

# NUTRITIONAL VALUE FOR BEES OF POLLEN SUBSTITUTE ENRICHED WITH SYNTHETIC AMINO ACIDS

## Part II. BIOLOGICAL METHODS

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### S u m m a r y

The objective of this study was to assess the biological value for the honey bee of pollen substitute formulations the chemical makeup of which was supplemented with DL and L isomers of synthetic amino acids and thus upgraded to the standard of natural pollen from pollen loads. The following feed component made up the pollen substitute: potato protein 32%, soybean cake 18%, rapeseed cake 6%, yeast *Candida utilis* 6%, wheat flour 14.8%, maize grits 17.5%, soybean oil 3.3%, lecithin 0.5% polfamix W 1.4%, Vitazol AD3EC 0.2%, glucose with vitamin C 0.1%. Prior to be fed to bees, the pollen substitute and pollen loads were mixed with powder sugar to bring protein level in all feeds to 21%. Biological methods were used as the measure of the nutritional value of the feed to test the condition of bees. The condition of bees was expressed in terms of the size of pharyngeal glands, the size of the fat body and the number of haemocytes in haemolymph. The poorest developed pharyngeal glands and fat body were recorded in bees which were fed a non-supplemented pollen substitute. The highest number of haemocytes in haemolymph was recorded in bees which received pollen substitute supplemented with all lacking L amino acids, the number being slightly lower when the amino acids were fed as DL isomers. There were no significant differences between the two groups. Bringing the amino acid composition of pollen substitutes with essential amino acids up to their level in natural pollen favourably influenced the nutritional value of the protein in terms of fat body size but the effect was weaker in terms of the development of pharyngeal glands. As the nutritional value of the pollen substitute protein increased there was an increase in the number of haemocytes in honeybee haemolymph. L isomers of the amino acids tested were demonstrated to be better utilized than their DL counterparts.

**Keywords:** honeybee nutrition, pollen substitute, nutritional value, amino acids, biological methods

### INTRODUCTION

Pollen is a food of complex chemical makeup, the protein being the ingredient of the greatest importance for bees. Breaks of prolonged duration in the supply of that food to bee colonies may negatively affect the development and the functioning of a bee colony. In such cases pollen collected with bee traps during high pollen flow or pollen substitutes should be fed to colonies (Doull 1980 a,b; Peng et al. 1984;

Chambers 1990; Szymaś 1994).

In the search of formulations of potential use as pollen substitutes individual feed ingredients or formulations made up of different high-protein feeds were tested (Herbert and Shimanuki 1978, Szymaś 1994, Szymaś and Maliszewska 1999).

Laboratory tests that exclude natural food sources provide the basis for the assessment of the nutritional value of pollen substitutes for honeybees (Maurizio

1954, Szymaś and Wójtowski 1974, Szymaś and Przybył 2000).

The objective of the study was to assess, by means of biological methods, the nutritional value of pollen substitutes the composition of which was supplemented with L and DL isomers of synthetic amino acids to bring them up to the standard of protein in pollen loads.

## METHODS

The preparation of pollen substitute consisted in blending feed ingredients which had been subjected to various treatments (Szymaś, Przybył 2002). The yeast *Candida utilis* and wheat flour were mixed and 4% of water was added to the mixture. The mixture was microwaved for 40 sec. in a 1,000 W microwave oven. Soybean flour, rapeseed cake and maize grits were subjected to thermobaric treatment – extrusion. When mixed, all components were ground in an impact grinder to particles 150 µm in diameter. The pollen substitute was made up of the following ingredients: potato protein 32%, soybean cake 18%, rapeseed cake 6%, yeast *Candida utilis* 6%, wheat flour 14,8%, maize grits 17.5%, soybean oil 3.3%, lecithin 0.5% polfamix W 1.4%, Vitazol AD3EC 0.2%, glucose with vitamin C 0.1%. Once ready the substitute was acidified with lactic acid to pH 4.8 i.e. to the pH of pollen loads, Subsequently, it was oiled with soybean oil. Pollen substitute, prior to be fed to bees, was mixed with powder sugar in such a ratio was that the protein level of all feeds was 21%.

The bees for the study were collected by placing frames with emerging brood from a single queen (*Apis mellifera carnica*) in incubators set to the temperature of 33°C and the relative humidity of 80%. Subsequently, small hives made of polystyrene and equipped with feeders and water feeders were colonized by 150 one day-old

bees. The bees were subjected to different feeding treatments for two weeks. Each year there were five replications of each treatment. The hives with bees were kept in incubators at 31°C and 40% relative humidity for two weeks. The experiment was run in 1998 and 1999. In each treatment the bees were fed pollen substitutes to which lacking amino acids were added to make the composition equal to that of pollen. The amino acids were purchased at SIGMA-ALDRICH. Even though the ingredient makeup of the pollen substitutes was the same, there were slight differences in amino acid composition due to some differences in the chemical makeup of the ingredients which came from different companies. For that reason they were treated as separate feeds in each of the two experiment years: as substitute A in 1998 and as substitute B in 1999.

