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Amino acids in honeybee worker haemolymph

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Summary. In honeybee workers proline was the predominant amino acid and comprised from 50% in newly emerged bees up to 80% of total amino acids from the 3rd day on. The overall concentration averaged at about 20 mM in newly emerging bees, rose to a maximum of about 25 mM at the 3rd–5th day and decreased in older bees. Essential amino acids decreased by 40% during the first 3 days and thereafter stayed constant. The bulk of amino acids with a lower concentration (from traces to about 2 mM) showed either no change in concentration or was higher in newly emerging bees and decreased during the lifespan of the insects. Forager bees, collected after flight, had significantly lower proline concentrations as compared to 22 day old bees collected from the colony, while the concentrations of the bulk of all other amino acids did not change significantly. There was a great variance in the concentration of all amino acids between different colonies but we could not prove dependency on relatedness.

Keywords: Amino acids – Apis mellifera – Free amino acids – DABS-Amino acids – Haemolymph – Age dependency

Introduction

Protein metabolism in honeybees has been studied thoroughly (DeGroot, 1953; Crailsheim, 1990). Both anabolic metabolism for the production of proteinacious jelly for brood rearing and nursing other bees and the capability to degrade the main protein source of honeybees, pollen, show a marked dependence on the age and function of the individual (Moritz and Crailsheim, 1987; Lass and Crailsheim, 1996). Honeybees, with their pronounced polyethism (Rösch, 1925), reflect in their physiological parameters not only their age but also their function in the colony. Compared to the protein metabolism, little is known about the role of free amino acids in the honeybee workers haemolymph. As honeybee haemolymph has a low content of inorganic cations of 93 mEq/litre, half of which is Na^+, free amino acids could play a role in osmoregulation (Florkin and Jeuniaux, 1974). Age dependent changes in the amount and composition of protein and minerals are

shown by ElShakaa and Shahein (1987), who reported a decrease in total crude protein content from newly emerged bees to 20 day old bees from 69% to 41% (% of dry weight). Correspondingly, the total mineral content reached its maximum on day 1 and decreased to day 20. The predominant amino acid in hydrolysates was glutamic acid, followed by alanine, except in 5 day old bees in whom alanine was highest (McLellan, 1976).

Age related changes in amino acid and protein content of the haemolymph were reported by Sinitzky and Lewtschenko (1971). An increase of haemolymph protein concentration from 3.0% (w/v) at the 1st day to 6.2% at the 10th–12th day was followed by a decrease to 2.5% at day 30 in summer bees. Bees from the autumn generation had a slightly higher maximum at 8.5% and when reared in a greenhouse had drastically lower protein concentrations.

In haemolymph pools analysed by paper chromatography stained with ninhydrin reagent the overall concentration of free amino acids in the haemolymph stayed constant from days 1 to 7 and thereafter decreased. Generally lower values were found in forager bees. Individual amino acids, however, showed higher variability of concentration than the overall content. Analysis of many of the free amino acids in a single class of honeybee workers (10 days) reported by Wang and Möller (1970) gave similar results to those reported by Sinitzky and Lewtschenko (1971), with one obvious exception: the proline content in the data from Wang and Möller (1970) comprised more than 50% of the amino acids whereas Sinitzky and Lewtschenko (1971) reported about 6%.

In spite of all the research work done to date on the dependence of the amino acid content in the honeybee haemolymph on age, the results are different and even contradictory. It is unclear whether these differences may be mainly due to a high variability in free amino acids, possibly as a result of using different races of honeybees, nutritional or seasonal differences, or obliteration of individual variability due to the need to analyse haemolymph pools for lack of sufficiently sensitive methods to analyse single specimens.

This study was designed to determine to which extent the concentration of individual free amino acids in the haemolymph of honeybee workers is dependent on the age of the bee, how strong concentrations can differ among individuals of the same age, and if there are differences between genetically related and unrelated colonies.

Material and methods

Animals

Six colonies of *Apis mellifera carnica Pollm.* in two to three store hives (20–30 frames) were kept in a garden in the city of Graz. Four colonies with single drone inseminated queens in their first year (hives designated colonies 1–4, colonies 1, 2 and 3, 4 were pairs of sister queens inseminated by pairs of sibling drones; the two pairs were not closely related to each other) and two colonies with naturally inseminated queens of the same age (colonies 5, 6) but different origin, were used for the experiments done between 3rd July and 27th July 1994. All colonies collected pollen and nectar from urban gardens and parks.

