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Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion

KIRK E. ANDERSON,*† § MARK J. CARROLL,*† § TIM SHEEHAN,‡ BRENDON M. MOTT,§ PATRICK MAES† and VANESSA CORBY-HARRIS§

*Center for Insect Science, University of Arizona, Tucson, AZ 85721, USA, †Department of Entomology, University of Arizona, Tucson, AZ 85721, USA, ‡Department of Microbiology, University of Arizona, Tucson, AZ 85721, USA, §USDA-ARS Carl Hayden Bee Research Center, Tucson, AZ 85719, USA

Abstract

Honey bee hives are filled with stored pollen, honey, plant resins and wax, all antimicrobial to differing degrees. Stored pollen is the nutritionally rich currency used for colony growth and consists of 40–50% simple sugars. Many studies speculate that prior to consumption by bees, stored pollen undergoes long-term nutrient conversion, becoming more nutritious 'bee bread' as microbes predigest the pollen. We quantified both structural and functional aspects associated with this hypothesis using behavioural assays, bacterial plate counts, microscopy and 454 amplicon sequencing of the 16S rRNA gene from both newly collected and hive-stored pollen. We found that bees preferentially consume fresh pollen stored for <3 days. Newly collected pollen contained few bacteria, values which decreased significantly as pollen were stored >96 h. The estimated microbe to pollen grain surface area ratio was 1:1 000 000 indicating a negligible effect of microbial metabolism on hive-stored pollen. Consistent with these findings, hive-stored pollen grains did not appear compromised according to microscopy. Based on year round 454 amplicon sequencing, bacterial communities of newly collected and hive-stored pollen did not differ, indicating the lack of an emergent microbial community co-evolved to digest stored pollen. In accord with previous culturing and 16S cloning, acid resistant and osmotolerant bacteria like *Lactobacillus kunkeei* were found in greatest abundance in stored pollen, consistent with the harsh character of this microenvironment. We conclude that stored pollen is not evolved for microbially mediated nutrient conversion, but is a preservative environment due primarily to added honey, nectar, bee secretions and properties of pollen itself.

Keywords: bee bread, fermentation, honey, *Lactobacillus kunkeei*, microbes, nutrition

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Introduction

Nutrition is fundamental for every organism, with effects ranging from proximate behaviour to the evolution of life history (Toth & Robinson 2005; Hartfelder *et al.* 2006; Hunt *et al.* 2010; Corona *et al.* 2013). In social insects, the collection, processing and consumption of nutrients are complex because individuals must continually compensate for shifting environments and colony level adjustments (Michener 1974). As an extreme example, overwintering workers store glycolipoprotein within their abdomens, a storage reserve used to nourish a spring cohort of developing larvae (Amdam & Omholt 2002). While nutrition stored within the body of an individual is protected by anatomical barriers and active immune physiology, nutrition stored outside the individual within the hive requires a different form of protection from microbial degradation. It is unknown to what degree an exposed and ageing nutrient source

Correspondence: Kirk E. Anderson, Fax: (520) 670-6493; Email: kirk.anderson@ars.usda.gov

contributes to the fitness of most social insects (Judd 2011).

Rates of food collection and consumption are key components of in-hive food storage, but the nutritional bookkeeping of many social insect colonies has an intimate association with microbes and must account for daily changes involving in-hive nutrient conversion, storage and spoilage (Guedegbe *et al.* 2009; Haeder *et al.* 2009; Judd 2011). Many species of ants and termites have co-evolved with a fungal food source that is fed collected plant matter, carefully pruned and nurtured and consumed only at maturity (Mueller & Gerardo 2002). The nutritional needs of such colonies require a continual accounting of the time period associated with microbial conversion of collected plant matter into the primary food source (Kang *et al.* 2011). Given the extended time periods associated with the microbial conversion of enzyme resistant plant polysaccharides, the integration of colony developmental state with microbial nutrient conversion is a critical component of colony fitness, involving complex communication and unique behavioural and/or physical castes (Hölldobler & Wilson 2008).

Nutrient conversion is readily distinguished from food preservation (storage). Although both processes have associated microbial communities, the primary function of food preservation is to prevent microbial degradation by inducing a state of molecular and enzymatic stasis. Although closer inspection of various social insect systems may yield many subtle processes that include simultaneous nutrient conversion and preservation, the present understanding of social insect nutrition places these two processes at extreme ends of a functional spectrum (Haeder *et al.* 2009; Rodrigues *et al.* 2011). Nutrient conversion involves an extended time component following the collection of plant matter, massive nutrient turnover prior to consumption and a vertically inherited mutualistic microbe or small community of microbes that emerges as the dominant force in the conversion of recalcitrant plant material (Cafaro *et al.* 2011). Conversely, preservation environments composed of plant material (e.g. silage) are typically dominated by *Lactobacillus* spp. and other acid tolerant microbes (Daeschel *et al.* 1987).

In this study, we focus on the bacterial communities of newly collected and hive-stored pollen of honey bees paying special attention to the behaviours and physical microenvironments that may influence nutrient conversion or preservation of hive-stored pollen or 'bee bread'. Hive-stored pollen is the source of proteins, lipids and minerals consumed by nurse bees, converted to nutrient rich royal jelly in modified salivary (hypopharyngeal) glands and distributed via trophallaxis to growing larvae and other hive members. In previous work, we determined that pollen taken from the hind leg corbicular baskets of returning foragers (newly collected) and hive-stored pollen contains incidental amounts of core hind-gut bacteria, suggesting that this core gut community does not contribute substantially to the conversion or preservation of pollen stores (Anderson *et al.* 2013; Corby-Harris *et al.* 2014a). This new finding contrasts markedly with the previous culture-dependent view that 'most of the organisms isolated from newly collected and hive-stored pollen were associated particularly with the guts of adult worker honey bees' (Gilliam 1997).

