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Nectar microbes can reduce secondary metabolites in nectar and alter effects on nectar consumption by pollinators

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Abstract

Secondary metabolites that are present in floral nectar have been hypothesized to enhance specificity in plant-pollinator mutualism by reducing larceny by non-pollinators, including microorganisms that colonize nectar. However, few studies have tested this hypothesis. Using synthetic nectar, we conducted

laboratory and field experiments to examine the effects of five chemical compounds found in nectar on the growth and metabolism of nectar-colonizing yeasts and bacteria, and the interactive effects of these compounds and nectar microbes on the consumption of nectar by pollinators. In most cases, focal compounds inhibited microbial growth, but the extent of these effects depended on compound identity, concentration, and microbial species. Moreover, most compounds did not substantially decrease sugar metabolism by microbes, and microbes reduced the concentration of some compounds in nectar. Using artificial flowers in the field, we also found that the common nectar yeast *Metschnikowia reukaufii* altered nectar consumption by small floral visitors, but only in nectar containing catalpol. This effect was likely mediated by a mechanism independent of catalpol metabolism. Despite strong compound-specific effects on microbial growth, our results suggest that the secondary metabolites tested here are unlikely to be an effective general defense mechanism for preserving nectar sugars for pollinators. Instead, our results indicate that microbial colonization of nectar could reduce the concentration of secondary compounds in nectar and, in some cases, reduce deterrence to pollinators.

Keywords: floral microbiology, nectar chemistry, plant-pollinator interactions, mutualism

Introduction

Many organisms express secondary metabolites to defend against predation or herbivory or to compete with other organisms (Rosenthal and Berenbaum 1992, Duffey and Stout 1996, Seigler 1996), but these same compounds can also negatively affect mutualists (Strauss et al. 2002). For example, chemical compounds expressed by plants for anti-herbivore defense are often present in floral nectar (Baker and Baker 1983, Detzel and Wink 1993), and these compounds can deter or harm pollinators (Detzel and

Wink 1993, Gegear et al. 2007). This contradiction in plant-pollinator mutualism has intrigued ecologists for decades (Rhoades and Bergdahl 1981, Stephenson 1981, Baker and Baker 1983, Adler 2000).

The presence of secondary compounds in nectar could be non-adaptive or adaptive. On one hand, these compounds may be expressed in nectar as an unintended consequence of anti-herbivore defense in other plant tissues (Adler 2000). On the other hand, expression of compounds in nectar could be adaptive (Rhoades and Bergdahl 1981). For example, the high concentration of secondary compounds in *Chelone* sp. and the distinct composition of cardenolides in *Asclepias* nectar suggest an adaptive role (Manson et al. 2012, Richardson et al. 2015). Experimental work has found that "toxic" nectar (Adler 2000) can enforce pollinator specificity (Stephenson 1981), manipulate pollinator behavior to improve pollination (Kessler et al 2012, Wright et al. 2013), and provide compounds that inhibit the growth of insect parasites (Manson et al. 2010, Richardson et al. 2015).

Because many secondary compounds exhibit general antimicrobial effects (Wink & Twardowski 1992, Wallace 2004), it has also been proposed that these compounds may reduce the growth of microorganisms in nectar (Adler 2000, Sasu et al. 2010, Heil 2011, Aizenberg-Gershtein et al. 2015). These microbes, including yeasts and bacteria, can reduce plant reproduction and alter pollinator foraging (Kevan et al. 1988, Vannette et al. 2013, Junker et al. 2014, but see Schaeffer & Irwin 2014). Bacteria and yeasts are dispersed to flowers by pollinators and other floral visitors (e.g. Belisle et al. 2012, Aizenberg-Gershtein et al. 2013, Samuni-Blank et al. 2014) and can attain high densities in the nectar of some plant species (Herrera et al 2008, Fridman et al. 2012). However, few studies have tested if compounds present as secondary metabolites exhibit antimicrobial activity in nectar (but see Fridman et al. 2012, Pozo et al. 2012).