The chemical makeup and amino acid composition of the experiment feeds and of pollen are shown in Table 1.

Biological methods were used as the measure of the nutritional value of the feed to test the condition of bees. The condition of bees was expressed in terms of the size of pharyngeal glands, the size of fat body and the number of haemocytes in haemolymph. The method by Maurizio (1954) was used to measure the size of pharyngeal glands, the size of fat body and the number of haemocytes in haemolymph. The measurements were made on 10% of bees picked at random upon the termination of the experiment. A four-degree score was used to characterize the size of the fat body and of the pharyngeal glands. The score of 4 was assigned to the maximum extent of organ development. The total number of haemocytes was investigated in a Bürker chamber using non-diluted haemolymph collected in vivo from bees. Haemolymph was collected from the dorsal sinus of the bees' circulatory system using an in-house made Pasteur's pipette

Table 1

Chemical composition (%) and amino acid composition (g/100g) of pollen substitutes and of natural pollen from pollen loads

Specification	Pollen substitute A (1998)	Pollen loads (1998)	Pollen substitute B (1999)	Pollen loads (1999)
Dry mass	91.57	91.36	92.88	90.71
Total protein	42.01	27.62	42.08	24.66
Crude fat	5.43	4.18	5.85	4.07
Crude fibre	2.37	2.59	2.51	2.59
Crude ash	3.57	2.81	3.41	3.85
NFE (Nitrogen free extractives)	38.19	55.48	39.03	55.54
Arg	4.80	4.79	5.13	4.18
His	2.77	3.26	2.52	2.97
Lys	6.14	7.00	6.56	7.23
Phe+Tyr	9.41	9.30	8.94	8.82
Met+Cys	3.63	4.07	3.01	3.26
Thr	4.57	4.67	4.59	4.68
Leu	8.35	6.86	8.08	6.57
Ile	4.52	4.04	4.33	3.97
Val	5.39	4.80	5.12	4.51

by puncturing the membrane between the 2<sup>nd</sup> and the 3<sup>rd</sup> abdominal tergite. Haemolymph was transferred to Bürker glass and haemocyte counts were made in five eight-day-old bees i.e. in 25 individuals per group.

The statistical analysis including ANOVA and Tukey's test at  $\alpha=0.05$  was performed to test differences between the weighed means of the score values for hypopharyngeal gland size, fat body size, and haemocyte counts.

## RESULTS

The development of pharyngeal glands and of the fat body in worker bees fed pollen substitute A in 1998 are shown in Table 2.

The poorest developed pharyngeal glands and fat body were recorded in bees which received the non-supplemented substitute (D1). The addition of DL-methionine and DL-lysine significantly raised the protein value of the pollen substitute in terms of fat body size. Upon supplementing the substitute with lacking essential amino acids as L and DL isomers (groups D3, D4, and D5) the scores of the fat body were very advantageous, similar to those in the pollen-fed group as no significant differences were found between the two groups. The highest scores of pharyngeal glands were recorded in bees fed pollen substitute supplemented with all lacking amino acids as L isomers.

The highest scores were obtained in bees fed pollen from pollen loads.

Table 2

Development of pharyngeal glands and of the fat body in bees fed substitute A and pollen loads in 1998 together with Tukey's test results at  $\alpha=0.05$

Group/food	Pharyngeal glands		Fat body	
	Bees with 3 <sup>rd</sup> and 4 <sup>th</sup> degrees of development of the examined organs in %	Mean classification degrees (1-4)	Bees with 3 <sup>rd</sup> and 4 <sup>th</sup> degrees of development of the examined organs in %	Mean classification degrees (1-4)
Kp/pollen loads	95	3.60 a*	83	3.15 a
D1/pollen substitute A	30	2.42 c	32	2.26 c
D2/pollen substitute A +DL-Met+ DL-Lys	52	2.44 c	65	2.66 b
D3/pollen substitute A +L-Met+L-Lys	54	2.59 c	66	2.98 ab
D4/pollen substitute A +DL-Met+ DL Lys+DL-His+ DL-Thr	62	2.80 bc	72	2.82 ab
D5/pollen substitute A +L-Met+L-Lys+ L-His+L-Thr	56	3.12 b	70	2.98 ab

\* abc – the means in columns are not significantly different when followed by at least one same character

Table 3

Development of pharyngeal glands and of the fat body in bees fed substitute A and pollen loads in 1999 together with Tukey's test results at  $\alpha=0.05$

