

## Research

### Priority effects can persist across floral generations in nectar microbial metacommunities



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The order of species arrival can influence how species interact with one another and, consequently, which species may coexist in local communities. This phenomenon, called priority effects, has been observed in various types of communities, but it remains unclear whether priority effects persist over the long term spanning multiple generations of local communities in metacommunities. Focusing on bacteria and yeasts that colonize floral nectar of the sticky monkey flower, *Mimulus aurantiacus*, via hummingbirds and other flower-visiting animals, we experimentally manipulated initial microbial dominance on plants (regarded as metacommunities) to examine whether its effects persisted across multiple generations of flowers (regarded as local microbial habitats). The experimental introduction of *Neokomagataea* (= *Gluconobacter*) bacteria and *Metschnikowia* yeasts into wild flowers showed that the effects of initial dominance were observable across multiple floral generations. Three weeks after introduction, corresponding approximately to three floral generations, *Neokomagataea* introduction led to exclusion of yeasts, whereas *Metschnikowia* introduction did not result in the exclusion of *Neokomagataea*. Our results suggest that, even when local habitats are ephemeral, priority effects may influence multiple generations of local communities within metacommunities.

Synthesis

How species affect one another in habitat patches can depend on the order in which species arrive, the phenomenon known as priority effects. Studies on priority effects have naturally focused on species interactions within habitat patches, but many ecosystems are characterized by repeated appearance and disappearance of many habitat patches that are rather ephemeral. Are priority effects short-lived and therefore unimportant in these systems? Not necessarily, according to our study. We found that priority effects in nectar-colonizing microbes persisted across several generations of their ephemeral habitat patches, flowers. Priority effects may have longer-lasting consequences for community assembly than generally thought.



## Introduction

The order of species arrival during community assembly can promote or limit species coexistence (Palmgren 1926, Sutherland 1974, Drake 1991). These effects, termed priority effects, have been reported in a range of organisms, including plants (Grman and Suding 2010), animals (Alford and Wilbur 1985), fungi (Kennedy and Bruns 2005), protists (Louette and De Meester 2007) and bacteria (Devevey et al. 2015). These studies have shown that species that arrive early influence community development by competitively suppressing later colonizers or by modifying habitat conditions to some species' favor and not others' (Fukami 2015).

Although local priority effects are well studied, relatively little is known about whether priority effects persist over time across ephemeral local communities (Pu and Jiang 2015). In most previous studies of priority effects, the observed communities are persistent (i.e. they do not disappear during the period of the study), but many natural metacommunities are characterized by a high turnover of local habitat patches with frequent patch formation and extinction (e.g. pitcher plants colonized by aquatic invertebrates and animal carcasses colonized by terrestrial invertebrates) (Ellis et al. 2006, Vanschoenwinkel et al. 2008a, Ripley and Simovich 2009). Knowledge of such patch dynamics is well recognized as essential for predicting species extinction resulting from habitat destruction that creates increasingly patchy landscapes (De Meester et al. 2005, Tschardt et al. 2007). Thus, studies that inform whether the effects of initial dominance by specific species persist across ephemeral communities over multiple generations of local habitat patches are not only of fundamental interest, but also have applied importance. To our knowledge, however, no experimental test has been conducted.

In this study, we examine whether priority effects persist across ephemeral communities of bacteria and yeasts that colonize floral nectar of wild plants. Nectar-inhabiting microbes disperse across flowers via flower-visiting animals (Brysch-Herzberg 2004, Herrera et al. 2010, Belisle et al. 2012, Jacquemyn et al. 2013, Schaeffer and Irwin 2014). Local communities of nectar microbes are highly ephemeral as the host flowers inevitably disappear as they wither, but the collection of flowers on a host plant functions as a microbial metacommunity that lasts longer than individual flowers while the plant is in bloom (Belisle et al. 2012). Previous studies have suggested that nectar microbial communities of our study plant, the sticky monkey flower *Mimulus aurantiacus* (Phrymaceae), is dominated by *Neokomagataea* bacteria (Acetobacteraceae) (formerly recognized as *Gluconobacter*) and *Metschnikowia* yeasts (Metschnikowiaceae) (Vannette et al. 2013, Tucker and Fukami 2014). We have also shown in an experiment that priority effects can be strong between *Neokomagataea* sp. and *Metschnikowia reukaufii* (Tucker and Fukami 2014). In this experiment, priority effects were mutually negative, and whichever species arrived first reduced the abundance of the other that arrived later, in both the presence and absence of temperature fluctuations. However, this study was conducted in

laboratory microcosms with artificial nectar, and it remains unclear how strongly priority effects may influence microbes in real nectar in the field. Here we report the results of a field experiment with wild *M. aurantiacus* plants, in which we experimentally introduced *Neokomagataea* bacteria or *Metschnikowia* yeasts into flowers early in the flowering season. We then investigated whether these microbes persisted despite frequent turnover of local communities.