In this paper, we report the results of three experiments that tested whether five compounds from three chemical classes limit microbial growth and degradation of nectar and whether the microbes

affect the way these compounds influence pollinator visitation. The first experiment examined the effects of these compounds on the growth of several species of fungi and bacteria, including those to which nectar is thought to provide a primary habitat and those to which it is not. The second experiment examined microbial modification of nectar constituents that may influence pollinator foraging, including sugar concentration, composition and compounds often isolated as secondary metabolites in nectar. The last experiment examined the interactive effects of chemical compounds and a common nectar specialist microbe on nectar consumption by pollinators.

Methods

Study organisms, artificial nectar, and secondary compounds

We obtained culturable microorganisms likely to encounter nectar, including those from floral nectar and hummingbird bills at Jasper Ridge Biological Preserve. We also included strains of *Pseudomonas* bacteria, which are frequently isolated from leaves and soil, but not found at abundance in nectar. We refer to species that attain high density in floral nectar as 'nectar specialists' and those that do not as 'non-specialists.' We chose five chemical compounds that have been documented to occur in nectar (or commercially available analogs of naturally-occurring compounds) for microbial growth assays. We used two complementary approaches to examine the effects of chemical compounds on microbial growth in liquid media. See Appendix A for details of the microbial species, artificial nectar, and secondary compounds used in the experiments.

Experiment 1: compound effects on microbial growth

We assessed the effects of five chemical compounds that occur in nectar on growth responses of several microbial species outlined above. We examined the effects of each compound on the growth parameters of single strains of each microorganism using a plate reader (Tecan, San Jose, CA), to quantify changes in microbial growth in liquid media and provide detailed growth curves. We assessed a broad range of concentrations, spanning the range of plausible concentrations in nectar (Appendix B, Table B1) and including higher concentrations than have been observed in nectar. Chemical compounds were dissolved in artificial nectar (Appendix A) and serial dilutions using artificial nectar were made to achieve desired concentrations.

Microbial suspensions were prepared from single-species cultures in artificial nectar to which no secondary compounds (Table B1) were added. Twenty µl of microbial suspension was mixed with 180 µl of artificial nectar in the wells of transparent flat-bottomed 96-well plates (BD, Franklin Lakes, NJ). Plates were wrapped in parafilm, maintained in the dark at 25^{o}C, and the optical density (OD) at 595 nm read for 65 hours at 15-minute intervals using a TECAN Infinite M200 microplate reader (Tecan Systems, San Jose, CA, USA), following Peay et al. (2012). We included between two and four replicates of each species x compound treatment combination at each concentration described above. Strains of *Pseudomonas syringae* pv. *maculicola* and *Erwinia/Pantoea* sp. did not increase in OD under any conditions in the TECAN experiment in any of the replicate trials.

We examined growth parameters, including maximum growth rate and density in nectar, that likely enhance competitive and dispersal ability of microbes in ephemeral habitat patches like flowers. Microbial growth curves were analyzed using the R package grofit (Kahm et al. 2010), as in Peay et al. (2012). Best-fit models were chosen using AIC (implemented in grofit) and the fit was visually confirmed. Estimated parameters for microbial growth rate (µ) or maximum OD (A) were extracted from the best-fit

models. In wells where no microbial growth was detected (no increase in OD) and growth curves could not be fit, μ and A were imputed as zero and the initial value (0.6), respectively. 'No growth' responses were consistent within a treatment, and nearly all of these were found in high nicotine treatments. Otherwise, species that we included in this analysis grew well in the 96 well plate format. Average parameters for each species in the control condition were used to calculate relative μ and A for each species at each concentration ((treatment-control)/control). To examine the effects of compounds on relative μ and A, we used a two-way ANOVA with microbial species identity and concentration of compound (as a factor) as predictors. Models were estimated for each compound separately.