Hive-stored pollen or 'bee bread' generally refers to flower pollen that has been mixed with nectar and packed by bees into hexagonal cells of wax comb. The final pollen storage product is highly acidic (pH 4) and contains 40–50% simple sugars (Anderson *et al.* 2011; Nicolson 2011). Despite this harsh environment, speculation that hive-stored pollen is the product of long-term microbial succession and nutrient conversion has been continually reinforced by the scientific community, but never documented. More specifically, it was hypothesized that co-evolved microbes orchestrate the long-term conversion of stored pollen into a more nutritious food source, a process involving microbial succession, anaerobic breakdown of materials, the release of pollen cell contents and/or predigestion by moulds (Herbert & Shimanuki 1978; Gilliam 1979, 1997; Loper *et al.* 1980; Gilliam *et al.* 1989; Anderson *et al.* 2011; Vásquez & Olofsson 2011; DeGrandi-Hoffman *et al.* 2012; Mattila *et al.* 2012; Lee *et al.* 2014). In general, direct methods used to quantify nutritional differences between newly collected and hive-stored pollen nutrition have proved problematic (Roulston & Cane 2000; Nicolson 2011). Consistent with such polarized claims and incomplete methods, the literature is limited and conflicting concerning nutritional changes ascribed to stored pollen, indicating either slightly increased or decreased nutrition, or no real change (Herbert & Shimanuki 1978; Loper *et al.* 1980; Standifer & McCaughey 1980; Fernandes-da Silva & Serrão 2000).

Here, we use a multifaceted approach to determine whether hive-stored pollen of honey bees involves significant nutrient conversion or 'pre-digestion' by microbes. To this end, we explore (1) the time period associated with pollen storage prior to ingestion by nurse bees, (2) the absolute number of bacteria in stored pollen, (3) the association between bacterial abundance and pollen storage time, (4) the pollen to microbe biomass ratio, (5) the degree of digestion of hive-stored pollen and (6) whether the differences in bacterial richness and diversity between newly collected and hive-stored pollen are consistent with a preservation or nutrient conversion environment.

Methods

Consumption time frame of stored pollen

If microbial processing of hive-stored pollen is a key component of honey bee nutrition, then the time frame associated with pollen storage should be sufficient to permit microbes acclimation, population growth and enzyme production to alter pollen grains. The existence of such a processing period would be supported by the biased consumption of pollen stored for an extended time, at least 3 days.

We recorded pollen storage time and consumption by recording newly emptied and newly filled pollen cells on the same frames every 24 h for 5 days. The week beginning 31 March 2014, we selected one frame near the centre of the brood nest from each of eight colonies. Criteria for frame selection included the presence of honey, open brood, sufficient stored pollen and empty cells to compare proportions stored and consumed. On day one, selected frames were labelled, cleared of bees and placed into a frame holder where all stored pollen cells were identified by tracing coloured circles on overlain transparent acrylic sheets. Day one represented stored pollen of unknown but 'older age'. On day two, using a different colour, newly deposited pollen cells were circled, and previously marked pollen cells that disappeared over the 24-h period were identified with an 'X' drawn within the circle. This protocol was repeated for three additional days using different colours, allowing us to record the proportion of 24- to 48-, 48- to 72- and 72- to 96-h-old stored pollen cells eaten on days three, four and five. We repeated this experiment two more times using different colonies located throughout the same apiary (April 14–18, and May 19–23). In total, we recorded 72 colony/days of both 24–48 h and 'older age' consumption (3 days × 8 colonies × 3 trials), 48 colony/days of 48–72 h consumption (2 days × 8 colonies × 3 trials) and 24 colony/days of 72–96 h consumption (1 day × 8 colonies × 3 trials). We compared these proportions to one another and to the proportion of consumed pollen of unknown but 'older age' using a *Z*-test for proportions.

Bacterial abundance in hive-stored pollen

Using standard methods agar (SMA), we determined the number of bacterial CFUs per gram of pollen stored <24, 24–48, 48–72, 72–96 and >96 h within the hive under natural conditions. Standard methods agar is nonselective growth medium commonly used in food microbiology to enumerate 'total' or viable bacterial growth. To explore relationship between nonselective and selective media, we replicate plated all samples on de Man Rogosa Sharp agar (MRS) media, selective for acid tolerant bacteria found in hive-stored pollen (Anderson *et al.* 2013).

During the third trial assessing the time frame of stored pollen consumption, we sampled five age classes of hive-stored pollen. At each of these five time periods, we collected three stored pollen cores per colony from each of four colonies using sterile cut pipette tips (1000 μL). Each core was immediately weighed to avoid dehydration, subject to a serial dilution series and spread plated in triplicate at concentrations of 10^{-3}, 10^{-4} and 10^{-5} on both media types. Stored pollen samples consisted of 1080 total plates: (4 colonies × 5 age classes per colony × 3 cores per age class = 60 × 2 growth media × 3 dilution factors × 3 technical replicates). We also sampled inbound corbicular pollen from the legs of six returning foragers per colony. Corbicular pollen was pooled by colony and cultured similarly. Colony-forming units were counted after 48 h of incubation at 35° C under aerobic conditions.

Replicate plates by media were averaged according to standard food safety methods (Sutton 2011), and CFU values were compared with the inclusion of counts <25 per plate. In reporting the absolute number of CFUs, we followed standard food safety methodology counting only those plates with >25 and <250 CFUs. Replicate averages below 25 were designated as <25 × the lowest dilution factor or $<2.5 \times 10^3$ CFUs per gram.

We explored the change in CFUs per gram over time with regression analysis. We used the Kolmogorov–Smirnov test to examine the normality of CFU data independently for each media type. To fit the assumptions of linear regression, we performed a common log transformation, typically applied to random count data like CFUs (Zar 1996). Following transformation, statistical outliers were determined according to extreme studentized deviate method ($n = 12$, $\alpha = 0.05$, Z critical value = 2.41) (Rosner 1983). We examined the relationship between the two media types by storage age class with a Mann–Whitney *U*-test with an adjustment for the number of tied ranks.

Estimating the bacteria to pollen grain ratio

Using a random subsample of our plated pollen samples, we estimated average pollen grains per gram using light microscopy at 400× and two analogous approaches that differed primarily in scale and depth of view; (1) a Neubauer-ruled counting chamber (hemocytometer) according to established guidelines (Jones 2012) and (2) a 22-mm^2 slide cover from which 12 fields of view uniformly distributed throughout the cover slip were counted, averaged and extrapolated to total cover slip area, which enclosed 10 μL of a stored pollen

solution at 0.1 mg/10 μL. These methods were compared with a two-tailed *t*-test.

Pollen grains per gram were used in combination with microbial counts per gram to estimate the absolute number of microbes per pollen grain. We then determined the biological potential for microbial function according to the ratio of pollen grain surface area to microbial surface area using 2 μm as the average width of a microbe and 40 μm as the average width of a pollen grain. We ignored exine sculpturing to provide a conservative estimate.

