Enhancing the Health and Lifespan of Honey Bees (*Apis mellifera* L.) with an Innovative Soybean-Based Diet Supplemented with Porcine Blood[1]

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J. Entomol. Sci. 60(3): 411–423 (July 2025)
DOI: 10.18474/JES24-49

Abstract This study investigated the effect of soy-based diets supplemented with porcine blood (PB) on the diet's external morphology, nutritional efficacy, and health impacts on honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). Scanning electron microscopy revealed that the control diet (maize bee bread) displayed ruptured granules with small particles, whereas the soy-based diets showed varying degrees of protein gelatinization and aggregation depending on the presence of PB. Nutritional analysis indicated that maize bee bread was used as a positive control contained 12.2% protein and provided 312.89 kcal/100 g, whereas the formulated soy-based diets with skim milk or PB showed 13–15% protein, meeting the required needs for optimal honey bee health and development. Survival and longevity assessments showed no significant difference in lifespan between honey bees fed with artificial diets and the control, but all treatment groups outlived the negative control, which fed only on syrup. Moreover, the diameter of the hypopharyngeal gland acini, a key indicator of the nutritional state and health in honey bees, was significantly larger in honey bees fed protein-enriched diets compared with those receiving only syrup. These findings underscore the potential of soy-based artificial protein diets, especially when enhanced with PB, to support honey bee health and longevity, comparable with natural pollen sources.

Key Words honey bee nutrition, artificial pollen diet, porcine blood, lifespan, hypopharyngeal gland

Honey bees (*Apis mellifera* L.) (Hymenoptera: Apidae) require both pollen and nectar to sustain their foraging flights, generate heat for hive thermoregulation, and support brood rearing. Nectar serves as a vital carbohydrate source, whereas

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pollen provides essential protein, lipids, vitamins, and minerals necessary for nurturing larvae. The potency of pollen directly affects the quantity of eggs laid by the queen and the proportion that successfully develop into adults (Danmek et al. 2021, Frizzera et al. 2020). In instances of pollen scarcity or absence, or when poor-quality pollen is prevalent, beekeepers frequently supplement colony diets with either pollen substitutes (devoid of pollen) or supplements (containing pollen) (Mortensen et al. 2019). Commercially manufactured bee diets offer a valuable means to augment existing pollen stores and offer a viable strategy for supporting brood rearing in honey bee colonies. Artificial pollen diets, comprising 10–20% or greater protein sources, exhibit exceptional palatability to honey bees and fulfill the nutritional demands crucial for their development and reproductive processes (Li et al. 2012, Zheng et al. 2014). These proteins play a crucial role in honey bee nutrition, particularly during the early stages of adulthood when worker bees consume significant amounts of pollen protein to support the growth of their mandibular glands and provide this protein-rich material to various members of the hive (Crailsheim 1991). For optimal hypopharyngeal gland (HPG) growth, newly emerged bees require a protein-rich diet. There is a direct correlation between pollen consumption and gland formation in nurse bees. This underscores the importance of assessing the development of the HPG system as a crucial measure for evaluating the suitability of natural pollen or protein supplements for young bees (Mohamed et al. 2023, Omar et al. 2017).

Both an artificial pollen diet and natural pollen are equally embraced by the honey bees, indicating that the substitute is as appealing as the natural counterpart and can be conveniently administered as patties to colonies in conventional hives (Dastouri and Maheri-Sis 2007, Hoover et al. 2006, Pernal and Currie 2000). Enhancing beekeeping efficiency through proteinaceous feed supplementation hinges, in part, on the advancement of a potent pollen substitute to nourish colonies during periods of pollen scarcity, particularly when natural pollen is insufficient (Jang et al. 2022, Saffari et al. 2010). Providing appropriate protein feed sources to bolster colony strength, thus, would aid in optimizing both honey production and crop pollination, mitigating the effects of pesticide exposure and improving resistance to parasites and diseases (Annoscia et al. 2017).

