Note

Omnivory in Bees: Elevated Trophic Positions among All Major Bee Families

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abstract: As pollen and nectar foragers, bees have long been considered strictly herbivorous. Their pollen provisions, however, are host to abundant microbial communities, which feed on the pollen before and/or while it is consumed by bee larvae. In the process, microbes convert pollen into a complex of plant and microbial components. Since microbes are analogous to metazoan consumers within trophic hierarchies, the pollen-eating microbes are, functionally, herbivores. When bee larvae consume a microbe-rich pollen complex, they ingest proteins from plant and microbial sources and thus should register as omnivores on the trophic "ladder." We tested this hypothesis by examining the isotopic compositions of amino acids extracted from native bees collected in North America over multiple years. We measured bee trophic position across the six major bee families. Our findings indicate that bee trophic identity was consistently and significantly higher than that of strict herbivores, providing the first evidence that omnivory is ubiquitous among bee fauna. Such omnivory suggests that pollen-borne microbes represent an important protein source for larval bees, which introduces new questions as to the link between floral fungicide residues and bee development.

Keywords: $\delta^{15}N$, compound-specific isotopic analysis, microbiome, pollen, trophic.

Introduction

Of the more than 20,000 bee species on Earth, virtually all are widely considered to be strict herbivores (Loper et al. 1980; Standifer et al. 1980; Vaudo et al. 2015), with the rare exception of highly specialized stingless bees that feed exclusively on carrion (Gilliam et al. 1985; Camargo and Roubik 1991) or fungi (Menezes et al. 2015). Adult bees are known to consume large amounts of nectar as a source of carbohydrates, while larval bees consume much more pollen than nectar during their development (Danforth 2007). Importantly, within bee-collected pollen provisions there are diverse and abundant microbial communities that not only play roles in pollen processing and preservation (Gilliam 1997; Martinson et al. 2011; Mattila et al. 2012; Corby-Harris et al. 2014; McFrederick et al. 2014, 2017; McFrederick and Rehan 2016; Graystock et al. 2017; Steffan et al. 2017*b*) but also may serve as significant protein sources for developing bees. Recent studies suggest that certain bee taxa may be consuming significant nonplant protein within pollen provisions (Chikaraishi et al. 2011; Steffan et al. 2017*a*).

Among social and solitary bee species, the pollen and nectar provisions are often aged (Standifer et al. 1980; Gilliam et al. 1989; Anderson et al. 2014), undergoing chemical breakdown and consumption by microbes (Loper et al. 1980; Standifer et al. 1980; Gilliam et al. 1989). This microbe-mediated process can alter the biochemical composition of pollen provisions while also serving as a "selective sieve" (Corby-Harris et al. 2014), shaping the microbial community within the pollen mass (Gilliam 1979*a*, 1979*b*, 1997; Loper et al. 1980; Gilliam et al. 1989). It has long been suggested that such microbes play important roles in the nutrition of larval bees as well as protect the bees from parasites and pathogens (Gilliam 1979*a*, 1979*b*, 1997; Loper et al. 1980; Gilliam et al. 1989; Anderson et al. 2011; Koch and Schmid-Hempel 2011; DeGrandi-Hoffman et al. 2012). When bees collect pollen, they moisten it with nectar and regurgitated enzymes (Cane et al. 1983), acids, and microbes before storing it for later consumption by developing larvae (Gilliam 1979*a*; Yoder et al. 2013). In the heat of summer, this blend of sugar-rich nectar and proteinaceous pollen is colonized thoroughly by microbes, which consume the pollen in successional stages (Gilliam