Microscopy of pollen from different environments

As an indicator of microbial predigestion occurring in hive-stored pollen, we determined by light microscopy whether stored pollen showed an intermediate morphology between newly collected and gut-digested pollen (Jones 2012). Using the same samples from which we obtained 454-amplicon data, we examined morphological differences between hive-stored ($n = 12$) and newly collected pollen ($n = 12$). To provide a realistic comparison of stored pollen with digested pollen, we examined twelve hind-gut contents of nurse bees 7–8 days old.

Samples from all environments were collected into 95% EtOH and diluted to approximately 0.1 mg/10 μL. Resulting solutions were quickly vortexed, homogenized by slow and steady pipetting, and 10 μL of this pollen solution was examined under 400× with a light microscope. For each of the 36 samples, we performed three technical replicates, each replicate representing an average of five fields of view uniformly distributed across the slide cover slip. From each field of view, we recorded the proportion of pollen grains that were >50% depleted of protoplasm (Crailsheim *et al.* 1992) or shape compromised due to a loss of protoplasmic pressure. The presence of fungal hyphae was also noted. Proportion of digested pollen in each sample type was compared among treatments using a Wilcoxon's test.

To verify observations made with the light microscope, we performed scanning electron microscopy of pollen grains from hive-stored pollen and the hindgut. Entire hindguts and pollen cells plus the surrounding wax were excised and placed in a solution of 4% formaldehyde and 5% glutaraldehyde in phosphate buffer at 4° C for 24 h. To maintain structure, tissues were placed inside a folded piece of filter paper and treated for 8–12 h for each step of a dehydration series of 10–100% ethanol in Milli-Q water. Wax was removed from stored pollen, and samples were placed inside a filter basket and critical point dried. Pollen masses remaining intact after this process were dissected and adhered to imaging stubs with conductive tape. Samples were sputter coated from five angles with platinum (60 s each) and imaged with a Hitatchi S-4800 SEM.

Amplicon sequencing and analysis

Pollen sampling/DNA extraction

Newly collected and hive-stored pollen was collected approximately every other month between November 2011 and October 2012 from two neighbouring colonies. At each of six sampling periods, 10–12 cells of stored pollen were collected from the central brood frame of each colony using a precut 1 mL-pipette tip. The same day, we sampled stored pollen, we also collected eight to twelve pollen foragers per colony. Foragers were captured with soft forceps and immediately submerged in 95% ethanol. Corbicular pollen was then separated from the legs of foragers. Newly collected and hive-stored pollen were pooled separately by colony and sampling event and stored in 95% ethanol until DNA extraction.

Pollen samples were vortexed to suspend pollen grains in solution, and 750 μL of this mixture was placed in a new 1.5-mL tube. This subsample of pollen was centrifuged on high for 5 min, and the ethanol was decanted. The process of vortexing, subsampling and centrifuging the pollen samples was repeated five times until there was approximately 500 μL of pollen in the new 1.5-mL tube. We added 1 mL of TE buffer (20 mM Tris-HCl, 2 mM EDTA, 1% Triton-X, pH 8.0) to the pollen. This mixture was vortexed for 5 min and pulse centrifuged for 5 s. The supernatant was removed and placed into another tube and spun down for 20 min (20 800 *g*, 4° C) to pellet bacterial cells. This wash cycle was repeated four times. On the final (fourth) wash cycle, the supernatant was retained and 350 μL of 0.5 mm silica beads was added to the mixture and bead-beaten for 30 s. The supernatant from these samples was then removed to a new tube and spun down for 30 min (20 800 *g*, 4° C) yielding a pellet used in subsequent DNA extraction. We extracted DNA using the GeneJet Genomic DNA Purification Kit (Fermentas) following the protocol for gram-positive bacteria.

Pyrosequencing preparation

The V1–V2 region of the 16S rRNA gene was PCR amplified using universal 16S rRNA primers fitted with 454 FLX Titanium adapter sequences (27F 5′-CCATCTC ATCCCTGCGTGTCTCCGACTCAG-NNNNNNNNNN-agagtttgatcctggctcag -3′; 338R: 5′- CCTATCCCCTGTGT GCCTTGGCAGTCTCAG-tgctgcctcccgtaggagt -3′; uppercase letters denote the adapter sequences, N's indicate library-specific barcodes, lowercase letters indicate universal 16S rRNA primers (Table S1, Supporting

information). PCR program was 2 min at 94° C followed by 35 cycles of 15 s at 94° C, 20 s at 50° C and 30 s at 72° C and a final elongation step of 2 min at 72° C. Amplicons were sequenced using Roche 454 GS FLX Titanium sequencing.

Analysis of reads and assignment of taxonomy

Sequence data were processed using Mothur v.1.26.0 (Schloss *et al.* 2009). Sequences in the.sff files were quality filtered using the trim.flows command, and all sequences <150 bp with more than two base mismatches to the 27F primer sequence or 1 mismatch to the 10-bp pyrotag after trimming were eliminated using the trim.seqs command. Pyrotags were removed and the sequences were aligned to SILVA SSUREF database (v102) using the align.seqs command. Sequences that did not align to the 27F primer position or that were shorter than 98% of the sequences were eliminated using the screen.seqs command. Sequences with 99% or greater similarity were clustered together using the pre.cluster command, which identifies and clusters sequences that may be the result of slight sequencing error (Huse *et al.* 2010) and condenses the data set to speed the analysis. Chimeras were removed using uchime (Edgar *et al.* 2011) in addition to any sequences that were mitochondrial, chloroplast, archaeal, eukaryote, or of unknown origin. The sequence libraries were concatenated and aligned as described above. A distance matrix was constructed for the aligned sequences using the dist.seqs command and the default parameters. Sequences were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity. Representative sequences from each OTU were characterized in two ways. First, the sequences were used to query the NCBI nucleotide database. Second, these sequences were classified using the RDP Naïve Bayesian Classifier using a manually constructed training set that contained sequences from the Greengenes 16S rRNA database, the RDP version 8 training set, and all full length honey bee associated gut microbiota listed in NCBI trimmed to the V1–V2 region of the 16S rRNA gene. Any remaining sequences that were of chloroplast or mitochondrial origin were removed as well as any sequences classified with <80% confidence at the phylum level according to the RDP Naïve Bayesian Classifier (Wang *et al.* 2007).

