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Temporal variation in fungal communities associated with tropical hummingbirds and nectarivorous bats

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ABSTRACT

Species of yeasts and other microfungi carried by pollinators are of general ecological interest because some of these microbial species can grow in floral nectar and affect plant–pollinator interactions. It is, however, not well understood how the composition of fungal species found on pollinators varies over space or time. The spatial and temporal distribution patterns in the microfungi found on the bills of hummingbirds and in the mouths of nectarivorous bats was investigated along a gradient of deforestation within a Costa Rican countryside landscape. The community composition of microfungi found on hummingbirds' bills and bats' mouths underwent substantial compositional turnover over a 2-month period and between 2 yr. In contrast, fungal community composition was not significantly correlated with spatial distance, habitat type, species of hummingbirds, nor the forest dependency of the hummingbirds sampled for microfungi. These findings suggest that, in this landscape, fungal communities on a nectarivous vertebrate vector might be influenced primarily by temporal factors such as plant and flower phenology rather than spatial environmental heterogeneity.

Introduction

Recent studies have shown that flower-visiting animals such as bees, beetles, ants and hummingbirds vector multiple species of microfungi from flower to flower as they forage for floral nectar (Brysch-Herzberg, 2004; Herrera et al., 2008, 2010; 2013; Belisle et al., 2012; Vannette et al., 2013; De Vega and Herrera, 2013). Once introduced into nectar, some of the fungi utilize the sugars and amino acids present in the nectar for growth (Vannette et al., 2013; Peay et al., 2012; Brysch-Herzberg, 2004; Herrera et al., 2008). These microbes are of general ecological interest because they have the potential to alter plant–pollinator interactions by changing the chemical properties of nectar (Herrera et al., 2008; Peay et al., 2012 De Vega and Herrera, 2013; Vannette et al., 2013) and, consequently, the attractiveness of flowers to pollinators (Herrera et al., 2013; Vannette et al., 2013). Recent research indicates that the effects of nectar-inhabiting microbes on

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plant–pollinator interactions depend on the identity of microbial species (Vannette et al., 2013; Good et al., 2014). If so, to improve our understanding of the implications of microbes in nectar, it is important to know how the community composition of fungi (and bacteria) found on pollinators may vary over space and time. However, few data are currently available to address this question.

In this paper, we report a survey of the community composition of fungal species found on the bills of hummingbirds and nectarivorous bats in Costa Rica. We hypothesized that the fungal communities would: (1) vary in species composition through time and (2) vary in both abundance and species composition through space across a gradient of deforestation in the countryside landscape. Our analysis for testing these hypotheses focused primarily on hummingbirds because we had more extensive data on hummingbirds than on bats.

Materials and methods

Study system

Hummingbird and microorganism sampling took place in and around the Las Cruces Biological Station (LCBS) in the Coto Brus Valley in southwestern Costa Rica (8° 47′ N, 82° 57′ W, 1 100 m above sea level). The LCBS encompasses ~280 ha of protected primary and mature secondary forest (Fig 1A). The climate of the region is characterized by a long rainy season (Stiles et al., 1989) and forests in the area are classified as premontane tropical wet forest (Holdridge, 1979). Over 60 % of the Coto Brus Valley, once forested, has been converted into cropland and pasture since the 1950s (Mendenhall et al., 2011; Sansonetti, 1995).

We sampled at 18 study sites within a 4 300 ha area (Fig 1). The study sites were classified into three types, as in Mendenhall et al. (2011): Las Cruces Forest Reserve (3 study sites), sun coffee plantations (6 study sites), and countryside forest elements (9 study sites). Sun coffee plantations had ~5–25 % seasonal canopy cover directly over the coffee shrubs, mostly consisting of nitrogen-fixing Poró trees (*Erythrina* spp.) and banana and plantain plants (*Musa* spp.). Countryside forest elements included clusters of trees of various sizes and qualities, live fences, hedgerows and riparian strips embedded in agricultural land, which were too complex in configuration to be considered isolated forest fragments (Mendenhall et al., 2011). Study sites were chosen to maximize spatial independence and avoid spatial clustering of a similar type (Fig 1A).