To obtain defined age classes of honeybee workers brood combs with sufficient emerging brood were taken out of the hives and put into separate well aerated boxes. They were transferred to the laboratory in insulated containers. In the laboratory the combs were kept in individual cages in an incubator at 35°C and approximately 60% rel. humidity.

To obtain honeybees of 0 days age the combs were kept in the laboratory at 28°C for about 1.5 hours and bees were taken during emergence, before they had had an opportunity to feed on honey or pollen. In the control experiment bees of 0 days age were collected as described above. Bees colour marked at emergence formed one group. They were returned to their colonies at once and then collected 24 hours and 3 days later. Another group of bees, marked at emergence, was kept on the isolated comb for 24 hours.

For another series of experiments honeybees that emerged overnight were individually marked for the day of emergence and their original colony with colour dots on the thoraces and reinserted into their colonies. Combs in the incubator were exchanged for combs with sufficient emerging brood as necessary. After defined time intervals (3, 5, 9, 12, 17, 22 days) bees were identified by their colour code and collected from the hives. One day old bees were collected from the isolated combs. A behaviour-defined class of pollen foragers was obtained from each hive by collecting pollen carrying bees from the entrance to each hive. The mothers and the ages of these bees were uncertain, usually pollen foragers are older than 17 days.

Collection of haemolymph samples

Honeybees were narcotised immediately after collection by gassing with CO_2 for about 1 min and kept on ice for a maximum of 1.5 hours. As it is known that both carbon dioxide and low temperature narcosis influence honeybee physiology (Ebadi et al., 1980; Bühler et al., 1983; Robinson and Visscher, 1984), we ran control experiments, to prove, that narcotising with CO_2 and cooling of the honeybees had no influence on the content of free amino acids during the experimental procedure. Results show no appreciable difference in the concentrations of free amino acids in the haemolymph of narcotised honeybees on ice for a maximum of 1.5 hours and in the concentrations found in freshly taken haemolymph (data not shown).

Narcotised honeybees were punctured between the 3rd and 4th abdominal segment slightly left of dorsal. The incision was done with a pair of surgical eyescissors. From the wound haemolymph was drawn into a calibrated 5 μl glass capillary by capillary force. Care was taken to obtain exactly 1 μl haemolymph.

Sample size for each age class and the behaviour-defined class (pollen foragers) was 6–8 in each of the 6 colonies. When sample size was lower (2 cases) it is indicated.

Sample preparation

The haemolymph was immediately expelled into 100 μl of 0.5 M ice-cold glucose solution in a 1.5 ml Eppendorf reaction vessel. Cells were centrifuged in a tabletop centrifuge at 8,000 g for 1 min. Of the supernatant, 95 μl were mixed with an equal amount of acetonitrile in a new reaction vessel. The protein precipitate was centrifuged at 8,000 g for 3 min. From this supernatant 47.5 μl (representing an aliquot of 0.25 μl honeybee haemolymph) were transferred into a 1.5 ml Eppendorf reaction vessel and lyophilised immediately or frozen at −70°C for further processing at a later time. To eliminate the possibility of biasing the concentrations of free amino acids in the haemolymph by contributions from disrupted haemocytes, we compared the amino acid concentration in aliquots of haemolymph samples where the haemocytes were disrupted osmotically (10 mM glucose, centrifuged at 8,000 g) to aliquots of the same haemolymph samples where the haemocytes were centrifuged off in a nondisruptive way (500 mM glucose, 800 g). Both measurements led to identical results, showing that the intracellular concentration of free amino acids is close enough to that in the haemolymph or not important,

and that, based on the disparity of cell volumes to haemolymph volume, intracellular free amino acids do not contribute to the overall concentration of free amino acids in the haemolymph (data not shown).

Lyophilised 0.25 µl haemolymph aliquots were redissolved in 20 µl 50 mM sodium bicarbonate buffer (Merck), pH 8.1. After adding 40 µl of a 4 mM solution of 4-(dimethylamino)-azobenzenesulfonyl chloride (DABS-Cl) (SIGMA D-7772 Lot 73H3489, recristallized twice in acetone at −20°C overnight) in acetonitrile (Biosolve LTD, HPLC gradient grade) the solution was heated at 70°C for 12 min in a waterbath. The reaction vessels were vortexed after 1 and 4 minutes of heating. During the first minutes of derivatisation the solution turned from red to yellow. Samples with no colour change were discarded. After the derivatisation the samples were diluted with 10 mM citrate buffer (Citrate monohydrate, Riedel-deHaen 33114) pH 4.6, containing 30% acetonitrile and 4% dimethylformamide (Riedl-deHaen) to a final volume of 500 µl (Lin and Lai, 1980; Knecht and Chang, 1986; Hughes et al., 1987; Stocchi et al., 1992). Analysis was performed on a Beckman System Gold automated binary HPLC-system (Pump: programmable solvent module 126, detector: programmable detector module 166 fitted with a tungsten lamp) combined with an autosampler (Spark Triathlon). Column used was a C18 reversed phase Ultrasphere XL-ODS 3 µm 4.6 mm × 7 cm Cartridge Column with an integrated precolumn (Beckman) and a prefilter RP18 5 µm (Merck). Separation of 10 µl injected sample was performed in a gradient of 12 mM to 17 mM citrate buffer ph 6.4 (depending on column age and performance, citrate concentration was varied in order to set the arginine peak between threonine and glycine), 4% DMF (Solvent A) against acetonitrile containing 30% 12 mM citrate buffer pH 6.4, 4% DMF (Solvent B).

