Video Article

Methods for Comparing Nutrients in Beebread Made by Africanized and European Honey Bees and the Effects on Hemolymph Protein Titers

Gloria Degrandi-Hoffman[1], Bruce Eckholm[2], Ming Huang[3]

[1]Carl Hayden Bee Research Center, USDA-ARS

[2]Coupeville, WA, USA

[3]Ecotoxicology Division, Eurofins Agroscience Services, Inc.

Correspondence to: Gloria Degrandi-Hoffman at gloria.hoffman@ars.usda.gov

URL: https://www.jove.com/video/52448
DOI: doi:10.3791/52448

Keywords: Molecular Biology, Issue 97, pollen, nutrition, microbes, protein, amino acids, Africanized bees, genotype, *Apis mellifera*, *scutellata*

Date Published: 3/17/2015

Citation: Degrandi-Hoffman, G., Eckholm, B., Huang, M. Methods for Comparing Nutrients in Beebread Made by Africanized and European Honey Bees and the Effects on Hemolymph Protein Titers. *J. Vis. Exp.* (97), e52448, doi:10.3791/52448 (2015).

Abstract

Honey bees obtain nutrients from pollen they collect and store in the hive as beebread. We developed methods to control the pollen source that bees collect and convert to beebread by placing colonies in a specially constructed enclosed flight area. Methods were developed to analyze the protein and amino acid composition of the pollen and beebread. We also describe how consumption of the beebread was measured and methods used to determine adult worker bee hemolymph protein titers after feeding on beebread for 4, 7 and 11 days after emergence. Methods were applied to determine if genotype affects the conversion of pollen to beebread and the rate that bees consume and acquire protein from it. Two subspecies (European and Africanized honey bees; EHB and AHB respectively) were provided with the same pollen source. Based on the developed methods, beebread made by both subspecies had lower protein concentrations and pH values than the pollen. In general, amino acid concentrations in beebread made by either EHB or AHB were similar and occurred at higher levels in beebread than in pollen. Both AHB and EHB consumed significantly more of the beebread made by AHB than by EHB. Though EHB and AHB consumed similar amounts of each type of beebread, hemolymph protein concentrations in AHB were higher than in EHB. Differences in protein acquisition between AHB and EHB might reflect environmental adaptations related to the geographic region where each subspecies evolved. These differences could contribute to the successful establishment of AHB populations in the New World because of the effects on brood rearing and colony growth.

Video Link

The video component of this article can be found at https://www.jove.com/video/52448/

Introduction

Nutrition plays a fundamental role in the health and vigor of honey bee colonies and in their establishment as populations. Nutrients from food provide energy and the biochemical components needed for brood rearing, thermoregulation, foraging and immune response. For honey bee colonies, the nutrients needed to grow colony populations and maintain their health come from nectar and pollen. Nectar provides carbohydrates and pollen supplies the remaining dietary requirements such as protein, lipids, vitamins and minerals [1].

Subspecies of honeybees can differ in nutritionally based colony-level parameters such as worker longevity, brood rearing, and mechanisms of social immunity [2-6]. These differences might be linked to how food, particularly pollen is processed by the colony and digested in individuals. Pollen is stored in comb cells and through microbially mediated lactic acid fermentation is chemically changed [7-10]. The fermented pollen is called beebread. There might be genotypic differences among subspecies in their ability to acquire nutrients from the pollen they collect and store and this could affect colony growth and survival. Genotypic effects on food processing and nutrient acquisition have been documented in other organisms causing some individuals to obtain more nutrients and calories than others while consuming the same foods [11-14].

Here we describe methods used to compare the composition and consumption of beebread made by different subspecies of honey bees. Methods to measure the resulting hemolymph protein titers in worker bees also are described. Previous studies on the nutritional composition of beebread were done with European honey bees (EHB) [10,15,16]. However, there may be differences in beebread made by bees of different subspecies even when they feed on the same pollen. EHB and AHB were compared because these subspecies have distinct behavioral and physiological differences that might be related to food processing and nutrient acquisition [17]. Some of the most notable differences are that AHB collect and consume more pollen than EHB and seem to convert it more readily into brood [18]. AHB colonies have higher swarming rates than EHB and abscond when food resources become limited [19-23]. Absconding is rare in EHB. AHB also have higher metabolic rates than EHB [24]. The nutritional basis for the colony-level differences between EHB and AHB might be related to the rate of pollen collection and also to its nutrient content (*e.g.*, amino acids and protein) after it is converted to beebread. Beebread consumption and the resulting protein acquisition also might play a role in the colony-level differences between EHB and AHB. Using the developed methods, EHB and AHB made beebread from the same pollen source. The beebread was then fed back to the bees of each subspecies and could determine if bees acquire protein from beebread in a manner distinctive to their subspecies or to the source of the beebread.