Hummingbird and bat sampling

Hummingbird bills were sampled for microfungi from Jan. 2, 2011 to Mar. 28, 2011 and from Jan. 7, 2012 to Feb. 28, 2012. In total we sampled 585 birds and bats in 2011 and 305 in 2012 (Table 1). Birds and bats were sampled by constant-effort mist netting. Mist-netting protocols consisted of twenty 12 × 2.5 m, ground-level mist nets with 32 mm mesh in a 3 to 5 ha plot haphazardly placed at each of the 18 study sites. After capture, hummingbirds and bats were fed sterile sugar water containing 50 % sucrose in Eppendorf tubes (Fig 1B), and the remaining sucrose solution which had come into contact with birds' beaks and tongues was refrigerated for further analysis.

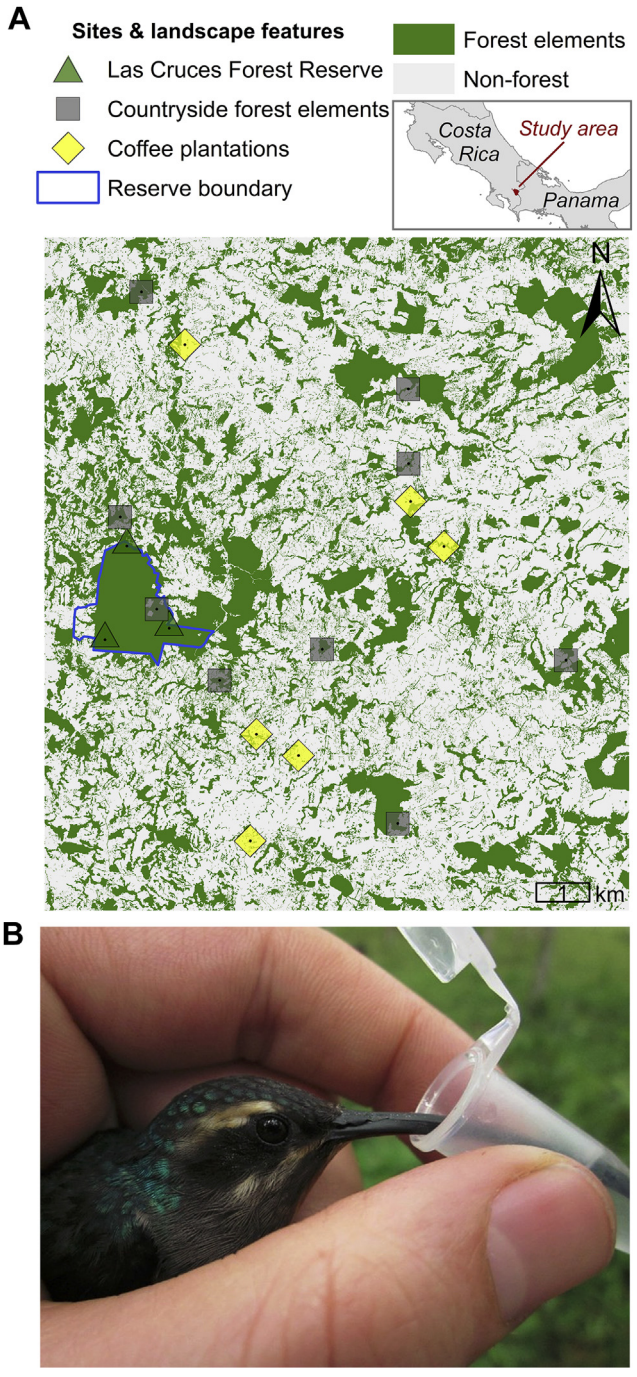

Fig 1 – (A) Map displaying study sites in Coto Brus, Costa Rica. The Organization for Tropical Studies Las Cruces Biological Station's Las Cruces Forest Reserve is outlined in blue. Each point represents one of 18 sites. (B) A Green Hermit being fed sterile sugar water (50 % sucrose) from an eppendorf tube.