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1997). It appears that microbes are integral to the successional use and breakdown of the pollen resource, increasing digestibility and enhancing the nutritional content of pollen provisions (Mattila et al. 2012). The extracellular secretions of pollen-borne microbes aid in the enzymatic digestion of the recalcitrant exines and intines of the pollen grain (i.e., the carbon-rich "shell" comprised largely of cellulose, lignins, and waxes), affording bees more efficient access to the protein-rich pollen cytoplasm (Loper et al. 1980; Gilliam et al. 1989; Anderson et al. 2013). Microbial digestion of the pollen may also preserve the nutrient-rich pollen substrate from opportunistic, pathogenic microbes, allowing larvae to feed over extended periods (Gilliam 1997; Hani et al. 2012). In honey bee hives, this conversion of raw pollen to a microbe-rich pollen provision has been referred to as the creation of "beebread" (Herbert and Shimanuki 1978; Gilliam et al. 1989). In an analogous fashion, other animal species inoculate their diet (e.g., carrion-feeding burying beetles) with specific bacteria and yeasts, facilitating biofilm formation around the surface of a cadaver, which preserve the carrion as the beetle larvae develop (Shukla et al. 2018).

Bees may also actively screen out certain microbes while aging the pollen provision. For example, in a study of honey bees Gilliam (1997) found that the bacterial presence declined during the transfer of pollen from flower to hive. Approximately 50% of the flowers in the foraging range of the honey bees were shown to have bacteria present, but by the time the pollen was fermenting in the hive only 4% of the provisions contained measurable levels of bacteria (Gilliam 1997). Beneficial fungi (yeasts and filamentous fungi), such as *Candida*, *Saccharomyces*, *Penicillium*, *Rhizopus*, *Cladosporium*, and *Aspergillus*, are commonly found in the pollen provisions of honey bees (Yoder et al. 2013), suggesting that fungi may be important constituents of the pollen-borne microbe community. While it is not entirely clear what functions these fungi and bacteria serve in their use of the pollen substrate, it is evident that there is an active microbial community within a pollen mass (Inglis et al. 1993; Anderson et al. 2013) and that fungi are often strongly represented (Gilliam 1997; Yoder et al. 2013).

Among solitary bees, the role of microbes within pollen provisions appears to be particularly important. Yeasts have been consistently found in the provisions of a variety of solitary bee species (Roberts 1971; Inglis et al. 1993; Pimentel et al. 2005). Colletid bee provisions, for example, were described by Roberts (1971) as having active, observable fermentation, to the extent that bubbles could be seen evolving from the soupy provisions within the brood cell, with the distinct odor of yeast fermentation evident. Two ground-nesting anthophorid bees were also found to have strong yeast associations in their pollen provisions (Rosa et al. 2003). Here, the yeast biomass was actually the dominant component of the pollen provision. Furthermore, these yeasts were quite specialized in terms of their ability to digest sugars, produce proteinases, and disperse defensive compounds against competing microbes (Rosa et al. 1999). Thus, the microbial communities within the pollen provisions of these bees were dominated by symbiotic yeasts that effectively conditioned the pollen and appeared to have kept it from being overtaken by undesirable microbes. Yeasts (and their metabolic products) may be critical components of native bee nutrition (Rosa et al. 1999) and are likely important to the defense of pollen provisions (Kaltenpoth 2009; Mattila et al. 2012). Importantly, these pollen-borne symbionts are distinct from those of the bee gut: in a recent study (Saraiva et al. 2015), the degree of overlap between the microbial community of beebread versus that of the gut was shown to be merely 7%, suggesting that for honey bees these two communities of symbionts are not interchangeable.

While feeding on pollen provisions, bees are consuming microbes within the fermented mass of pollen, which should elevate bee trophic position (TP_{bee}) to values greater than 2.0 (the expected TP of strict herbivores; Steffan et al. 2015). Such trophic inflation occurs because the aging pollen mass gradually transforms into a complex of trophic groups (consumer biomass enmeshed throughout diet biomass), which elevates the trophic position of the entire substrate (Steffan et al. 2017*a*). If bees derived all of their protein from pollen alone, then they would, as strict herbivores, register at TP $\sim$ 2.0. Any significant elevation above this trophic position (i.e., $TP_{bee} > 2.0$) would indicate that bees had assimilated substantial protein from heterotrophic food sources (in this case, microbial sources) and thus would be measurably omnivorous. Recent findings suggest that select bee taxa may not be strictly herbivorous (Chikaraishi et al. 2011; Steffan et al. 2017*a*) and that, perhaps as larvae, they are assimilating heterotrophic proteins via rampant microbivory. Given the abundance of bacteria and fungi within fermenting pollen provisions, microbivory among larval bees should be ubiquitous and should significantly elevate bee trophic position. We investigated this hypothesis by measuring the trophic positions of a broad diversity of wild-collected bees. These bee specimens represented the six major extant bee families on Earth.