Group/food	Pharyngeal glands		Fat body	
	Bees with 3 <sup>rd</sup> and 4 <sup>th</sup> degrees of development of the examined organs in %	Mean classification degrees (1-4)	Bees with 3 <sup>rd</sup> and 4 <sup>th</sup> degrees of development of the examined organs in %	Mean classification degrees (1-4)
Kp/pollen loads	78	3.08 a*	88	3.06 a
D1/pollen substitute B	56	2.70 b	26	2.24 c
D2/pollen substitute B +DL-Met+ DL-Lys	58	2.80 ab	50	2.64 b
D3/pollen substitute B +L-Met+L-Lys	62	2.76 ab	56	2.66 b
D4/pollen substitute B +DL-Met+ DL-Lys+ DL-His+DL-Thr	46	2.74 b	58	2.72 b
D5/pollen substitute B +L-Met+L-Lys+ L-His+L-Thr	54	2.96 ab	62	2.92 ab

\* abc – the means in columns are not significantly different when followed by at least one same character

In Table 3, the effect of feed B formulated in 1999 and supplemented with DL and L amino acids on the development of pharyngeal glands and of the fat body is shown vs. the effect of pollen. The data show that the best pharyngeal gland scores were obtained in bees fed natural pollen (Kp) and the lowest scores were recorded in groups D1 and D4. The addition of synthetic amino acids to the substitute failed to bring about any significant differences among groups D1 – D5. However, the addition of synthetic amino acids to the substitute in groups D2, D3 and D5 brought the nutritional value of the substitute (groups D2, D3 and D5) closer to the value of pollen (Kp).

The fat body was best developed in bees which were fed natural pollen (Kp) and was worst developed in bees fed the non-supplemented substitute (D1). The addition of all lacking amino acids as L isomers to the substitute brought the value of the substitute close to the value of natural pollen.

From the distribution of the ratings of 3 and 4 for the development of pharyngeal glands and of the fat body it can be

inferred that supplementing the substitutes with essential amino acids beneficially affected the nutritional value of the protein with respect to the fat body and had less effect on the development of pharyngeal glands. The impact of natural pollen with respect to the development of pharyngeal glands was greater in 1988 and it was slightly lower in 1999 with respect to the fat body (Tables 2 and 3).

The effect of the feeding treatments on the haemocyte content of bee haemolymph is shown in Table 4.

The highest counts of haemocytes in haemolymph were recorded for bees which received the substitute supplemented with all lacking amino acids as L isomers. They were slightly lower when DL amino acids were added but no significant differences were found between the two groups. The lowest haemocyte counts were recorded in the haemolymph of bees of the control group (Kp). No significant differences were found when compared with the means of groups D1, D2 and D3.

Table 4

Haemocyte counts of bee haemolymph in the experiment of 1998 together with Tukey's test results at  $\alpha=0.05$

Group/food	Total haemocyte count (per $\mu\text{L}$ )
Kp/pollen loads	11540 c*
D1/pollen substitute A	16542 bc
D2/pollen substitute A +DL-Met+DL-Lys	12164 c
D3/pollen substitute A +L-Met+L-Lys	17170 bc
D4/pollen substitute A +DL-Met+DL-Lys+DL-His+DL-Thr	22139 ab
D5/pollen substitute A +L-Met+L-Lys+L-His+L-Thr	23570 a

\* abc – the means in columns are not significantly different when followed by at least one same character

## DISCUSSION

According to many sources pharyngeal glands, organs secreting enzymes and royal jelly, quickly respond to changes in the nutritional value of feed protein (Rutz et al. 1976; Brauwers 1982; Knecht and Kaatz 1990; Huang 1990). In this study, pharyngeal glands were one of the parameters to describe the nutritional value of protein feeds. For their assessment the method by Maurizio (1954) was used that consisted in the rating of different degrees of gland development based on size and the shape of alveoli. The suitability of that method was repeatedly questioned due to the fact that it was based on the subjective evaluation of the classifier. Crailsheim and Stolberg (1989) and Lass and Crailsheim (1996) used the micrometric eyepiece scale of the microscope whereas Szymaś et al. (2003) used for the purpose a special computer software that substantially facilitated the measurement and allowed the data to be stored as computer files already at the moment of making the measurement and, subsequently, to process them. The above mentioned authors demonstrated by means of statistical analysis that Maurizio's method is reliable and less time-consuming than the methods based on metric scales or computer techniques. Because of that, in this study Maurizio's (1954) method was used to assess the nutritional value of feed protein. The results from this study indicate that the feed formulations beneficially affected the physiological condition of bees expressed in terms of the high degree of development of the pharyngeal glands and of the fat body. The better balanced the amino acid composition was by adding synthetic amino acids the higher the values were. Bees that scored 3 and 4 on the fat body development scale were more numerous than the bees assigned the same scores on the rating of pharyngeal glands. The same conclusion

was arrived at by Szymaś and Maliszewska (1999) when they added methionine and lysine to the substitute. The supplementation of of pollen substitute with L isomers of amino acids increased the value of the feed substantially, and the results were comparable to those from the control group in which bees were fed natural protein food.