Protocol

1. Obtaining Beebread from AHB and EHB Colonies

1. Place pollen traps on honey bee colonies and collect pollen. Grind the pollen into a fine powder (similar to pollen shed from anthers) using a coffee grinder.
2. Establish 5 colonies each of AHB and EHB in an enclosed flight area (EFA) so that bees forage only on the pollen provided. To prevent workers from drifting between EHB and AHB colonies, divide the EFA into separate sections so that bees cannot cross between them. Place individual EHB or AHB colonies with 3,500-4,000 worker bees, wax comb with nectar, honey, immature brood and empty comb in each section of the EFA.
   NOTE: The colonies do not have stored pollen when established. The rate that pollen is stored can be increased by not including a laying queen in the colonies.
3. Feed ground pollen to colonies by placing a tray with the pollen in each section of the EFA. Spreading about 60 g of pollen on each tray so that foraging bees can collect it as corbicular loads and store the pollen in their colonies as beebread. Continue providing fresh pollen on each tray daily for 3 weeks.
4. Refer to beebread from the European colonies as European beebread (EBB), and from Africanized colonies as Africanized beebread (ABB).

2. Feeding Bees in Cages

1. Place frames of sealed worker brood from AHB and EHB colonies in separate emergence cages in an environmental room set at 32-34 °C and 40% relative humidity..
2. When the workers emerge and are about 24 hr old, establish 12 Plexiglas bioassay cages (dimensions = 11.5 x 7.5 x 16.5 cm^3) and add either 100 newly emerged EHB or 100 newly emerged AHB worker bees to each cage. Place a section of comb with a known number of either EBB or ABB cells (24-30 cells per cage) in each cage to generate the following treatment combinations: AHB fed ABB, EHB fed ABB, AHB fed EBB and EHB fed EBB. (4 treatments; 6 cages per treatment; 24 cages in total).
3. Add vials of water and a 50% honey and water solution formulated by volume to each cage. Refill the honey and water vials daily for the 11 day study period.

3. Sampling Worker Bees and Beebread and Estimating Consumption

1. Sample 10 newly emerged EHB and AHB workers prior to placing them in the cages. Refer to these as Day 0 bees and have them serve as a baseline for hemolymph protein concentrations.
2. Remove 10 bees from each cage after they fed on EBB or ABB for 4, 7, and 11 days.
3. Place the live bees in individual microcentrifuge tubes and set on ice packs. Select a subsample of four bees for analysis of hemolymph protein concentration.
4. After sampling bees on Day-11, count the number of comb cells that still contain beebread. This is a relative measure of beebread consumption.
5. Remove the remaining beebread from the cells in each cage and store in separate microcentrifuge tubes according to cage. Keep the beebread samples at -80 °C until analyzed for pH, soluble protein concentration, and amino acid content.

4. Estimating pH of Pollen and Beebread

1. Take six random 0.3 g samples of the pollen fed to bees in the EFA and dissolve it in 300 µl of distilled water. Measure the pH using a waterproof double junction pH spear with an accuracy of +0.01.
2. Take 0.3 g sample of beebread that remained after the 11-day feeding period in each cage. Dissolve the beebread in 300 µl of distilled water and measure pH as described for pollen (4.1).

5. Protein Analysis

1. Take six samples of the pollen and a sample of EBB and ABB from each cage. Store samples at -20 °C until analyzed for soluble protein concentration.
2. Mix 20 mg of either pollen or beebread with 1,000 µl of 0.1 M phosphate buffer solution (PBS).
3. Vortex the mixture for 10 sec and centrifuge at 571.2 x g for 1 min.
4. Remove a 10 µl sample of the supernatant and place in wells of a 96 well flat bottom EIA/RIA polystyrene plate. Replicate each sample in three wells.
5. Draw hemolymph from bees collected from each cage by inserting a 20 µl capillary tube (that had been heated and pulled to a needle-sharp point) into the right lateral portion of the thorax near the point of attachment of the wings. Collect additional hemolymph, if needed, by inserting the same tube into the membrane between the abdominal tergites.
6. Add 1 µl of hemolymph to 9 µl of 0.1 M PBS. Store the hemolymph solution at -20 °C until analysis for soluble protein.
7. Determine total soluble protein concentrations in pollen, beebread, and hemolymph samples using a commercial Bradford protein assay kit. Follow the manufacturer's instructions.
8. Establish a standard curve to estimate soluble protein concentration in the samples by measuring protein absorbance with known protein concentrations in bovine serum albumin (BSA). Measure protein absorbance at 595 nm using a spectrophotometer.