Methods

Sample Collection and Preparation

To resolve the question of bee trophic identity, we examined taxa spanning six bee families (fig. 1). The specimens sampled in this study ($N = 54$) represented 14 species across 12 genera. Specimens were collected in the United States, primarily in Ithaca, New York, and central Wisconsin (cranberry marshlands of Wood County); *Ptiloglossa* specimens were collected in Arizona. Bees were collected and curated between June and September, 2008–2015. All bees collected

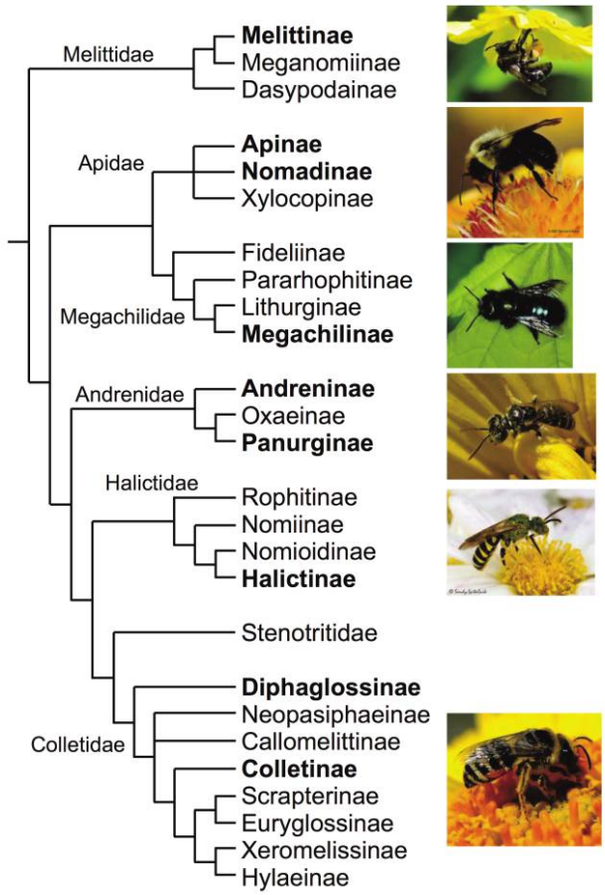

Figure 1: Family- and subfamily-level phylogeny for bees (based on Danforth et al. 2013). Subfamilies sampled in this study are in bold. Pictured bees (from top to bottom): *Macropis nuda* (Melittidae), *Bombus impatiens* (Apidae), *Osmia lignaria* (Megachilidae), *Calliopsis rhodophila* (Andrenidae), *Agapostemon virescens* (Halictidae), and *Colletes* (Colletidae). Photos courtesy of J. D. Gardner (Melittidae), Donna K. Race (Apidae), Lynette Elliott (Megachilidae), Ron Hemberger (Andrenidae), Sanwdra Spitalnik (Halictidae), and Hartmut Wisch (Colletidae).

in Wisconsin were caught in pan traps with dilute soapy water after the cranberry bloom in 2008, 2010, and 2011 (Day 2013); stored in 70% alcohol; and then dried, pinned, labeled, and identified to the species level. Specimens collected in New York were netted on flowers, placed individually in Eppendorf tubes stored on ice, identified to the species level, and placed in a −80°C freezer. We also collected pollen provisions from two independent nesting tubes of *Osmia cornifrons* to quantify the trophic identity of aged pollen, which forms the sole diet for the developing bees. Pollen provisions had been aged for a minimum of 10 days prior to preparation for isotopic analysis (see the "Supplemental Methods" section of the appendix, available online).