Pork represents a major fraction of the meat consumed worldwide, but only 30% of the blood generated in slaughterhouses is reused as raw material for food and feed. Porcine blood (PB) is a by-product from the meat industry produced in large volumes. In fact, blood represents up to 4.0% of the animal's weight and, for each slaughtered animal, around 3.0 L of blood is produced (Marques et al. 2024). PB is composed of 79.14% water, 19.4% organic matter, and 1.46% inorganic matter. It contains 18.22% protein, including albumin (2.08%), globulin (1.99%), fibrinogen (0.12%), and hemoglobin (14.02%), thus enhancing the palatability and acceptance of the feed (Jin et al. 2020). PB is primarily obtained through the chemical or enzymatic hydrolysis of proteins found in agroindustrial by-products from animals, rendering its application appealing from both environmental and economic perspectives (Jin et al. 2020).

In recent years, several studies have highlighted the advantageous impacts of PB on growth, yield, and various physiological and biochemical traits in livestock (Middelkoop et al. 2023) and aquaculture (Gisbert et al. 2012). Additionally, in insects, it was noted that utilizing blood meal from chickens as a protein source

proved effective as a substitute for conventional use in artificial diets for silkworms (*Bombyx mori* L.) (Matsura 1994) and crickets (*Gryllus bimaculatus* De Geer) (Orinda et al. 2017). Although the formulation and production of PB diets have seen advancements in recent years, with several commercial options available, the inclusion of PB in artificial diets has only been partially realized in a very limited number of insect species, particularly application in honey bee diets. Therefore, one strategy for improving the formulation of an artificial pollen diet for honey bees involves incorporating specific nutrients, such as PB, to boost the diet's nutritional profile. Consequently, it is imperative to characterize the effects of each new potential raw material source of PB that could be utilized in an artificial pollen diet.

The objective of this research was to evaluate the effectiveness of a novel soy-based artificial pollen diet enriched with PB in promoting the health and longevity of honey bee colonies. We aimed to compare the health outcomes and lifespan of honey bees fed this new diet against those fed a traditional diet or pollen. Specifically, we assessed parameters such as HPG acini development and overall bee vitality to gauge the potential benefits of incorporating PB into honey bee nutrition.

Materials and Methods

PB preparation. The PB chosen for this study was obtained from commercially seasoned PB, which can be purchased in supermarkets throughout Thailand. The nutritional values of the sample were 10.0% protein, 2% sugar, and 1.25% salt. The sample was used after ensuring microbiological safety by analyzing the total viable cell count, total coliforms, *Escherichia coli*, and *Salmonella* species on a plate count according to the Association of Official Analytical Chemists (AOAC) (AOAC 1984). After this, PB was preserved and stored at −20°C until further use.

Experimental dietary interventions for honey bees. We examined the effects of 12.0% protein derived from four diets composed of soybean, PB, and bee-collected maize pollen, commonly used as feed sources for honey bees in Thailand. Table 1 presents the dietary formulations and approximate compositions of four isocaloric test diets (referred to as diets 1, 2, 3, and control), along with maize bee bread (maize pollen:syrup at a ratio of 2:1) serving as the control diet. In brief, all ingredients were blended to achieve a uniform slurry consistency. The PB sample obtained in the preceding steps underwent hydrolysis using commercial acid protease (iKnowZymeTM®) purchased from Rechbiotechnology Co., Ltd. (Pathum Thani, Thailand). Subsequently, the diets were incubated at 60°C for 3 h and then subjected to protease deactivation by boiling in water for 15 min. Finally, all diets were placed in containers and heated until approximately 80–85% of the original volume remained, ensuring that the diets reached a soft consistency suitable for consumption by the honey bees.

Characterization of diets. The microstructures of all diets were examined using scanning electron microscopy (SEM). Before analysis, the samples were sputter-coated with gold and observed under a Prisma E SEM (Thermo Fisher Scientific, Waltham, MA). Biochemical analysis of diets was conducted in triplicate. Diets were ground through a 1.0-mm screen and assessed for dry matter, crude protein, crude fat, crude fiber, and ash content using procedures outlined by the AOAC (AOAC 2016).

Table 1. Ingredients and chemical compositions (g/110 g) of the experimental diets.

Ingredients	Positive Control	Diet 1	Diet 2	Diet 3
Maize pollen	68.0	5.0	5.0	5.0
Porcine blood*	—	—	38.0	38.5
Skim milk	—	5.0	—	—
Soybean powder	—	25.0	20.0	20.0
Protease*	—	1.0	1.0	1.0
Distilled water*	32.0	28.0	—	—
Sucrose	—	30.0	30.0	30.0
Palm oil*	—	5.0	5.0	5.0
Vitamin premix	—	0.5	0.5	0.5
Mineral premix	—	0.5	0.5	—

* Weight measurements were taken according to raw material conditions and 50% sucrose solution was used as a negative control.