Experiment 2: microbial effects on nectar chemistry

In a second experiment, we assessed the effects of compounds on nectar modification by microorganisms isolated from nectar ('specialist strains'). Microbial activity in nectar can alter sugar chemistry, often decreasing sugar concentration and increasing the proportion of monosaccharides (glucose and fructose), which can influence pollinator foraging (e.g. Waller 1972, Stiles 1976). To assess if compounds prevent microbial modification to nectar, we examined microbial effects on nectar sugar concentration and composition, and also concentrations of secondary metabolites. Concentrations of compounds used were higher than concentrations typically found in nectar, but were ecologically plausible (Appendix B, Table B1). Microbial suspensions were prepared, cell titer assessed using a hemocytometer, and suspensions adjusted individually to 400 cells/μl. Single strains and a mix of all 'nectar specialist' strains were used. To mimic realistic nectar volumes, each replicate involved 9 μl of nectar in a round-topped 200 μl PCR tube (BioExpress, Kaysville, UT), to which 0.5 μl of the microbial suspension (approximately 200 cells) was added. Tubes were capped and incubated at 25^{o}C for 5 days, which roughly corresponds to the average lifespan of a *M. aurantiacus* flower. Evaporation was minimal.

This experiment was conducted twice, and each treatment was replicated six times, except for the *Gluconobacter* treatment, which was replicated three times due to difficulty in maintaining its growth in culture.

At harvest, samples from each tube were split for further analyses. A subset of the nectar was diluted in sterile 20% sucrose solution and then 50 µl of nectar dilutions was plated. For plating, two dilution factors were used for each nectar sample (i.e., 0.5 and 0.05 µl of undiluted nectar, each serially diluted in 20% sucrose solution). Bacteria-inoculated samples were plated on R2A agar, yeast-inoculated samples on yeast media agar (YMA), and mixed cultures on both. Plates were incubated for 3-5 days and colony-forming units (CFUs) of each species counted. Colony morphotypes of each species were easily distinguished from one another and from common laboratory contaminants, so we could be confident in the species identity of the microorganism growing in the nectar. Contamination by unwanted microbial colonies occurred in less than 5% of plates, and no contaminated samples were used for downstream analyses.

From the remaining nectar (~7 µl), we quantified the concentration of sugars and compounds using UPLC and UV or ELS detection, targeting only the compound that was added for each nectar type. Specifically, the remaining nectar was diluted at a ratio of 1:4 in water and filtered through a 0.22-µm centrifugal filter (Millipore, Billerica, MA). A subset of each filtered sample was further diluted in 100 µl for sugar analysis. Chromatographic conditions and detector conditions for all analytes are contained in Appendix C (Table C1). Compounds were identified and quantified using external standards. Peak areas were converted to concentration in the original sample (mM).

To examine the overall effects of compounds on microbial growth, we first used a two-way ANOVA. Microbial density was log-transformed ($\log_{10}$(CFU/µl nectar +1)) to homogenize the variance among groups and used as the response variable. Microbial species, compound identity, and their

interaction were included as predictors. Second, separate one-way ANOVAs were performed for each compound using microbial species as a predictor of the log ratio of CFU density ($\log_{10}$((CFU/µl nectar$_{treatment}$ +1)/(CFU/µl nectar$_{control}$ +1))). Tukey HSD post-hoc tests were conducted to determine differences among species within a nectar treatment.

To examine if the presence of compounds altered the effect of microbial growth on sugar degradation, we used ANOVA to examine microbial effects on the relative change in total sugar concentration (as calculated above) and the ratio of monosaccharides:total saccharides in nectar, which have been linked to pollinator preference (Martínez del Rio et al. 1992, Petanidou 2005). Predictors for each model included compound identity, species identity, and their interaction. Pearson correlations were used to examine the relationship between $\log_{10}$-transformed microbial CFU density in nectar and the concentration of each saccharide (sucrose, glucose and fructose) separately. To examine if microbial species differentially affected the concentration of non-saccharide compound, one-way ANOVAs were performed separately for each compound.