All specimens (pollen and bees) were desiccated in a drying oven for ~7 days at a temperature of 45°–60°C. Each fermented pollen provision was placed in a separate clean glass vial. Each bee was cleaned with 70% ethanol (to rinse off trace amounts of foreign material), and the head and legs were separated, consolidated, and homogenized for subsequent isotopic analysis. Analysis of the head and six legs effectively integrated all major tissue types of the bee (e.g., musculature, exoskeleton, nervous tissue, hemolymph) while precluding the possibility that excessively decayed gut tissues could skew the isotopic composition of the sample. Bee samples were homogenized, packaged in glass vials, and along with the aged pollen provisions shipped to the Japan Agency for Marine-Earth Science and Technology for compound-specific isotopic analysis (CSIA) using previously established protocols (Chikaraishi et al. 2007).

Measurement of Bee Trophic Position

The TP values of the aged pollen provisions and bee specimens were calculated using CSIA of amino acids, a relatively new technique that has provided novel insights into the trophic ecology of diverse phylogenetic groups from multiple ecosystems (Chikaraishi et al. 2009; Steffan et al. 2013, 2015). Sample preparation involves HCl hydrolysis and *N*-pivaloyl/isopropyl derivatization of the amino acids within each sample (Chikaraishi et al. 2009). Amino acids are extracted from samples, and the abundance of each compound is measured via gas chromatography before being analyzed for its isotopic (^{15}N) composition (see the appendix for further methodological details). By measuring the isotopic signatures (‰) of two particular amino acids, glutamic acid ($\delta^{15}N_{glu}$) and phenylalanine ($\delta^{15}N_{phe}$), the TP of the organism could be determined using a proven equation for terrestrial C_3 plant-based food webs (Chikaraishi et al. 2009; Steffan et al. 2013): $TP = [(\delta^{15}N_{glu} - \delta^{15}N_{phe} + 8.4‰)/TDF] + 1$. Here, the TDF parameter represents the trophic discrimination factor (net intertrophic enrichment of ^{15}N between the two amino acid pools: glutamic acid and phenylalanine). The TDF has recently been shown to be centered near 7.2‰ among a broad diversity of terrestrial organisms (Steffan et al. 2015); hence, a TDF of 7.2 was used for all $TP_{aged\ pollen}$ and TP_{bee} calculations. TP_{bee} estimates were analyzed based on family affiliation and mode of provisioning (i.e., mass vs. progressive provisioners; Michener 2007; appendix). Trophic position estimates were analyzed using both parametric and nonparametric tests, as dictated by heteroscedasticity and normality of data (SPSS 18.0; IBM, Chicago, IL). Comparisons of TP_{bee} were conducted using one-sample single-tailed *t*-tests.

Results

Bee trophic position was measured for 14 different species (12 genera) distributed among six families. Mean TP_{bee} among bee specimens ($N = 54$) was 2.60 ± 0.06 (mean ± 1 SE),

which was significantly higher (Wilcoxon signed rank, $Z = 1476$, $P < .001$) than would be expected of strict herbivory (TP = 2.0). Within each family, TP_{bee} was significantly greater than 2.0 ($TP_{Andrenidae} = 3.09 \pm 0.15$, $N = 6$, $P = .02$; $TP_{Apidae} = 2.62 \pm 0.09$, $N = 21$, $P < .001$; $TP_{Colletidae} = 2.11 \pm 0.05$, $N = 8$, $P = .03$; $TP_{Halictidae} = 2.78 \pm 0.19$, $N = 6$, $P < .01$; $TP_{Megachilidae} = 2.75 \pm 0.12$, $N = 7$, $P < .001$; $TP_{Melittidae} = 2.14 \pm 0.04$, $N = 6$, $P = 0.01$), with significant among-family differences (Kruskal-Wallis $\chi^2_5 = 30.84$, $P < .001$; table A1, fig. 2; tables A1, A2 are available online). While there was no statistical difference between mass-provisioning (2.61 ± 0.08, $N = 38$) and progressive-provisioning (2.50 ± 0.08, $N = 16$) bees, the TP_{bee} of each group was significantly higher than 2.0 ($P < .001$). Data have been archived in the Dryad Digital Repository (https://dx.doi.org/10.5061/dryad.b6063f5; Steffan 2019).

