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## Amino acid and lipid spectra of larvae of honey bee (*Apis cerana* Fabr) feeding on mustard pollen

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**Summary** — Amino acid and lipid compositions of the larvae of honeybees (*Apis cerana*) confined to (i) caged foraging on mustard (*Brassica campestris* L cv Toria) pollen only (CM), (ii) uncaged foraging in open abundantly flowering mustard plants (UCM), and (iii) caged foraging in non mustard plants (CNM), are reported. Both the content and quality of the amino acids and lipids of the larvae were affected by the type of pollen nutrition of the bees. The CM larvae appeared to contain greater amounts of total amino acids and lipids compared to the UCM and CNM larvae. Proline was the amino acid present in the greatest amount. Triglycerides and phospholipids form the major lipid classes of the larvae irrespective of the type of pollen nutrition of the bees, the former being present in the greatest amount. The concentrations of all the biochemical constituents, the brood area and the larval weight varied in the order CM > UCM > CNM. The concentration of protein amino acids in the CM was about twice as high as that in the CNM, but free amino acids varied in the order CNM > CM > UCM. As such, mustard is a superior pollen source of bee nutrition.

***Apis cerana* / feeding / pollen / *Brassica campestris* / amino acid / lipid**

### INTRODUCTION

The Toria cultivar of mustard is an important oil seed crop of India. We have previously reported that the yield and biochemical composition of the seed of this cultivar are influenced by bee pollination (Singh and Singh, 1992). Honeybees depend on the collection and storage of pollen as a primary and rich source of proteins, amino

acids, lipids, minerals and vitamins. Pollen is also an important dietary component for young bees and, of course, developing and growing larvae (Southwick, 1990). Caged worker honeybees feeding on colony-stored pollen in their combs have a lower mortality rate than those feeding on laboratory-stored pollen (Vorst and Jacobs, 1980). The brood-rearing capacity of *Apis mellifera* is known to be improved by the addition of pollen ash

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to a chemically defined diet (Herbert and Shimanuki, 1978; Herbert et al, 1980). The nutritional status and biochemical composition of the royal jelly, as influenced, to a large extent, by the type of pollen nutrition (Stanley and Linskens, 1974), may affect the composition of honeybee larvae.

Thus far, most research appears to have been confined to the effect of pollen nutrition of bees on their brood-rearing capacity, particularly during the later stages of development of young bees, when pollen nutrition effects may be masked by other secondary nutritional factors such as nectar and honey feeding. To our knowledge no attempt has been made to monitor nutrition-mediated growth and metabolic changes during early larval development when secondarily elicited nutritional effects are non-existent. We have reported previously the biochemical composition of pollen of the Toria cultivar of mustard (Singh and Singh, 1991). The objective of this paper was to ascertain the effects of feeding Toria pollen to worker honeybees on their brood production and growth, and the amino acid and lipid composition of their larvae.

## MATERIALS AND METHODS

Mustard plants (*Brassica campestris* L cv Toria) were used as the test source of pollen. Pollen from some naturally growing non-mustard companion plants such as *Ageratum conyzoides*, *Cynodon dactylon* and *Antigonon leptopus* was also used to serve as controls. The details of the experimental set up, sampling and methods of analyses of biochemical constituents were as follows:

### *The experimental set-up*

The total experimental plot area measuring 18 x 5 m was divided into three equal-sized consecutive plots, each measuring 6 x 5 m. The following three treatment setups were employed to

provide three different types of pollen nutrition for the bees and their developing and growing larvae: i) the beehives and flowering mustard plants alone were caged together in fine iron wire netting (6 x 5 x 2 m) and all non-mustard plants were weeded out (CM); ii) beehives were kept uncaged (with identical distances and number of flowers) in a field of flowering mustard plants along with some non-mustard plants, described above, which were growing naturally as a mixed population (UCM); and iii) beehives and non-mustard plants (*A conyzoides*, *C dactylon* and *A leptopus*) only were caged in iron wire netting (6 x 5 x 2 m) (CNM).

