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Connecting the nutrient composition of seasonal pollens with changing nutritional needs of honey bee (*Apis mellifera* L.) colonies

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ABSTRACT

Free-ranging herbivores have yearly life cycles that generate dynamic resource needs. Honey bee colonies also have a yearly life cycle that might generate nutritional requirements that differ between times of brood rearing and colony expansion in the spring and population contraction and preparation for overwintering in the fall. To test this, we analyzed polyfloral mixes of spring and fall pollens to determine if the nutrient composition differed with season. Next, we fed both types of seasonal pollens to bees reared in spring and fall. We compared the development of brood food glands (i.e., hypopharyngeal glands – HPG), and the expression of genes in the fat body between bees fed pollen from the same (in-season) or different season (out-of-season) when they were reared. Because pathogen challenges often heighten the effects of nutritional stress, we infected a subset of bees with *Nosema* to determine if bees responded differently to the infection depending on the seasonal pollen they consumed. We found that spring and fall pollens were similar in total protein and lipid concentrations, but spring pollens had higher concentrations of amino and fatty acids that support HPG growth and brood production. Bees responded differently when fed in vs. out of season pollen. The HPG of both uninfected and *Nosema*-infected spring bees were larger when they were fed spring (in-season) compared to fall pollen. Spring bees differentially regulated more than 200 genes when fed in- vs. out-of-season pollen. When infected with *Nosema*, approximately 400 genes showed different infection-induced expression patterns in spring bees depending on pollen type. In contrast, HPG size in fall bees was not affected by pollen type, though HPG were smaller in those infected with *Nosema*. Very few genes were differentially expressed with pollen type in uninfected (4 genes) and infected fall bees (5 genes). Pollen type did not affect patterns of infection-induced expression in fall bees. Our data suggest that physiological responses to seasonal pollens differ between bees reared in the spring and fall with spring bees being significantly more sensitive to pollen type especially when infected with *Nosema*. This study provides evidence that seasonal pollens may provide levels of nutrients that align with the activities of honey bees during their yearly colony cycle. The findings are important for the planning and establishment of forage plantings to sustain honey bees, and in the development of seasonal nutritional supplements fed to colonies when pollen is unavailable.

1. Introduction

The ability of free-ranging herbivores to find resources to meet seasonal nutritional needs is crucial for their health and survival. Those needs are dependent on seasonal activities, reproduction, growth, and stress. To sustain herbivore populations, habitats must be dynamic in nutrient content, and have a vegetation structure that provides appropriate nutrition during the different stages of the herbivore's yearly cycle. In the spring, high-quality resources such as plant shoots meet the elevated nutritional demands of pregnancy and lactation. Staple resources like stems and leaves provide the bulk of the diet during the summer growing season, and in the fall, lipid-rich seeds and fruit supply high-calorie reserves that can be stored in fat tissue during plant dormancy in winter (Owen-Smith and Cooper, 1989).

Though many vertebrate species are active throughout the year, most insects overwinter as eggs or in a state of diapause (Leather et al.,

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1995). One exception is the honey bee (*Apis mellifera* L.). Colonies of honey bees are active throughout the year and, like active vertebrates, have an annual cycle (Winston, 1987). The cycle begins in the spring with brood rearing, colony growth and reproduction by swarming. Brood rearing and population growth continue in the summer. As fall approaches, egg laying and brood rearing decline and bees store resources in preparation for confinement during winter.

While herbivores can meet their changing nutritional needs by consuming different plant parts, honey bees feed only on pollen and nectar. Pollen supplies proteins, lipids, vitamins and minerals (Roulston and Cane, 2000), and nectar provides carbohydrates (Baker, 1977). Honey bees of all ages feed on nectar, but pollen is consumed primarily by young adult workers (nurse bees). Nurse bees convert pollen to worker jelly in specialized organs called hypopharyngeal glands (HPG) (Crailsheim et al., 1992). Worker jelly is a nutrient-rich food source that is fed to larvae and the queen and is required for brood rearing.

Though the dietary needs of individual bees are well defined by caste, age and task, these individuals are part of a colony, a superorganism that through the coordinated physiology and behaviors of individuals share the characteristics of an organism (Hölldobler and Wilson, 2009). What is yet unclear is whether colonies, like other organisms with yearly cycles, also have seasonal nutritional requirements. In areas where honey bees flourish, spring pollen flows fuel brood rearing, colony expansion, and swarming. Pollen flows from fall flowers though are stored as brood areas contract, and colonies prepare for winter. The expansion and contraction of the brood area are due to the rate of egg laying by the queen, but also the combinations and ratios of nutrients available in seasonal pollens (Avni et al., 2014; Liolios et al., 2015; Di Pasquale et al., 2016; Filipiak et al., 2017). Spring pollens may have nutrients that support brood rearing. Fall pollens may be rich in nutrients that can be stored and mobilized, or that are key components of immune pathways to address challenges from pathogens during winter confinement (DeGrandi-Hoffman and Chen, 2015). There is evidence that foragers show preference for certain micronutrients and those choices vary with season (Bonoan et al., 2017, 2018). If there are seasonal requirements for certain nutrients, the physiological responses of bees consuming seasonal pollens may differ depending on the season when the pollen is produced and the bee is reared.

We investigated if seasonal differences in nutritional requirements occur in honey bees by conducting two experiments. First, we analyzed polyfloral pollen mixes collected during the spring and fall and determined if there were differences in nutritional composition that might align with seasonal colony activities. Then, we fed the pollen to worker bees reared in either spring or fall and compared responses between those fed pollen from the same (in-season) or different (out-of-season) season from when the bee was reared. Since pathogen challenges heighten responses to nutritional stress, we infected a subset of spring and fall bees with *Nosema* and measured responses after feeding them in- vs. out-of-season pollen. *Nosema* infections cause nutritional stress (Mayack and Naug, 2009) because this pathogen damages the midgut, and reduces nutrient absorption (see Paris et al., 2018). Also, *Nosema* is a microsporidium and because of reduced metabolic capacities, relies heavily on its host to furnish energy for growth and reproduction (Williams, 2009; Martín-Hernández et al., 2011). The effects of feeding on in- vs. out-of-season pollen were evaluated by measuring consumption and protein digestion as a means to estimate total protein consumed and then measuring resulting HPG size for each pollen type. We determined levels of gene expression in bees fed in- vs. out of season pollen using the fat body transcriptome. We chose the fat body because it is the storage organ with a role in a variety of metabolic and immune processes associated with seasonal activities (Arrese and Soulages, 2010).

We found that spring and fall pollens were similar in total protein and lipid concentrations, but spring pollens had higher concentrations of amino acids and fatty acids that support HPG growth and brood care. Bees differed in their response to in- versus out-of-season pollen. Most notable was that spring bees fed spring (in-season) pollen developed larger HPG than those fed fall pollen even though the bees consumed the same amount of protein from each pollen type. HPG in fall bees fed either in- or out-of-season pollen were similar in size. There also were differences in the expression of genes related to development and metabolic functions particularly between spring bees fed in-season vs. out-of-season pollen. This study provides the first evidence that bee responses to polyfloral pollen mixes differ depending on the season when the pollens are produced and the bee is reared. The responses may be driven by differences in the nutritional composition of seasonal pollens and may reflect changing nutritional needs of colonies during their yearly cycle.

2. Materials and methods

The study was conducted at the USDA-ARS Carl Hayden Bee Research Center in 2015–2016 with European honey bees (*A. mellifera ligustica*). Source colonies were headed by marked Pendell queens (Pendell Apiaries, Stonyford, CA). Two trials were conducted: one in the June (spring bees) and another in the October (fall bees). Pollen used in both trials was collected from colonies in apiaries located at the University of Arizona, West Campus Agricultural Center. Pollen was collected as corbicular pellets from February to April 2015 (spring pollen) and from September to November 2015 (fall pollen) using pollen traps at the entrances of hives. Six hives located at two sites were used for pollen collection. Pollen was collected and immediately frozen at -20 °C until fed to bees.