Honey bee health and lifespan. Honey bee colonies were obtained from the bee farm at the apiary located at the Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Thailand (N 19°4′21.432″, E 99°53′3.6234″) in February 2024 and were maintained in accordance with established beekeeping protocols. For the purposes of this investigation, sealed brood frames were removed from the honey bee colonies and transferred to an insect growth chamber set at a temperature of 33°C ± 1.0°C with a relative humidity of 60% ± 1.0% and maintained in darkness until pupae metamorphosed into emerged adult honey bees, following the methods outlined by Hsu et al. (2021).

Honey bee lifespan. The experiment involved four groups, each composed of 30 newly emerged adult honey bees. These honey bees were housed in transparent polypropylene plastic cages with a volume of 600 mL. Air circulation within the experimental cages was facilitated through the lid, which was equipped with a nylon grid with openings of 5.0 mm or smaller. All cages were kept within the growth chamber maintained in darkness, and a 50% (w/w) sucrose solution totaling 10 mL was provided in the syrup feeder. The diets (as detailed in Table 1) were provided to the colonies at 2.0 g of each diet in the form of prepared feed patties. These patties, wrapped in standard waxed paper, were positioned in the cages. Both the sucrose solution and patties were monitored and replaced every 3 d. Honey bee mortality was documented, with the number of dead bees recorded daily until 21 d. Each day, dead bees were removed from each cage and counted. The longevity and survivorship of worker bees from the initiation of the experiment were assessed for each treatment group (Jang et al. 2022).

HPG acini. The measurement of HPG acini was modified following the protocol described in Corby-Harris and Snyder (2018). Three honey bee workers from each treatment were selected on day 6 to assess HPG acini development. The

HPG acini were removed and placed in a Petri dish with wax depressions, each containing a droplet of ice-cold normal saline solution (0.85%, isotonic to the hemolymph). Micrographs of the HPG acini were captured utilizing a microscope outfitted with a stereo microscope (Leica Microsystems Fluorescence Stereo Microscopes Leica M205 FCA and Leica M205 FA, Wetzlar, Germany) and a three-dimensional digital microscope camera (Hirox HRX-01 and RX-100, Oradell, NJ). Regarding the HPG acini, the diameters of 20 randomly selected acini with clearly defined borders were assessed in pixels and subsequently converted to millimeters (three replications). The means of the measurements obtained from the 20 individual acini per honey bee head were used for subsequent statistical analysis.

Statistical analysis. The statistical analysis involved a one-way analysis of variance, followed by Tukey's honestly significant difference, with a significance level set at $P < 0.05$, conducted using SPSS version 26.0 (IBM Co., Armonk, NY). Kaplan–Meier survival analysis was used to evaluate the impact of PB supplements on the lifespan of honey bees. The statistical significance of differences in time distributions between groups was assessed.

Results and Discussion

SEM was used to investigate the morphologic structures of soy-based artificial pollen diets with the addition of PB, which were compared with a control diet and are presented in Fig 1. Before SEM analysis, maize bee bread (control diet) was dried and ground, resulting in ruptured pollen granules with smaller particles, whereas the maize pollen consisted of regularly oval and round particles with a smooth surface. The soy-based artificial pollen diet without PB exhibited a gelatinized protein structure, forming a compact matrix with irregular and rough structures. This phenomenon was supported by Wang et al. (2023), who observed protein aggregation and protein folding in soy-based substrates caused by high temperatures. However, soy-based diets with PB (Diets 2 and 3) did not exhibit a fully gelatinized protein structure like the soy-based artificial pollen diet without PB (Diet 1). Instead, small fragmented, lumpy, and wrinkled structures were found to be embedded on the surface of both Diets 2 and 3. This suggests a morphologic characteristic of diets containing PB influenced by PB structure, high temperature, and protease activity (Wang et al. 2023).