A set of five beehives of *Apis cerana*, each with four full frame colonies (40 000 bees per beehive) were placed equidistant from each other in each of the three experimental plots of plants under similar ambient environmental conditions (11 h day and 13 h night cycle; temp 24 h cycle, min 12 °C, max 18 °C; RH 70%, rain 0; wind speed not measured but usually conducive to normal foraging).

### *Sampling and methods of analyses*

Triplicate samples of five larvae from each treatment were randomly collected eight days after egg laying. As the fresh weights of larval samples in the three treatments were different (table I), the final volumes of the larval extracts were adjusted accordingly to a uniform weight:volume relationship prior to chromatographic analysis. Free amino acids and amides from hot ethanol-extracted (80% v/v) fresh larval samples and protein amino acids from 6N-HCl hydrolysates of the ethanol-extracted residue were resolved by two-dimensional paper chromatography (Consden et al, 1944; Partridge, 1948). The one- and two-dimensional runs of the chromatograms were completed in the solvent systems phenol/water/ammonia (80:20:3 v/v) and *n*-butanol/acetic acid/water (4:1:5 v/v) respectively. The different ninhydrin positive substances were detected by spraying the chromatograms with 0.1% ninhydrin in *n*-butanol and were measured quantitatively with a Spectronic 20D spectrophotometer against authentic reference compounds (BDH). Proline was measured separately (Wren and Wiggall, 1965) as follows. Basic amino acids from the ethanol extract were removed using a permutit cation exchanger followed by extraction with benzene which was previously washed with strongly

**Table I.** Effect of feeding mustard pollen to workers of *Apis cerana* on brood area and fresh weight of their larvae.

	CM larvae $\pm$ SD	UCM larvae $\pm$ SD	CNM larvae $\pm$ SD
Brood area per beehive (cm <sup>2</sup> )	1 600 $\pm$ 20.4	1 550 $\pm$ 40.8	940 $\pm$ 32.6
Fresh wt per larva (mg) <sup>a</sup>	70.0 $\pm$ 2.4	68.0 $\pm$ 2.4	62.1 $\pm$ 2.8

<sup>a</sup> Average of 25 larvae.

acidified 40% ninhydrin solution. This benzene/ninhydrin (2.5:5.0 mL) solution was centrifuged (5 000 rpm) to a clear benzene layer. Proline was quantitatively measured from the colour-intensity of the benzene layer as usual against a reference standard of authentic proline (BDH).

Lipids were extracted and purified from fresh larval samples ten times the size used for amino acid analyses (Blight and Dyer, 1959). Thin layer chromatography (TLC) of neutral lipids was performed using the solvent system petroleum ether/diethyl ether/acetic acid (90:10:1 v/v). The separation of phospholipids was performed by TLC (Gentner et al, 1981) involving three step-wise one-dimensional developments using a solvent system of petroleum ether/diethyl ether/acetic acid (90:10:1 v/v), acetone, ethyl acetate/2-propanol/water (50:35:15 v/v) respectively for runs I, II and III. All lipids were detected by exposure of the TLC plates to iodine vapours (Sims and Larose, 1961). Neutral lipids were quantified using 2.5% acid dichromate reagent (Amenta, 1964) and phospholipids eluted from TLC plates (Biezanski, 1967) were quantified by phosphorus estimation (Fiske and Subarow, 1925). The quantities of lipids were obtained from standard curves of reference compounds (Sigma Chemical Company). The standard deviations of data from triplicate analyses were determined (Panse and Sukhatme, 1985).

## RESULTS

A visual examination suggested increased egg laying within three days of feeding mustard pollen to worker honeybees. A week

after the commencement of pollen feeding the brood area and larval weight were higher in the CM sample compared to the UCM and CNM (table I).