6. Amino Acid Analysis

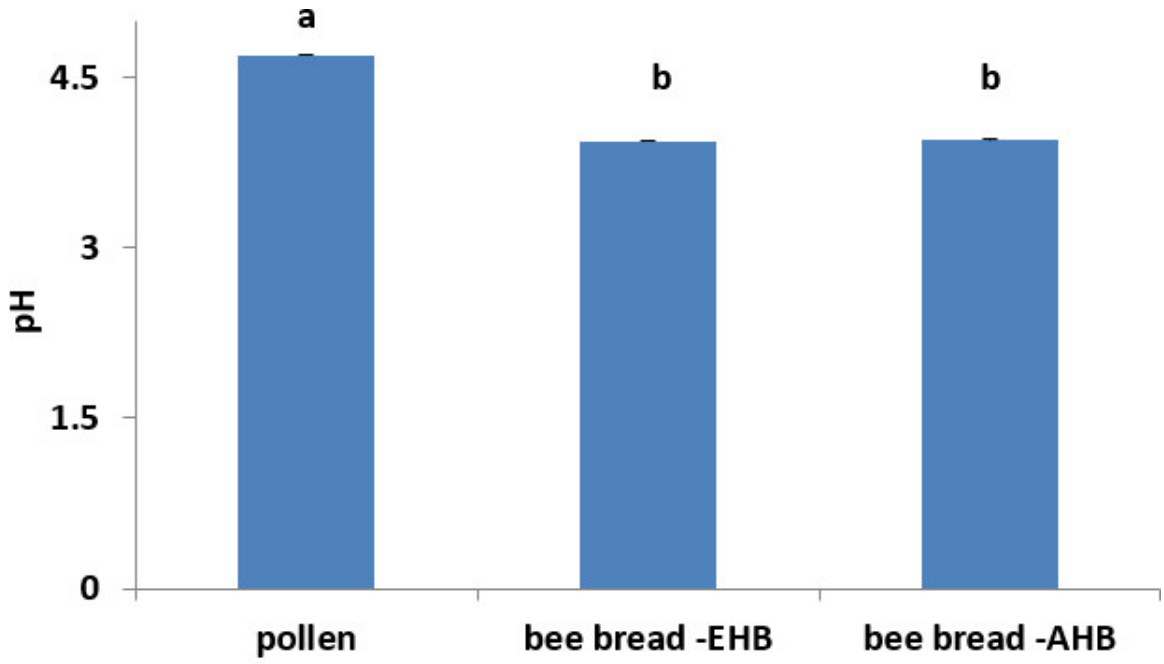
1. Pool individual samples from comb cells of each colony to create a representative sample of EBB and ABB for analysis.
2. Take a 50 mg pollen or beebread sample weighed into autosampler vials, and add 1 ml of distilled water to the vial, along with 100 µl of a 50 ng/µl internal standard solution consisting of d_4-alanine, d_{23}-lauric acid, $^{13}C_6$-glucose and d_{39}-arachidiac acid.
3. Cap the sample and sonicate for 5 min.
4. Condition an HLB cartridge by adding 1 ml of methanol, equilibrated by adding 1 ml of distilled water followed by the addition of 1 ml of the beebread or pollen sample. Wash the cartridge with 1 ml of 5.0% MeOH/H_2O and elute with 1 ml of 80% MeOH/H_2O.
5. Evaporate the sample to dryness under a stream of nitrogen. Reconstitute the sample with 50 µl of Pyridine and 100 µl of N,O-Bis (trimethylsilyl)trifluoroacetamide + Trimethylchlorosilane (BSTFA + TMCS).
6. Cap and incubate the sample at 70 °C for 30 min.
7. Allow the sample to cool and transfer it to a clean autosampler vial.
8. Cap and place the sample into a Mass Selective Detector interfaced to a gas chromatograph to analyze the samples both for volatile compounds and organic acids. Separate the sugar and organic acids following TMS derivatization with BSTFA + TMCS using a column (30 m x 0.25 mm i.d.) with a 1.0 µm film thickness.
9. Set the column oven at 50 C for 2 min, then increase the temperature linearly to 290 °C at 5 C/min. and hold for 7 min. Set the GC injector and GC/MS interface to 250 °C and 290 °C, respectively.
    1. Use Helium as a carrier at a flow rate of 1.0 ml/min. Set the MS source temperature to 230 °C.
10. Tune and calibrate the mass spectrometer daily with Perfluorotributylamine (PFTBA). Use a 1 µl injection of PFTBA in the full scan (35-700 amu) positive ion mode to obtain data on the presence and concentrations of amino acids.

Representative Results

Beebread was stored in -80 °C for less than a month before being analyzed for pH and protein concentration, and for about 4 months before amino acid analysis. Beebread differed from the pollen in pH and protein concentration (**Figure 1**). The pH of beebread was lower than the pollen as was the protein concentration. Both EHB and AHB consumed more ABB than EBB (**Figure 2**).

Levels of soluble protein in the hemolymph of AHB were significantly higher than EHB regardless of the type of beebread they consumed (**Figure 3**). These differences in hemolymph protein levels occurred even though EHB and AHB consumed similar amounts of each type of beebread. The age of the bees at the time of sampling significantly affected soluble protein concentrations in the hemolymph. Protein concentrations were significantly lower in day-4 bees compared with day-7 or 11 which did not differ.