The main natural protein source for honey bees is pollen, which they primarily use in the form of bee bread to feed developing larvae and young bees. Pollen provides essential building blocks for muscles, glands, and other tissues. The protein contained in pollen is crucial for the development of brood and is also utilized in the production of royal jelly, which is secreted from the HPG of nurse bees (Crailsheim et al. 1992). Honey bees commonly utilize maize pollen as a natural food resource. The composition of maize pollen includes 17.71% protein, 14.01% moisture, 4.42% fat, 2.85% crude fiber, 2.12% ash, and 61.74% carbohydrates, providing an energy content of 357.58 kcal/100 g. Similarly, research conducted by Hsu et al. (2021) determined the nutritional values of maize pollen from Taiwan to be 17.2% protein, 21.9% moisture, 2.8% fat, 2.0% ash, and 78.1% carbohydrates, providing 443.4 kcal/100 g of energy. This study suggests that honey bee colonies require a dietary bee

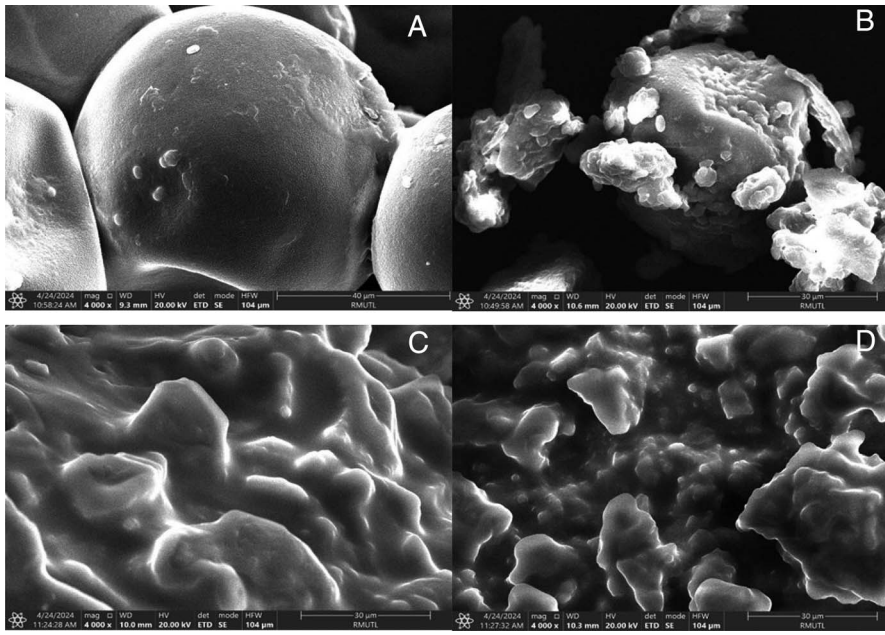

Fig 1. Scanning electron microscopy (4,000×) of maize pollen (A), maize bee bread (B), soy-based diet without porcine blood (PB) (C), and soy-based diet with PB (D).

bread with a protein content of 12% (after mixing with syrup at a ratio of 2:1) to achieve maximum population growth and ensure optimal worker quality.

The artificial diets and dietary formulations of this experiment can be used to provide honey bees with a completely nutritious, easily digestible, and complex variety of nutrition. At this stage of the study, we calculated the proportions of raw materials to be used in the artificial diet formula to achieve nutritional values (Table 1), particularly protein, equal to 12%. However, the resulting diets had a rather liquid consistency. Consequently, all diets were placed in containers and heated until approximately 80–85% of the original volume remained. This process ensured that the diets reached a soft consistency suitable for consumption by the honey bees and exhibited a protein content ranging from 13% to 15% (Table 2). Herbert et al. (1977) reported that honey bees require a minimum level of 23–30% crude protein (dry mass) in pollen for brood rearing but the protein levels of diets in our study were 12–15%. This disparity may be attributed to the fact that honey bee protein requirements under laboratory conditions are lower than those under natural conditions. This finding is consistent with the conclusions of Zheng et al. (2014), who recommended a dietary crude protein content of 29.5–34.0% mixed with sugar syrup at a 2:3 ratio by weight (resulting in a calculated protein content of the diet averaging 11.8–13.6%) to maximize the brood production of honey bee colonies in early spring. Manning et al. (2007) supported the results that caged honey bees fed solely soy flour had a shorter lifespan compared with those fed soy flour mixed