Feeding different pollen types to worker bees influenced the amino acid, neutral lipid and phospholipid composition of their larvae (tables II–IV). The amino acids commonly detected in the larvae comprised leucine, isoleucine, valine, tyrosine, glutamic acid, threonine, arginine, aspartic acid, glycine, serine, lysine, histidine, cystine, proline,  $\alpha$ -alanine and  $\beta$ -alanine. However, the CM and UCM larvae lacked glutamic acid, arginine,  $\alpha$ -alanine and leucine and isoleucine. Whereas the concentrations of the free amino acids followed the order CNM > CM > UCM, the protein amino acids varied in the order CM > UCM > CNM. However, the total of free and protein amino acids varied in the order CM > UCM > CNM. Proline was by far the most predominant amino acid, both in the free amino acid pool and also in the protein fraction.

Neutral lipids of larvae comprised sterol, free fatty acids, triglycerides, sterol esters, fatty acid methyl esters and a fair amount of hydrocarbons (table III). Triglycerides were the major lipids of the larvae, being present in the greatest proportion irrespective of the type of pollen nutrition of bees. The total amount of phospho- and neutral lipids in the larva varied in the order CM > UCM > CNM. Of the four phospholipids, viz

**Table II.** Effect of feeding mustard pollen to worker bees of *Apis cerana* on their larval amino acids ( $\mu\text{g}$  per 100 mg fresh weight).

Amino acids	Free amino acids			Protein amino acids		
	CM larvae $\pm$ SD	UCM larvae $\pm$ SD	CNM larvae $\pm$ SD	CM larvae $\pm$ SD	UCM larvae $\pm$ SD	CNM larvae $\pm$ SD
Leucine + isoleucine	—	—	28.5 $\pm$ 2.4	19.5 $\pm$ 0.8	19.2 $\pm$ 3.2	—
Valine	55.5 $\pm$ 4.4	56.6 $\pm$ 4.1	39.9 $\pm$ 3.2	186.5 $\pm$ 3.3	185 $\pm$ 3.2	79.8 $\pm$ 3.2
Tyrosine	44.0 $\pm$ 4.0	39.9 $\pm$ 3.2	—	120.0 $\pm$ 4.0	115.3 $\pm$ 4.0	57.1 $\pm$ 11.9
Glutamic acid	—	—	39.9 $\pm$ 1.6	—	—	—
Threonine	49.9 $\pm$ 3.2	39.9 $\pm$ 3.2	85.6 $\pm$ 1.2	155.7 $\pm$ 4.0	153.8 $\pm$ 2.7	114.0 $\pm$ 7.7
Arginine	—	—	22.8 $\pm$ 1.6	—	—	—
Aspartic acid	16.5 $\pm$ 0.8	16.6 $\pm$ 1.6	2.8 $\pm$ 0.6	63.5 $\pm$ 2.4	64.1 $\pm$ 3.2	64.4 $\pm$ 3.2
Glycine + Serine	26.7 $\pm$ 1.6	26.6 $\pm$ 1.7	39.9 $\pm$ 3.9	94.5 $\pm$ 3.2	96.1 $\pm$ 3.8	79.8 $\pm$ 6.9
Lysine + Histidine	54.2 $\pm$ 3.2	53.2 $\pm$ 2.0	39.9 $\pm$ 2.0	190.5 $\pm$ 4.0	173.0 $\pm$ 5.2	102.6 $\pm$ 4.8
Cystine	1.5 $\pm$ 0.2	1.6 $\pm$ 0.1	2.8 $\pm$ 0.4	30.5 $\pm$ 1.6	25.6 $\pm$ 4.0	5.7 $\pm$ 0.8
Proline	110.5 $\pm$ 2.0	108.3 $\pm$ 4.8	105.6 $\pm$ 4.1	610.0 $\pm$ 8.1	600.0 $\pm$ 12.2	284.9 $\pm$ 11.4
$\alpha$ -Alanine	—	—	17.1 $\pm$ 1.6	—	—	—
$\beta$ -Alanine	10.5 $\pm$ 1.6	9.9 $\pm$ 0.8	—	19.0 $\pm$ 3.2	20.0 $\pm$ 4.0	5.7 $\pm$ 0.8

—: not detected.