The two nesting tubes provisioned by *Osmia cornifrons* yielded five and six pollen provisions. Mean $TP_{aged\ pollen}$ across all specimens ($N = 11$) was 1.46 ± 0.06 (mean $\pm$ 1 SE). This value was significantly higher than the well-established trophic position of autotrophic (plant) biomass: $TP_{autotroph} = 1.0$ (one-sample t-test, $t_{10} = 7.28$, one-tailed $P < .0001$; table A2).

Discussion

Our findings reveal both the breadth and the magnitude of bee reliance on pollen-borne microbes as significant dietary resources. Bee specimens representing six of the seven extant bee families all exhibited significantly higher trophic positions than that of a purely herbivorous consumer (fig. 2), suggesting a consistent tendency toward omnivory. Foraging female bees are clearly prodigious harvesters of pollen, which serves as the primary nutritional "currency" for immature bees (Anderson et al. 2014; Vaudo et al. 2015); however, the degree to which bee larvae directly consume pollen appears to be highly variable. Our results suggest that bees often feed secondarily on pollen. Specifically, we show that bee larvae consume significant nonplant protein during development, which means that the bees we examined were omnivorous, feeding on heterotrophic organisms as well as pollen.

When microbes consume an aged, plant-based matrix such as pollen, they multiply rapidly and become enmeshed within the plant substrate. This detrital complex may register at a higher trophic position than an uncolonized substrate because the complex represents a mix of multiple trophic groups—both diet and consumer biomass (Steffan et al.

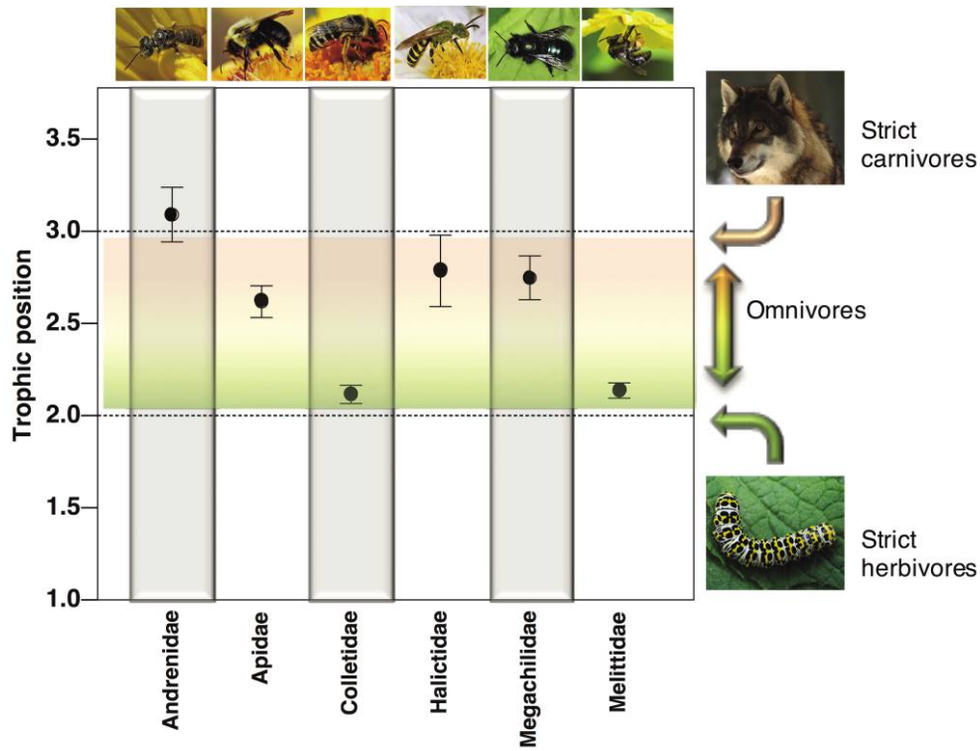

Figure 2: Trophic positions (mean $\pm$ SE) observed in bees representing the six major extant bee families. Elevated trophic positions were observed among all specimens ($N = 54$), placing each family significantly above trophic level 2.0 (the trophic level associated with strict herbivory). Caterpillar photo (CC BY) by Ian Kirk (https://commons.wikimedia.org/wiki/File:Mullein_moth_caterpillar_(9087934004).jpg).