To minimize contamination, great care was taken to introduce only the hummingbirds' bills or the bats' tongue and the associated microbes into the sugar water, and the Eppendorf tubes closed immediately after inoculation. Even though some contamination may have been inevitable despite the care taken,

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Table 1 – Bird and bat species observed in this study. NA indicates not enough data available to estimate forest dependency

Bird or bat species		Number of birds and bats sampled		% Yeast		Forest dependency (−1 to 1)
		2011	2012	2011	2012	
Amazilia tzacatl	Rufous-tailed hummingbird	186	116	66.2	87	−0.58
Phaethornis guy	Green hermit	99	57	68.7	86	0.47
Glossophaga soricina	Pallas's long-tongued bat	66	0	89.4	–	NA
Anoura geoffroyi	Geoffroy's tailless bat	26	0	88.5	–	NA
Amazilia edward	Snowy-bellied hummingbird	23	22	39	86.2	−0.47
Phaethornis striigularis	Stripe-throated hermit	22	16	59.1	93.8	0.51
Campylopterus hemileucurus	Violet sabrewing	17	16	70.1	100	0.68
Thalurania colombica	Violet-crowned woodnymph	17	12	64.8	83.3	0.65
Lonchophylla robusta	Orange nectar bat	16	0	81.3	–	NA
Heliodoxa jacula	Green-crowned brilliant	15	5	93	100	0.98
Amazilia decora	Charming hummingbird	14	5	79	100	0.47
Elvira chionura	White-tailed emerald	13	6	30.8	100	0.92
Phaeochroa cuvierii	Scaly-breasted hummingbird	12	1	50	100	−0.73
Eutoxeres aquila	White-tipped sicklebill	11	8	72.3	100	0.44
Chlorostilbon assimilis	Garden emerald	9	9	89	89	−0.78
Glossophaga commissarisi	Commissaris's long-tongued bat	8	0	87.5	–	NA
Heliomaster longirostris	Long-billed starthroat	8	5	100	100	−0.88
Hylonycteris underwoodi	Underwood's long-tongued bat	6	0	83.3	–	NA
Phyllostomus discolor	Pale spear-nosed bat	5	0	100	–	NA
Heliothryx barroti	Purple-crowned fairy	3	1	67	100	0.16
Florisuga mellivora	White-necked jacobin	2	2	100	100	NA
Doryfera ludovicae	Green-fronted lancebill	1	0	100	–	NA
Klais guimeti	Violet-headed hummingbird	1	0	100	–	NA
Lampornis viridipallens	White-throated mountaingem	1	0	100	–	1
Threnetes ruckeri	Band-tailed barbthroat	1	0	100	–	NA
Archilochus colubris	Ruby-throated hummingbird	0	1	–	100	NA
Lampornis castaneoventris	White-throated mountaingem	0	2	–	100	NA
Total		585	305			

the same method of microbial sampling was used throughout this study, making all samples standardized with respect to the potential chance of contamination. Therefore, our sampling protocol should be valid for the main purpose of this study, i.e., the examination of the temporal and spatial variation in microbial communities. We do suggest, however, that it would be informative in the future to include negative controls, i.e., samples where every procedure is followed the same as other replicates, except allowing the sterile sugar solution to come into contact with a hummingbird's bill and a bat's tongue.

There was no significant temporal trend in the species composition of hummingbirds captured, according to an analysis of a linear model to predict the number of captures by Julian date while allowing each hummingbird species to have a random intercept in a linear model.