Gradient profile:

Time	Solvent b	Flow
Start to 8.60	30% to 56%	1.2 ml/min
8.60 to 10.60	56%	
10.60 to 12.34	56% to 86%	
12.34 to 13.68	86%	
13.68 to 14.18	86% to 100%	
14.18 to 17.10	100%	1.5 ml/min
17.10 to 17.40	100% to 38%	
17.40 to 20.00	38%	
20.00 to 20.30	38% to 30%	1.2 ml/min
20.30 to 22.00	30%	

DABS amino acids were detected at 436 nm. At a flow of 1.2 ml/min each separation took 22 min. The operating backpressure was between 1.8 kPSI with new columns up to 2.5 kPSI when prefilter, precolumn or column were exchanged. Under our conditions prefilters were usually replaced after 200–400 samples, precolumns lasted for 500–600 samples and the main separation column was discarded after about 1,500 separations. After about 200 separations (2 × autosampler capacity) columns were flushed for 1 hour with 100% solvent B which usually restored pressure to normal values.

Peak identification was done by comparison with an external standard, quantification by integration of the peak areas. Asparagine, glutamine, serine, threonine, arginine, glycine, alanine, proline, valine, methionine, isoleucine, leucine, tryptophane, phenylalanine, cystine, lysine, histidine and tyrosine were identified by individual standards. Of those arginine, valine, methionine, isoleucine, leucine, threonine, phenylalanine, lysine, histidine and tryptophane are reported as essential for honeybees (DeGroot, 1953). Concentration of the samples was set to obtain a maximum quantifiable range of about 0.1 nmol/µl to 50 nmol/µl amino acid in the haemolymph.

We determined the free amino acids in 3 pools of honeybee-worker haemolymph with PITC (phenylisothiocyanate) and OPA (orthopthalaldehyde) derivatisation in parallel to our method (DABS). The results were in good agreement except for proline, which is known not to be quantifiable with the OPA method (Fallon et al., 1993) (data not shown).

Statistical analysis

Text and tables give mean values and standard errors of means. Significances of differences in parameters of different groups of bees were tested with the Mann Whitney U-test, the chosen level was $p < 0.05$ (Sachs, 1972).

Results

In the HPLC separations 18 amino acids, including the 16 listed as principal and most permanent constituents of insect haemolymph (Florkin and Jeuniaux, 1974), were identified in a concentration range of 0.01 nmole/μl up to a maximum of 50 nmole/μl per single amino acid. No unidentified substances over a concentration of 1.5% of the overall content (total peak area of DABS derivatives) were present in the chromatographs (data not shown). Honeybee workers from 6 colonies in 8 age-defined classes and one behaviour-defined class (pollen foragers) were analysed. Table 1a shows the results from the above experiment. Amino acid concentrations given are means of all analysed bees from 6 colonies (34–47 samples at each age class and the behaviour-defined class) and the standard deviations. The total concentration of free amino acids and the total concentration of essential free amino acids are also provided.

Included in Table 1b,c are data for free amino acids by other authors. For the sake of clarity the dimensions given by these authors were recalculated into nmol free amino acid/μl haemolymph. Data given by Wang and Möller (1970) are free amino acids measured in a haemolymph pool of 10 day old