Experiment 3: microbial and compound effects on pollinator foraging

In the last experiment, we investigated if microbes and compounds interactively altered nectar consumption by potential pollinators. We used catalpol and caffeine because they occur in the nectar of plants growing in California, including *Citrus* species (Wright et al. 2013) and *M. aurantiacus* (M.D. Bowers, unpublished data), and are likely to be encountered by pollinators at our field site. Artificial nectar solutions were prepared as described above, and the concentrations of compounds mimicked naturally occurring concentrations in nectar (Appendix B, Table B1). The control solution contained neither compound. Aliquots were stored at -80^{o}C, which has no measurable effect on nectar chemical composition (Peay et al. 2012). In preparation for each experimental replicate (day), thawed aliquots

were inoculated with either a suspension of *M. reukaufii* to an initial concentration of approximately 20 cells/µl (as above), to approximate microbial density following bird visitation (15 ± 4 yeast cells, 561 ± 82 bacterial cells, mean ± SEM, Vannette et al. unpublished data), or an equal volume of control artificial nectar. Nectar solutions were incubated for 5 days at 25^{o}C, after which nectar pH was examined to assess if microbial growth had occurred (Vannette et al. 2013) and experiments performed.

The experiment was conducted in the field at the Plant Growth Facility on Stock Farm Road on the Stanford University campus in July-August 2014 (See Appendix D for dates). Artificial flower arrays were designed to attract either hummingbirds (Array A) or bees (Array B), as in Vannette et al. (2013) and Good et al. (2014), respectively. Array A consisted of six blocks, each separated by 3-5 meters. Each block contained 6 stakes. Each stake contained five tubes of a single nectar treatment plus one tube covered with small white organza bag (ULINE, Pleasant Prairie, WI). The bagged tube was used to estimate the loss of nectar volume by evaporation. Individual nectar samples were contained in 200-µl PCR tubes, and tubes were placed in pipette tips covered in orange tape and arranged vertically on a stake (Appendix D, Fig D1). Our observations indicate that Anna's hummingbirds (*Calypte anna*) tend to begin foraging on the uppermost flowers on our artificial plants and, after sampling nectar from 1-2 flowers, either continue foraging on lower flowers on the same plant or leave the plant. To estimate the contribution of bees and other small-bodied visitors, we caged stakes from two of the blocks using 2 cm square garden netting, as in Belisle et al. (2012), which made them inaccessible to hummingbirds (see further details in Appendix D). All blocks were positioned near potted *M. aurantiacus* plants. We observed frequent visitation by multiple *C. anna* individuals to uncaged stakes, while caged flowers in array A received infrequent visits by honeybees (*Apis mellifera*).

A separate array (array B) was designed to attract bees and was positioned near honeybee hives maintained at the Plant Growth Facility. Array B consisted of four blocks, and each nectar treatment was replicated once per block. Within each block, each of 3 stakes supported 4 artificial flowers, constructed from yellow 'petals,' as in Good et al. (2014), with treatments assigned to stakes haphazardly within a block. Nectar was contained in a cap from a 1.5-ml centrifuge tubes affixed to the 'flower' using putty (see Appendix D, Fig D2). We observed honeybee (*A. mellifera*) visitation to array B, with virtually no observations of consumption by non-target organisms, as in Good et al. (2014).

In each array, nectar was exposed to potential pollinators during peak foraging hours (approximately 10 am to 1 pm), and after 3-4 hours, tubes were capped. Individual tubes were prepared daily and the experiment replicated 12 times for array A and 8 times for array B. Remaining nectar volume was quantified using graduated microcapillary tubes. We estimated nectar loss to evaporation using bagged control tubes on each day for both arrays (9±7% of initial volume, mean ± SD). The proportion of nectar consumed was calculated by subtracting nectar remaining from the average volume of evaporation controls and dividing by the volume remaining in the controls. Nectar-inhabiting yeast populations have a doubling time of 1-2 hours or longer under optimal conditions (Peay et al. 2012), so it is unlikely that microbes introduced during the experiment influenced pollinator foraging substantially enough to be detected. We also analyzed sugars and secondary compounds in a subset of conditioned nectar using methods described above, and results did not differ qualitatively (data not shown).