Microbial identification

We plated 50 μl of each of the remaining sugar water samples prepared as above on yeast-malt agar (YMA; Difco, Sparks, MD, USA), mixed with 50 μl of deionized water for spreading. Plates were incubated for 48 hr at room temperature (22–25 °C) and colony forming units (CFUs) counted to estimate microbial abundance. This method of estimating microbial abundance only reveals culturable taxa. It should also be noted that some bacterial taxa, in addition to fungal taxa, may have formed colonies on YMA. However, most species of microfungi found in nectar had been shown to form colonies on YMA (Brysch-Herzberg, 2004), providing an informative, though not precise, estimate of microbial abundance for the purpose of this study. One colony was chosen from each YMA plate at random for DNA amplification and sequencing for species identification. If colonies had visibly different morphologies, multiple colonies were chosen. Each hummingbird or bat was only sampled for 1–2 colonies because a previous study indicated that up to 6 colonies of microbes sampled from the same individual yielded identical DNA matches (Belisle et al., 2012). In the present study, there were 19 hummingbird samples that had visibly different colony morphologies. When sequenced, only 4 out of the 19 samples had more than 1 species of microfungus.

DNA was extracted using the Sigma Extract-N-Amp tissue polymerase chain reaction (PCR) kit and amplified using the MyTaq red mix (Bioline). We used the primers NL1 (5′-GCA TATCAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG GAC GG-3′) to amplify the D1/D2 domains of the large subunit nuclear ribosomal RNA gene (Brysch-Herzberg, 2004; Kurtzman and Robnett, 1998; Lachance et al., 2003; Herrera et al., 2010). PCR samples that produced a visible band on a 1.25 % sodium boric acid gel (Brody and Kern, 2004) were sequenced by Elim Biopharm (Hayward, CA). This commonly used microbial identification method produces an informative representation of nectar-inhabiting fungal species composition (Brysch-Herzberg, 2004).

Sequences were trimmed and aligned using the MAFFT plugin in the program Geneious Pro (Biomatters Ltd.,

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Auckland, New Zealand). Aligned sequences were grouped into operational taxonomic units (OTUs) using a 98 % sequence similarity cutoff (Kurtzman and Robnett, 1998). Use of 99 % and 97 % sequence similarity cutoffs yielded virtually identical results. The consensus sequence of each OTU was input into the National Center for Biotechnology Information GenBank, and species names assigned using Basic Local Alignment Search Tool (BLAST) matches in combination with a phylogenetic approach. A total of 189 sequences in 2012 and 303 sequences in 2011 were identified to species spanning 38 fungal OTUs (Table 2).

Forest dependency scores

Hummingbird species' forest dependency was scored from complete forest dependency (1) to complete forest avoidance (−1), and was determined by comparing a species' relative abundance in the Las Cruces Forest Reserve versus coffee plantations (Fig 1). The relative abundance proportions of each hummingbird species in each habitat was calculated by summing the number of individuals captured from 2007 to 2009 and controlling for sampling effort among habitat types (Mendenhall et al., 2012).

Statistical analysis

To calculate the effect of spatial distance on microfungal communities we created pairwise similarity matrices of geographic distances between the sites at which hummingbirds were captured and the abundance (CFUs μl^{-1}) of the species of microfungi detected on each hummingbird. Statistical significance of correlations between these variables was tested using a Mantel test in the R package vegan (Oksanen et al., 2008).

All non-metric dimensional scaling plots were created using the vegan package in the statistical software, R (Oksanen et al., 2008), supplemented by the permutational multivariate