**Table III.** Effect of feeding mustard pollen to worker bees of *Apis cerana* on their larval lipid classes ( $\mu\text{g}$  per 100 mg fresh weight).

Lipid classes	CM larvae $\pm$ SD	UCM larvae $\pm$ SD	CNM larvae $\pm$ SD
Phospholipids	520.0 $\pm$ 16.3	518.0 $\pm$ 6.5	400.0 $\pm$ 16.3
Sterols	46.5 $\pm$ 4.8	16.2 $\pm$ 1.8	46.2 $\pm$ 4.8
Free fatty acids	120.5 $\pm$ 8.5	116.2 $\pm$ 4.5	80.7 $\pm$ 4.0
Triglycerides	940.5 $\pm$ 30.5	938.0 $\pm$ 10.8	830.2 $\pm$ 18.8
Sterol esters	75.2 $\pm$ 5.2	77.0 $\pm$ 5.7	65.3 $\pm$ 2.4
Methyl esters of free fatty acids	85.2 $\pm$ 5.1	83.4 $\pm$ 2.4	70.7 $\pm$ 3.7
Hydrocarbons	90.0 $\pm$ 6.4	91.0 $\pm$ 4.8	35.9 $\pm$ 2.9

phosphatidyl ethanolamine, lyso-phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl choline, the latter two constituted the major phospholipids. Both the individual and the total phospholipid contents varied in the order CM > UCM > CNM (table IV). In the CM larvae the concentration of phosphatidyl choline (lecithin) was ~1.4 times higher than in the CNM larvae.

## DISCUSSION

Despite pollen being a poor energy source, the dependence of honeybees on it in several ways is well documented (Stanley and Linskens, 1974; Wille et al, 1985; Southwick, 1990). Pollen is used primarily as a source of essential amino acids required by

**Table IV.** Effect of feeding mustard pollen to worker bees of *Apis cerana* on individual phospholipids ( $\mu\text{g}$  per 100 mg fresh weight) of their larvae.

<i>Individual phospholipids</i>	<i>CM larvae <math>\pm</math> SD</i>	<i>UCM larvae <math>\pm</math> SD</i>	<i>NM larvae <math>\pm</math> SD</i>
Phosphatidyl ethanolamine	55.5 $\pm$ 4.0	52.5 $\pm$ 4.7	40.2 $\pm$ 7.7
Lysophosphatidyl ethanolamine	65.8 $\pm$ 3.6	60.5 $\pm$ 4.0	50.5 $\pm$ 4.8
Phosphatidyl serine	160.7 $\pm$ 4.0	150.5 $\pm$ 3.3	130.5 $\pm$ 2.9
Phosphatidyl choline	238.0 $\pm$ 11.0	254.5 $\pm$ 4.0	178.8 $\pm$ 8.5

honeybees (De Groot, 1952, 1953) in protein synthesis. The hypopharyngeal glands of honeybees secrete royal jelly with a high concentration of protein (Rembold, 1974). In the light of these reports our results are significant in that the brood production, brood development and larval growth of bees, as indicated by increased brood area and larval weight, follow the order CM > UCM > CNM. As such, monophagous feeding of bees with mustard pollen (CM) was found to be nutritionally superior to polyphagous feeding of pollen from a mixed populations including non-mustard plants (UCM), or from non-mustard plants only (CNM).