Of the 10 amino acids that are essential for honey bees, all but histidine were detected in the pollen. In most cases, amino acid concentrations measured in beebread were higher than in the pollen (**Figure 4**). For example, concentrations of leucine and threonine were about 60% higher in beebread compared with pollen, and valine concentrations were about 25% higher. Alanine, aspartic acid, glutamine, and methionine levels also were higher in beebread than in pollen. Amino acids concentrations did not differ greatly between ABB and EBB with the exception of phenylalanine and cysteine. Phenylalanine levels were about twice as high in ABB compared with either EBB or pollen. Cysteine concentrations were lower in EBB compared with ABB or pollen. Tryptophan was the only amino acid present in higher concentrations in pollen than in EBB or ABB. Concentrations of proline in pollen and ABB were higher than in EBB.



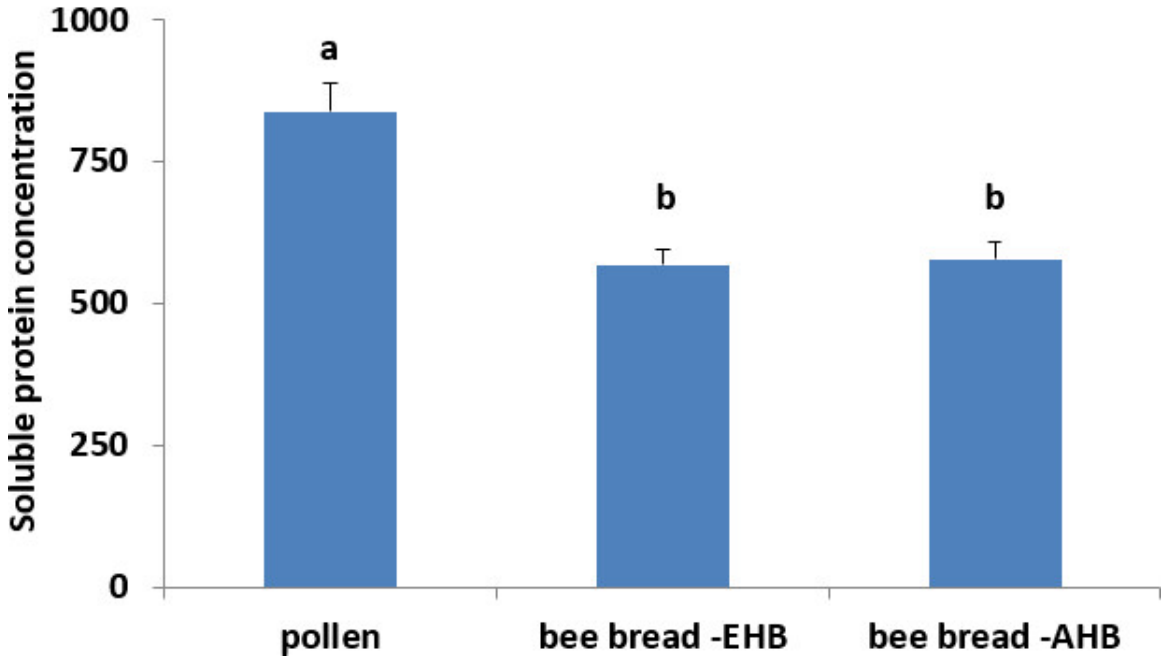


Figure 1: Comparisons of pH **(A)** and soluble protein concentrations **(B)** in pollen and the beebread made by European (EHB) or Africanized (AHB) honey bees. The pH of pollen was significantly higher than the beebread as determined by analysis of variance ($F_{2,12}$ = 3725, p <0.0001) followed by a Tukeys W- multiple comparison test. The protein concentration in pollen was significantly higher than in beebread made by EHB (EBB) or AHB (ABB) ($F_{2,27}$ = 16.49; p <0.0001). Means followed by the same letter are not significantly different at the 0.05 level.