## Material and methods

### Experiment

The experiment was conducted at the Turchet Leasehold land owned by Stanford University, located in the Santa Cruz Mountains of California, USA (37°38'8"N, 122°19'4"W). This site mainly consisted of oak woodland and chaparral habitats similar to those found at the nearby Jasper Ridge Biological Preserve of Stanford Univ. (JRBP), described in Belisle et al. (2012) and Vannette et al. (2013). Within a 2.4-ha area at the site, 24 individuals of *Mimulus aurantiacus* were tagged and monitored through the plant's flowering season in 2013, which lasted from April to June (Supplementary material Appendix 1). Each of the 24 *Mimulus* individuals was assigned to one of the following three experimental treatments: *Neokomagataea* bacterium introduction treatment (eight individuals), *Metschnikowia* fungus (yeast) introduction treatment (eight individuals), and control (sham introduction of species) treatment (eight individuals).

The treatments were assigned to plants in a stratified random fashion so as to have the treatments evenly spread spatially (Supplementary material Appendix 1). We regarded plant individuals as each representing a microbial metacommunity consisting of multiple flowers each harboring a local community of nectar microbes. Bacterial and fungal species disperse among local communities (flowers) by Anna's hummingbird *Calypte anna*, the primary pollinator of *M. aurantiacus* at the study site (Belisle et al. 2012, Vannette et al. 2013), and other flower visitors. Besides Anna's hummingbird, Allen's hummingbird *Selasphorus sasin*, Rufous hummingbird *Selasphorus rufus*, bees (e.g. *Bombus vosnesenskii*, *Ceratina acantha* and *Xylocopa micans*), and thrips visit *M. aurantiacus* flowers (Vannette et al. 2013). Nectar microbes could also disperse from other external environments, e.g. pollen, phylloplane, and air (Belisle et al. 2012, Vannette and Fukami 2017). The three treatments represented metacommunities with *Neokomagataea* priority-effects, those with *Metschnikowia* priority-effects, and those with no experimental priority effects, respectively.

To examine possible effects of floral visits by hummingbirds (and other large pollinators), we divided flowers of each plant individual into two categories: caged (some branches on each plant were enclosed in a cylindrical cage covered with 2.5-cm black plastic mesh) and exposed (control) flowers. The cages were used to exclude hummingbirds in order to evaluate their effect on microbial communities in flowers.

Smaller animals such as bees and thrips could enter the cages and access flowers in them. We found previously that microbial densities were lower in caged *M. aurantiacus* flowers than in exposed (control) flowers, indicating that the lack of hummingbird visits resulted in reduced microbial dispersal (Belisle et al. 2012). The cages were placed on the plants early in the flowering season, on 22–26 April 2013.

Before anthesis, we had tagged each harvested flower with a small piece of adhesive address label that had a unique identification number printed on it. We monitored each tagged flower bud to check when they opened. This way, we could tell the age of flowers, and in order to standardize for flower age, we harvested all flowers when they were four or five days old. The first sampling of flowers was conducted from 7 to 10 May (week 0) to compare microbial community composition between caged and exposed flowers. The harvested flowers were immediately placed in a cool box, brought to the laboratory, and nectar extracted, diluted, and plated as described below within 3 h of the harvesting. After plating, the remainder of the diluted nectar samples was stored at  $-80^{\circ}\text{C}$  until DNA extraction was conducted as described below. Colony-forming units (CFUs) were recorded for the plate samples, but the CFU data did not show statistically significant differences in total or colony morphotype abundances among the inoculation treatments, presumably due to low taxonomic resolution and accuracy. In this paper, we focus on data from the Illumina sequencing.