Table 2. Proximate analysis of the experimental diets.*

Parameters (g/100 g)	Treatments				*P* Value	*F* Value
	Positive Control	Diet 1	Diet 2	Diet 3		
Moisture	28.07 ± 1.34b	31.68 ± 1.40a	33.70 ± 0.86a	32.77 ± 1.16a	0.002	12.506
Protein	12.20 ± 0.59c	13.91 ± 0.78b	15.49 ± 0.38a	15.66 ± 0.57a	0.000	22.032
Carbohydrate	55.40 ± 1.51a	48.67 ± 1.34b	45.37 ± 1.54bc	43.58 ± 1.88c	0.000	32.567
Fat	2.76 ± 0.11c	3.62 ± 0.17b	5.43 ± 0.42a	5.17 ± 0.24a	0.000	71.212
Fiber	1.93 ± 0.11c	2.11 ± 0.10bc	2.68 ± 0.23a	2.62 ± 0.34ab	0.006	8.901
Ash	1.78 ± 0.10b	2.35 ± 0.28ab	2.56 ± 0.12a	2.52 ± 0.37ab	0.026	5.358
Energy (kcal/100 g)	312.89 ± 10.90a	272.34 ± 12.48b	282.51 ± 6.21b	279.87 ± 4.98b	0.003	11.316

* Data were analyzed by analysis of variance. Mean values ± standard deviations. Means followed by the same lowercase letter in the same row are not significantly different at $P < 0.05$ as determined by Tukey's honestly significant difference.

with other ingredients. On the basis of the findings, it can be concluded that soy-based artificial pollen diets supplemented with PB can prolong the survival of honey bees in cages and increase their survival rates to levels comparable with those observed in honey bees fed bee bread.

The developmental period and growth rates of insects were influenced by the type of feed they consumed (Elora and Sarkar 2018, Orinda et al. 2017). The impact of feeding artificial pollen diets on honey bee longevity is primarily assessed by confining newly emerged workers, providing them with different protein diets, and comparing honey bee longevity among the various treatment groups. Studies have shown that workers have a longer lifespan when provided with diets compared with those fed syrup alone (Almeida-Dias et al. 2018). Our study demonstrates that providing honey bees in cages with a supplemental artificial pollen diet increases their median lifespan. The negative control group, which received no diet, had a median lifespan of 8.5 d, whereas honey bees provided with diets had a median lifespan exceeding 21 d, with these treatment groups retaining 80% of their population (Fig. 2). According to the survival analysis, there was no significant difference ($P \geq 0.05$) in survival between honey bees fed maize bee bread (control) and those fed soy-based artificial pollen diets (with and without PB) ($P \geq 0.05$). Additionally, honey bees fed diets with a higher protein ratio (15%) exhibited no significant difference ($P \geq 0.05$) in mean lifespan compared with those fed maize bee bread (12% protein). After a 21-d feeding period, the overall survival rate of bees across the diet supplements ($n = 30$) averaged >80.0%. Honey bees fed Diet 2 exhibited the highest survival rate (92.22% ± 1.92%), followed by those fed Diet 3 (88.89% ± 1.92%), the control group provided with maize pollen (87.78% ± 1.92%), and Diet 1 (83.33% ± 3.33%). Conversely, honey bees fed only sucrose syrup (the negative control) experienced a mortality rate of 70.00% ± 13.33% by day 10 of the experiment. In addition to protein, other nutrients also play a role in promoting honey bee health. Dietary fiber can alter the microbiota of animals and influence the physiology of the host animal. This experiment showed that these artificial diets can improve honey bee health (Ricigliano et al. 2022).

The diameter of the HPG acini serves as a well-established measure for assessing the physiologic state of honey bee health, as it changes with age, nutritional factors, and environmental conditions (Jang et al. 2022, Ueno et al. 2015). The comparison between bees receiving no diet and those receiving diet supplements throughout the experiment highlighted significant differences in PB supplementation. The studies that included a negative control showed that honey bees that consumed an artificial pollen diet had better HPG acini development than honey bees that did not. On day 5, honey bees exclusively fed syrup without protein supplementation exhibited significantly smaller HPG acini ($F_{4,95} = 25.561$; $P = 0.000$), measuring 0.056 ± 0.010 mm, compared with those fed protein patties, as illustrated in Fig 3. When compared with the group receiving protein patty treatments ($F_{3,76} = 2.157$; $P = 0.100$), the largest HPG acini were observed in the groups fed with maize pollen (0.098 ± 0.011 mm), Diet 2 (0.096 ± 0.014 mm), Diet 3 (0.093 ± 0.016 mm), and Diet 1 without PB (0.086 ± 0.022 mm). However, there were no significant differences ($t = 0.733$; $P = 0.472$) between the groups fed Diet 2 with mineral premix and Diet 3 without mineral premix using a paired *t* test.