Multivariate analysis of newly collected and hive-stored pollen

We compared the bacterial community composition of newly collected and hive-stored pollen. As above, a distance matrix was constructed for the aligned sequences using the dist.seqs command with default parameters. Sequences were grouped together, and representative sequences from each (97%) OTU were obtained. The abundance of each OTU in each library was calculated using the cluster and make.shared commands in Mothur (Schloss *et al.* 2009). The representative sequences from each OTU were aligned using the align.seqs command. This alignment was filtered using the filter.seqs command (vertical = T, trump=.) and the resulting alignment, sequence abundances per OTU in each library, and a file describing the experimental treatments were analysed using the phangorn, pegas and GUNIFRAC packages in R (Paradis 2010; Schliep 2011; Chen *et al.* 2012; R Core Team 2013). A pairwise distance matrix was constructed from the sequence alignment, and a midpoint-rooted neighbour-joining phylogeny was constructed in R using the phanghorn (Schliep 2011) and ape (Paradis 2010) packages and the phylogenetic reconstruction methods of (Saitou & Nei 1987). Variance adjusted weighted (VAW) and generalized ($\alpha = 0.5$) pairwise UniFrac distances between each library were calculated using the GUNIFRAC package (Chen *et al.* 2012). A permutational multivariate analysis of variance (using PERMANOVAG in the GUNIFRAC package) was used to determine whether season, colony nested within season and source (i.e. newly collected or stored pollen) nested within colony and season significantly affected the combined VAW and generalized UniFrac distance matrices. We also determined whether the core hind-gut bacterial community (Moran *et al.* 2012; Sabree *et al.* 2012) was at significantly greater relative proportion in newly collected vs. stored pollen with a Mann–Whitney *U*-test using an adjustment for the number of tied ranks.

Results

Consumption time frame of stored pollen

Nurse bees prefer to consume newly collected pollen (Fig. 1). Bees consumed proportionally more pollen aged between 24 and 72 h than they did older pollen (24- to 48-h-old pollen: $Z = -3.43$; two-tailed $P = 0.0006$, 48- to 72-h-old pollen: $Z = -3.56$; two-tailed $P = 0.0004$). This comparison is conservative because some subproportion (<5%) of stored pollen categorized as unknown (but older) age and then consumed was newly collected, aged between 48 and 72 h.

Bacterial abundance in hive-stored pollen

Following replicate assessment, a majority of the samples (75 of 120 or 62.5%) produced colony-forming units (CFUs) $<2.5 \times 10^3$ CFU per gram (Table S3, Supporting

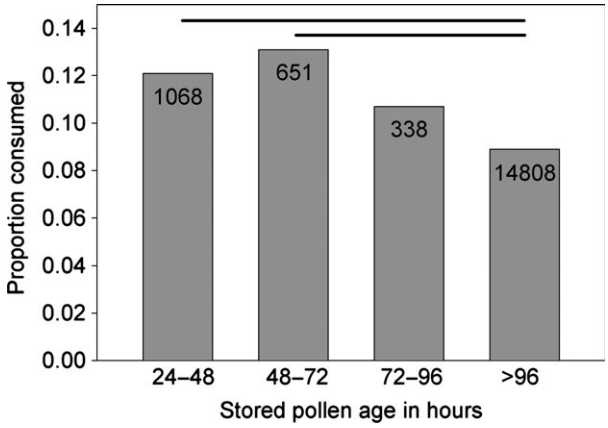

Fig. 1 Honey bees prefer to consume pollen stored less than 72 h. Graph depicts combined results from 23 colonies, and three separate week long trials. Within each vertical bar is the total number of pollen containing cells from each storage age available for consumption. Horizontal bars are significant differences between pollen age groups. Bees preferentially consumed recently collected pollen based on a *Z*-test for proportions (24- to 48-h-old pollen; $Z = -3.43$; two-tailed $P = 0.0006$, 48- to 72-h-old pollen; $Z = -3.56$; two-tailed $P = 0.0004$).

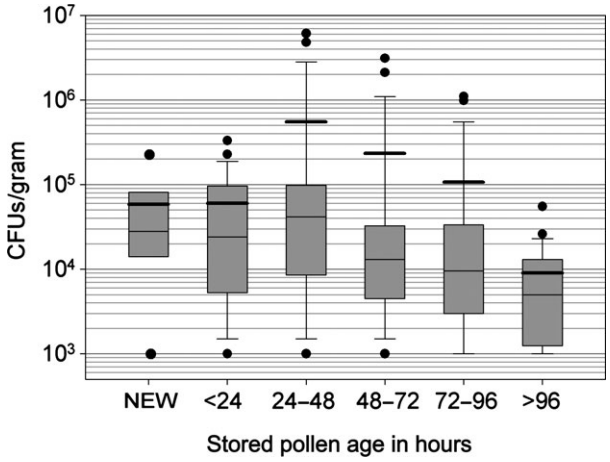

Fig. 2 Bacterial abundance decreases with increased pollen storage time. *Y*-axis is colony-forming units (CFUs per gram) of pollen stored for 1–5 days within active colonies. NEW represents corbicular pollen removed from the legs of returning foragers ($n = 8$). Values that compose each stored pollen boxplot ($n = 24$) include average replicate counts from both media types and all plates showing growth above the lowest dilution factor (10^{-3}). All outliers are shown, the median is a horizontal line within the grey box, and the bold black line represents the mean. Regression analysis revealed a significant negative relationship between bacterial counts and pollen storage time following data normalization and removal of statistical outliers (Adj Rsq = 0.15; $F = 21.2$; $P < 0.0001$). See Figure S1 (Supporting information) for results partitioned by media type.

information). In the interest of comparison, we report the actual number of CFUs $\geq 10^3$ per gram, as well as the standard methodology for counting and comparing only those plates with >25 and <250 CFUs. We replicate plated at $10^{-3} - 10^{-5}$, so the lower limit we report is 1 CFU at a dilution factor of 10^{-3} (Table S3, Supporting information).

From both media types, CFUs per gram of pollen were generally below 10^5 across all age classes (Fig. 2). Only four of 60 samples revealed population spikes $>10^5$ per gram. These occurred in pollen aged 24–96 h, skewing the mean microbial count well above the CFU median for each of these three age classes (Fig. 2). Each instance of CFUs above 10^5 per gram was characterized by MRS media revealing CFU counts roughly equal to those cultured on SMA. At values below 5×10^4 per gram, this trend disappeared (Fig. 3). For the two oldest storage age classes, MRS media returned significantly lower CFU counts than did SMA media according to a Mann–Whitney *U*-test (>96 h: Adj-$H = 13.9$; 1 d.f.; $P = 0.0002$, 72–96 h: Adj-$H = 7.1$; 1 d.f.; $P = 0.008$). Thus, at higher bacterial numbers, the two media equally represented population spikes of colonies identified as *Lactobacillus kunkeei*, but at lower bacterial numbers, MRS failed to represent the greater diversity of microbes produced on SMA media (Fig. 3).