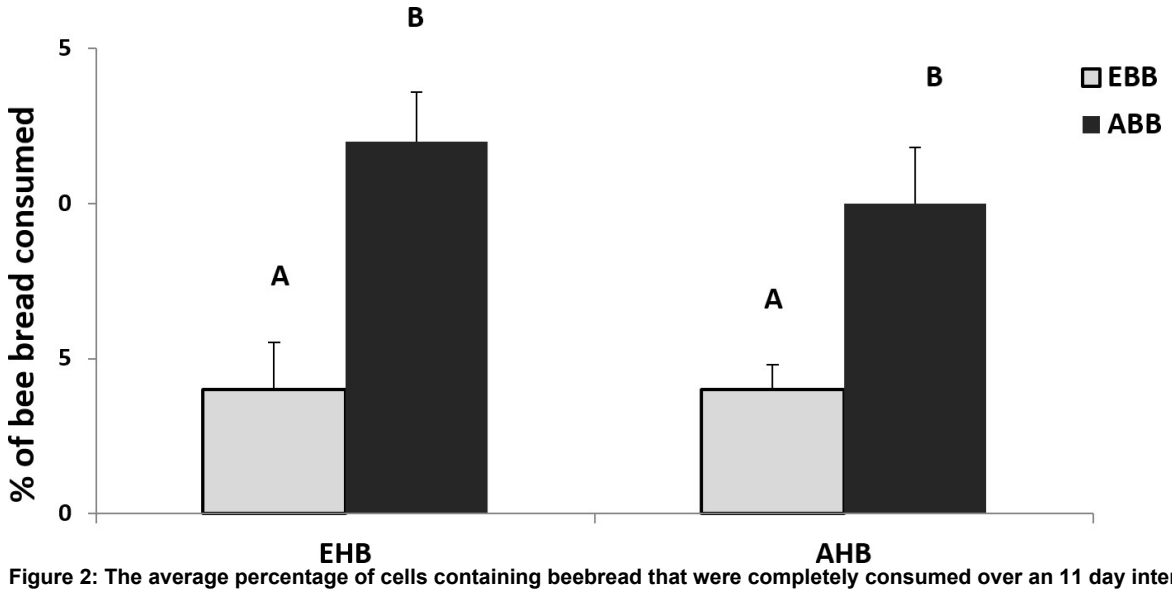


Figure 2: The average percentage of cells containing beebread that were completely consumed over an 11 day interval by caged bees. The beebread was made by either European (EHB) or Africanized (AHB) bees using the same pollen source. Means were estimated from five cages of each treatment; those with the same letter are not significantly different at the 0.05 level as determined by a one way analysis of variance ($F_{3,16}$ = 7.3, p = 0.003) and Tukey's W test. This Figure has been modified from [25].

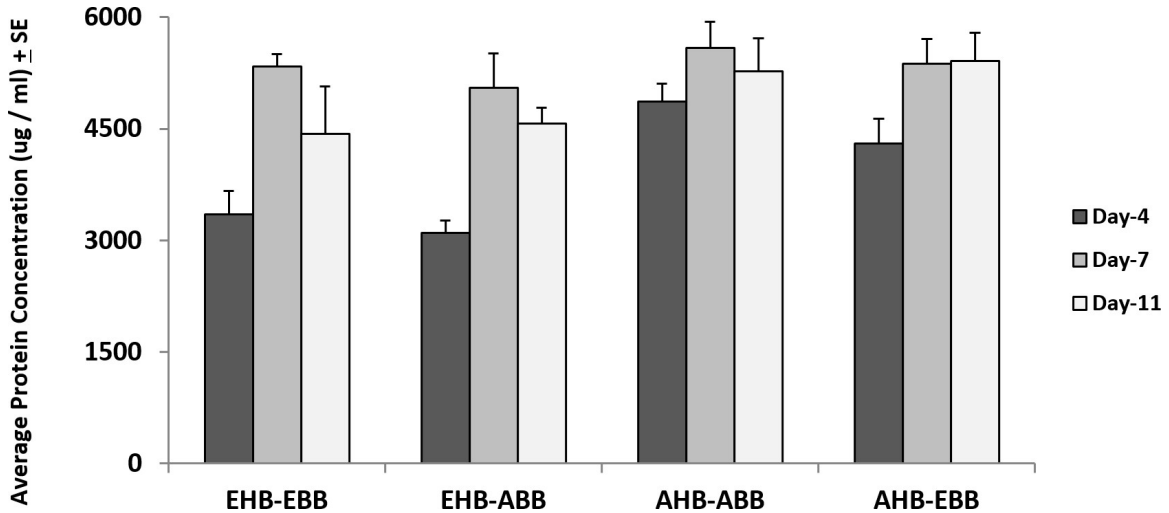


Figure 3: The average concentration of protein in hemolymph from European (EHB) or Africanized (AHB) honey bees fed beebread made by European (EBB) or Africanized (ABB) bees for 4, 7, and 11 days. A repeated measures analysis of variance indicated significant differences among the 4 treatment groups ($F_{3,20}$ = 19.7, p <0.001). Levels of soluble protein in AHB fed ABB were significantly higher than EHB fed ABB (p = 0.008) or EBB (p = 0.018). The age of the bees at the time of sampling significantly affected soluble protein concentrations in the hemolymph. Levels were significantly lower in day-4 bees compared with day-7 (p <0.0001) or 11 (p = 0.001). Day 7 and day11 bees did not differ (p = 0.149). This figure has been modified from[25] .