We introduced a single strain of *Neokomagataea* sp. and one of *Metschnikowia reukaufii* into the flowers of the plants pre-assigned to *Neokomagataea* and *Metschnikowia* treatments, respectively, on 7 to 10 May, immediately after the week-0 harvesting of flowers. For this introduction, we used a strain of *Neokomagataea* sp. collected in 2012 at JRBP and similar to the one described in Vannette et al. (2013), which we referred to as *Gluconobacter* sp. in this previous paper ([www.ncbi.nlm.nih.gov/nuccore/JX437138](http://www.ncbi.nlm.nih.gov/nuccore/JX437138)), and a strain of *M. reukaufii* collected in 2010 at JRBP and similar to the one described in Peay et al. (2012) ([www.ncbi.nlm.nih.gov/nuccore/JF809868](http://www.ncbi.nlm.nih.gov/nuccore/JF809868)). For the *Neokomagataea* and *Metschnikowia* treatments,  $4\ \mu\text{l}$  of  $10^4$  cells  $\mu\text{l}^{-1}$  in 20% sucrose was added to all caged and exposed flowers on the focal plant individuals. For the control treatment,  $4\ \mu\text{l}$  of 20% sucrose was added to all flowers. Seven days (week 1) after the introduction treatment, up to 14 flowers were harvested per plant individual in the field, using the same procedure as for week 0. The same sampling was conducted again 21 days (week 3) after the introduction treatment (up to 11 flowers per plant individual). The flowers sampled in weeks 1 and 3 were different from the original flowers into which *Neokomagataea* bacteria or *Metschnikowia* yeasts were introduced in week 0. Flowers of *M. aurantiacus* usually wither 6 to 10 days after opening (Peay et al. 2012). Over the three sampling periods, a total of 513 flowers were sampled (Supplementary material Appendix 3). A widespread incidence of midge infestation of *M. aurantiacus* flower buds in week 3 limited flower availability as flowers did not develop once infested. For this reason, we harvested fewer flowers in week 3 than in weeks 0 and 1.

## Illumina sequencing of bacteria and fungi

From the nectar of each flower sample, microbial genomic DNA was extracted using TRIzol reagents (ThermoFisher Scientific). We then PCR-amplified bacterial 16S ribosomal DNA region and fungal internal transcribed spacer 1 (ITS1) region using the specific primer pairs, 515f (5'- GTG YCA GCM GCC GCG GTA A -3') – 806rB (5'- GGA CTA CNV GGG TWT CTA AT -3') (Caporaso et al. 2012) and ITS1-F\_KYO1 (5'- CTH GGT CAT TTA GAG GAA STA A -3') – ITS2\_KYO2 (5'- TTY RCT RCG TTC TTC ATC -3') (Toju et al. 2012), respectively. Each of the forward and reverse primers was fused with 3–6-mer Ns for improved Illumina sequencing quality (Lundberg et al. 2013) and a Illumina sequencing primer region (forward, 5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG- [3–6-mer Ns] – [515f or ITS1-F\_KYO1] -3'; reverse, 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G [3–6-mer Ns] - [806rB or ITS2\_KYO2] -3'). The multiplex PCR of the 16S and ITS regions was conducted using the MyTaq HS DNA polymerase Mastermix (Bioline) with a temperature profile of  $95^{\circ}\text{C}$  for 2 min, followed by 37 cycles at  $95^{\circ}\text{C}$  for 20 s,  $50^{\circ}\text{C}$  for 20 s,  $72^{\circ}\text{C}$  for 50 s, and a final extension at  $72^{\circ}\text{C}$  for 10 min. The ramp rate was set to  $1^{\circ}\text{C}\ \text{s}^{-1}$  to prevent the generation of chimeric amplicons (Stevens et al. 2013). P5/P7 Illumina adaptors were then added in the subsequent PCR using fusion primers with 8-mer index sequences for sample identification (Hamady et al. 2008) (forward, 5'- AAT GAT ACG GCG ACC ACC GAG ATC TAC AC - [8-mer tag] - TCG TCG GCA GCG TC -3'; reverse, 5'- CAA GCA GAA GAC GGC ATA CGA GAT - [8-mer tag] - GTC TCG TGG GCT CGG -3'). The temperature profile was  $95^{\circ}\text{C}$  for 2 min, followed by 8 cycles at  $95^{\circ}\text{C}$  for 20 s,  $50^{\circ}\text{C}$  for 20 s,  $72^{\circ}\text{C}$  for 50 s, and a final extension at  $72^{\circ}\text{C}$  for 10 min (ramp rate= $1^{\circ}\text{C}\ \text{s}^{-1}$ ). The PCR amplicons of the samples were pooled with equal volume after a purification/equalization process with AMPure XP Kit (sample:AMPureXP = 1:0.6). The pooled library was sequenced using the Illumina MiSeq sequencer of the Stanford Functional Genomics Facility ( $2 \times 250$  cycle sequencing kit) with 15% PhiX spike-in.