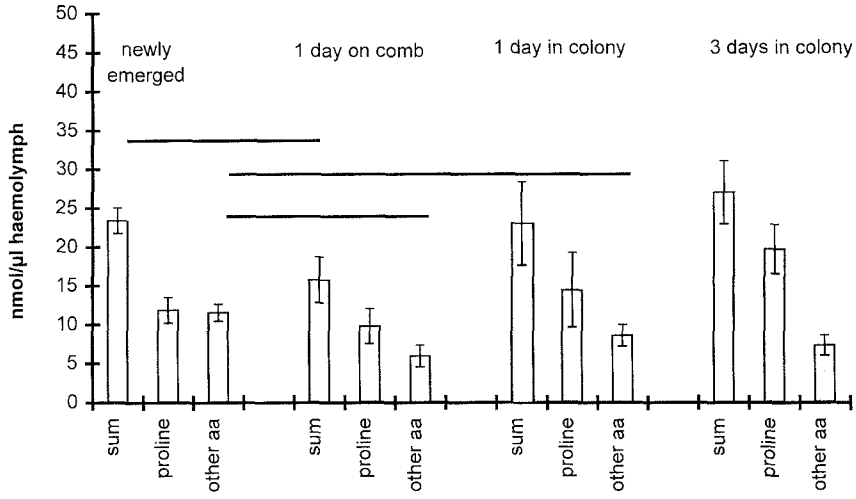

Fig. 1. Concentrations of total free amino acids, free proline and the bulk of other free amino acids: in the haemolymph of newly emerged honeybees – in the haemolymph of 1-day-old honeybees kept for one day on isolated combs – in the haemolymph 1-day-old honeybees kept in the colony for one day – in the haemolymph of 3-day-old honeybees kept in the colony for three days – ($n = 12$ for each data point). Horizontal lines indicate significance of differences

Table 1. Free amino acids in the haemolymph of honeybee workers

	Free amino acids in the haemolymph of Apis mellifera workers																			
	a		**a**		**a**		**a**		**a**		**a**		**a**		**a**		**b**	**c**			
Amino acid nmol/μl	0 days mean	*std*	3 days mean	*std*	5 days mean	*std*	9 days mean	*std*	12 days mean	*std*	17 days mean	*std*	22 days mean	*std*	pollen foragers mean	*std*	10 day	1 day	5–7 d	10–12 d	foragers
Asp	0.00	*0.00*	0.00	*0.00*	0.00	*0.00*	0.00	*0.00*	0.00	*0.00*	0.00	*0.00*	0.00	*0.01*	0.00	*0.00*	0.63	1.01	0.64	0.90	0.28
Glu	0.09	*0.06*	0.01	*0.02*	0.00	*0.01*	0.00	*0.00*	0.00	*0.00*	0.00	*0.00*	0.00	*0.01*	0.00	*0.01*	1.34	1.30	1.38	1.02	1.13
Ser	0.65	*0.21*	0.44	*0.13*	0.42	*0.16*	0.28	*0.10*	0.29	*0.13*	0.29	*0.17*	0.23	*0.13*	0.14	*0.15*	1.41	1.94	2.20	1.84	1.68
Thr*	0.42	*0.16*	0.15	*0.09*	0.18	*0.09*	0.09	*0.07*	0.16	*0.12*	0.16	*0.16*	0.09	*0.08*	0.06	*0.18*	1.11	1.63	1.60	1.66	1.01
Arg*	0.52	*0.21*	0.25	*0.14*	0.20	*0.15*	0.12	*0.07*	0.09	*0.06*	0.07	0.07	0.06	*0.05*	0.07	*0.06*	2.41	1.46	1.66	1.18	0.59
Gly	1.35	*0.32*	0.92	*0.24*	0.84	*0.20*	0.55	*0.17*	0.67	*0.26*	0.59	*0.32*	0.43	*0.18*	0.36	*0.36*	2.26	2.20	2.02	1.94	1.29
Ala	0.49	*0.13*	0.54	*0.19*	0.49	*0.15*	0.37	*0.16*	0.41	*0.19*	0.39	*0.21*	0.35	*0.20*	0.26	*0.17*	3.13	1.72	3.04	3.09	2.44
Pro	10.70	*5.54*	19.67	*9.65*	19.98	*6.94*	14.89	*4.29*	16.31	*4.04*	16.06	*5.26*	15.17	*4.23*	9.49	*3.91*	25.70	1.50	1.60	1.51	0.87
Val*	1.50	*0.41*	1.26	*0.95*	1.10	*1.09*	1.06	*0.83*	0.62	*0.58*	0.84	*0.65*	0.62	*0.67*	1.27	*0.86*	0.94	1.42	0.96	0.74	0.51
Met*	0.07	*0.09*	0.06	*0.06*	0.05	*0.06*	0.06	*0.09*	0.09	*0.11*	0.08	*0.10*	0.11	*0.12*	0.08	*0.12*	0.19	0.33	0.15	0.35	0.14
Ile*	0.82	*0.48*	0.26	*0.16*	0.31	*0.17*	0.27	*0.11*	0.35	*0.21*	0.38	*0.33*	0.27	*0.14*	0.31	*0.45*	0.72	2.42	1.55	1.65	0.83
Leu*	0.94	*0.53*	0.51	*0.39*	0.47	*0.26*	0.29	*0.20*	0.35	*0.31*	0.43	*0.52*	0.24	*0.18*	0.23	*0.63*	0.61	n.d.	n.d.	n.d.	n.d.
Try*	traces		traces		traces		traces		traces		traces		traces		traces		0.00	0.79	0.83	0.92	0.61
Phe*	0.59	*0.48*	0.33	*0.18*	0.42	*0.24*	0.43	*0.17*	0.37	*0.18*	0.36	*0.18*	0.44	*0.20*	0.45	*0.29*	0.57	1.30	0.99	1.19	0.64
Cys	0.02	*0.06*	0.01	*0.01*	0.01	*0.01*	0.01	*0.01*	0.01	*0.01*	0.01	*0.01*	0.01	*0.01*	0.01	*0.04*	0.00	0.92	0.74	0.71	0.53
Lys*	1.13	*0.68*	0.35	*0.27*	0.32	*0.22*	0.22	*0.13*	0.39	*0.53*	0.32	*0.40*	0.37	*0.39*	0.25	*0.40*	3.57	1.67	3.28	2.90	1.86
His*	0.26	*0.28*	0.23	*0.22*	0.15	*0.08*	0.18	*0.21*	0.38	*0.59*	0.29	*0.44*	0.52	*1.00*	0.60	*1.20*	1.17	2.35	2.25	2.60	0.98
Tyr	0.84	*0.82*	0.55	*1.22*	0.30	*0.86*	0.04	*0.07*	0.25	*0.83*	0.33	*0.90*	0.09	*0.15*	0.37	*0.91*	0.47	1.13	0.45	0.49	0.25
ess.a.a.	6.26	*2.12*	3.40	*1.22*	3.20	*1.40*	2.67	*0.77*	2.72	*1.29*	2.89	*1.88*	2.68	*1.56*	3.37	*2.83*					
Sum	20.61	*4.84*	25.79	*9.62*	25.32	*7.42*	18.99	*4.70*	20.72	*4.98*	20.57	*6.41*	18.94	*5.52*	14.17	*5.94*	46.22	25.07	25.34	24.70	15.64