Table 2 – Taxonomic assignments of the microfungi observed in this study

	Number of sequences		Number of sequences from bats	Number of sequences from birds	Top Blast match	% Match	GenBank accession#
	2011	2012					
Clavispora lusitaniae	106	0	24	82	AJ539564	100	KF830171
Aureobasidium pullulans	2	70	0	72	FN868849	100	KF830172
Candida metapsilosis	20	41	7	54	FJ746055	100	KF830173
Candida quercitrusa	45	0	34	11	DQ655691	100	KF830174
Cryptococcus flavescens	18	21	1	38	AM748528	100	KF830175
Candida intermedia	22	0	1	21	DQ377635	100	KF830176
Candida rancensis	12	5	1	16	JN642531	100	JN642531
Lodderomyces elongisporus	16	0	3	13	AM397853	100	KF830177
Metschnikowia koreensis	6	8	0	14	AF296438	100	KF830178
Candida oleophila	10	0	5	5	HM627133	100	KF830179
Candida sp. MB1	5	4	0	9	JX409495	100	KF830182
Candida leandrae	3	5	0	8	FJ527048	100	KF830180
Kodamaea ohmeri	8	0	1	7	AB617938	100	KF830181
Rhodotorula mucilaginosa	7	1	1	7	EU563932	99.8	KF830200
Candida sp. MB2	0	6	0	6	AY520407	100	KF830183
Candida albicans	0	5	0	5	KC139704	100	KF830184
Candida saopaulonensis	5	0	4	1	AY695398	99.6	KF830185
Debaryomyces hansenii	4	0	1	3	EU596437	100	KF830186
Hyphopichia burtonii	0	4	0	4	U45712	100	KF830187
Meyerozyma guilliermondii	4	0	0	4	EF694600	100	KF830188
Sporobolomyces carnicolor	0	4	0	4	JN940713	100	KF830189
Penicillium brevicompactum	0	3	0	3	AY484921	100	KF830190
Cryptococcus sp. MB1	0	3	0	3	AF444699	100	KF830199
Yarrow lipolytica	3	0	0	3	AM268457	100	KF830192
Cryptococcus nemorosus	2	1	0	3	FN428910	100	KF830191
Candida boidinii	0	1	0	1	GU225763	100	KF830193
Candida parazyma	0	1	0	1	EU273475	97.1	KF830194
Pleospora sp.	0	1	0	1	KC510071	99.8	KF830195
Pseudozyma hubeiensis	1	0	0	1	DQ008953	100	KF830196
Starmerella bombicola	0	1	0	1	U45705	100	KF830197
Wickerhamomyces sp.	1	0	0	1	FN908198	100	KF830198
Cryptococcus podzolicus	1	0	0	1	FN428938	99.8	KF830201
Hannaella luteola	1	0	0	1	KC798402	100	KC798402
Hannaella zeae	1	0	0	1	AJ965480	100	KF830202
Cryptococcus carnescens	0	1	0	1	HM145912	100	KF830203
Schwanniomyces polymorphus	0	1	0	1	U45836	100	KF830204
Cryptococcus sp. MB2	0	1	0	1	JX242210	100	KF830205
Rhodosporidium babjevae	0	1	0	1	AF389828	100	KF830206
Total	303	189	83	409			

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analysis of variance (PERMANOVA) on abundance-based Bray–Curtis dissimilarity coefficients using the function ADONIS in the vegan package (Oksanen et al., 2008). Microfungal species were pooled by the hummingbird species on which they were found to analyze the effects of time (Fig 2A and B) and of hummingbird's forest dependency (Fig 2B) on microbial community composition. The variables used in the multivariate analyses were the composition of fungal OTUs, as represented by the CFU μl^{-1} of each OTU observed, which were each averaged across all samples for each hummingbird species (Fig 2A), each sampling period (Fig 2B), or each forest element type (Fig 3B). In 2012, hummingbirds were sampled for an additional month, but to standardize for seasonal effects, only samples acquired from Jan. 7 to Feb. 28 in each year were included in the analysis of time of the year. In addition, microfungal species were also pooled by the bat species on which they were found, in order to compare differences in community composition between birds and bats. Hummingbird species and microfungal species were pooled by the study site in which they were found, to analyze the effects of habitat type on microbial community composition (Fig 3A and B).

All analysis was conducted in R v. 2.15.2 (R Development Core Team 2012).