The Illumina sequencing data (DNA Databank of Japan (DDBJ) BioProject, PRJDB4972) were processed through the bioinformatic pipeline detailed in Supplementary material Appendix 2. The obtained data matrix depicting samples in rows and operational taxonomic units (OTUs) in columns (sample  $\times$  OTU matrix: Supplementary material Appendix 3) was zero-inflated: i.e. most elements in the matrix were zero, which represented the absence of an OTU in a sample. Because community ecological patterns are generally obscured in the statistical analyses of zero-inflated matrices (Martin et al. 2005), we prepared another matrix, in which the sequencing read counts of bacterial and fungal OTUs (Supplementary material Appendix 4) were summed at the class level (sample  $\times$  class matrix; Supplementary material Appendix 3). Although the class-level data may obscure functional diversity, these data still provide adequate

information for studying priority effects. These data enable a conservative test of priority effects, in the sense that detection of a priority effect at this broad taxonomic level would indicate a strong priority effect, affecting class composition, whereas failing to detect a priority effect at this taxonomic level would not necessarily mean that a priority effect did not exist at finer taxonomic scales. Bacterial and fungal sequences were amplified in the same PCR tubes (i.e. multiplex PCR). Therefore, the proportion of bacterial and fungal sequencing reads in the matrix varied among samples, presumably reflecting the relative abundance of bacteria and fungi in the samples.

In the sample  $\times$  class matrix, the number of obtained reads varied considerably among flowers (Supplementary material Appendix 3) probably due to variation in bacterial and fungal (yeast) density in nectar samples. Therefore, the data matrix was rarefied to 300 reads per flower sample using the *vegan* ver. 2.2-3 package (Oksanen et al. 2012) of R ver. 3.3.1 (< [www.r-project.org](http://www.r-project.org) >). Three-hundred reads per sample were sufficient to describe the class-level composition of the bacterial and fungal communities (Supplementary material Appendix 5). The matrix contained two archaeal, 25 bacterial, and 11 fungal classes (including 'unidentified') (Supplementary material Appendix 3). Due to the lack of nectar or low microbe density in many flowers, the number of samples with the information of class-level community structure was reduced to 189 (Supplementary material Appendix 3).

The most frequently observed bacterial OTU belonging to the bacterial family Acetobacteraceae (1103:13204:3353\_467; Supplementary material Appendix 3) was allied to the sequence of a species in a recently proposed genus, *Neokomagataea* (Yukphan et al. 2011) (Supplementary material Appendix 6a). In the fungal data, 18 OTUs were allied to *Metschnikowia reukaufii* (Supplementary material Appendix 6b).

### Statistical analysis

In the sample  $\times$  class matrix representing bacterial and fungal community structure (for a total of 189 samples), week-1 and week-3 flower samples were separated into two data matrices to construct models testing for priority effects in respective sampling weeks. For each of the week-1 and week-3 data, we first constructed a generalized linear mixed model (GLMM) to predict the proportion of Alphaproteobacteria reads, of which 87.8% were allied to *Neokomagataea* reads (those belonged to the family Acetobacteraceae) (Supplementary material Appendix 3). The proportion of Saccharomycetes (*Metschnikowia*) reads was too small to be used as a response variable. The GLMM models included the introduction treatment and the presence/absence of the experimental cage as fixed effects and plant individuals as a random effect (models with treatment  $\times$  cage interactions failed to converge). To take into account the spatial positions of the sampled plant individuals relative to one another, we also included the first three vectors derived from the principal coordinate analysis of a truncated distance matrix (PCNM) (Borcard and Legendre 2002) as fixed effects. The *glmmML* ver. 1.0 package (Broström 2016) of

R (< [www.r-project.org](http://www.r-project.org) >) was used in the GLMM analysis (family = binomial).