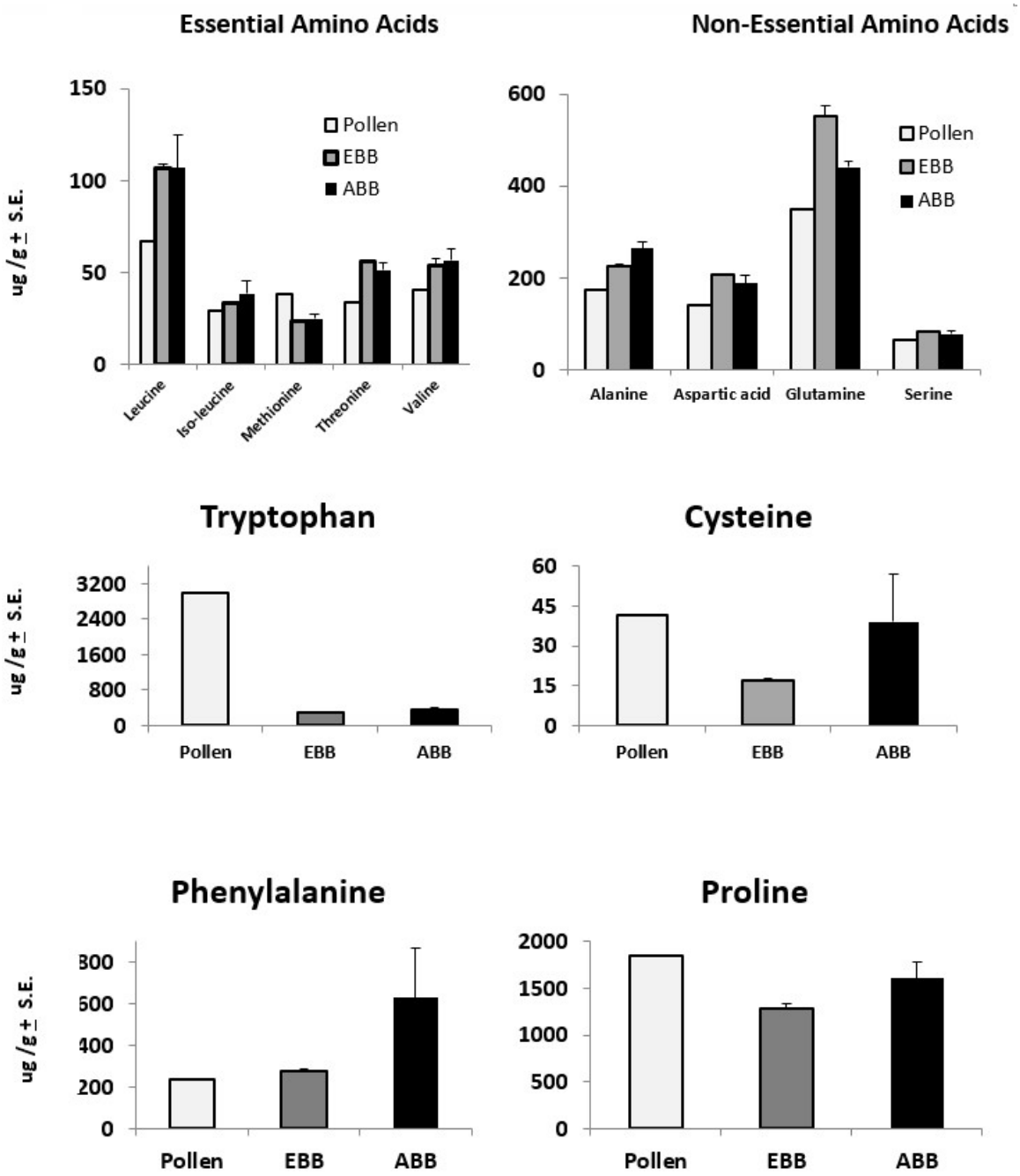


Figure 4: Concentrations of amino acids (µg per gram of pollen or beebread) in pollen or the beebread made from it. EBB is beebread made by European bees and ABB was made by Africanized bees. Tryptophan, cysteine, phenylalanine and proline were plotted separately for purposes of clarity in presenting their amounts. This figure has been modified from [25].

Discussion

Using the methods described above, we found that the beebread made by AHB was consumed in greater amounts by both AHB and EHB. Though EHB and AHB consumed similar amounts of each type of beebread, AHB had higher hemolymph protein titers. Findings based on our methods were similar to previous reports where hemolymph protein levels in AHB were higher than in EHB though both were fed the same diets [26] . By measuring consumption of EBB and ABB which were consumed at different rates by both EHB and AHB, it was determined that hemolymph protein concentration in each subspecies could not be raised by increasing food consumption. There seems to be a plateau for hemolymph protein concentration in workers of nurse bee age and that the set point for the plateau is higher in AHB than EHB.

There are several important conditions for establishing colonies for beebread production that will optimize the rate of pollen storage. First, the colonies need frames with open brood. Without open brood to feed, workers will not collect much pollen. Secondly, the colony must be queenless so no additional brood is produced. Brood rearing requires large amounts of pollen, and only excess pollen is stored. In the small colonies established in EFA, there would be little pollen to be stored as beebread if brood areas were expanding so colonies need to be queenless.