2.1. Cage setup and sampling

Cages containing newly emerged worker bees (< 24h old) were established for spring and fall trials. The newly emerged bees were obtained from sealed brood frames placed in a temperature-controlled dark environmental room (32–34 °C, 30–40% relative humidity) (DeGrandi-Hoffman et al., 2010). Upon emergence, the bees were placed abdomen first into individual 2-ml centrifuge tubes (modified with a 1.6-mm diameter air hole in the lid). Bees were fed 2-µl of a 50% sucrose solution with either 10^6 *Nosema* spp. spores/µl (treatment: +*Nosema*) or sucrose alone (control: −*Nosema*). The *Nosema* spores used to infect +*Nosema* bees were obtained from the gut contents of caged bees heavily infected with the pathogen. Gut contents alone could affect gene expression so to control for this possibility we fed a separate set of newly emerged bees 2-ul of a 50% sucrose solution with ground guts from uninfected bees (−*Nosema* + gut). The −*Nosema* + gut bees were used only for the transcriptome analysis of the fat body. After feeding the bees sucrose, *Nosema* spores or gut contents alone, they remained in the individual tubes for 2 h (Fries, et al., 2013). Then, 50 bees from each group were placed in Plexiglas cages (dimensions: 11.5 by 7.5 by 16.5 cm^3). Twenty-four cages were established for each trial: (2 pollen types (spring or fall) × 3 treatments (−*Nosema*, +*Nosema*, −*Nosema* + gut) x 4 replicate cages per treatment group.

The spring and fall pollens were ground separately into powder with a coffee grinder (Mr. Coffee model 1DS77) before feeding them to the bees. The ground pollen (10 g) was inserted into a plastic tube (2.2 cm in diameter) positioned on the side of each cage. Each cage also had a bottle with 30 mL of 50% sucrose solution and another with 30 mL of water. Pollen, sucrose and water were fed *ad libitum*. A piece of wax foundation hung in the middle of each cage near the sugar solution and water vials and the bees clustered on it. Cages were kept in the environmental room throughout the study period at a temperature of 32–34°.

Pollen consumption was measured by weighing the tube containing pollen as a single unit prior to feeding (initial weight) and after days 4 and 7 to estimate consumption. Fresh pollen was added on d-4. Five bees were sampled from each cage on d-7. These bees were used to

estimate protein digestion, HPG acini size, and *Nosema* spore numbers.

2.2. Pollen identification

Spring and fall pollens were identified to genus using ITS gene sequencing. Following Wilson et al. (2010), a 30 mg sample of either spring or fall pollen was first exposed to 120 µL of cyclohexane for 20 min, crushed with a pestle, and extracted using the DNeasy Plant Mini Kit (Qiagen). The eluted DNA was subjected to PCR using the ITS primers and PCR protocol described by Little et al. (2004) in a 25ul reaction using GoTaq (Promega). The PCR product was visualized on a 1% agarose gel stained with SYBR Safe DNA gel stain (ThermoFisher), cut out, and then purified using a Gel/PCR DNA Fragments Extraction Kit (IBI Scientific). The product was cloned into the PGEM-T Easy Vector (Promega) and transformed into JM109 chemically competent cells (Promega). At least 100 clones were picked from selective agar plates and subjected to a colony PCR with M13F (5′-CGCCAGGGTTTT CCCAGTCACGAC-3′) and M13R (5′-TCACACAGGAAACAGCTATGAC -3′) primers. This PCR product was directly sequenced at UAGC (http://uagc.arl.arizona.edu/). Upon sequencing, the vector was removed and the resulting sequence was characterized to the family or genus level using NCBI's BLAST algorithm (Altschul et al., 1990).

2.3. Protein and amino acid concentrations in pollen

Corbicular pollen collected in the spring and fall from all colonies was pooled to create single sources of spring and fall pollen to feed to bees. Soluble protein in pollen was measured by taking three random samples of spring and fall pollens and analyzing them for soluble protein with a BCA protein assay (Thermo Scientific) as described in (DeGrandi-Hoffman et al., 2015). The pollen was not dried prior to analysis to more closely capture protein concentrations in corbicular loads consumed by the bees.

Amino acids (AAs) were quantified using six random samples of the spring or fall pollen. AA were extracted and quantified from each pollen sample after fluoroalkyl chloroformate derivatization using an EZ-FAAST amino acid hydrolysate kit (Hušek et al., 2008; Phenomenex, Inc.). All essential amino acids except tryptophan and cysteine were characterized after acid hydrolysis. Glutamine and asparagine were converted by acidic conditions to their acid equivalents glutamic acid and aspartic acid. As a result, each amine/acid pair was indistinguishable. 10 mg of pollen was sealed under nitrogen gas in a crimp vial and digested in 500µL 6 M HCl with 4% thioglycolic acid at 70 °C for 24 h. 50 µL of the acid hydrosylate was filtered and dried down in a Savant 2200 Speed Vac (Thermo Scientific Inc.) to remove acid residues. The dried material was then resolvated, derivatized, and separated by the EZ-FAAST kit protocol. Tryptophan was extracted separately after base hydrolysis and characterized by fluoroalkyl chloroformate derivatization (Yust et al., 2004). 10 mg pollen was sealed under nitrogen gas in a crimp and digested in 1.5 mL 4 M NaOH at 90 °C for 4 h. 500µL of the base hydrosylate was carefully acidified to pH 4–5 with 6 N HCl with 4% thioglycolic acid and dried. The residue was reconstituted, derivatized, and separated by the EZ-FAAST kit protocol.

Chloroformate-derivatized amino acids were analyzed by EI GC–MS on an HP 7890A gas chromatograph coupled to a HP 5975D mass spectrometer detector (Agilent, Inc.). 1µL sample was injected at 250 °C onto a Phenomenex ZB50 column (30 m × 0.25 mm × 0.25 µm film; Phenomenex, Inc.) with helium as a carrier gas at 1.2 mL/min. Compounds were separated by oven temperatures programmed at 30 °C/min from 110 °C with an initial 1 min hold to 320 °C with a 5 min final hold. Derivatized amino acids were characterized by comparison of mass spectra and retention times (RT) with derivatized authentic standards. Compounds were quantified by comparison of characteristic mass fragments (m/z) with known amounts of standards. The fraction of the total sample present in each injected sample was estimated from the amount of norvaline internal standard recovered.

Cysteine residues were quantified after phenylisothiocyanate (PITC) derivatization adapted from Manneberg et al. (1995). Cysteine residues present in 10 mg of pollen were completely digested and oxidized to cysteic acid in 1 mL 6 M HCl with 0.02% phenol and 0.2% NaN_3 at 70 °C for 24 h. 25µL of the acid hydrosylate was mixed twice with 50 µL 2:2:1 MeOH: H_2O: TEA (triethylamine) and dried down. The residue was derivatized in total darkness with 50µL PITC reagent (7:1:1:1 MeOH: H_2O: TEA: PITC) for 20 min at 25 °C and dried down. The product was resolvated twice in 50µL MeOH, then dried down and reconstituted in 200µL 95:5 5 M sodium phosphate pH 7.4: acetonitrile. 10 µL of the reactant was injected on a Thermo Spectra System AS 3000 HPLC with a Finnegan Surveyor PDA. Amino acid residues were separated on a Waters Pico Tag column (3.9 mm × 150 mm; Waters Corporation, Milford, MA, USA) with a step gradient from 100% sodium acetate buffer (150 mM sodium acetate with 6% ACN and 0.05% TEA) to 18:28:54 H_2O: ACN: sodium acetate buffer in 5.5 min, then to 40:60 H_2O: ACN in 10.0 min, followed by a 2.5 min hold. The amount of PITC-derivatized cysteic acid (oxidized cysteine) present in samples was calculated by comparison of RT and peak areas with known amounts of derivatized standards.