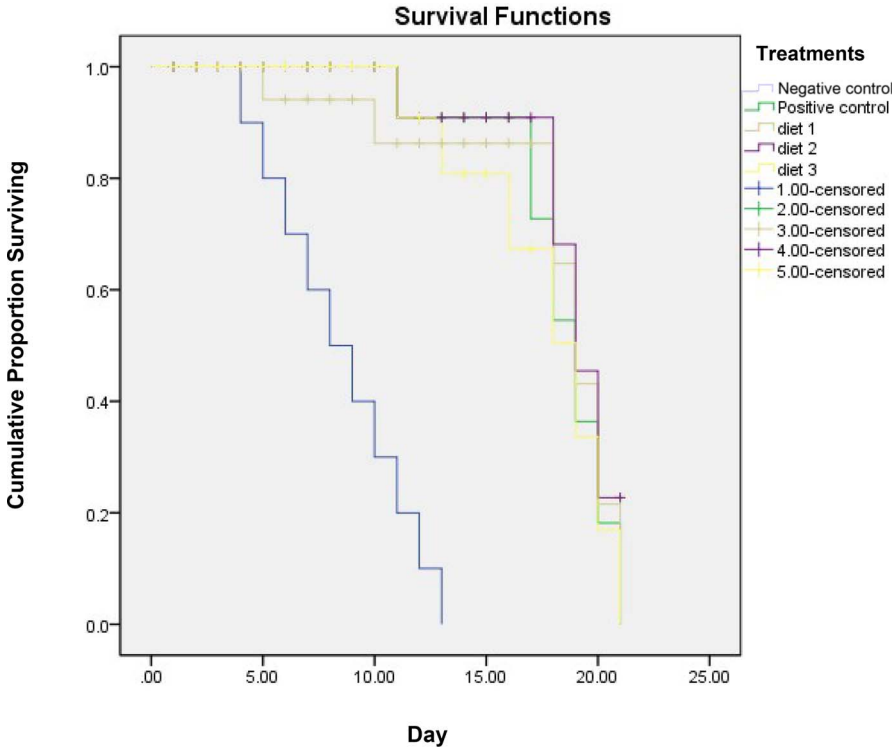

Fig 2. Survival of worker honey bees fed on 50% sucrose solution (negative control), maize pollen (positive control), soy-based diet without porcine blood (PB) (Diet 1), soy-based diet with PB (Diet 2), and soy-based diet with PB but no mineral premix (Diet 3) from day 1 of emergence under laboratory conditions of 25°C and 75% relative humidity.

Our research is supported by previous studies that demonstrated changes in the size of honey bee HPG acini when provided with high-quality artificial pollen diets compared with control groups (Jang et al. 2022, Omar et al. 2017). Comparable outcomes were observed in honey bees fed pollen patties versus those fed a commercial diet devoid of pollen. It seems that an artificial pollen diet stimulated the development of HPG acini in a manner similar to natural bee bread (DeGrandi-Hoffman et al. 2010). Additional data supporting the results of this experiment are that PB normally contains essential amino acids (EAAs) necessary for honey bees, including higher levels of leucine, valine, and lysine (Mahan and Shields 1998). This finding is consistent with the work of de Groot (1953) and Ghosh et al. (2020), who specify the essential amino acids required by honey bees as leucine, lysine, valine, threonine, arginine, phenylalanine, isoleucine, methionine, and histidine. Moreover, EAAs significantly affect honey bee health by enhancing the size of HPG acini, as indicated in the study by Hendriksma et al. (2019). Our results suggest that supplementing a soy-based artificial pollen diet with PB to achieve an approximate protein content level of 15% promotes the health of honey bees similarly to maize bee bread.