Finally, for beebread to be made, pollen must be collected as corbicular loads and stored in comb cells. If the pollen is collected in pollen traps, it must be ground to a fine powder before presenting it to the bees so they can collect it as corbicular loads.

The methods to measure the consumption of beebread generated qualitative rather than absolute estimates. The only consumption that was counted was when cells were completely emptied of bee bread. A more accurate estimate of total bee bread consumption might be obtained by removing the bee bread from the cells and making it into a patty that could be weighed before and after the study period. However, we wanted to keep the bee bread in the cells so that the bees could feed on it as they would in a colony and perhaps continue processing it during the study period. The difference in weight of the comb sections before and after the study was not used as an estimate of consumption because the weight might have increased because bees put the diluted honey fed to them in some cells.

The workers also might have added some of the diluted honey to the bee bread. For these reasons, cells containing approximately equal amounts of bee bread before and after the feeding period were counted and generated a qualitative measurement. Still, there was a striking difference between the two types of beebread in the number of empty ABB cells counted compared with EBB after 11 days.

Determining when stored pollen becomes beebread can be difficult because bees continually add pollen to cells. The colonies used for producing beebread were established with frames of open brood so bees would collect pollen. However, the colonies were queenless so there were larvae to feed for only about 9 days after the colony was established. For the remainder of the 3 week period when colonies were in the EFA, the pollen that the bees collected was stored and being converted to beebread. Keeping the stored pollen in the comb cells for an additional 11 days when feeding it to bees in cages also may have continued the processing of pollen to beebread. The conversion of pollen to bee bread takes about 7 days [8]. The beebread fed to EHB and AHB had lower pH and reduced protein concentrations compared with the pollen fed. Similar findings of changes in pollen after conversion beebread have been reported by others [7,10,27]. Our results differed from previous reports however, in that there were differences in concentrations of certain amino acids between beebread and pollen. The changes in both protein and amino acid concentrations could be due to the activity of proteolytic enzymes, the source of which might be the bees themselves or the microbial communities established in the beebread [7,8,28,29].

The methods used to measure protein concentration were similar to those previously described to determine the effects of dietary protein on Africanized and European bees [26]. As an extension of the methods, we were able to estimate soluble protein in the pollen and beebread. Those methods generated similar findings to previous reports [7,10,27]. Our findings provide additional evidence that AHB more efficiently assimilate dietary protein than EHB, and that this could be a key factor in the ecological dominance of AHB in most regions where it has immigrated and become established [30-32].

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors thank Mona Chambers, Geoffrey Hidalgo and Maurissa Two Two for their technical assistance. We also thank Dr. Earl White for amino acid analysis, Dr. Roger Simonds for analysis of pollen for pesticides, and Drs. Stanley S. Schneider, Vanessa Corby-Harris and Kirk Anderson for their reviews and discussion of earlier versions of the manuscript and many helpful suggestions.

References

1. Brodschneider, R., Crailsheim, K. Nutrition and health in honey bees. *Apidologie.* **41**, (3), 278-294 (2010).
2. Spivak, M. The relative success of Africanized and European honey-bees over a range of life-zones in Costa Rica. *J. Appl. Ecol.* **29**, (1), 150-162 (1992).
3. Schneider, S. S., McNally, L. C. Spatial foraging patterns and colony energy status in the African honey bee Apis mellifera scutellata. *J. Insect Behav.* **6**, (2), 195-210 (1993).
4. Becerra-Guzman, F., Guzman-Novoa, E., Correa-Benitez, A., Zozaya-Rubio, A. Length of life, age at first foraging and foraging life of Africanized and European honey bee (Apis mellifera) workers, during conditions of resource abundance. *J. Api. Res.* **44**, (4), 151-156 (2005).
5. Saltykova, E. S., Lvov, A. V., Ben'kovskaya, G. V., Poskryakov, A. V., Nikolenko, A. G. Interracial differences in expression of genes of antibacterial peptides, Abaecin, Hymenoptaecin, and Defensin, in bees Apis mellifera mellifera and Apis mellifera caucasica. *J. Evol. Biochem. Phys.* **41**, (5), 506-510 (2005).
6. Decanini, L. I., Collins, A. M., Evans, J. D. Variation and heritability in immune gene expression by diseased honeybees. *J. Hered.* **98**, (3), 195-201 (2007).
7. Gilliam, M. Microbiology of pollen and beebread: the genus Bacillus. *Apidologie.* **10**, (3), 269-274 (1979).
8. Gilliam, M. Microbiology of pollen and beebread: the yeasts. *Apidologie.* **10**, (3), 43-53 (1979).
9. Gilliam, M. Identification and roles of non-pathogenic microflora associated with honey bees. *FEMS Microbiology Letters.* **155**, (1), 1-10 (1997).
10. Loper, G. M., Standifer, L. N., Thompson, M. J., Gilliam, M. Biochemistry and microbiology of bee-collected almond (Prunus dulcis) pollen and beebread. I. Fatty acids, sterols, vitamins, and minerals. *Apidologie.* **11**, (1), 63-73 (1980).
11. Khachatryan, Z. A., *et al.* Predominant role of host genetics in controlling the composition of gut microbiota. *PLoS ONE.* **3**, (8), e3064 (2008).
12. Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., Gordon, J. I. An obesity associated gut microbiome with increased capacity for energy harvest. *Nature.* **444**, (7122), 1027-1031 (2006).
13. Ley, R. E., *et al.* Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci.* **102**, (31), 11070-11075 (2005).
14. Ley, R. E., Peterson, D. A., Gordon, J. I. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell.* **124**, (4), 837-848 (2006).