2.4. Total lipid and fatty acid concentrations

Total lipid contents of pollen samples were quantified using a chromic acid oxidation assay (Amenta, 1970). 10 mg of pollen was extracted by 1 mL Folch reagent (2:1 chloroform: methanol partitioned against a KCl solution) and homogenized for 3 × 30 sec in a Bead Beater (BioSpec Products, Bartlesville, OK, USA) (Bligh and Dyer, 1959). 500 µL of the chloroform: methanol layer was removed, dried, and reacted with 1 mL chromic acid at 95 °C for 30 min. Oxidized lipids were detected by the reduction of hexavalent chromium to trivalent chromium at 620 nm in a Gen-5 plate reader (Biotek, Inc., Winooski, UT, USA). The amount of total lipids present in each sample were quantified by comparison against an oleic acid standard curve.

Fatty acids were analyzed by FAME (fatty acid methyl ester) analysis after conversion to their methyl ester equivalents (Seppänen-Laakso et al., 2002). 10 mg pollen was extracted with 1 mL Folch reagent (2:1 chloroform: methanol) and homogenized for 30 sec in a Bead Beater. The homogenate was partitioned against 210µL 0.25% KCl. 160 µL of the chloroform: methanol layer was removed and dried. The sample was reconstituted in 100µL toluene then esterified with 900µL methanolic HCl (8% HCl in MeOH) at 45 °C for 16 h. FAME compounds were recovered by partitioning the reactant solution against hexane. 600µL hexane and 300µL H_2O was added to the sample, then the hexane layer was transferred and washed 3× with DI H_2O to remove acidic residues. The hexane layer was then analyzed for FAME compounds by EI GC–MS on an HP 7890A gas chromatograph coupled to a HP 5975D mass spectrometer detector (Agilent, Inc.). 1µL sample was injected at 220 °C onto a HP-5MS column (30 m × 0.25 mm × 0.25 µm film; Agilent, Inc.) with helium as a carrier gas at 1.2 mL/min. Compounds were separated by oven temperatures programmed at 10 °C/min from 35 °C with an initial 1 min hold to 230 °C with an 8 min hold, then to 320 °C with a 0.5 min final hold. FAME compounds were elucidated by comparison of mass spectra and retention times with esterified standards. FAME compounds were quantified by comparison of characteristic mass fragments (m/z) with known amounts of authentic standards. The fraction of the total sample injected was calculated from the amount of internal standard (pentadecanoic acid) detected in each injected sample.

2.5. Estimating pollen protein digestion

Protein concentration was measured in the hindgut contents of 7d old bees fed in- or out-of-season pollen and that were ± *Nosema* using methods described in (DeGrandi-Hoffman et al., 2016). Briefly, hindguts were removed from the abdomens of five bees per cage for 16

cages (2 pollen types × 2 *Nosema* infection levels (±) × 4 replicate cages per treatment group). An incision into the hindgut was made and a 1ul sample of the gut contents was taken. The contents were transferred to a 2 mL microcentrifuge tube containing 99-ul of phosphate buffered saline (PBS) with 1% EDTA-free Halt Protease Inhibitor Cocktail (Thermo Scientific). The gut contents from the five bees were pooled to generate a single sample for the cage. Samples were stored at −80 °C until analysis by a BCA protein assay (Thermo Scientific). The proportion of digested pollen protein was estimated as: protein concentration in the hindgut/protein concentration in the pollen. Higher levels of protein in the hindgut indicated lower levels of protein digestion.

2.6. Measuring hypopharyngeal glands

HPG were measured in 7-day old bees fed in- or out-of-season pollen and that were ± *Nosema*. Five bees were collected from each of the 16 cages (see above), flash frozen in liquid nitrogen, and maintained at −80 °C until their HPG were measured using previously described techniques (DeGrandi-Hoffman et al., 2010; Corby-Harris et al., 2016). HPG were removed from head capsules and placed into PBS (37 mM NaCl, 2.7 mM KCl, and 10 mM PO4, pH 7.4). The HPG were examined microscopically at 60× magnification, and the area (mm^2) of five randomly selected acini per bee was measured using the Leica Applications Suite v.3.8.0 software. Only acini with clear borders were measured. Acini areas were averaged among individuals to obtain an estimate of HPG size for the cage.

2.7. Quantifying Nosema spores per bee

Five bees were sampled from each cage on d-7 after the trial began. For each bee, the abdomen was removed and placed individually into Eppendorf tubes containing 1 mL of ultrapure water and crushed with a small pestle. A 10-μL sample was pipetted into a hemocytometer and covered with a glass slip. *Nosema* spores were counted at 40X magnification and converted to total spores per bee using methods described in Fries et al. (2013).

2.8. RNA extraction and sequencing

To better understand how bees responded to different pollen types and states of infection (± *Nosema*), we measured differential expression of genes in the adult fat body. Transcriptomes were obtained for bees fed in- or out-of-season pollen that were ± *Nosema* and – *Nosema*-gut. Two separate data sets were obtained, one for the spring trial and another for the fall. In the spring and fall trials, five bees from each cage were flash frozen in liquid nitrogen and stored at −80 °C prior to the dissection of their fat bodies. For the spring trial, the fat bodies from five, 11-day-old bees were dissected and pooled from 24 cages, yielding 24 samples. In the fall, the bees experienced higher mortality and so fat bodies were removed from five, 7-day-old bees and pooled yielding a total of 24 samples (2 pollen types × 3 levels of infection × 4 replicates = 24).

Total RNA was extracted from each pooled fat body sample using Qiagen's RNeasy kit according to the manufacturer's specifications. RNA integrity was confirmed using Agilent's 2100 Bioanalyzer. 4 μg of total RNA from each the 24 RNA sequencing (RNA-Seq) libraries were prepared using Illumina's TruSeq RNA Sample Preparation Kit. The sequencing libraries were validated and run through a 6% TBE PAGE gel. 350 bp fragments were isolated and run through the Illumina HiSeq 2000/2500 sequencer for 2 × 150 cycles at a starting concentration of 12 pM per library.

2.9. RNA sequence analyses

The two transcriptome data sets (one set for the fall bees, one for the spring bees) were analyzed separately. The paired-end reads were processed as described in Corby-Harris et al. (2014) and were aligned to version 4.5 of the *A. mellifera* genome (Elsik et al., 2014) with TopHat (Trapnell et al., 2009) version 2.0.8.b. The alignment data were analyzed using the edgeR (Robinson et al., 2010, McCarthy et al., 2012) and DESeq2 (Love et al., 2014) packages in R (version 3.4.2). Separate PCA plots were constructed to visualize the relationships among the transcriptome libraries generated from the fall and spring bees. The read count data were transformed using the regularized-logarithm (rlog) transformation (Love et al., 2014). A principal components analysis was performed on the transformed data and the first two principal components were graphed on the x- and y-axes.

For each data set, two analyses were performed to understand how spring and fall bees respond to (1) in- or out-of-season pollen (i.e., pollen type) and (2) how the response to *Nosema* is impacted by pollen type. To address the first question, a one-way analysis was first performed on the transcriptomes of the uninfected bees fed in- or out-of-season pollens to identify genes where expression was influenced by pollen type. To address the second question, a two-way analysis was used to investigate the influence of the main effect of pollen type and infection and also the pollen type x infection interaction. The goal with this analysis was to identify genes with similar infection-induced gene expression across both diets (main effect of infection) or with similar diet-induced changes in gene expression in both the + and −*Nosema* bees (main effect of pollen type). Additionally, this model identified genes with different patterns of infection-induced expression that depended on pollen type (i.e., the pollen type × *Nosema* interaction). We controlled for the effect of feeding bees other bees' *Nosema*-free guts by including three levels of infection in the model (−*Nosema*, −*Nosema* + guts, or +*Nosema*). The impact of pollen type, infection, or pollen type x infection was investigated according to the edgeR (Robinson et al., 2010, McCarthy et al., 2012) and DESeq2 (Love et al., 2014) package vignettes and included only comparisons between −*Nosema* and +*Nosema* treatments, even though the impact of the guts-only inoculation was controlled for in the full model. The significance of the gene expression differences was adjusted using a Benjamini-Hochberg correction (Hochberg and Benjamini, 1990). The final set of differentially expressed (DE) genes represented those that were significant in both the edgeR and DESeq2 analyses (Zhang et al., 2014) and, in the case of the main effects, that exhibited a $\log_2$ fold change $\geq |1|$. Where possible, each DE gene was assigned a BeeBase identifier according to the latest *Apis mellifera* genome annotation (Elsik et al., 2014). These BeeBase gene identifiers were used to query the DAVID Bioinformatics Database 6.8 Functional Annotation Clustering Tool [9] for informative functions and KEGG pathways. Functions and KEGG pathways that were enriched at a Benjamini-Hochberg adjusted significance value of ≤5% are reported.