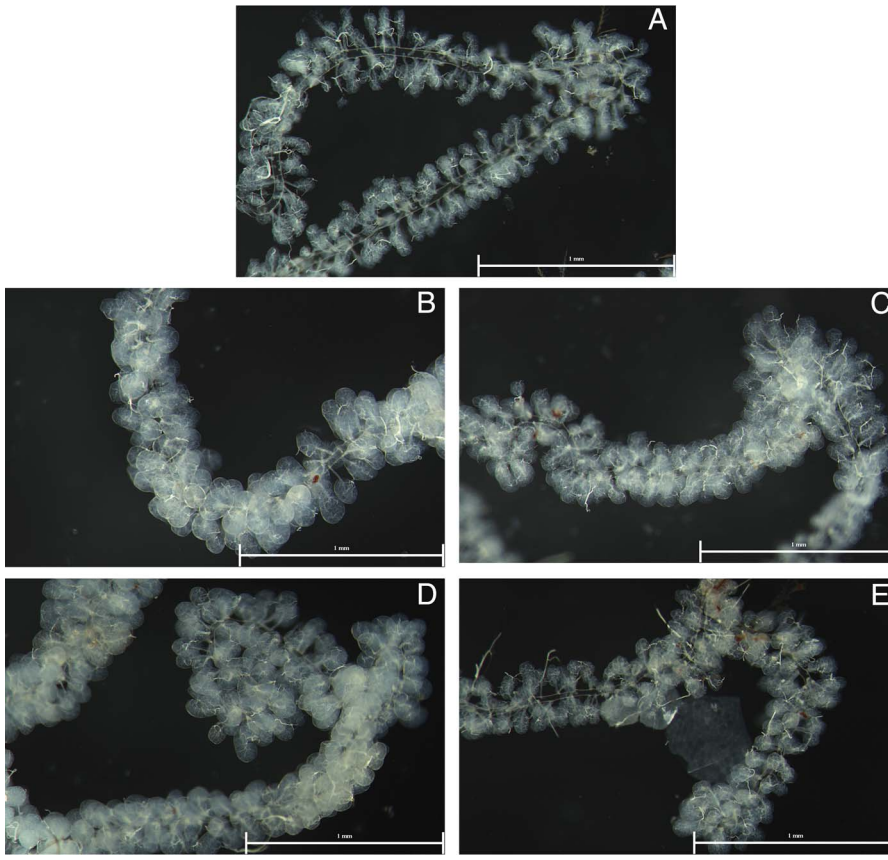

Fig 3. Development of hypopharyngeal gland acini in honey bees fed different diets: sugar syrup only (A), maize bee bread (B), soy-based diet without porcine blood (PB) (C), soy-based diet with PB (D), and soy-based diet with PB but no mineral premix (E).

In conclusion, honey bees rely on both pollen and nectar to sustain their foraging flights, generate heat for nest thermoregulation, and support brood rearing. Pollen serves as a vital protein source, providing essential nutrients necessary for nurturing larvae and maintaining overall colony health. Commercially manufactured honey bee diets offer a valuable means to augment existing pollen stores and support brood rearing in honey bee colonies. Artificial pollen diets, comprising 10–20% or greater protein sources, have been shown to exhibit exceptional palatability to honey bees and fulfill the nutritional demands crucial for their development and reproductive processes. Porcine blood, a by-product from the pig industry produced in large volumes, presents a promising protein source for honey bee diets.

Our results indicate that supplementing a soy-based artificial pollen diet with PB to achieve an approximate protein content level of 15% promotes honey bee health similarly to maize bee bread. Honey bees fed with the soy-based artificial

pollen diet supplemented with PB exhibited improved HPG acini development and lifespan compared with honey bees fed with traditional diets or pollen. Furthermore, our findings suggest that soy-based artificial pollen diets supplemented with PB can prolong the survival of honey bees, increasing their survival rates to levels comparable with those observed in honey bees fed bee bread. In conclusion, incorporating PB into honey bee artificial pollen diets represents a promising strategy for enhancing colony health and productivity, which could ultimately optimize both honey production and crop pollination, mitigate the effects of pesticide damage, and combat resistance to parasites and diseases.

Acknowledgments

This research has received financial support from the 2024 NRCT-NRF Scientist Exchange Program and National Research Council of Thailand NRCT 2024, grant no. N11E670082 and was partially supported by Chiang Mai University. We also acknowledge the support from Andong National University supported by the National Research Foundation of Korea (NRF-2018R1A6A1A03024862). Special thanks to Nattaporn Koktrakool and Worrakamon Ruealom from the School of Agriculture and Natural Resources, University of Phayao, for their assistance. We gratefully acknowledge Michael Burgett for reviewing the English.

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