2017*a*). The herbivorous microbes within the complex register predictably at or near trophic position 2.0, which means that they are trophically (functionally) equivalent to "meat" in the food chain, no different from grazing caterpillars or fish (Steffan et al. 2015). Raw pollen or any plant substrate that is colonized and consumed by heterotrophic organisms can be transformed into a microcosm of the broader food web (Steffan and Dharampal 2019), and as the detritus ages the trophic identity of the detrital mass will often become progressively elevated (Steffan et al. 2017*a*). The aged pollen provisions in our study exemplify this phenomenon, exhibiting significantly elevated trophic positions ($TP_{\text{aged pollen}} > 1.0$). We show that the mean trophic position of the aged pollen provisions ($TP_{\text{aged pollen}} \sim 1.5$) was significantly higher than that of plant tissue alone (table A2), indicating that the fermented pollen had been transformed into a detrital complex composed of multiple trophic groups.

When fauna such as bee larvae consume a detrital complex, they are ingesting microbial meat along with the entire detrital mass. As a result, the detritivorous animal feeds as both a carnivore and a herbivore, assimilating heterotrophic (microbial) and autotrophic (plant) proteins, respectively, and exhibiting an elevated trophic position compared with strict herbivory (Steffan et al. 2017*a*). The mixing of carnivory and herbivory represents canonical omnivory, and while common among many animal species, such omnivory has never been reported among bees. The elevated trophic positions observed across all bee taxa ($TP_{\text{bee}} \sim 2.6$) in our study is consistent with that of detritivores feeding on a mixture of autotrophic and heterotrophic dietary components (Steffan et al. 2017*a*). While all 54 bee specimens in our study registered at trophic positions greater than 2.0, the modest within-family sample sizes preclude characterization of family-specific trophic tendencies. Our conclusions, therefore, apply to bees as a group and emphasize the consistency of omnivory across families. Given the well-documented abundance and diversity of microbiota within aged pollen substrates (Gilliam 1997; Yoder et al. 2013; Steffan et al. 2017*b*), our evidence of elevated bee trophic positions strongly suggests that as larvae, bees assimilate significant amounts of microbe-derived amino acids. These pollen-borne organisms tend to be distinctly different from those found in the midgut and hindgut of bees (Corby-Harris et al. 2014; Saraiva et al. 2015), which is quite interesting because pollen-borne microbes are clearly ingested and would ultimately be mixed with gut microbes. However, the gut microbiota of insects appear to have coevolved with their hosts and often thrive within a true mutualism (Ayayee et al. 2016). As such, these gut-borne microbes thrive within the gut because it is kept hospitable for them, while the pollen-borne symbionts are largely digested and assimilated. As further evidence of this "dinner/mutualist" hypothesis, the trophic positions of all strict herbivores examined in previous CSIA studies (Chikaraishi et al. 2009; Steffan et al. 2013, 2015) registered very close to $TP \sim 2.0$ despite having digestive tracts full of microbes.

Recent studies of stingless, social bees (*Scaptotrigona depilis*) in Brazil revealed that certain bee species rely heavily on fungal proteins to survive (Menezes et al. 2015). In this tight symbiosis between bees and their fungal symbiont (*Monascus*), the bee larvae feed largely on the fungal mycelia within brood cells. Thus, for *S. depilis*, their fungal symbiont represents the primary protein source for the larvae, making this bee-microbe symbiosis analogous to that of leaf-cutter ants and their "fungus gardens" (Currie et al. 1999). Leaf-cutter ant larvae develop almost exclusively on fungal proteins and register trophically as strict carnivores (Steffan et al. 2015). *Scaptotrigona depilis* bees, therefore, are also likely to register as carnivores (at or near trophic position 3.0) given their fungivorous habit. There are numerous examples of specialized nutritional symbioses between insects and microbes that allow the former to subsist on otherwise low-quality diets (Moran et al. 2008; Martinson et al. 2011). These examples of tight animal-microbe symbioses underscore the ubiquity and importance of microbes in animal ecology and provide broader context for our finding that heterotrophic microbes represent a significant protein source for bees.