Lastly, to link the gene expression data obtained from the ± *Nosema* fall and spring bees fed in or out of season pollen, we compared the expression of several immunity genes – *defensin 1*, *pelle*, *apidaecin 1*, and *hymenoptaecin* (LOC40614). Previous studies showed that these genes are differentially expressed with *Nosema* infection in fat body and abdominal tissue (Holt et al., 2013, Li et al., 2018).

2.10 Statistical analysis

Average protein, lipid, amino and fatty acid concentrations were compared between spring and fall pollens using t-tests. Three sets of comparisons were made to evaluate the effects of consuming in- vs. out-of-season pollen by ± *Nosema* bees. First, pollen consumption, digestion, total protein consumed and HPG size were compared between uninfected (−*Nosema*) bees that consumed either spring or fall pollen. Comparisons were made using separate one-way ANOVA for each factor. Separate analyses were conducted for the spring and fall trials. Similar ANOVA were conducted on data from +*Nosema* bees consuming in- vs out-of-season pollen. A 2-way ANOVA with pollen type

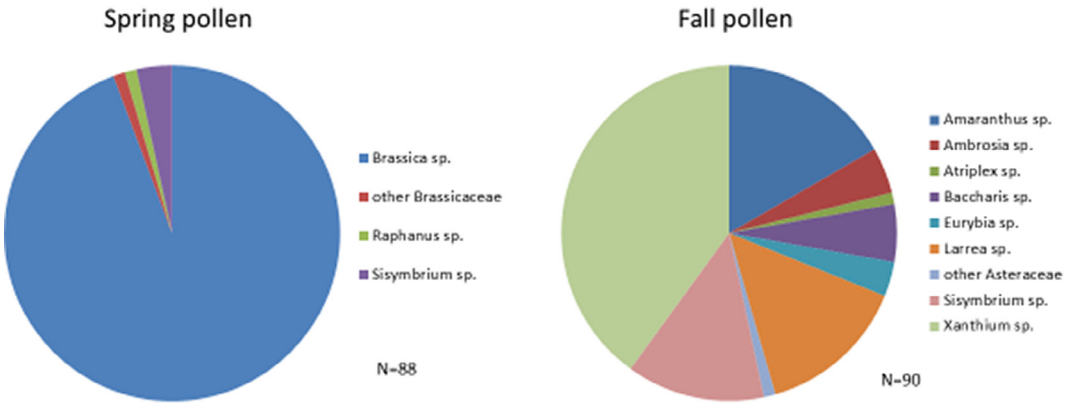

Fig. 1. Composition of spring and fall pollens fed to honey bees. Pollens were collected by colonies located in the Sonoran Desert region of Pima County Arizona, USA. Pollens were identified to genus using ITS gene sequencing following protocols from Wilson et al. (2010). For each seasonal pollen mix, 'N' represents the number of quality ITS DNA sequences obtained from each library.

(in- vs out-of-season) and ± *Nosema* infection as factors in the general linear model was conducted to compare ± *Nosema* bees fed each pollen. Separate 2-way ANOVAs were conducted for each factor we measured and for each trial.

All *Nosema* spore count data were $\log_{10}$ transformed prior to analysis. Spore counts from individual d-7 bees were averaged for each cage to generate a single value. Separate analysis was conducted for each trial. Comparisons between ± *Nosema* bees and those fed in- vs. out-of-season pollen were made using an F-test followed by Tukey's pairwise comparisons. All data were analyzed with JMP (SAS Institute, Cary NC) and Minitab (Minitab Inc., State College, PA).

3. Results

3.1. Protein and lipid composition of spring and fall pollens

Spring pollens were comprised primarily of *Brassica* spp. (Fig. 1). Other Brassicaceae (i.e., mustard) species (*Sisymbrium* (tumbling mustard), and *Raphenus* (wild radish) also were present. Fall pollens had greater diversity and were composed of high levels of *Xanthium* spp. (cocklebur) and *Amaranthus* spp. (pigweed). Both seasonal pollen mixtures had some *Sisymbrium*, but levels were higher in the fall.

The total protein concentration of spring and fall pollens was similar (spring pollen: 421 ± 28 μg/mg, fall pollen: 425 ± 30 μg/mg; $t_{17} = 0.1$, $p = 0.92$). The pollens differed however, in the concentrations of certain amino acids: spring pollen had significantly higher concentrations of tryptophan, valine, isoleucine, serine, asparagine and glutamine, while fall pollen was higher in proline and hydroxyproline (Fig. 2).

Total lipid concentrations did not differ between spring and fall pollens (spring pollen: 142.3 ± 12 μg/mg, fall pollen: 133.9 ± 7.8 μg/mg; $t_8 = 0.59$, $p = 0.57$). Spring pollens had significantly higher concentrations of y-linolenic, arachidic, lignoceric, lauric, and myristic acid than fall pollens (Fig. 3). Fall pollen had higher concentrations of capric and elaidic acid than spring pollens.

3.2. Consumption, protein digestion, total protein consumed and hypopharyngeal gland size in uninfected bees fed in- or out-of-season pollen

After 7 days of feeding, spring bees consumed significantly more in-season (spring) pollen than out-of-season (fall) pollen ($F_{1,7} = 18.46$, $p = 0.004$), but digested more protein from out-of-season (fall) pollen ($F_{1,6} = 16.1$, $p = 0.007$) (Fig. 4). We estimated the total protein from each pollen type obtained by the bees after 7 days (μg of protein per mg of pollen × mg of pollen consumed × proportion of pollen protein digested) and found no significant difference between the amounts of in- vs out-of-season protein spring bees consumed ($F_{1,6} = 0.01$, $p = 0.94$). Though similar amounts of protein from in- and out-of-season pollen were consumed, spring bee HPG were significantly larger in those fed in-season pollen ($F_{1,6} = 11.1$, $p = 0.016$).

In the fall trial, bees consumed similar amounts of each pollen type ($F_{1,7} = 1.12$, $p = 0.33$), digested significantly more protein from in-season (fall) pollen ($F_{1,4} = 741.4$, $p < 0.0001$), consumed similar amounts of protein from in- and out-of-season pollen ($F_{1,5} = 2.2$, $p = 0.2$), and had HPG that were similar in size whether they consumed in- or out-of-season pollen ($F_{1,6} = 2.46$, $p = 0.168$).

3.3. Mapping RNA-seq reads to the Apis mellifera genome

On average, 38 and 20% of the reads in the libraries were successfully mapped to the *A. mellifera* genome in spring and fall bees, respectively. Upon further inspection of the unmapped reads, we found that many transcripts in all libraries mapped to *Apis*-associated RNA viruses specifically, deformed wing virus (Table S1). All reads were deposited in the NCBI SRA under BioProject SRP136261.