Mustard pollen is a rich protein source (21.7%) (Kosonocka, 1990). The pollen amino acids in the royal jelly serve as sources of nutrition to the growing and developing larvae. A majority of the essential amino acids required by honeybees (De Groot, 1952, 1953) are present in the Toria pollen, proline being present in the greatest amount (70% of the free amino acid pool), apart from total amounts of 4.0% amino acids, 9.2% lipids, 4.6% sugars and 1.4% phospholipids (Singh and Singh, 1991). Special importance may be attached to proline as it is highly soluble in water and its energy content is also very high. As such it may serve as an excellent energy source, providing easily utilized food for the growing and developing larvae. The total proline concentration of the insect larvae (table II) as well as the Toria pollen ( $9 \times 10^2 \mu\text{g}$  per

100 mg dry wt) (Singh and Singh, 1991) is very high compared to that of the other amino acids present. Pollen proline, thus, appears to be the major source of larval proline, being taken up and utilized by the larvae from the royal jelly. Also, the relatively high value of total protein, to the extent of 34  $\mu\text{g}$  per 100 mg dry wt in the Toria pollen (Singh and Singh, 1991) suggests that pollen protein may be the primary source of larval amino acids. The order CM > UCM > CNM of concentration of amino acids (the sum of free and protein amino acids) in the larvae apparently indicates mustard again as a superior pollen source. The presence of the lowest amount of protein amino acids in the CNM larvae indicates a low rate of protein synthesis from CNM pollen, poor in amino acids.

Not all pollen species have the same nutritive value for bees. Our data indicate that entomophilic mustard pollen, with a larger number and greater amount of amino acids (Singh and Singh, 1991), is nutritionally superior to anemophilic (Stanley and Linskens, 1974) or non-mustard pollen, as the low amino acid values of the UCM and CNM larvae indicate. Bees, with their observed inclination to visit mustard flowers (Boch et al, 1978) may take advantage of the superior nutritive value of mustard pollen.

The contents of triglycerides and phospholipids as the two major lipid classes of the insect larvae (tables III and IV) also vary in the order CM > UCM > CNM. The usual

role of triglycerides is that of storage of nutritive compounds, and that of phospholipids is that of providing structural components of larval cell membranes. The role of sterols in the prepupal development of honeybees and increased brood rearing efficiency of honeybees by pollen lipids is known (Svoboda et al, 1980; Herbert et al, 1980). Compared to other pollens (Stanley and Linskens, 1974) Toria pollen (Singh and Singh, 1991) is rich in lipids. The presence of high levels of lipids and sterols in the CM larvae in this study further suggests mustard is a nutritionally superior pollen source.

From the foregoing discussion it is tempting to suggest that monophagous feeding with nutritive pollen such as mustard may have a greater effect on improving brood production and brood development of bees than polyphagous feeding with apparently nutritionally inferior pollen from several non-mustard plants. Although mustard pollen may serve as a good, solitary source of nutrition, by optimal foraging, the bees may mix other pollen to obtain the very best diet possible. However, this requires more critical evaluation by future experiments involving feeding several different pollen types to honeybees both from single and mixed plant populations.

**Résumé — Composition en acides aminés et en lipides des larves d'abeilles (*Apis cerana* Fabr) nourries au pollen de moutarde.** On a étudié la composition en acides aminés et en lipides de larves d'abeilles (*Apis cerana*): i) encagées (6 x 5 x 2 m) avec de la moutarde (*Brassica campestris* L cv Toria) en fleurs (CM), ii) butinant en plein air un champ de moutarde en fleurs (UCM) et iii) encagées avec des plantes autres que la moutarde (CNM). L'analyse des acides aminés des larves a été faite 8 jours après la ponte par chromatographie sur papier, celle des lipides par chromatographie sur couche mince. La quantité de proline a été déterminée par