Although biologically relevant, these population spikes were not representative of the storage age population, producing an excessive influence on R-squared value. Two of these data points, sample 3.3 and 11.1

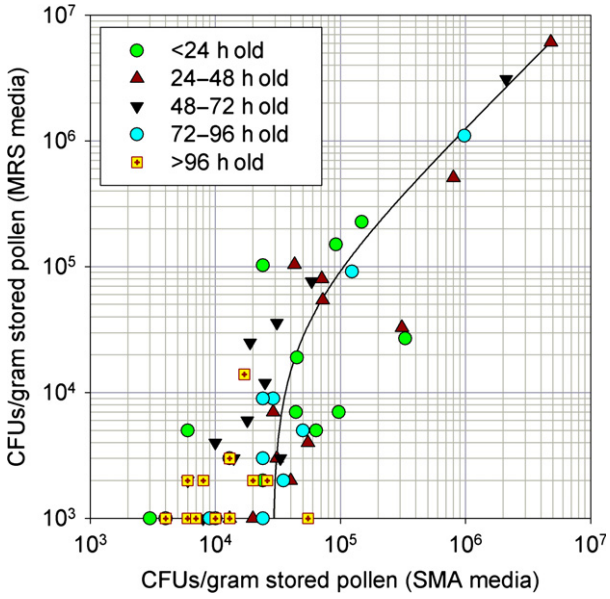

Fig. 3 Selective and nonselective media produce similar results above 10^5 CFUs/gram. Total bacterial counts expressed as colony-forming units (CFUs per gram) of stored pollen aged from 1 to 5 days (see key). Graph is scaled by common log. Bacteria were grown on both nonselective (SMA) and *Lactobacillus* selective media (MRS). Each data point (symbol) represents the average plate count from three replicate plates and two media.

from the groups aged 48–72 and 72–96 h, respectively, were statistical outliers based on our criteria (Table S3, Supporting information). As these data points represented the same sample grown on different media types, their removal resulted in a normal distribution for both media types (SMA: K-S statistic = 0.13; $P = 0.29$, MRS: K-S statistic = 0.15; $P = 0.13$). Regression analysis on the normalized data sets revealed a significant negative relationship between bacterial counts and pollen storage time for each media type individually (Fig. S1, Supporting information, SMA: Adj Rsqr = 0.14; $F_{1,57} = 10.5$; $P = 0.002$, MRS: Adj Rsqr = 0.16; $F_{1,57} = 12.0$; $P = 0.001$), and when replacing all CFU counts of $<2.5 \times 10^3$ per gram with 2.5×10^3 per gram in line with standard protocols for food microbiology (SMA: Adj Rsqr = 0.15; $F_{1,57} = 10.5$; $P = 0.002$, MRS: Adj Rsqr = 0.09; $F_{1,57} = 6.3$; $P = 0.02$).

Estimating the bacteria to pollen grain ratio

The Neubauer-ruled counting chamber (hemocytometer) showed an average of 89 million pollen grains per gram of stored pollen (Table S4, Supporting information). The cover slip area method revealed an average of 98 million pollen grains per gram. The difference between the two methods of estimation was not significant based on a *t*-test ($t = -0.918$; $P = 0.3635$).

In determining the microbe to pollen grain surface area ratio, we considered average pollen width = 40 μm (Knight *et al.* 2010) and the average bacterial width = 2 μm. Solving for the surface area of a sphere, an average pollen grain is over 5000 μm^2, 400× larger than the surface area of a bacterium (12.6 μm^2). Assuming 9000 bacterial CFUs/gram in pollen stored >96 h and 90 million pollen grains per gram, the numerical ratio of bacteria to pollen grains is 1:10 000. The biologically relevant ratio expressed in terms of surface area (10 000 × 400) is 1:4 000 000.

Microscopy of pollen from different environments

There were no discernible morphological differences between newly collected and hive-stored pollen (Table S5, Supporting information; Wilcoxon $Z = 0.6$; two-tailed $P = 0.54$). The proportion of pollen grains categorized as digested averaged <1% for both hive-stored pollen (0.73 ± 0.08, range 0.2–1.2%) and newly collected pollen (0.78 ± 0.09, range 0.2–1.2%). We detected no fungal hyphae in any sample. As expected (Oliveira 2002), the control pollen from the hindgut of nurse bees differed significantly from both newly collected and stored pollen sample types (hind-gut proportion digested; 83.4 ± 5.8, range 74–91%) and provided necessary perspective on the appearance of pollen that has been somehow compromised or completely digested. Of the grains examined from the hindgut, 83 per cent were clearly shape distorted and <50% filled.

Scanning electron microscopy revealed that microbes were conspicuously absent in hive-stored pollen. Shown for contrast, the hindgut of a nurse honey bee is an environment packed with both pollen and active bacteria (Fig. 4).

Amplicon sequencing and analysis

Analysis of reads and assignment of taxonomy

Following initial quality trimming, a total of 433 247 reads remained across the 24 libraries. Of these reads, 0.75% were removed as chimeras. From each of the two sampled colonies, 80% and 54% of the sequences were removed as chloroplast origin (cpDNA), yielding a total of 141 787 sequences across the 24 libraries. Further culling of nonbacterial DNA and poorly defined sequences left a total of 100 769 sequences across the 24 libraries that grouped into 4104 OTUs (97%) (Table S6, Supporting information). Rarefaction curves indicate