3.4. Transcriptomic responses to pollen type in uninfected bees

Transcriptome analysis revealed DE genes in the fat body of bees fed in- vs. out of season pollen. More genes were differentially expressed due to pollen type in spring bees (228 genes) compared with fall bees (4 genes). Of the 228 genes differentially expressed in spring bees due to pollen type, 128 were more highly expressed in bees fed in-season (spring) pollen, and 100 in those fed out-of-season (fall) pollen (Table S2). The list of genes with higher expression in spring bees fed in-season pollen was not enriched for any function or pathway. The 100 genes more highly expressed in spring bees fed out-of-season pollen were significantly associated with the fatty acid biosynthesis KEGG pathway ($p = 0.023$). In the fall bees, *vitellogenin*, a glycolipoprotein involved in diverse functions such as nutrition and caste determination in the honey bee, was the only gene more highly expressed in bees fed out-of-season (spring) pollen. *Cdk4*, a serine/threonine protein kinase, *N*, a protein in the notch signaling pathway, and ND4L were more highly expressed in fall bees fed in-season (fall) pollen. A summary of the gene expression changes due to pollen type in spring and fall bees is presented in Fig. 5.

3.5. Nosema spore counts in infected bees fed in- season or out-of-season pollen

+*Nosema* bees were successfully infected by the spores we fed them. Spores were detected in all +*Nosema* bees and the averages per cage were significantly higher by day-7 than −*Nosema* bees in both trials (spring bees: $F_{3,12} = 211.4p < 0.0001$; fall bees: $F_{3,12} = 29.1$, $p < 0.0001$). Spore numbers in + *Nosema* spring bees did not differ

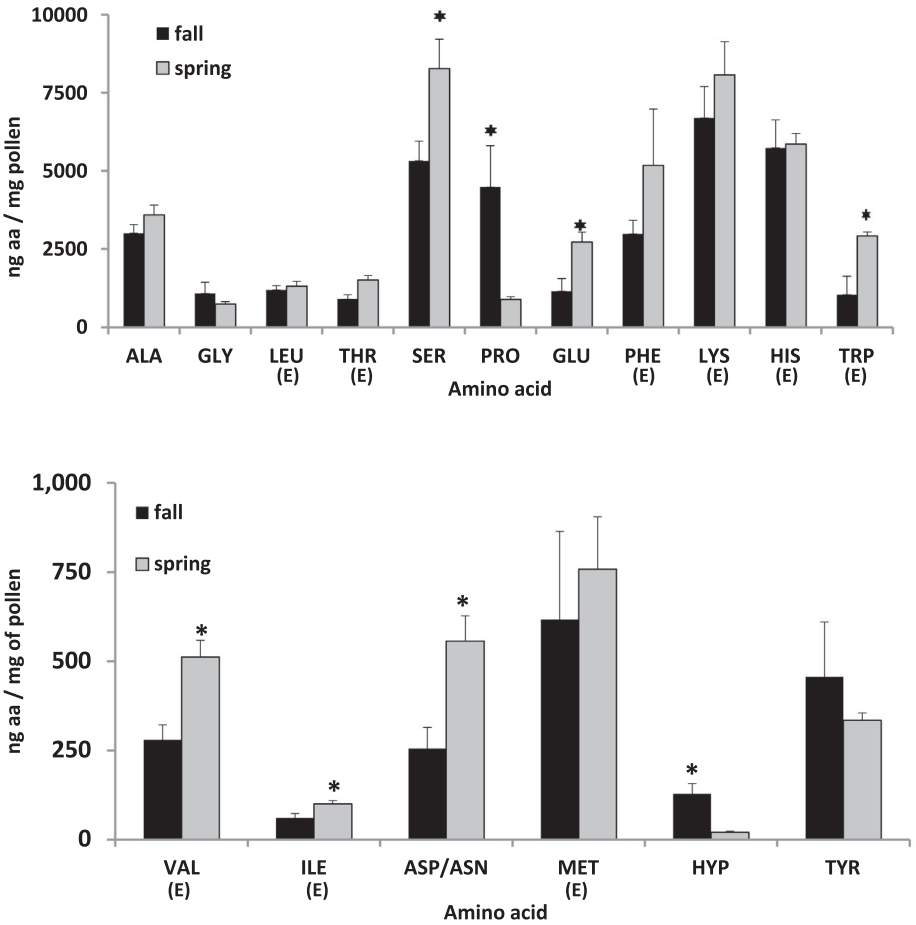

Fig. 2. Amino acids present in spring and fall pollen mixtures collected in the Sonoran Desert of Pima County, Arizona, USA. Concentrations of specific amino acids marked with an asterisk indicate significant differences between spring and fall pollens as determined by a *t*-test ($p < 0.05$). Those with an (E) are essential amino acids. Amino acids are shown on separate plots due to differences in concentrations.

between those fed in- or out of season pollen and were 14-fold higher than −*Nosema*. Similar results occurred with +*Nosema* fall bees, and these had spore numbers that were 6-fold higher than −*Nosema*.

3.6. Effects of pollen type and Nosema infection on consumption, protein digestion and hypopharyngeal gland size

An analysis with +*Nosema* bees indicated that infected spring bees consumed similar amounts of spring and fall pollen ($F_{1,6} = 1.68$, $p = 0.24$), digested equivalent percentages of protein from both pollen types ($F_{1,6} = 4.12$, $p = 0.09$), and had significantly larger HPG when fed spring pollen compared with fall pollen ($F_{1,6} = 13.1$, $p = 0.011$). +*Nosema* fall bees consumed equivalent amounts of spring and fall pollen ($F_{1,6} = 1.29$, $p = 0.3$), digested higher amounts of protein from fall pollens ($F_{1,5} = 10.1$, $p = 0.025$), and had similar size HPG when fed either pollen type ($F_{1,6} = 0.36$, $p = 0.57$).

Comparisons between – and +*Nosema* bees indicated that pollen consumption was not significantly different between bees with and without *Nosema* fed either pollen type (spring bees: $F_{3,12} = 1.1$, $p = 0.385$, fall bees: $F_{3,13} = 0.86$, $p = 0.486$). +*Nosema* bees digested higher percentages of protein in spring pollen than −*Nosema* bees (spring bees: $F_{3,12} = 9.37$, $p = 0.006$, fall bees: $F_{3,9} = 25.15$, $p < 0.0001$). However, HPG in +*Nosema* bees were significantly smaller than in −*Nosema* bees fed the same pollen source (spring bees: $F_{3,12} = 14.52$, $p < 0.0001$; fall bees: $F_{3,12} = 27.65$, $p < 0.0001$).

An analysis that included pollen type, ± *Nosema* and interaction terms in spring and fall bees indicated that HPG in spring and fall bees fed either in- or out of season pollen were smaller when the bees were infected with *Nosema* (Table 1). This occurred even though pollen consumption, and total protein consumed were similar between ± *Nosema* bees in both trials. A difference between trials was that +*Nosema* spring bees digested significantly more in-season pollen than −*Nosema* bees. Protein digestion in fall bees was not affected by *Nosema* infection. Interaction effects between pollen source and *Nosema* infection were not significant for any factor in either trial.

3.7. Transcriptome-level responses to infection depending on pollen type

The PCA plots of spring bee libraries showed some clustering based on infection, with −*Nosema* bees forming a distinct group from −*Nosema* + gut and +*Nosema* bees (Fig. S1). There also were slight differences in the magnitude of separation between these two clusters for bees fed either in-season or out-of-season pollen. In contrast, the three treatment groups overlapped in the fall bees fed in- and out-of-season pollen.