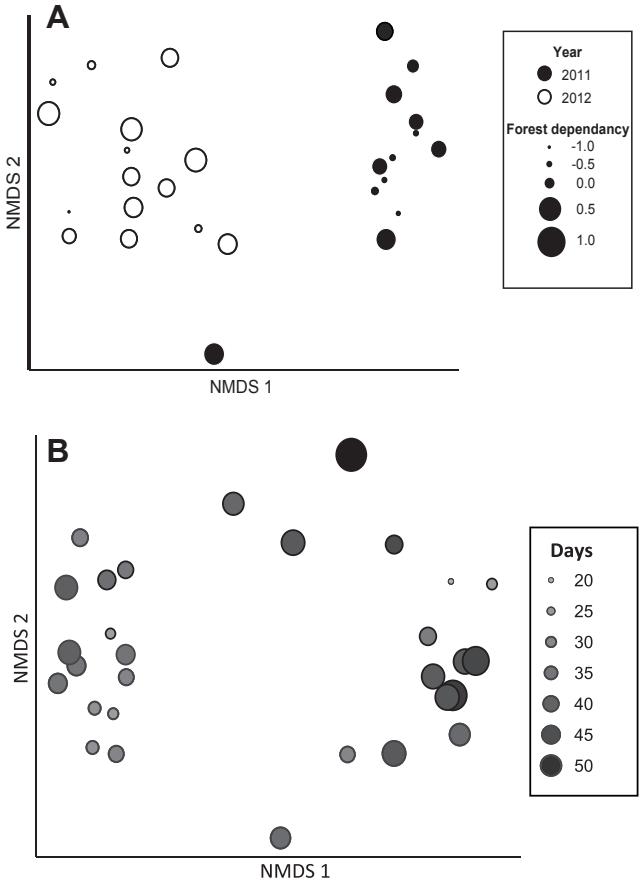

Fig 2 – Non-metric multidimensional scaling (NMDS) results based on Bray–Curtis species abundance-based similarity coefficients. (A) Plot indicates similarities between hummingbird species in the species of microfungi found on their bills. Hummingbird species are categorized as either having been sampled in 2011 (closed circles) or 2012 (open circles). Circle sizes correspond to the index of forest dependency, in which birds that were completely forest dependent had a score of +1 and those that were completely non-forest dependent had a score of −1 (see the Materials and Methods for detail of the forest dependency index). (B) Each data point represents a hummingbird species, and the locations of points relative to one another represent how similar birds are in the fungal species they carried. The shading and size of each data point indicate the average sampling time, from Jan. 7th to Feb. 28th, of that hummingbird species for the duration of the survey. We separated samples by year as microfungal communities varied drastically by year.

Results

Out of 583 hummingbirds sampled in 2011, 414 had microfungi on their bills (71 %). In 2012, 270 out of 305 birds (88.5 %) sampled had microfungi (Table 1). Birds had an average of 664 CFUs μl^{-1} of sucrose solution, whereas bats had average of 382 CFUs μl^{-1}.

The composition of microfungal species on hummingbirds was significantly different between the two years sampled (PERMANOVA result $F_{(1,31)} = 6.1077$, $p < 0.001$), with only 6 of 31 identified fugal species found in both 2011 and 2012 (Fig 2A, Table 2). In addition, the species of microfungi on hummingbirds varied significantly during a standardized two-month sampling period (PERMANOVA $F_{(1,26)} = 1.8296$, $p = 0.025$), with birds sampled earlier in the season tending to have a more similar fungal community composition to each other than birds sampled later in the season (Fig 2B).