Additionally, to examine the effects of the introduction treatment on the composition of the entire microbial community, we constructed GLMM models in which the axes of principal coordinate analyses (PCoAs) summarizing the microbial community structure quantified at the family level were used as response variables. In each model of PCoA1 or PCoA2 axis in week-1 or week-3 data, the same fixed and random effects as described above were incorporated. In another set of GLMM models, we also evaluated the effects of the introduction treatment on the community structure of microbes other than Alphaproteobacteria, using PCoA on all sequence reads included except Alphaproteobacteria reads. Because Gaussian family models were unavailable with the *glmmML* package, we used the *glmmPQL* function of the MASS ver 7.3-45 package of R.

### Data deposition

Data available from the Dryad Digital Repository: < <http://dx.doi.org/10.5061/dryad.0j4b2> > (Fukami et al. 2017).

### Results

The GLMMs indicated that the introduction of *Neokomagataea* and *Metschnikowia* influenced microbial community structure three weeks (but not one week) after the introduction treatment (Table 1; PCoA2 in Table 2; Fig. 1–2). In week 3, few *Metschnikowia* or other fungal sequences were detected from the plants to which *Neokomagataea* bacteria were introduced in week 0 (Fig. 1). The *Neokomagataea* treatment samples were dominated by Alphaproteobacteria (mostly *Neokomagataea*) reads. In contrast, the *Metschnikowia* and control treatment samples contained both *Metschnikowia* (Saccharomycetes) and Alphaproteobacteria (Fig. 1).

The proportion of Alphaproteobacteria reads was influenced by the presence/absence of cages, but the effects of cages (exclusion of hummingbirds) differed between week 1 and week 3: the presence of cages increased Alphaproteobacteria in week 1, whereas it reduced the relative abundance of the nectar bacterial clade in week 3 (Table 1). In addition, the GLMMs also showed that the introduction treatment influenced the community structure of microbes other than Alphaproteobacteria (PCoA 1 in Supplementary material Appendix 7) in week 3, but not in week 1 (Supplementary material Appendix 7). Effects of the caging on the entire microbial community structure were detected only in week 1 (Table 2).

We also found that the proportion of samples with Alphaproteobacteria were higher in the *Neokomagataea*-introduced plants and lower in the *Metschnikowia*-introduced plants than they were in the control plants in week 3 (Supplementary material Appendix 3 and 8). In addition, Saccharomycetes reads were obtained from none of the samples of *Neokomagataea*-introduced plants but were present in other treatment groups in week 3 (Supplementary material Appendix 8).

Table 1. Effects of the experimental introduction of *Neokomagataea/Metschnikowia* on the proportion of sequence reads mapping to Alphaproteobacteria. In each GLMM model of week-1 or week-3 data, the first three PCNM vectors of the sampling points (spatial structure), the introduction treatment, and the presence/absence of cages were included as fixed effects and plant individuals as random effects.

Week	Explanatory variable	Coefficient	SE	z	p
Week 1	(Intercept)	-1.71	0.50	-3.45	0.0006
	PCNM 1	0.64	3.35	0.19	0.8480
	PCNM 2	2.97	3.25	0.92	0.3600
	PCNM 3	4.46	3.77	1.18	0.2370
	Treatment ( <i>Neokomagataea</i> )	0.37	0.66	0.57	0.5700
	Treatment ( <i>Metschnikowia</i> )	0.69	0.63	1.09	0.2750
Week 3	Cage (Exposed)	0.18	0.05	3.55	0.0004
	(Intercept)	1.23	1.31	0.94	0.3470
	PCNM 1	4.08	12.71	0.32	0.7480
	PCNM 2	-18.19	10.30	-1.77	0.0773
	PCNM 3	-7.85	15.43	-0.51	0.6110
	Treatment ( <i>Neokomagataea</i> )	6.17	2.20	2.81	0.0050
	Treatment ( <i>Metschnikowia</i> )	0.57	1.70	0.33	0.7390
	Cage (Exposed)	-5.47	0.25	-21.97	0.0000

## Discussion

Our results indicate that the effects of initial dominance can persist across multiple generations of ephemeral local habitats in this nectar microbial system. Given our experimental design,

a higher relative abundance of *Neokomagataea* in flowers on *Neokomagataea*-inoculated plants compared to those on *Metschnikowia*-inoculated or control plants could have simply reflected increased dispersal of *Neokomagataea* among flowers on *Neokomagataea*-inoculated plants. However, we found