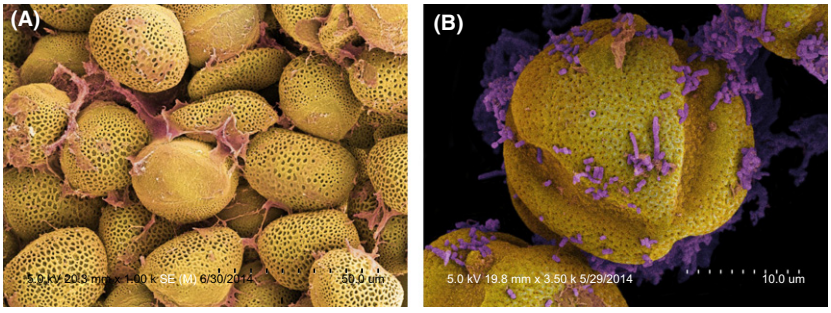

Fig. 4 Hive-stored pollen lacks the microbial biomass needed to alter pollen nutrition. Scanning electron micrograph of hive-stored pollen (A), which contains 40–50% simple sugars by weight (pink hue), but only one microbe for every 2500 pollen grains (3.6×10^4 bacteria per gram), many orders of magnitude below that required for pollen predigestion. Shown for contrast and mathematical rigour, images from the hindgut (B) reveal hundreds of bacteria (purple) per pollen grain (4.9×10^9 bacteria per gram, see also; Rada *et al.* 1997; Kacániová *et al.* 2004).

that the libraries did not fully capture the sequence diversity present in either sample type. A total of 703 OTUs were shared between the newly collected and stored pollen libraries, and the majority of OTUs were unique to either stored (2462 OTUs) or newly collected (939 OTUs) libraries. However, the vast majority of sequences (85% of stored pollen sequences and 95% of newly collected pollen sequences) belonged to the 703 OTUs that were shared between the two sample types (Fig. S2, Supporting information).

An average of 12% of sequences across all 24 libraries were classified as core hind-gut bacteria (Table S7, Supporting information): Alpha 2.1, *Lactobacillus* sp. Firm 4, *Lactobacillus* sp. Firm 5, *Frischella perrara* (Gamma 2), *Gilliamella apicola* (Gamma 1), *Snodgrassella alvi* (Beta) and *Bifidobacterium* sp. (Cox-Foster *et al.* 2007; Martinson *et al.* 2011; Ahn *et al.* 2012; Moran *et al.* 2012; Sabree *et al.* 2012; Anderson *et al.* 2013; Engel *et al.* 2013; Corby-Harris *et al.* 2014a). The relative abundance of core hindgut bacteria varied widely among samples (minimum = 0.3%, maximum = 89%; Fig. 5). Stored pollen libraries contained a significantly lower proportion of core gut microbial sequences (mean = 3% ± 1% SE) than newly collected pollen libraries (mean = 21% ± 8% SE, Kruskal–Wallis test $X_1^2 = 12.0$; $P = 0.0005$). More than half of the reads found across all 24 libraries matched to nongut bacteria *Lactobacillus kunkeei* (26%), Actinobacteria (11%), Acetobacteraceae Alpha 2.2 (8%) and an Enterobacteriaceae tentatively classified in the genus *Pantoea* (7%) (Table S7, Supporting information; Fig. 5).

Multivariate analysis of newly collected and hive-stored pollen

We performed a PERMANOVAG analysis to test whether the distribution of microbial taxa differed according to season, pollen storage state (newly collected or stored pollen) or colony. Only the effect of season was significant ($F_{5,23} = 2.66$; $P = 0.001$). Colony nested within season and source nested within colony among seasons were both nonsignificant (Fig. 5).

Discussion

Our combined results do not support the hypothesis that hive-stored pollen of honey bees involves nutrient conversion or predigestion by microbes prior to consumption. (1) The preferential consumption of freshly collected pollen indicates that bees have not evolved to rely on microbes or other time-related factors for pollen predigestion (Fig. 1). (2) Relative to other plant material involving microbial digestion or extensive fermentation, hive-stored pollen contains very few microbes. (3) The absolute number of bacteria in hive-stored pollen decreases with storage time, indicating that it is not a suitable medium for microbial growth (Fig. 2). (4) The microbe to pollen grain ratio is many orders of magnitude removed from that required to alter hive-stored pollen (Fig. 4). (5) Regardless of sampled season or the taxonomic character of microbial communities, microscopic examination revealed no intermediate stage of pollen digestion in hive-stored pollen (Table S5,

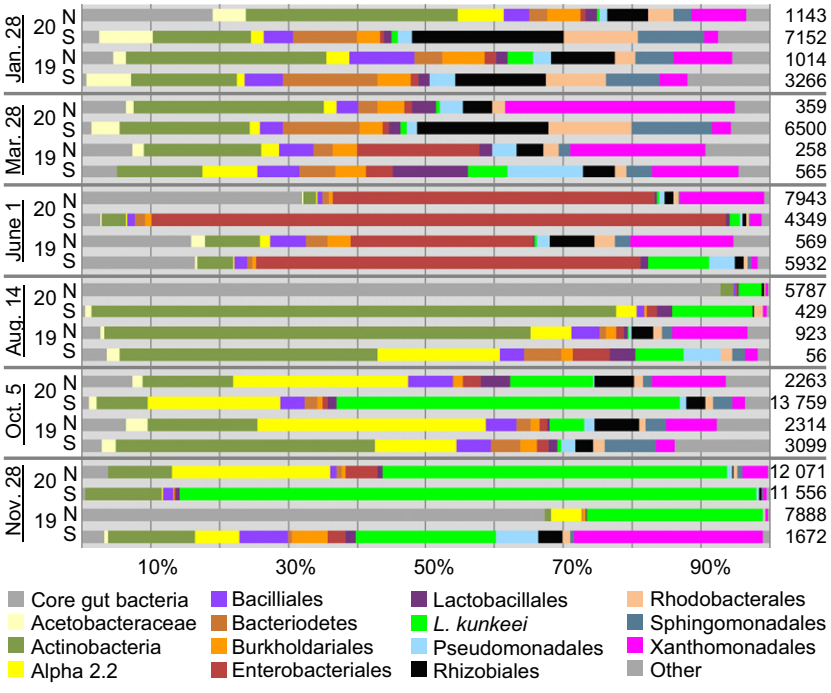

Fig. 5 Bacterial communities of newly collected and hive-stored pollen are most similar by season. Relative proportions of bacterial taxa in newly collected (N) and hive-stored (S) pollen from two colonies (19 and 20) sampled at six discrete time points over the course of a year. For each of the pollen associated bacterial libraries, the proportion of sequences belonging to various bacterial taxa is shown as different colours. Grey represents both core gut bacteria (far left) and bacteria occurring at low frequency (far right). Total read number is displayed to the right of each individual library. PERMANOVAG analysis based on variance adjusted weighted and generalized pairwise unifrac distances reveals a significant effect of season on bacterial community composition ($F_{5,23} = 2.66$; $P = 0.001$).