We assessed the interactive effects of compound addition and yeast inoculation on nectar removal using the proportion of nectar consumed from each tube as a response variable. For array A, stake-level averages were used to reduce the probability of Type I error. Linear mixed effects models (Pinheiro et al. 2012) were used with the microbial treatment, nectar type, the presence of cage (in

array A) as predictor variables. Individual observations were used for array B because each treatment was only replicated once per block. Both models included as random effects both the experimental block nested within experiment date. Tukey's HSD tests implemented in the multcomp package (Hothorn et al. 2008) examined pairwise differences between treatments. In addition, the proportion of flowers that had been completely emptied by pollinators was modeled using a mixed effects binomial GLM implemented in the lme4 package (Bates et al. 2011) with experimental block and date used as random effects. Likelihood ratio tests were used to compare nested models with and without the microbial treatment x nectar type interaction and main effects (Zuur et al. 2009). The significance of random effects was not examined.

All statistical analyses were performed using R (R Core Development Team 2012).

Results

Experiment 1: compound effects on microbial growth

Chemical compounds present in artificial nectar affected microbial growth in a species- and compound-specific manner. Both growth rate (μ) and maximum optical density (A) were affected by chemical compounds in some cases, although μ was more variable than A. In general, compounds had the largest effect on microbial growth parameters at high concentrations (Fig 1). For example, increasing concentration of caffeine decreased maximum optical density attained by microbial species (A), aucubin increased A in most species, and catalpol generated species-specific responses. The alkaloid caffeine reduced microbial maximum growth rate (μ) and A in most species (Fig 1), particularly at high concentrations. However, species varied in their growth parameters (Appendix E, Fig E1) and susceptibility to compounds: caffeine decreased μ and A for most species, but doubled μ for *P. syringae*

pv. *pisi* at high concentrations (Fig 1c). In some cases, species responded nonlinearly to increasing compound concentration. For example, nicotine increased μ and A in some species at intermediate concentrations (Fig 1), but decreased μ and A in most species at high concentrations. The cardenolide ouabain did not significantly affect microbial growth parameters (Fig 1).

Experiment 2: microbial effects on nectar chemistry

At concentrations similar to or higher than that found in floral nectar, all compounds tended to decrease microbial density in nectar (Fig 2), but species varied substantially in average CFU density (Appendix E, Fig E2) and in their response to the presence of compounds (Fig 2). In the most extreme case, nicotine drastically reduced culturable cell density of yeast *Starmerella bombicola* from 1.1×10^4 to 10 CFU μl^{-1} but increased CFU density of the bacterium *Erwinia* sp. from no measurable microbial growth (control) to nearly 5.8×10^4 CFU μl^{-1}. However, microbial responses to other compounds were more modest (Fig 2) and largely negative. *Gluconobacter* sp. and *M. reukaufii*, both common in catalpol-containing *M. aurantiacus* nectar, maintained high CFU densities in the presence of catalpol.

Microbial species reduced total sugar concentration by an average of ~22% in control nectar, although the extent of reduction depended on species identity and ranged from 6-25% of initial sugar concentration (Species $F_{6,151}=5.01$, $P<0.0001$). The presence of most compounds did not significantly alter the proportion of total sugars metabolized by microorganisms (Fig 3a), except ouabain, which reduced the percentage of sugar consumed by microbes (Fig 3a). In addition, microbial growth increased the proportion of monosaccharides in nectar, from less than 1% in solutions with no microbes to 3-8% in inoculated solutions. The extent of this effect varied among microbial species (Species $F_{6,151}=34.29$, $P<0.0001$). In most cases, compounds increased the proportion of nectar sugars comprised by monosaccharides following microbial growth (Fig 3b), although nicotine decreased the proportion of monosaccharides compared to microbial growth in the control condition (Fig 3b). Although effect of

compounds on microbial growth varied in magnitude among species (Species x Nectar $F_{28, 151}$=3.55 P<0.0001, Appendix F, Fig F1), the direction was largely consistent with the main effects shown in Fig 3a-b. As in previous studies, the concentration of fructose, a product of sucrose hydrolysis and less preferred monosaccharide, was positively correlated with microbial density (r=0.40, P<0.001, Fig 3c).