The two-way model afforded an opportunity to look at the main effects of infection and diet in spring and fall bees. We also compared the expression of several immune genes (Holt et al., 2013; Li et al., 2018) in + and −*Nosema* spring and fall bees fed either pollen type. In the spring bees, the main effect of *Nosema* did not impact the expression of the immunity genes. However, the main effect of diet resulted in the differential expression of 21 genes (Table S3). An example of how diet influenced the expression of four genes is shown in Fig. S3. In fall bees, the main effect of *Nosema* did not impact the expression of any genes,

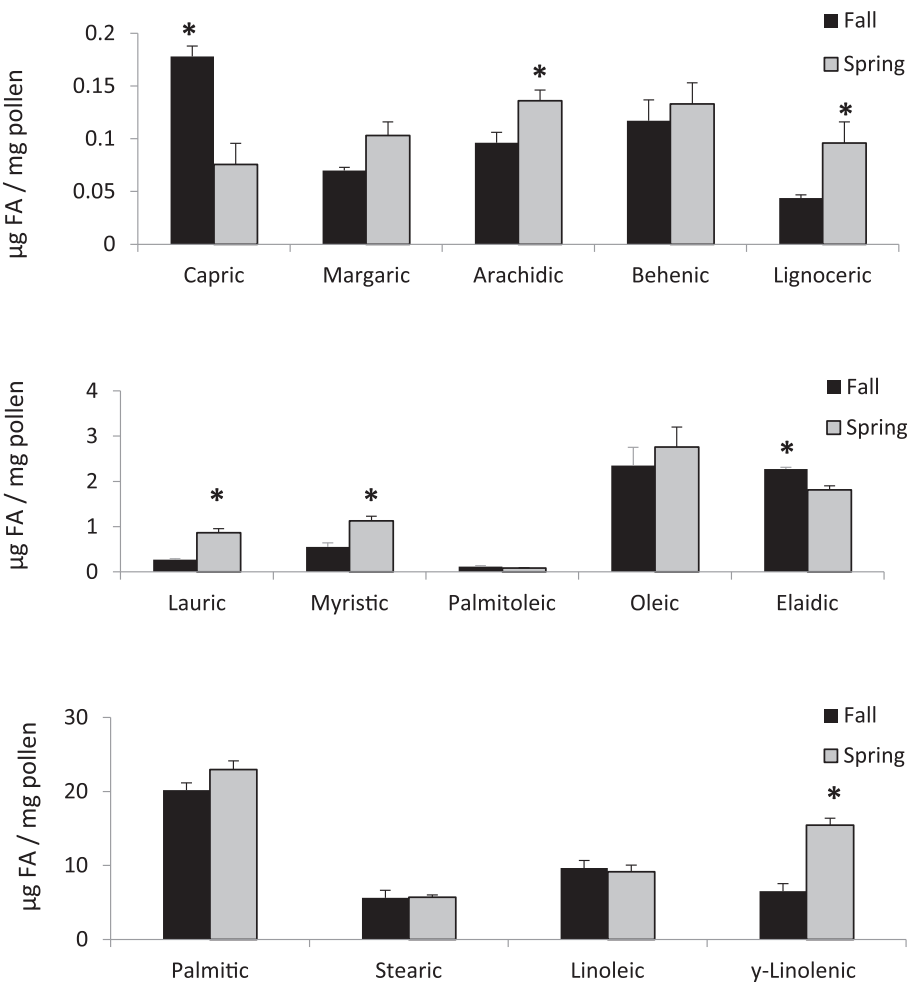

Fig. 3. Fatty acids in spring and fall pollen collected by bees in the Sonoran Desert, Pima County, Arizona. Concentrations of specific fatty acids marked with an asterisk indicate significant differences between spring and fall pollens as determined by a *t*-test ($p < 0.05$). Fatty acids are shown on separate plots due to differences in concentrations.

while the main effect of diet resulted in the differential expression of 12 genes (Table S4). In the two-way model, the expression of the four immune genes that were tested – *defensin-1*, *pelle*, *apid1*, *LOC40614* – did not change with infection or diet in either the spring or fall bees (Fig. S3).

Genes that showed different patterns of *Nosema*-induced expression depending on pollen type (i.e., the pollen type × *Nosema* interaction) also were identified. For the spring bees, this list contained a total of 399 genes (Table S5). The interaction effect is illustrated for four genes in Fig. S2. The list of genes for which the response to *Nosema* was impacted by pollen type included those associated with several KEGG pathways (TCA cycle, $p = 8.9 \times 10^{-3}$; carbon metabolism, $p = 9.7 \times 10^{-4}$; pyruvate metabolism, $p = 4.4 \times 10^{-3}$ Table 2) and lipid metabolism (Table 3). In the fall bees, no genes exhibited different patterns of infection-induced expression depending on pollen type. In other words, the fall bees were identical in their transcriptional response to infection when fed either in- or out-of-season pollen.

4. Discussion

We compared the nutritional composition of pollen mixtures collected in the spring and fall, and the responses of honey bees that consumed them. Spring and fall pollen mixes had similar protein and lipid concentrations, but spring pollens had higher concentrations of amino acids required for HPG growth and brood rearing. Concentrations of fatty acids required for HPG development, learning, and that have antimicrobial activity against brood pathogens also were higher in spring compared with fall pollen. Bee responses to seasonal pollens differed depending on whether the pollen was in or out of season relative to when the bee was reared and if the bees were infected with *Nosema*. Spring bees had larger HPG when fed in-season pollen, while HPG in fall bees were not impacted by pollen type. The fat body transcriptomes of spring bees with and without *Nosema* infection showed many genes differentially expressed due to pollen type, but this was not the case for fall bees. The functions of the affected genes suggest that spring bees fed out-of-season pollen may experience nutritional stress due to a reduced ability to generate energy through the TCA cycle or to synthesize amino acids. Signs of malnutrition were heightened, especially as expressed in HPG size, when spring bees were infected with *Nosema* and fed out of season pollen.

Our investigation began with an analysis of seasonal pollens. Spring and fall pollens were collected from colonies in the Sonoran Desert. The protein and lipid concentrations however were similar to those reported previously from other geographic regions, elevations and climates (Odoux et al., 2012; Negrão et al., 2014; Avni et al., 2014; Di Pasquale et al., 2016). Fall pollen had greater diversity than spring pollen. Where spring and fall pollens differed was in species composition and resulting amino and fatty acid profiles. The differences can be germane to the responses of spring bees to in- vs out of season pollen, and to the seasonal behaviors and activities of colonies. For instance, tryptophan is required for HPG development and occurred at higher concentrations in spring compared with fall pollens (Fengkui et al., 2015). Others have

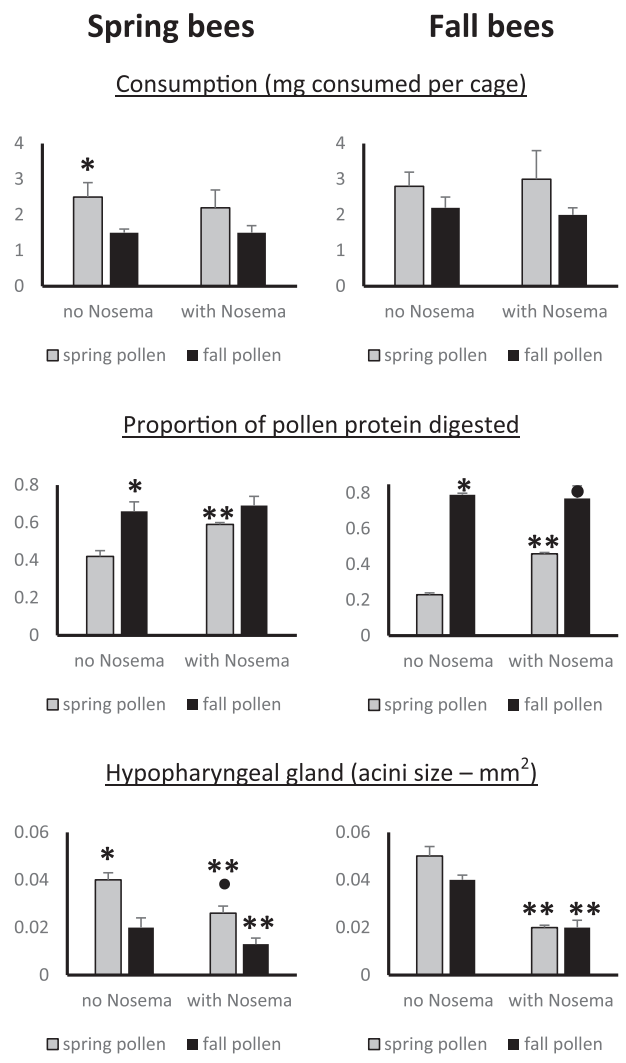

Fig. 4. Average amounts of spring and fall polyfloral pollen mixes consumed over 7 days by spring and fall worker bees with and without *Nosema*, the proportion of the pollen protein bees digested, and size of hypopharyngeal glands in d-7 bees. Averages with an asterisk (no *Nosema*) or dot (with *Nosema*) indicate significant differences between pollen types for either spring or fall bees. Averages with a double asterisk (**) indicate significant differences between bees with and without *Nosema* for the pollen type.