Supporting information). (6) The bacterial communities found in hive-stored pollen did not differ from those of newly collected pollen, but both sample types varied significantly by season (Fig. 5). This result indicates the lack of an emergent 'core' bacterial community co-evolved to predigest pollen. Based on these collective findings, we suggest that stored pollen is a preservative environment governed largely by nonmicrobial additions of nectar, honey and bee glandular secretions (Herbert & Shimanuki 1978; Human & Nicolson 2006; Judd 2011; Nicolson 2011).

Bee prefers freshly stored pollen

The bees in our study showed a significant preference for the consumption of pollen aged <72 h (Fig. 1, Table S2, Supporting information).The tendency to eat fresh vs. older pollen stores may involve the strength or type of chemical signal emanating from stored pollen of variable age. Capabilities for distinguishing variation in pollen storage state may have been amplified compared to those of a solitary bee and perhaps modified for in-hive tasks involving food storage. Seasonal fluctuations may have selected for the quick turnover of the most readily available pollen nutrients into a 'nutritional reservoir' of living tissue (i.e. larva and worker fat bodies). In such a state, nutritional reserves are better protected from microbial digestion, more quickly shared among hive members and easier to digest than hive-stored pollen.

Stored pollen contains few bacteria

Our results indicate that hive-stored pollen is not a suitable growth medium for microbes (Fig. 2). After 96 h of storage, mean bacterial counts in hive-stored pollen fell below 10^4 CFUs per gram. In fact, 62.5% of all samples over 24 h old revealed $<2.5 \times 10^3$ CFUs per gram indicating that a dilution series of 10^{-2}–10^{-5} per gram would more accurately represent the sparse number of bacteria present in hive-stored pollen (Table S3, Supporting information). Critical to the argument that hive-stored pollen is not evolved for microbial predigestion, CFU number decreased significantly over the sampled time period (Fig. 2). This decrease was significant even when bacteria were cultured on selective media (MRS), designed to favour the growth of acid tolerant bacteria like *Lactobacillus* (Fig. S1, Supporting information). Despite this general trend, our enumeration results detected sporadic bacterial growth from four of 60 total samples and three different stored pollen age classes (Fig. 3). Based on bacterial colony morphology, the microbe associated with these infrequent population spikes was *L. kunkeei* (Endo *et al.* 2012). Although its growth was seemingly subdued following >96 h of storage, *L. kunkeei* may produce enough acid via fermentation to affect pollen storage potential (Pain & Maugenet 1966). Alternatively, this rather short window of time may reflect acid production by many different microbes attempting to acclimate to the acidic and sugar-rich conditions of hive-stored pollen (Cotter & Hill 2003).

Stored pollen has a very low microbe to pollen grain ratio

While culturing can only represent a portion of the microbial community, most of the frequently occurring OTUs found in bee bread are cultivable, as are the core hind-gut bacteria (Kwong & Moran 2013; Anderson *et al*. 2013). In fact, with a relatively moderate culturing effort, 50% of the total sequences in stored pollen were shared between culture-based and nonculture-based approaches at 99% 16S rDNA sequence similarity (Anderson *et al*. 2013). This indicates that our measures of bacterial abundance in stored pollen are reliably underestimated by half. Honey bee-collected pollen intended for human consumption contains bacterial counts similar to our 96 h sample and a 1:1 ratio of fungal to bacterial OTUs (Bonvehı & Escola 1997; Brindza *et al*. 2010; Feás *et al.* 2012; Nogueira *et al*. 2012). Applying these coefficients to estimate total microbes per gram in our >96-h-old stored pollen, we double our CFU bacterial count (9000) to account for culture bias and double it again to account for fungi. This expression yields 3.6×10^4 total microbes per gram of stored pollen. To place this in perspective, one gram of hive-stored pollen typically contains >90 million pollen grains (Table S4, Supporting information), thus in stored pollen aged 96 h; we estimate one microbe for every 2500 pollen grains. Surface area is the physical and microbiological reality of this microbial interface, and the much larger pollen grains have conservatively 400× the surface area of an average sized microbe. Therefore, the most meaningful biological ratio is best stated in terms of surface area available for microbial metabolism. This ratio is 1:1 000 000; for every one million square microns of real estate available in stored pollen, only one is occupied by a microbe. Contrast this environment with the hindgut of the nurse honey bee wherein a single pollen grain can be covered by hundreds or thousands of bacteria (Fig. 4).

Stored pollen maintains structural integrity

Although our year-long pyrosequencing analysis revealed that pollen microbial communities contained taxonomic groups known to digest complex plant polymers, virtually none of the pollen grains associated with

newly collected or hive-stored pollen were scored as digested (Table S5, Supporting information). Despite a wide variety of pollen types, >99% of pollen grains across all samples appeared largely intact and retained their protoplasm and structural integrity independent of season. Although readily discernible at 400×, our microscopic assay revealed no fungal hyphae in any pollen sample regardless of storage status (Table S5, Supporting information). There is no standard methodology to assess pollen digestion, but the use of 95% EtOH as a storage and dilution medium did not alter pollen integrity, and other approaches revealed little if any predigestion in stored pollen (Fernandes-da Silva & Serrão 2000).

Bacterial taxonomy of stored pollen suggests preservation, not nutrient conversion

The bacterial communities of newly collected pollen were similar to those of hive-stored pollen (Fig. 5), demonstrating the lack of an emergent 'core-digestive' bacterial community co-evolved to predigest pollen. Further, many of the bacterial groups abundant in stored pollen were acid resistant and osmotolerant bacteria, similar to those isolated from honey, indicating that bees have evolved to use both sources of stored food in a preserved state that does not require nutrient breakdown or conversion by microbes. (Table S7, Supporting information, Snowdon & Cliver 1996; Olaitan *et al.* 2007; Ruiz-Argueso & Rodriguez-Navarro 1975; Tajabadi *et al.* 2011; Aween *et al.* 2012; Tajabadi *et al.* 2012; Anderson *et al.* 2013). A small subset of bacteria were present in both newly collected and stored pollen regardless of season, suggesting lasting associations with the hive environment or 'core-hive' bacteria (McFrederick *et al.* 2012; Anderson *et al.* 2013; Corby-Harris *et al.* 2014b). Our results show that at least two acid resistant and osmotolerant microbes, *Lactobacillus kunkeei* and the newly identified *Parasaccharibacter apium*, are found at high relative proportions in stored pollen (Fig. 5). It was recently shown that both of these bacteria are intimately associated with the acidic and antimicrobial environments of the foregut, honey and royal jelly (Ruiz-Argueso & Rodriguez-Navarro 1975; Vojvodic *et al.* 2013; Corby-Harris *et al.* 2014b) and may play key roles in hive hygiene, including food storage and larval health (Anderson *et al.* 2013; Corby-Harris *et al.* 2014b).