Microbes also decreased the concentration of some compounds in nectar (Fig 4). This effect depended on the identity of both compounds and microbial species. *Gluconobacter*, *A. astilbes*, and *M. reukaufii* decreased the concentration of aucubin ($F_{6,37}$=8.53, P<0.001, Fig 4a), and microbial density was negatively associated with the concentration of aucubin in nectar (r =-0.029±0.01, P=0.02). The magnitude of this effect was large in some cases: *Gluconobacter* decreased the concentration of aucubin by more than 50%. Species varied in their effects on nicotine concentration ($F_{7,29}$=3.81, P=0.0047, Fig 4d), where both *Erwinia* and *Gluconobacter* decreased nicotine concentration. Additionally, *S. bombicola* and *C. rancensis* marginally decreased the concentration of cardenolide ouabain ($F_{6, 30}$= 2.13, P=0.06, Fig 4e). Individual microbial species did not significantly reduce the concentration of caffeine or catalpol, but within the catalpol nectar type, microbial density was negatively correlated with the final concentration of catalpol with marginal significance (r =-0.02±0.01, P=0.08).

Experiment 3: microbial and compound effects on pollinator foraging

Nectar containing catalpol was strongly deterrent to both large- and small-bodied floral visitors, which consumed between 17 and 60% less catalpol-containing nectar than control or caffeine-containing nectar, and also emptied fewer of the flowers (Fig 5, Tables G1-3). In array A, large-bodied pollinators removed more nectar than did small visitors (caging $F_{1,325}$=59.2, P<0.001, Table G1). Microbial inoculation did not significantly influence pollinator consumption in array A ($F_{2,325}$=0.86, P=0.42). However, incubation with *M. reukaufii* increased consumption of catalpol-containing nectar in Array B ($F_{2,362}$=3.44, P=0.03, Fig 5b, Table G2). Similarly, *M. reukaufii* increased the proportion of catalpol-

containing flowers that were emptied by honey bees (Array B $\chi^{2=}$6.61, *P*=0.036, Fig 5c, Table G3). Nectar that contained caffeine was consumed to the same extent as control nectar in both arrays, and *M. reukaufii* did not alter the volume of caffeine-containing nectar consumed in either array (Fig 5).

Discussion

Overall, our results suggest that compounds found as secondary metabolites in nectar can alter microbial growth, but that growth inhibition or promotion is highly species- and compound-specific. Furthermore, although compounds commonly reduced microbial density, they did not decrease microbial sugar metabolism in most cases (Figs 2-3, Appendix F, Fig F1). At some concentrations, compounds (e.g., aucubin, caffeine, nicotine) increased microbial density, growth rate, and conversion of sucrose to monosaccharides (Figs 1&3). Growth stimulation or tolerance and detoxification mechanisms (Wink and Twardowski 1992) may in part explain the maintenance of sugar metabolism by microorganisms in the presence of compounds. Taken together, the results presented here suggest that these particular compounds in nectar may not be a primary or general ecological filter for the establishment of microorganisms or an effective defense for nectar sugars (Fridman et al. 2012).

Increasing evidence suggests that specialized nectar-dwelling microorganisms can tolerate osmotic stress (Herrera et al. 2010), oxidative stress (Álvarez-Pérez et al. 2012, Vannette et al. 2013) and, as suggested here, some compounds present as secondary metabolites. It is possible that some species of microbes may contain intraspecific genetic variation in their response to chemicals found in nectar, but this remains to be examined. In addition, other nectar components including volatile compounds, proteomic defenses (González-Teuber et al. 2009), or biochemical conditions created by enzymes in nectar (Carter and Thornburg 2004) may be more effective at preventing microbial growth. It is also possible that the compounds and enzymes described above may act synergistically. Only a few plant

species and compounds have been examined to date, and the question of which mechanisms are responsible for microbial growth inhibition in nectar is worthy of further inquiry.