15. Standifer, L. N., McCaughey, W. F., Dixon, S. E., Gilliam, M., Loper, G. M. Biochemistry and microbiology of pollen collected by honey bees (Apis mellifera L.) from almond, Prunisdulcis. II. Protein, amino acids and enzymes. *Apidologie.* **11**, (2), 163-171 (1980).
16. Human, H., Nicolson, S. W. Nutritional content of fresh, bee-collected and stored pollen of Aloe greatheadii var davyana (Asphodelaceae). *Phytochem.* **67**, (14), 1486-1492 (2006).
17. Schneider, S. S., DeGrandi-Hoffman, G., Smith, D. The African honeybee: Factors contributing to a successful biological invasion. *Ann. Rev. Entomol.* **49**, 351-376 (2004).
18. Winston, M. The biology and management of Africanized honey bees. *Annu. Rev. Entomol.* **37**, 173-193 (1992).
19. Woyke, J. Brood-rearing efficiency and absconding in Indian honeybees. *J. Apic. Res.* **15**, (3/4), 133-143 (1976).
20. Fletcher, D. J. C. Brood rearing and absconding of tropical honey bees. *African Bees.* 96-102 Apimondia Pretoria, South Africa (1977).
21. Winston, M. L., Otis, G. W., Taylor, O. R. Absconding behavior of the Africanized honey bee in. *South America. J. Api. Res.* **18**, (2), 85-94 (1979).
22. Schneider, S. S., McNally, L. C. Factors influencing seasonal absconding in colonies of the African honey bee, Apis mellifera scutellata. *Insectes Soc.* **39**, (4), 402-423 (1992).
23. Hepburn, H. R., Reece, S. L., Neumann, P., Moritz, R. F. A., Radloff, S. E. Absconding in honeybees (Apis mellifera) in relation to queen status and mode of worker reproduction. *Insectes Soc.* **46**, (4), 323-326 (1999).
24. Harrison, J. F., Fewell, J. H., Anderson, K. E., Loper, G. M. Environmental physiology of the invasion of the Americas by Africanized honeybees. *Integr. Comp. Biol.* **46**, (6), 1110-1122 (2006).
25. DeGrandi-Hoffman, G., Eckholm, B. J., Huang, M. H. A comparison of bee bread made by Africaized and European honey bees (Apis mellifera) and its effects on hemolymph protein titers. *Apidologie.* **44**, (1), 52-63 (2013).
26. Cappelari, F. A., Turcatto, A. P., Morais, M. M., DeJong, D. Africanized honey bees more efficiently convert protein diets into hemolymph protein than do Carniolan bee (Apis melliferacarnica). *Genet. Mol. Res.* **8**, (4), 1245-1249 (2009).
27. Human, H., Nicolson, S. W. Nutritional content of fresh, bee-collected and stored pollen of Aloe greatheadii var davyana (Asphodelaceae). *Phytochem.* **67**, 1486-1492 (2006).
28. Bonvehi, J. S., Jorda, R. E. Nutrient composition and microbiological quality of honeybee-collected pollen in Spain. *J. Agric. Food Chem.* **45**, (3), 725-732 (1997).
29. Anderson, K. E., *et al.* Microbial ecology of the hive and pollination landscape: Bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (Apismellifera). *PLoS ONE.* **8**, (12), e83125 (2013).
30. Southwick, E. E., Roubik, D. W., Williams, J. M. Comparative energy balance in groups of Africanized and European honey bees: ecological implications. *Comp. Biochem. Physiol. A.* **97**, (1), 1-7 (1990).
31. Spivak, M. The relative success of Africanized and European honey-bees over a range of life-zones in Costa Rica. *J. Appl. Ecol.* **29**, (1), 150-162 (1992).
32. Francoy, T. M., *et al.* Morphometric and genetic changes in a population of Apis mellifera after 34 years of Africanization. *Genet. Mol. Res.* **8**, (2), 709-717 (2009).