Such animal-microbe associations may be especially significant in bees, since the raw pollen they collect often represents a refractory substrate and is seldom fed to the larvae without some degree of microbial fermentation (DeGrandi-Hoffman et al. 2012; Anderson et al. 2013; Corby-Harris et al. 2014). The TP_{pollen} of aged pollen observed in this study ($TP \sim 1.5$) suggests that at the time of collection approximately half of the original plant proteins within the pollen provision had already been consumed and converted into heterotrophic proteins. As a result, when larval bees consume such a detrital complex, their TPs will likely reflect the magnitude of microbial conversion of the detritus. In our study, all bee trophic positions were greater than 2.0; indeed, the andrenid trophic tendency registered near 3.0, suggesting that most proteins consumed by these particular bees were heterotrophic (see the "Supplemental Discussion" section in the appendix). Given the microscale integration of plant and microbial biomass within an aged pollen provision, strict herbivory is a highly improbable scenario for larval bees. Consumption of microbes within aged pollen appears to be both unavoidable and beneficial for the developing bee larvae.

Our findings reframe the trophic identity of the dominant global pollinator group—bees. This casts bees as omnivorous animals that actively farm microbial "livestock" within their aged pollen provisions. Bees are clearly more than pollinators; pollination can be viewed as an incidental outcome of their efforts to collect pollen and nectar to maintain an adequate resource base for their microbial symbionts. In fact, several bee groups can be neutral or even antagonistic to plant reproduction (Parker et al. 2016; Quinalha et al. 2017). Their

microbial symbionts, however, clearly exploit the pollen substrate, enzymatically digesting the pollen and consolidating the amino acids within microbial proteins. These microbial activities appear to protect the pollen provision and enhance the protein availability for young bees. Interestingly, it may be microbes (more so than bees) that are the primary global consumers of pollen. Considering bee-microbe symbioses from the microbial perspective, microbes can be viewed as avid beekeepers, facilitating and assisting their faunal symbionts in the annual pollen harvest. Such insect-microbe symbioses, exemplified here in the case of bees, form one of the dominant trophic paradigms in terrestrial systems (Kaufman et al. 2000; Moran et al. 2008).

Bee-microbe symbioses, then, may be an underappreciated aspect of pollination ecology. It stands to reason that the widespread use of fungicides on flowering crops has the potential to alter the hive or brood cell microbiome, particularly for beneficial fungal species. Indeed, pathogen incidence in honey bee hives has been tempered by the presence of yeasts and molds in beebread (Gilliam et al. 1988). Given that fungicides are known to constrain the fungal communities of pollen provisions (Yoder et al. 2013) and that such antifungals can influence the gut microbial communities of solitary bees (McFrederick et al. 2014), it is conceivable that fungicide-mediated disruptions of the pollen microbiome may represent a stressor for bees. Recent findings indicate that certain fungicides have been linked to major colony declines in bumble bees (Bernauer et al. 2015; Steffan et al. 2017*b*). It is possible that such fungicides compromise bee health by constraining access to microbial proteins. In light of our finding that microbial protein is a major nutritional element for most, if not all, bees, the pervasive use of certain fungicides may be related to widely reported colony losses. Further research will better illuminate how fungicides may be mediating bee-microbe symbioses.

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A, Larval bees (*Osmia*, Megachilidae) feeding on microbe-colonized pollen masses. The black arrow indicates the distinctly U-shaped bee larva, situated atop its pollen provision within a hollow reed. *B*, Older megachilid larvae feeding on aged pollen masses. The white arrow indicates a highly fermented pollen-microbe complex. Photo credit: Shawn A. Steffan.