This study also yielded experimental support for the possibility that microbial growth alters the effects of secondary compounds on nectar consumption by potential pollinators (Fig 5), although the strength of these effects varied with compound and visitor identity (Fig 5). Both honey bees and hummingbirds were strongly deterred by the presence of catalpol in nectar (Fig 5, Tables G1-G2), but inoculation with *M. reukaufii* increased consumption of catalpol-containing nectar by small-bodied visitors. Although results from Array A trend in this direction, *M. reukaufii* did not significantly influence pollinator consumption of catalpol-containing nectar. This result may indicate that microbial effects on nectar chemistry are less important for large-bodied pollinators, or that variation in microbial abundance does not strongly influence foraging on fine scales (within a plant or on different branches of the same plant). Future research should examine if pollinator species vary in their response to nectar characteristics, including secondary chemistry and microbial presence in nectar.

Microbes may alter nectar consumption through multiple mechanisms. Some microbes may directly weaken the deterrent effect of secondary compounds on pollinators. For example, *Gluconobacter* sp. reduced the concentration of aucubin to the greatest extent of any of the microbes tested. The specific mechanism of degradation was not examined, but the closely related bacterium *Gluconobacter oxydans* expresses β-glucosidase (Kostner et al. 2015), which can initiate the degradation of glycosides, including aucubin. These effects on the concentration or toxicity of secondary chemicals may extend to influence pollinator foraging (Detzel and Wink 1993, Kessler and Baldwin 2006, Köhler et al. 2012), which is largely dependent on concentration (Singaravelan et al. 2005, Wright et al. 2013, Tiedeken et al. 2014). Through their effects on naturally occurring or synthetic compound concentration, microbes may also indirectly modify pathogen loads in pollinators (Manson et al. 2010, Richardson et al.

2015) or various aspects of pollinator behavior (Kessler et al. 2012, Wright et al. 2013, Kessler et al. 2015). Microbial effects on pollinator foraging depend on their density in nectar (Junker et al. 2013), which is likely affected by floral longevity or the time microbes have access to floral nectar. How floral longevity mediates microbial effects on plant-pollinator interactions is a question worthy of further investigation.

Other possible mechanisms by which microbes alter nectar consumption by pollinators are independent of assimilation or metabolism of secondary compounds. For example, *M. reukaufii* did not affect the concentration of catalpol in either the laboratory (Fig 4b) or field experiment, yet significantly increased foraging on this nectar in Array B (Fig 5). Iridoid glycosides taste bitter to humans, and their action as feeding deterrents suggests that they may act similarly in insects (Biere et al. 2004). Both wild and cultivated yeasts produce a variety of flavor-modifying enzymes, including those with proteolytic, polygalacturonase, and β-glucosidase activity (Lilly et al. 2000, Swiegers et al. 2005). Although perception of bitterness varies among pollinators (de Brito Sanchez 2011), chemical modifications to nectar could modify pollinator perceptions of and foraging on bitter-tasting nectar. Additional work will be required to determine if such modifications may outweigh the deterrent effects of some microbes (Vannette et al. 2013, Good et al. 2014, Junker et al. 2014) and if abiotic factors mediate the strength or direction of these effects.