Even though bird species composition in both 2011 and 2012 showed strong association with habitat type (Fig 3A, PERMANOVA $F_{(2,15)} = 5.113$, $p < 0.001$), no such pattern was found between fungal species community composition and habitat type where the birds were captured and the fungi sampled (Fig 3B, PERMANOVA $F_{(2,15)} = 0.790$, $p = 0.789$). Results were similar when 2011 and 2012 were analyzed separately (2012: $F_{(2,15)} = 0.789$, $p = 0.753$, and 2011: $F_{(2,15)} = 0.801$, $p = 0.765$). Bird species richness at a particular site did not explain fungal species composition (2011: $p = 0.15$, $R^2 = 0.145$, 2012:$p = 0.792$, $R^2 = 0.008$). Neither did spatial distance between the sites where hummingbirds were sampled (2011:Mantel statistic $r = 0.190$, $p = 0.089$; 2012: Mantel statistic $r = 0.058$, $p = 0.292$).

Hummingbird species were not significantly different in the species of fungi found on their bills (PERMANOVA $F_{(4,63)} = 0.077$, $p = 0.367$). However, in 2011, when both hummingbirds and nectarivorous bats were sampled, significant differences were suggested between the community composition of fungal species found on birds' bills and bats' mouths (Fig 4, PERMANOVA $F_{(1,21)} = 1.6671$, $p = 0.038$).

Discussion

Taken together, our results support our first hypothesis that the fungi found on hummingbird bills vary in species composition across time. Although we only sampled one colony per hummingbird capture, possibly underestimating the contribution of rare fungal species, there was a clear

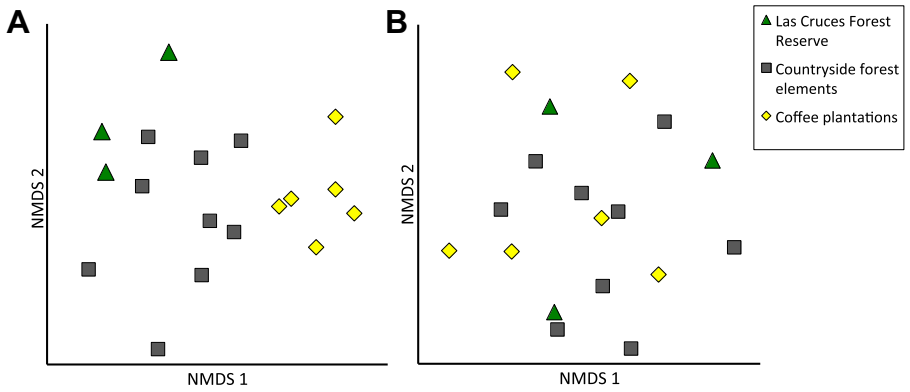

Fig 3 – Non-metric multidimensional scaling (NMDS) results based on Bray–Curtis abundance-based similarity coefficients and categorized by site type (symbols are as in Fig 1). (A) Points represent hummingbird species categorized by the plot in which they were captured. (B) Each data point represents microfungi community composition at a single plot pooling all microfungal species identifications from birds captured at that plot.

delineation in the composition of microfungal species between the two years sampled (Fig 2A, Table 1), and a significant difference in fungal species found on birds between the beginning and the end of the sampling period each year (Fig 2B). There was also a significant difference between the species of microfungi found on bats and hummingbirds (Fig 4), although the cause of this difference remains unclear.

Our second hypothesis was not supported by our data, with no significant association of fungal composition with birds' forest dependency (Fig 2A), spatial distance, habitat type (Fig 3B), or the species of hummingbird sampled (Fig 4). With the exception of rare fungal species, identified species of fungi were evenly distributed throughout the 18 study sites within the 4 300 ha study area. Previous research indicates that microbial communities are typically geographically limited by the activities of their vectors (Starmer and Lachance, 2011; Rosa et al., 1994; Belisle et al., 2012; Miller and Phaff, 1962). From these previous studies, it seemed reasonable to expect that hummingbird's strong preference for a particular habitat type (Fig 3A) would signify barriers to microfungal dispersal and that microfungal communities found on their bills would show a spatial distribution pattern that mimics that of hummingbirds or bats. However, there was little indication of microfungal species occurrences partitioned by habitat type or spatial distance (Fig 3B). If dispersal limitation at the spatial scale we measured was a factor driving observable patterns, a geographic pattern in microfungal communities would be expected (Martiny et al., 2006; Whitaker et al., 2003), but this was not the case.