des mesures colorimétriques en comparaison avec un standard de référence. Au bout d'une semaine la surface de couvain et le poids des larves des ouvrières nourries uniquement avec du pollen de moutarde (CM) avaient le plus augmenté (tableau I). La concentration totale en acides aminés a varié en fonction du traitement (CM > UCM > CNM) et le mode de nourrissage a influencé la composition quantitative aussi bien que qualitative en acides aminés (tableau II). La proline est l'acide aminé prédominant et semble être prélevé et utilisé par les larves principalement à partir de la gelée royale. Les larves d'abeilles renfermaient des lipides neutres (acides gras libres, triglycérides, esters de stérols, esters méthyliques d'acides gras, hydrocarbures) et des lipides polaires (phospholipides) dont la teneur variait en fonction du traitement : CM > UCM > CNM (tableaux III, IV). On peut déduire de ces résultats le rôle nutritionnel joué par le pollen de moutarde, donné en nourrissage à des ouvrières, dans la croissance et le métabolisme de leurs larves. Les acides aminés du pollen semblent être la source première des acides aminés des larves. L'accroissement du poids larvaire suite au nourrissage des ouvrières avec du pollen de moutarde reflète le rôle des triglycérides, en tant que lipides de réserve, et celui des phospholipides, comme composants des membranes cellulaires des larves. On en conclut qu'un nourrissage des ouvrières basé uniquement sur du pollen de moutarde est susceptible de favoriser plus la ponte et le développement du couvain qu'un nourrissage à base d'un mélange de pollens de qualité nutritionnelle inférieure.

***Apis cerana* / nourrissage / pollen / *Brassica campestris* / acide aminé / lipide**

**Zusammenfassung — Spektrum der Aminosäuren und Lipide in Larven der indischen Honigbienen (*Apis cerana* Fabr)**

**nach Fütterung mit Senfpollen.** Die Zusammensetzung der Aminosäuren und Fette in Larven von Honigbienen (*Apis cerana*) wurde bei drei Versuchsgruppen bestimmt: 1) die Bienen befanden sich in einem Käfig (6 m x 5 m x 2 m) und konnten nur Senfpollen sammeln (CM); 2) die ungekäfigten Bienen konnten an den zahlreich blühenden Senfpflanzen und an anderen Pflanzen sammeln (UCM); und 3) die Bienen wurden im Käfig mit anderen Pflanzen als Senf gehalten. Die Aminosäuren und Fette der Larven wurden mit Papier- und Dünnschichtchromatographie 8 Tage nach der Eilage analysiert und colorimetrisch gegen Standardreferenzen quantifiziert. Bei monophag mit Senfpollen als einziger Eiweißnahrung versorgten Arbeiterinnen wurde eine Steigerung der Eilegerate beobachtet, entsprechend nahm nach 3 Tagen die Brutfläche zu, das Larvalgewicht wurde ebenfalls höher. Die Gesamtkonzentration der Aminosäuren unterschied sich in der Reihenfolge CM > UCM > CNM. Sowohl der Gehalt als auch die Qualität der Aminosäuren wurde von der Pollenart als Nahrung der Bienen beeinflusst. Die Aminosäure Prolin stellte den höchsten Anteil und schien vor allem aus dem Königinnenfuttersaft aufgenommen und verarbeitet zu werden. Die Larven enthielten neutrale Lipide (freie Fettsäuren, Triglyceride, Sterolester, Fettsäuremethylester, Kohlenwasserstoffe) und polare Lipide (Phospholipide), die in der Reihenfolge CM > UCM > CNM variierten. Die Ergebnisse weisen auf einen hohen Nährwert des Senfpollens für das Wachstum und den Metabolismus der Larven hin. Die Aminosäuren der Pollen schienen die Primärquelle der Aminosäuren der Larven zu sein. Die Bedeutung der Triglyceride als Speicherfett und der Phospholipide als strukturelle Komponente der larvalen Zellmembranen wurde durch den Anstieg des Larvalgewichts nach dem Füttern mit Senfpollen deutlich. Aus diesen Ergebnissen wurde geschlossen, daß die ausschließliche Fütte-

rung der Arbeiterinnen mit dem als Nahrung hochwertigen Senfpollen einen günstigeren Einfluß auf die Eilage und die Brutentwicklung hat als eine vielseitige Fütterung mit minderwertigem Pollen aus einer gemischten Pflanzenpopulation.

### **Aminosäuren / Lipide / *Apis cerana* / Pollen / *Brassica campestris***

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