
L	55	2	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	-
L	56	18	Ascomycota		Unknown member of Pezizomycotina		
E	57	91	Ascomycota	Sordariomycetes	Hypocreales	-	<i>Myrothecium</i> sp.
H	58	242	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	<i>Alternaria</i> sp.
A, L, E, H	59	339	Basidiomycota	Microbotryomycetes	Sporidobolales	-	-
L, E, H	60	195	Basidiomycota	Microbotryomycetes	Sporidobolales	-	<i>Rhodotorula</i> sp.
L, E, H	61	894	Ascomycota	Dothideomycetes	-	-	-
H	62	244	Ascomycota	Dothideomycetes	-	-	-
A	63	166	Basidiomycota	Agaricomycetes	Polyporales	Ganodermataceae	<i>Ganoderma</i> sp.

Figure captions

Figure 1. Number of fungal OTUs assigned to either the Ascomycota, or Basidiomycota by sample. Several OTUs were identified as fungi but were unable to be assigned a phylum (shown as Unk. phylum).

Figure 2. Venn diagram showing similarity in fungal assemblages among life history stages of *L. melissa* and foliar tissue of its host *A. canadensis*. Counts shown are of OTUs.

Figure 3. Non-metric multidimensional scaling ordination of the fungal assemblage recovered from each life history stage of *Lycaeides melissa* and leaves of *Astragalus canadensis*. Axis labels refer to ordination dimension (only two dimensions were used).

Figure 1

Figure 2

Figure 3

(a)