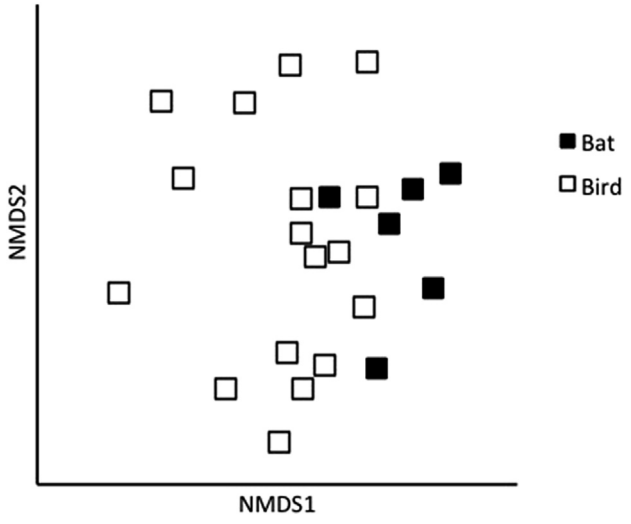

Fig 4 – Non-metric multidimensional scaling (NMDS) results summarized by whether identified microfungal species were collected from birds (open squares) or bats (closed squares). Each data point represents pooled microfungal species found on a particular species of bird or bat.

The temporal changes in the microfungal communities found on hummingbird bills may have been caused by changing environmental characteristics, such as the type of food available in different years and at different times in a season. In addition, changing microfungal communities on hummingbirds may be a reflection of microfungi-host dynamics. Due to competition and shifts in resources, microfungal communities on any given substrata are also likely to change through time (Miller and Phaff, 1962; Morais et al., 1995). Some species of microfungi may proliferate on a temporarily abundant food source and consequently become widely dispersed in the landscape. Alternatively or additionally, these findings may indicate that priority effects are prevalent in host substrata-pollinator community dynamics (Peay et al., 2012; Pozo et al., 2012. Tucker and Fukami, 2014). The ability of different species of microfungi to proliferate to a level suitable for subsequent dispersal may be more dependent on their arrival order and timing to a particular substratum than environmental constraints. Priority effects occurring within flowers may possibly scale up to collectively account for the rapid turnover, occurring at a landscape scale, of microbes found on hummingbirds' bills and the high variation in community structure across time points. The importance of priority effects at the landscape scale remains speculative and awaits further investigation.

Several studies have examined temporal changes in microfungal community composition (Latham, 1998; Lachance et al., 1988, 2005), and found that microfungal communities remain relatively stable over a long time. However, many of these surveys were conducted with years between sampling periods

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and over extensive periods (e.g., 11–17 yr). Although this study was originally designed to examine spatial patterns in microfungi distribution and we did not sample over a continuous period of time, this study is different from many previous studies in that it examined microfungal community turnover on both a short and relatively long time scale. As the turnover in microfungal communities may be rapid and happening in close association with changing environmental characteristics, future studies should examine microfungal communities on several time scales to determine which time scale is most relevant. Additionally, future studies should sample both the pollinators and the environment, particularly floral nectar of different plant species, not only those mainly visited by birds and bats, but also those primarily visited by insects.

Conclusion

In conclusion, even though hummingbird species co-varied closely with habitat type and available tree cover in the areas in which they were captured, the microfungi associated with these birds did not. Instead, there was a nearly complete temporal turnover of the microfungal community in the 2 yr sampling of hummingbird's bills. Moreover, during the two-month sampling period (Jan. and Feb. each year), there was a significant shift in the microfungal community. We conclude that, in this landscape, temporal changes in the environment may be more important in shaping the fungal communities on hummingbirds than spatial environmental gradients.

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