The bacterial communities of hive-stored pollen are highly dissimilar to those found in the honey bee midgut or hindgut or those found with limited sampling on the body surface of foraging bees (Table S7, Supporting information, Ahn *et al.* 2012; Moran *et al.* 2012; Martinson *et al.* 2012; Disayathanoowat *et al.* 2011; Aizenberg-Gershtein *et al.* 2013; Corby-Harris *et al.* 2014a). Found at low relative abundance in hive-stored pollen, the core gut bacterial community was at significantly greater relative proportion in newly collected pollen (Fig. 5, Table S7, Supporting information). This suggests that the stored pollen microenvironment quickly selects against the survival of hind-gut bacteria, but may facilitate interindividual transmission over the short term (Anderson *et al.* 2013). More direct collection methods (Corby-Harris *et al.* 2014a) found similar relative amounts of the hind-gut community in corbicular pollen, suggesting that this type of inoculation occurs naturally during the consolidation of newly collected pollen into corbicular pellets. This may occur through the contact of minute amounts of bacterial dense defecates found near the posterior of the forager abdomen.

Hive-stored pollen had 3× the number of unique bacterial sequences as did newly collected pollen, suggesting immigration and/or inoculation within the hive or admixtures from multiple source phyllospheres (Fig. S2, Supporting information). The much lower bacterial richness of newly collected corbicular pollen may be due to the influence of fewer source phyllospheres, because we collected all pollen foragers (8–12 bees) within <5 min, and individual colonies tend to key in on particular sources of forage over the short term (Waser 1986). However, the total OTUs shared between newly collected and hive-stored pollen (17%) account for the vast majority of total sequence reads across all libraries (90%). While a few of these major OTUs may be part of a 'core-hive' community, many of the shared taxa have unexplained proximal origins. Interesting among these are Bradyrhizobiaceae, Xanthomonadaceae, Enterobacteriaceae, Rhodobacterales, Pseudomonadales, Bacteriodetes and many groups of Actinobacteria (Fig. 5, Table S7, Supporting information). While it is tempting to attribute much of this unknown community to floral nectar or the phyllosphere (Jackson *et al.* 2006; Telias *et al.* 2011; Yashiro *et al.* 2011; Alvarez-Pérez *et al.* 2012; Fridman *et al.* 2012; Aizenberg-Gershtein *et al.* 2013; Aleklett *et al.* 2014), there are many unexplored microenvironments scattered throughout the hive, and within the mouth, pharynx and salivary glands of honey bees. Much like humans (Cotter & Hill 2003), honey bees may harbour a complex bacterial flora associated with their extended mouthparts.

Mechanisms of pollen preservation

All substances collected or produced by bees are antimicrobial to varying degrees including plant resins, nectar, pollen, wax, honey, royal jelly, propolis and bee salivary excretions (Kujumgiev *et al.* 1999; Ohashi

et al. 1999; Thornburg *et al.* 2003; Mundo *et al.* 2004; Vardar-Ünlü *et al.* 2007; Simone *et al.* 2009; Kwakman *et al.* 2010; Vojvodic *et al.* 2013). Given the high concentration of simple sugars (honey and nectar) in corbicular and hive-stored pollen (40–50%), we suggest that 'bee bread' has evolved to be a preservative environment. Honey with its added enzymes (amylase, alpha glucosidase, invertase and glucose oxidase) represents four of five major food preservation strategies adopted by human society; low water activity, acidic pH, high oxidation-reduction potential (hydrogen peroxide) and the presence of competitive microorganisms like lactic acid bacteria (Leistner 2000; Kwakman *et al.* 2010). The antimicrobial properties of honey likely operate in synergy to preserve stored pollen. For example, one consistent change detected in stored pollen is a loss of starch (Herbert & Shimanuki 1978), a substance rapidly converted by amylase secreted by the hypopharyngeal glands of foragers. Glucose results and is further digested by glucose oxidase forming gluconic acid and H_2O_2 in the presence of sufficient water activity (Hrassnigg *et al.* 2003). Particularly at the atmospheric interface, increased water activity in stored pollen will lead to higher pH and encourage the evolution of hydrogen peroxide by glucose oxidase, thwarting microbial growth via the production of reactive oxygen species (Bang *et al.* 2003; Kwakman *et al.* 2010). The SEM image of hive-stored pollen suggests an additional preservative mechanism associated with water activity (Fig. 4). Most of the stored pollen grains are swollen in appearance as compared to uncollected pollen, presumably due to rehydration following collection and consolidation with nectar (see also Human & Nicolson 2006). The selective uptake of water by pollen grains will increase the concentration of all solutes (e.g. sugar) in the surrounding microenvironment, resulting in an additional desiccating effect on microbes.

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K.E.A. conceived of and designed the study. K.E.A., M.J.C., T.H.S. and P.M. performed the experiments. K.E.A., T.H.S., B.M.M. and V.C-H. analysed the data. K.E.A. wrote the manuscript.

Data accessibility

All sequence data were deposited in the Sequence Read Archive (SRA) under study PRJNA259199 (SRA study accession no SRP045707). See supplemental files (Table S1) for barcodes linking the sample type to the read library.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Bacterial CFUs by media type and storage age class.

Fig. S2 Venn diagram of OTUs shared and unique to each sampled environment.

Table S1 Library-specific barcodes used in 454 pyrosequencing of bacterial 16S rRNA genes from newly collected and hive-stored pollen samples.

Table S2 Proportion of stored pollen available and eaten by storage age across 3 weeks of observations performed during spring colony growth.

Table S3 Bacterial plate counts of pollen stored 1–5 days under natural hive conditions. CFU's per gram from three cores of stored pollen from each of four hives plated in triplicate on two different media.

Table S4 Two independent estimates of pollen grain number per gram of stored pollen.

Table S5 Proportion of pollen digested from newly collected, hive-stored and hind-gut samples.

Table S6 Summary of 454 amplicon sequence processing.

Table S7 OTUs and sequence read number for 4104 OTUs (97%) in newly collected and hive-stored pollen samples.