Table 2. Effects of the experimental introduction of *Neokomagataea/Metschnikowia* on the entire microbial community structure. In each of the week-1 and week-3 microbial community data, a principal coordinate analysis (PCoA) was performed and the resultant PCoA1 and PCoA2 axes were used as response variables of the GLMM models examining the effects of the introduction treatment and experimental cages. In each GLMM model, the first three PCNM vectors of the sampling points (spatial structure), the introduction treatment, and the presence/absence of cages were included as fixed effects and plant individuals as random effects.

Week	PCoA	Explanatory variable	Coefficient.	SE	t	p
Week 1	PCoA 1	(Intercept)	0.108	0.110	0.98	0.3315
		PCNM 1	0.378	0.566	0.67	0.5077
		PCNM 2	0.230	0.549	0.42	0.6774
		PCNM 3	0.444	0.816	0.54	0.5894
		Treatment ( <i>Neokomagataea</i> )	0.082	0.102	0.80	0.4324
		Treatment ( <i>Metschnikowia</i> )	0.064	0.100	0.64	0.5313
		Cage (Exposed)	-0.204	0.098	-2.08	0.0437
Week 1	PCoA 2	(Intercept)	0.064	0.092	0.70	0.4887
		PCNM 1	-0.522	0.473	-1.10	0.2761
		PCNM 2	0.199	0.458	0.43	0.6672
		PCNM 3	0.356	0.681	0.52	0.6044
		Treatment ( <i>Neokomagataea</i> )	-0.081	0.085	-0.96	0.3528
		Treatment ( <i>Metschnikowia</i> )	0.024	0.083	0.28	0.7795
		Cage (Exposed)	-0.058	0.082	-0.71	0.4832
Week 3	PCoA 1	(Intercept)	0.167	0.208	0.80	0.4317
		PCNM 1	-0.568	1.020	-0.56	0.5834
		PCNM 2	-0.078	0.878	-0.09	0.9301
		PCNM 3	-0.339	1.278	-0.27	0.7935
		Treatment ( <i>Neokomagataea</i> )	0.200	0.173	1.16	0.2746
		Treatment ( <i>Metschnikowia</i> )	0.001	0.136	0.01	0.9946
		Cage (Exposed)	-0.259	0.185	-1.40	0.1749
Week 3	PCoA 2	(Intercept)	-0.297	0.157	-1.89	0.0725
		PCNM 1	-0.870	0.767	-1.13	0.2698
		PCNM 2	0.642	0.660	0.97	0.3421
		PCNM 3	1.097	0.962	1.14	0.2670
		Treatment ( <i>Neokomagataea</i> )	-0.134	0.130	-1.03	0.3279
		Treatment ( <i>Metschnikowia</i> )	0.292	0.102	2.86	0.0170
		Cage (Exposed)	0.217	0.139	1.57	0.1323