reported that HPG size increases with greater quantities of *Brassica* pollen perhaps because of the relatively high levels of tryptophan (Di Pasquale et al., 2016, Fengkui et al., 2015). In our study, *Brassica* was the predominant genera in spring pollen mixes. Valine and serine concentrations also were higher in spring pollens. These amino acids are constituents of apisimin, an alpha-helical peptide that promotes the assembly of major royal jelly protein – 1 (MRJP-1) (Bilikova et al., 2002). MRJP are the main constituents responsible for the specific physiological role of royal jelly, the main food source for brood (1–3 day old larvae), and queens (Simuth, 2001). Isoleucine is a brood pheromone carrier, and occurred in higher concentrations in spring pollens (Briand et al., 2002). Brood pheromone is released by larvae and communicates their need for food to nurse bees (LeConte et al., 1995; Pankiw, 2004; Forcone et al., 2011). Glutamine also was in higher concentrations in spring pollens. This amino acid may be a nutritional driver for the upregulation of genes associated with the mTOR signaling pathway (Münch and Amdam, 2010; Alaux et al., 2011; Zhai et al., 2015). The mTOR pathway affects the aging of individuals, caste determination, and division of labor that are critical components of colony expansion in the spring.

The only amino acids occurring in higher concentrations in fall pollens were proline and hydroxyproline. Higher levels of these amino acids in fall pollens would support activities that are essential to winter survival, specifically thermoregulating the winter cluster. Bees vibrate their flight muscles to generate heat and proline serves as a carbon shuttling molecule between lipid reserves in the fat body and flight muscles (Teulier et al., 2016; Arrese and Soulages, 2010). Hydroxyproline also contributes to the mobilization of stored metabolites (e.g., lipids, carbohydrates and proline) from the fat body into the hemolymph because it is a component of adipokinetic hormone that activates lipase and glycogen phosphorylase (Gäde et al., 2011).

As reported previously, palmitic, linoleic and linolenic were the predominant fatty acids in spring and fall pollen mixes (Manning, 2001; Szczęsna, 2006; Avni et al., 2014). Steric acid also was detected at high concentrations similar to reports by Human and Nicolson (2006). Concentrations of palmitic acid in our samples were within the averages reported by Avni et al. (2014), Human and Nicolson (2006) and Szczęsna (2006) (percentage of the total fatty acids in the sample). Our linolenic acid levels were low compared with those reported by Avni et al. (2014), but linoleic acid concentrations were similar to those reported by Human and Nicolson (2006) and Szczęsna (2006). Concentrations of other fatty acids also were similar to those reported by Szczęsna (2006) for spring and summer pollens collected in Poland, Korea and China, and for *Brassica* spp. reported by Manning (2001). From a seasonal perspective, concentrations of certain fatty acids differed between spring and fall pollens in our samples. Linolenic acid was found at higher concentrations in spring pollens and is required for HPG development, cognitive function specifically olfactory and tactile associative learning required for efficient foraging, and it has antimicrobial properties (Arien et al., 2015). Myristic and lauric acid concentrations also were higher in spring pollen, and both have bactericidal properties particularly to *Paenibacillus larvae larvae*, the causative agent of American foulbrood disease in honey bees (Feldlaufer et al., 1993; Manning, 2001; Manning and Harvey, 2002). The only fatty acid we found in higher concentrations in fall pollen was capric acid, that also has antimicrobial activity against *P. larvae larvae* (Feldlaufer et al., 1993).

The differences in nutrient composition between spring and fall pollens may have contributed to the responses we detected in spring bees fed either in- or out of season pollen. Spring bees were highly sensitive to pollen type and both HPG size and gene expression patterns were affected (Fig. 5). Of particular interest, spring bees that consumed in-season pollens had higher expression of three lipase genes (two triacylglycerol lipase genes and a phospholipase A1 gene) that may be involved in lipid mobilization and nutrient transport (Arrese et al., 2006; Arrese and Soulages, 2010) and contributed to greater HPG growth in spring bees fed in-season pollen.

The effects of consuming in vs. out of season pollen were enhanced by *Nosema* infection as +*Nosema* bees had smaller HPG especially in spring bees fed out of season pollen. These effects occurred even though +*Nosema* bees appeared to digest more protein from spring pollen. Our estimates of protein digestion were based on protein concentrations in the hindgut. Lower protein concentrations were associated with greater digestion levels. If *Nosema* increased protein digestion, we would not expect to see reduced HPG size. However, if lower protein concentrations in the hindgut of infected bees were from *Nosema* appropriating nutrients from the bee, then nutrient-sensitive structures such as HPG would be negatively affected (Corby-Harris et al., 2016).

The deleterious consequences of *Nosema* infection on HPG size were not mitigated by diet. However, pollen type did impact *Nosema*-induced gene expression for 399 genes in spring bees. This list included genes involved in the TCA cycle, pyruvate metabolism, and fatty acid biosynthesis. These results support earlier studies that *Nosema* impacts metabolism (Holt et al., 2013; Li et al., 2018) and suggest that pollen type could play a role in the host response to *Nosema*. This was the case

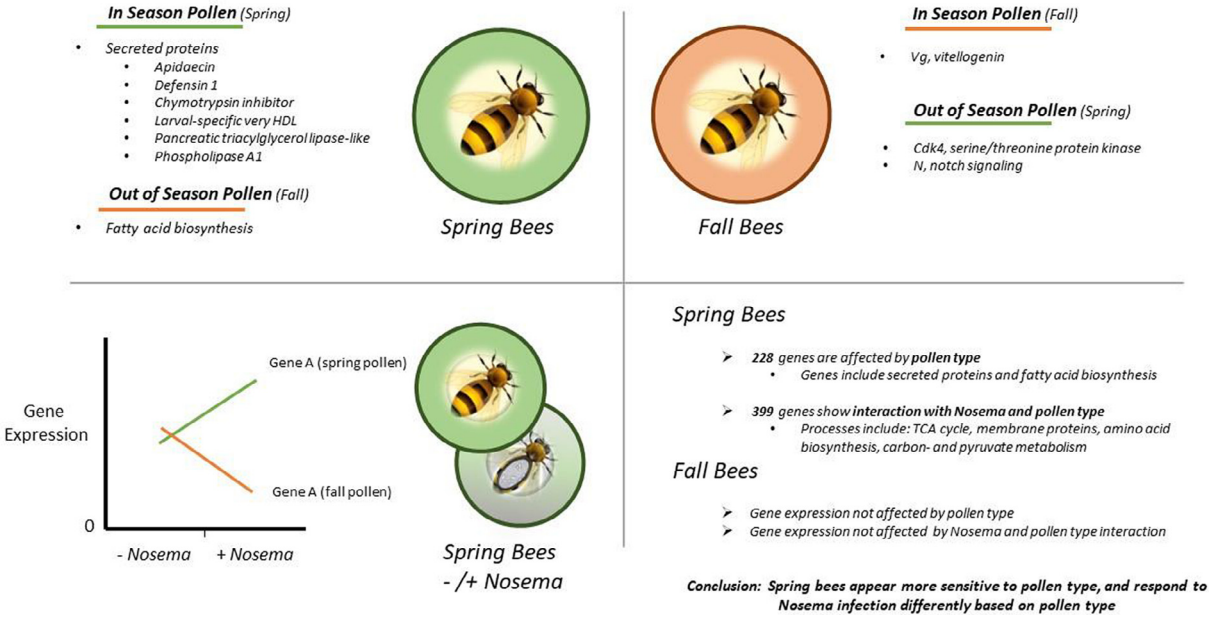

Fig. 5. Summary of the gene expression changes observed in spring and fall bees that were not inoculated with *Nosema* (top panels) and how the response to infection was altered by pollen type (bottom panels). The graph in the bottom left panel illustrates how, for a hypothetical gene of interest, infection-induced gene expression might change depending on diet for spring bees fed either in-season (spring) pollen or out-of-season (fall) pollen. Infection-induced expression is the change in gene expression between uninfected (*-Nosema*) and *Nosema*-infected (*+Nosema*) bees.