Insect haemolymph including the haemolymph of the honeybee performs many important functions, including osmoregulatory functions, protein and free amino acid transport (Atmowidjojo et al. 1999). Haemolymph glucose is utilized by insects as the source of energy for flying (Gmeinbauer and Crailsheim 1993). The composition of haemolymph is not constant. The counts of the known haemolymph cell types and their ratio were found to vary over the successive development stages (Gilliam 1973).

Szymaś and Jędruszek (2003) investigated the effect of different diets on the haemolymph of adult honeybee workers and determined the number and metabolic activity of haemocytes. They found the natural protein food to be an important factor in the functioning of the haemolymph cellular system of bees. The pollen substitute in their study which had an ingredient makeup very similar to the surrogate pollen investigated in this study turned out to be a very valuable food comparable to natural pollen. However, the metabolic activity of haemocytes was lower than that in bees that were fed natural pollen.

The lack of protein in the feed caused major disturbances in the structure and functioning of the haemolymph cellular system. Based on this study it can be said that the number of haemocytes in bee haemolymph increased with the increased nutritional value of substitute protein i.e. after the substitute was supplemented with synthetic amino acids.

## CONCLUSIONS

The supplementation of the amino acid composition of pollen substitutes with essential amino acids to bring it up to the natural pollen standard beneficially affected the nutritional value of the protein with respect to the fat body but had a lesser effect on the development of pharyngeal glands.

The number of haemocytes in honeybee haemolymph increased along with the increase in the nutritional value of pollen substitute protein.

Bees were shown to better utilize the L-isomers than the DL-isomers of amino acids.

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## WARTOŚĆ ODŻYWCZA DLA PSZCZOŁY MIODNEJ SUROGATU PYŁKU KWIATOWEGO WZBOGACONEGO AMINOKWASAMI SYNTETYCZNYMI Część II. METODY BIOLOGICZNE

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### S t r e s z c z e n i e

Celem niniejszej pracy było określenie metodami biologicznymi wartości odżywczej dla pszczoły miodnej namiastki pyłku kwiatowego, której skład uzupełniono aminokwasami syntetycznymi w formie izomeru DL i L do standardu jakim było białko pyłku kwiatowego z obnóży. W skład namiastki weszły następujące komponenty paszowe: białko ziemniaka -32%, śruta sojowa-18%, śruta rzepakowa-6%, drożdże *Candida utilis*-6%, mąka pszenna- 14,8%, grys kukurydziany -17,5%, olej sojowy-3,5%, lecytyna-0,5%, polfamixW-1,4%, Vitazol AD3EC-0,2%, glukoza z witaminą C-0,1%. Namiastkę pyłku kwiatowego oraz pyłek kwiatowy z obnóży przed podaniem pszczołom wymieszano z cukrem pudrem w takich ilościach by poziom białka wynosił we wszystkich pokarmach 21%. Do oceny kondycji pszczoł, jako miernika wartości odżywczej wytworzonej paszy, zastosowano metody biologiczne. Kondycja pszczoł wyrażona została na podstawie wielkości gruczołów gardzielowych, ciała tłuszczowego i liczby hemocytów w hemolimfie. Najślabiej rozwinięte gruczoły gardzielowo i ciało tłuszczowe odnotowano u pszczoł, które spożywały namiastkę nie uzupełnioną. Najwięcej hemocytów w hemolimfie odnotowano u pszczoł, które otrzymywały namiastkę uzupełnioną o wszystkie brakujące aminokwasy w formie L, a nieco mniej gdy uzupełnioną ją aminokwasami w formie DL. Między tymi grupami nie było statystycznie istotnych różnic. Uzupełnienie składu aminokwasowego namiastek pyłku kwiatowego aminokwasami egzogennymi do standardu jakim był ich poziom w pyłku kwiatowym, korzystnie wpłynęło na wartość odżywczą białka w odniesieniu do ciała tłuszczowego, a słabiej na rozwój gruczołów gardzielowych. Wraz ze wzrostem wartości odżywczej białka namiastki pyłku kwiatowego, zwiększyła się liczba hemocytów w hemolimfie pszczoły miodnej. Wykazano, że pszczoły lepiej wykorzystują aminokwasy w formie izomeru L niż DL.

**Słowa kluczowe:** żywienie pszczoły miodnej, namiastka pyłku kwiatowego, wartość odżywcza, białka namiastki, aminokwasy, metody biologiczne.