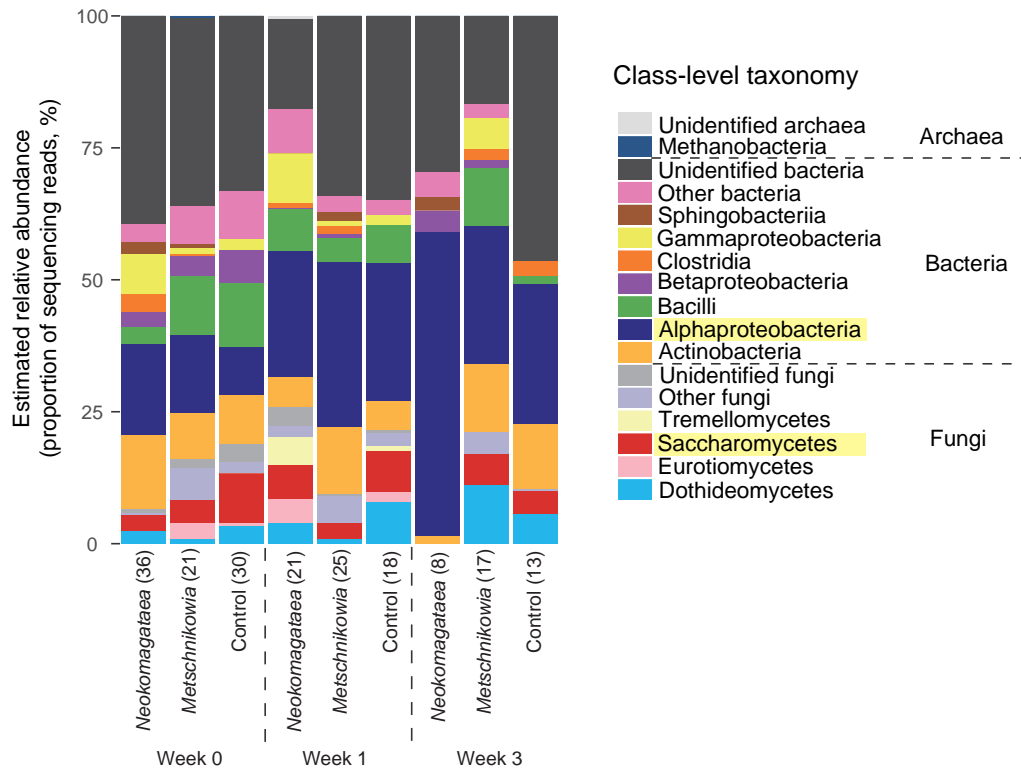


Figure 1. Comparison of community composition among microbial introduction treatments. Results are shown separately for the three introduction treatments (introduction of *Neokomagataea* sp., introduction of *Metschnikowia reukaufii*, and 20% sucrose as control), each separately for weeks 0, 1, and 3. Note that *Neokomagataea* and *Metschnikowia* account for 87.8% of Alphaproteobacteria and 100% of Saccharomycetes sequencing reads, respectively. The number of flowers analyzed is shown in a parenthesis for each group.

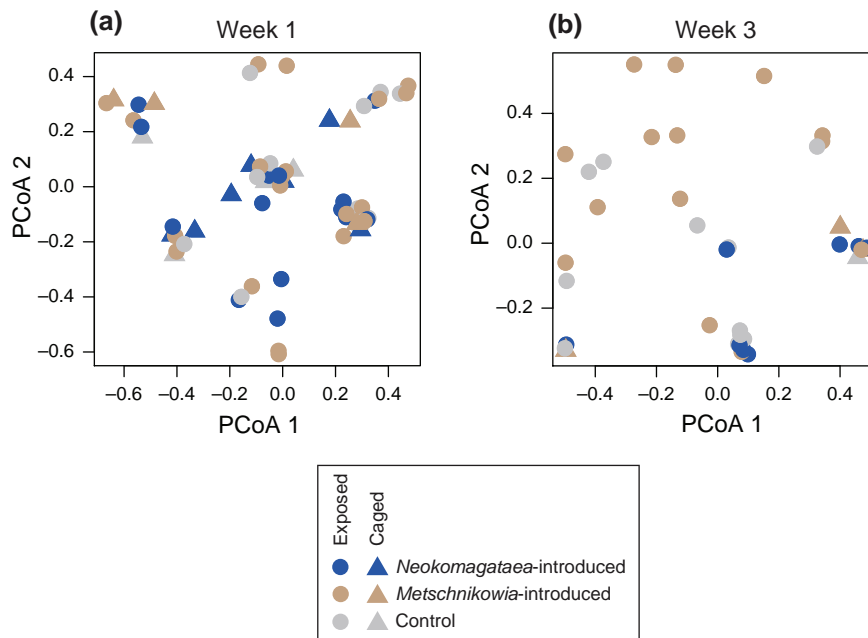


Figure 2. Microbial community structure in flowers, as characterized by PCoA on week-1 (a) and week-3 (b) sequence reads. Treatments where *Neokomagataea* (blue), *Metschnikowia* (beige), or no microbe (control) (grey) was initially introduced in week 0 are shown. Each data point represents a flower. Circles and triangles indicate exposed and caged flowers, respectively. Data points were jittered slightly (no more than 0.03) for better presentation of overlapping points.

that both *Neokomagataea* and *Metschnikowia* were present in at least some of the flowers sampled from control plants. We also found that *Metschnikowia* relative abundance was lower in *Neokomagataea*-inoculated plants than in *Metschnikowia*-inoculated or control plants in week 3, though not in week 1 (Fig. 1). Together, these two findings suggest competitive suppression of *Metschnikowia* by *Neokomagataea* in *Neokomagataea*-inoculated plants. We do not have direct evidence for such competitive interactions occurring in wild flowers (but see Tucker and Fukami 2014). Nevertheless, our results are consistent with the hypothesis that priority effects, along with other factors influencing pollinator behavior and plant physiological status, structure nectar microbial communities within and across floral generations.