Table 1
Effects of pollen type (spring or fall pollens), *Nosema* infection, and the interaction of both factors on average pollen consumption, digestion of protein in the pollen, total protein consumed and hypopharyngeal gland size in 7-day old worker bees. Data were analyzed using a two-way ANOVA.

Trial	Response	Pollen type			± Nosema			Pollen type * ± Nosema			Total d.f.
		F	d.f.	p	F	d.f.	p	F	d.f.	p	
Spring	pollen consumption	10.7	1	0.006	0.56	1	0.57	0.59	1	0.47	16
	protein digestion	19	1	0.001	6.2	1	0.03	2.9	1	0.11	15
	total protein consumed per bee	0.24	1	0.63	0.1	1	0.75	0.17	1	0.69	15
	hypopharyngeal gland size	23.9	1	< 0.0001	20.9	1	0.001	0.49	1	0.50	15
Fall	pollen consumption	2.5	1	0.13	0.001	1	0.97	0.19	1	0.67	16
	protein digestion	41.5	1	< 0.0001	3.14	1	0.11	4.39	1	0.066	12
	total protein consumed per bee	3.22	1	0.10	0.22	1	0.66	0.01	1	0.91	13
	hypopharyngeal gland size	0.66	1	.43	70.5	1	< 0.0001	2.41	1	0.146	15

in *+Nosema* spring bees where HPG in those fed in-season pollen were larger than those fed out-of-season pollen. However, there were limitations on the benefits of pollen type as HPG in *+Nosema* bees were smaller than *–Nosema* bees fed in-season pollen.

Fall bees were less sensitive to pollen type than spring bees in both physical responses and gene expression patterns. HPG were similar in fall bees fed either in- or out-of-season pollen. Gene expression data also showed little differential gene expression with pollen type; only 4 genes were differentially expressed due to pollen type. In the two-way gene expression analysis, the main effect of diet impacted the expression of 12 genes. In addition, we saw that the cost of *Nosema* infection to HPG size was not sensitive to pollen type because the interaction between pollen type and *Nosema* infection was not significant. This was again consistent with the gene expression data, where no genes were

Table 2
For spring bees, KEGG pathways containing genes that responded to infection differently depending on the pollen type that the bees consumed.

KEGG pathway	# genes DE	Genes (BEEBASE IDs)	p^A
ame01130: Biosynthesis of antibiotics	16	GB41912, GB54661, GB51042, GB43964, GB42526, GB52753, GB44983, GB47436, GB44897, GB46290, GB50902, GB43150, GB55537, GB54720, GB44039, GB45258	0.002
ame01200: Carbon metabolism	12	GB52753, GB44983, GB47436, GB46290, GB43150, GB50902, GB51042, GB55537, GB54720, GB42526, GB44039, GB45258	9.7×10^{-4}
ame00620: Pyruvate metabolism	7	GB44983, GB47436, GB46290, GB51042, GB54720, GB42526, GB44039	0.004
ame00020: Citrate cycle (TCA cycle)	7	GB52753, GB44983, GB51042, GB54720, GB42526, GB44039, GB45258	0.009
ame01100: Metabolic pathways	31	GB54661, GB42855, GB51042, GB42526, GB40119, GB54448, GB40727, GB52753, GB44983, GB55948, GB46290, GB46291, GB50902, GB55537, GB42385, GB44039, GB41912, GB51913, GB43964, GB47405, GB47436, GB48351, GB44897, GB47736, GB55466, GB43150, GB54720, GB45258, GB43362, GB42481, GB50993	0.034

[A] Benjamini-Hochberg adjusted *p*-value.

Table 3
Lipid metabolism genes that, in spring bees, responded to infection differently depending on the type of pollen that the bees consumed.

BEEBASE ID	gene
GB43362	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha-like (LOC724995)
GB40727	CDP-diacylglycerol–inositol 3-phosphatidyltransferase (LOC411408)
GB42217	acyl-CoA Delta(11) desaturase-like (LOC727333)
GB40681	elongation of very long chain fatty acids protein 1-like (LOC100578829)
GB54396	elongation of very long chain fatty acids protein AAEL008004-like (LOC724552)
GB47273	lipase 1-like (LOC552588)
GB49323	putative fatty acyl-CoA reductase CG5065 (LOC100578814)

impacted by this interaction term.

Although *Nosema* infection reduced HPG size in both the spring and fall bees, infection did not impact gene expression consistently across diets. In other words, there was not a main effect of infection on gene expression in either spring or fall bees. In the spring bees, this is explained primarily by strong diet x infection interaction effects on gene expression. For example, *Nosema* infection may have affected gene expression in bees fed either the spring or fall pollen, but the influence of infection was different depending on the type of pollen they consumed especially for spring bees. In the fall bees, there was no effect of infection or the interaction between diet and infection on fat body gene expression. Although it is difficult to determine from our analysis, this could be a timing issue – we did not capture any infection-induced changes because we may have sampled too late into the infection process – there was low mapping success of the fall samples to the *A. mellifera* genome.

Though our findings support the concept of seasonal nutritional requirements in honey bee colonies, interpretations of our results have limitations and additional studies are needed. Perhaps the most obvious limitation is that the study was conducted with caged bees. Although the bees were reared in colonies during different seasons, and showed characteristics that suggest differences in the processing and response to seasonal pollens, nestmate interactions and the presence of brood and young adult bees influence the physiology, gene expression, aging and life history of worker bees (Münch and Amdam, 2010; Traynor et al., 2017). The findings from our study are based on nutrition alone, and whether similar trends occur in colonies whose populations are expanding and contracting will require further study. Another factor requiring consideration is that our study was conducted in the Sonoran Desert with pollens collected from plants grown in the area. Though brood rearing declines in the fall and colonies experience periods of confinement in winter, those periods do not last for several months as in temperate areas. To determine if our findings are applicable to areas with longer periods of overwinter confinement, the study should be repeated with bees from temperate areas that are fed seasonal pollens from those regions.

Placing honey bee nutrition within a theoretical construct of the colony as an organism with a yearly lifecycle and seasonal nutritional requirements captures the dynamic relationships between honey bees and their environment. There are indications from our study that seasonal pollens are tuned to address the nutritional needs of colonies particularly as they relate to brood rearing in the spring and nutrient storage in fall and winter. Findings from our study have practical applications for establishing pollinator habitats and highlight the need for flowering plants to be available from spring through fall. Habitats that provide resources throughout the yearly cycle of a colony also may benefit other pollinators. For example, bumblebee colonies have cycles of growth and contraction and perhaps seasonal nutritional requirements. Our findings also should be considered when developing protein supplements that are fed to colonies during times of pollen shortages. The supplements often fall short of maintaining colony health or assuring survival (e.g., DeGrandi-Hoffman et al., 2016), perhaps because they lack sufficient amounts of specific nutrients required by the colony to sustain activities at a given time of year. Ideally, supplements should be formulated for the season when they will be fed to colonies. Our results also suggest that findings from nutritional studies should be interpreted within a framework that considers when the bees are reared since responses may differ depending on season.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jinsphys.2018.07.002.

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