Secondary metabolites in plants have traditionally been considered important in mediating antagonistic interactions. However, these metabolites may also play an underappreciated role in facilitative interactions. As our findings suggest, some nectar-colonizing microorganisms previously thought to be commensals or even parasitic to plants may in some cases indirectly facilitate the plants' mutualistic interactions with pollinators by modifying nectar chemistry. Such indirect facilitation would occur if microbial colonization increased nectar removal and in turn improved plant reproduction,

although nectar removal does not always confer a reproductive advantage to the plants (Kessler et al. 2008). We propose that, over evolutionary time, microbial activity in nectar may in some cases weaken the selective pressure for plants to reduce the concentration of secondary compounds in nectar, which may in part explain the puzzling prevalence of "toxic nectar" (Adler 2000). In addition, microbes may be under selective pressure to make nectar attractive to birds and bees that help microbes disperse among flowers. This pressure may provide an evolutionary reason behind microbial alleviation of the deterrent effects of secondary compounds in nectar.

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Figure legends

Figure 1. Response of microbial growth to increasing concentrations of compounds in Experiment 1. Exact compound concentrations are listed in Table B1. Column 1 displays the relative growth rates (μ) and column 2 the relative maximum optical density (A) of single-species microbial inoculations with increasing concentration of chemical compound. Growth parameters were standardized by individual species' average growth in the control condition; values above and below 1 indicate a higher and lower value of μ or A compared to the control, respectively. Open points represent 'nonspecialist' microbial species and filled points represent 'specialists' (see methods for more details). The availability of chemical materials limited the number of species that could be tested for some compounds (e.g. panels a & b). Points represent the replicate means for growth experiment (N=2-4) ± Standard error of the mean (SEM). No growth was detected in control (no inoculation) solutions. Statistical results are summarized in each panel as follows: Species (Sp), Compound concentration (Conc.), NS P>0.10, +*P*<0.10, **P*<0.05, ***P*<0.01, *** *P*<0.001.

Figure 2. Effects of compound addition on microbial density in Experiment 2. Bars indicate the relative change in microbial density in the treatment compared to growth in the control (no compound), calculated as $\log_{10}(CFU\mu l^{-1}_{treatment} / CFU\mu l^{-1}_{control}+1)$. Letters indicate significant differences among species within a nectar type. No microbes were detected in the uninoculated control solutions. +*P*<0.10, **P*<0.05, ***P*<0.01, *** *P*<0.001.

Figure 3. Effects of compounds on microbial modification of nectar sugars in Experiment 2, including a) reduction in total sugar concentration and b) total concentration of monosaccharides in nectar. In a) bars represent the standardized difference between microbe-added and control treatment, averaged across all microbe addition treatments ± SEM. In b) bars represent the average percent of total sugars comprised by fructose and glucose in microbe addition treatments ± SEM. Letters indicate nectar treatments that differ significantly using a Tukey HSD post hoc test, where $P<0.01$. See Appendix E for ANOVA tables. In c) microbial density is correlated with fructose concentration in the artificial nectar (Pearson's r=0.40, $P=0.0056$). Points represent the average values for each species x nectar treatment.

Figure 4. Effect of microbial growth on the concentration of secondary compounds in Experiment 2, for a) aucubin, b) catalpol, c) caffeine, d) nicotine, and e) ouabain. Letters indicate results of Tukey HSD tests ($P<0.05$), presented when ANOVA indicated a significant difference among treatments. Dotted lines indicate initial compound concentrations. Bars indicate means ± SEM. P-value indicates significance of *F*-statistic for species effect on compound concentration from ANOVA. +$P<0.10$, *$P<0.05$, **$P<0.01$, *** $P<0.001$.

Figure 5. Interactive effects of the inoculation with the yeast *Metschnikowia reukaufii* and the presence of compounds on synthetic nectar consumption (a-b) or the proportion of flowers emptied after foraging bouts (c). In Array A, flowers were designed to attract hummingbirds. In a) bars represent mean ±SEM in uncaged flowers (large floral visitors), and points represent means in caged flowers (small floral visitors), based on stake-level averages. In array B, flowers were designed to attract bees. In b), bars indicate mean ± SEM. *P*-values in figures are calculated from mixed models using *F*-tests (a-b) and χ^2 tests (c). Asterisks indicate significant ($P<0.05$) differences between control and yeast-inoculated treatment within a nectar type.