The strength of priority effects was asymmetric in the sense that introduction of *Neokomagataea* bacteria resulted in the lowered relative abundance of yeasts and other fungi in week 3 (Fig. 1), whereas introduction of *Metschnikowia* yeasts had only minor effects, if any, on *Neokomagataea* (Supplementary material Appendix 8). The reasons for this asymmetry are uncertain, but it might reflect species-specific efficiency of dispersal. In our experiments, the effects of the exclusion of hummingbirds differed between sampling weeks (Table 1–2), suggesting idiosyncratic contributions of pollinators to *Neokomagataea* abundance in floral nectar. Regardless, persistent priority effects could in turn influence pollination and plant fitness, as we found previously that *Neokomagataea* (= *Gluconobacter*), but not *Metschnikowia reukaufii*, could negatively affect pollinator foraging and seed set (Vannette et al. 2013, Good et al. 2014).

Priority effects were detected in week 3 but not in week 1 (Table 1–2), even though the larger number of samples analyzed for week 1 (Fig. 1) afforded higher statistical power compared to week 3. This result suggests that priority effects do not necessarily decrease monotonically over time, but that their magnitude can show some temporal fluctuation. Factors generating such potential fluctuation remain unclear, but one possibility is ambient temperature. If bacterial and yeast populations grow more rapidly at higher temperature (Tucker and Fukami 2014), priority effects may become stronger later in the flowering season as temperature increases. Consistent with this expectation, air temperature (daily means and maxima) appears to have been higher in week 3 than in week 1 (Supplementary material Appendix 9), although actual causality remains uncertain. Another possibility is pollinator abundance. Anecdotally, we have observed more insects visiting flowers later in the flowering season, which may increase the rate of among-flower dispersal of the nectar microbes. However, both factors remain no more than speculation at this point. Moreover, we used only one strain per species for the experimental inoculation, but strains can be phenotypically variable (Herrera 2014) and may therefore show different priority effects, which remains to be tested.

Metacommunities consisting of ephemeral local patches are found not only in nectar microbes, but are prevalent across a variety of habitats and organisms (Hanski 1998). Examples of metacommunities consisting of ephemeral habitats include

aquatic insects in phytotelmata (Ellis et al. 2006), symbiotic microbes in plant hosts (Jousimo et al. 2014), freshwater plankton in rock pools (Vanschoenwinkel et al. 2008b), and epiphyllous (leaf-inhabiting) bryophytes in tropical forests (Zartman and Nascimento 2006). Because priority effects within patch generations have now been documented widely, further research on priority effects across patch generations in different systems seems worthwhile for a better understanding of species coexistence. Moreover, habitats are becoming increasingly patchy and ephemeral because of human activity (Didham 2010). For this reason, knowledge of priority effects across multiple generations of ephemeral habitat patches will only become more important for biodiversity conservation.

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Supplementary material (available online as Appendix oik-04243 at < [www.oikosjournal.org/appendix/oik-04243](http://www.oikosjournal.org/appendix/oik-04243) >). Appendix 1. Spatial positions of the 24 *M. aurantiacus* individuals analyzed in this study. Appendix 2. Bioinformatic pipeline. Appendix 3. Data matrices. Appendix 4. Nucleotide sequences of the OTUs obtained in the sequencing analysis. Appendix 5. Relationship between the number of sequencing reads and that of bacterial and fungal classes per flower sample. Appendix 6. Phylogeny of *Neokomagataea* and *Metschnikowia* OTUs. Appendix 7. Effects of the experimental introduction of *Neokomagataea*/*Metschnikowia* on the structure of the microbial community except for Alphaproteobacteria. Appendix 8. Treatment effects on the proportion of samples with Alphaproteobacteria or Saccharomycetes reads. (a) Alphaproteobacteria. (b) Saccharomycetes. Appendix 9. Air temperature during the sampling periods.