a mean concentrations (mM) for individual free amino acids in the haemolymph of honeybees of different age and pollen foragers collected from 6 different colonies (n = 34–37). Included are total free amino acids (*sum*) and the total of essential amino acids (*ess a.a.*). Asterisk indicates essential amino acids for honeybees (DeGroot, 1953). **b, c** data recalculated from Wang and Möller (1970) (**b**) and Sinitzky and Lewtschenko (1971) (**c**). In (**c**) data for isoleucine and leucine are pooled.

worker honeybees by automated amino acid analysis. In the data from Sinitzky and Lewtschenko (1971) pooled haemolymph samples were determined by paper chromatography, and leucine and isoleucine were given as one value.

Our young honeybees kept for up to 24 hours after emergence on isolated combs had significantly lower values for the bulk of free amino acids other than proline compared to newly emerged bees. The proline content did not differ significantly from newly emerged bees. In an experiment where bees marked at emergence were kept on the combs for 1 day and other bees were put into the appropriate colony immediately after emergence, it was shown that the bees kept in the colony had increased proline concentrations, which, outweighing the decrease of concentration in the bulk of other amino acids, led to an increase in the total amino acid concentration, as would be expected from the curve from 0 to 3 day old bees (Fig. 1). Therefore the data from 1 day old bees kept on isolated combs were not included in Table 1.

Proline averaged lowest in newly emerged bees in whom this amino acid comprises 50% of all free amino acids. After 3 days the free amino acid pool reached its peak at 25.8 nmol/μl, 80% of which was proline at this time. From the 5th to the 22nd day the overall free amino acids decreased. Proline was still at about 80%. Methionine, at an average concentration between 0.06 and 0.11 nmole/μl, alanine (0.26–0.49 nmol/μl) and phenylalanine (0.33–0.59 nmol/μl) showed no marked, systematic dependence of concentration on age. All other amino acids started high at emergence and decreased in concentration during the lifespan of the insect (Table 1, Fig. 2).

The total concentration of essential free amino acids was highest at emergence, decreased significantly to 60% of the maximum after 3 days and thereafter showed no significant change throughout the observation

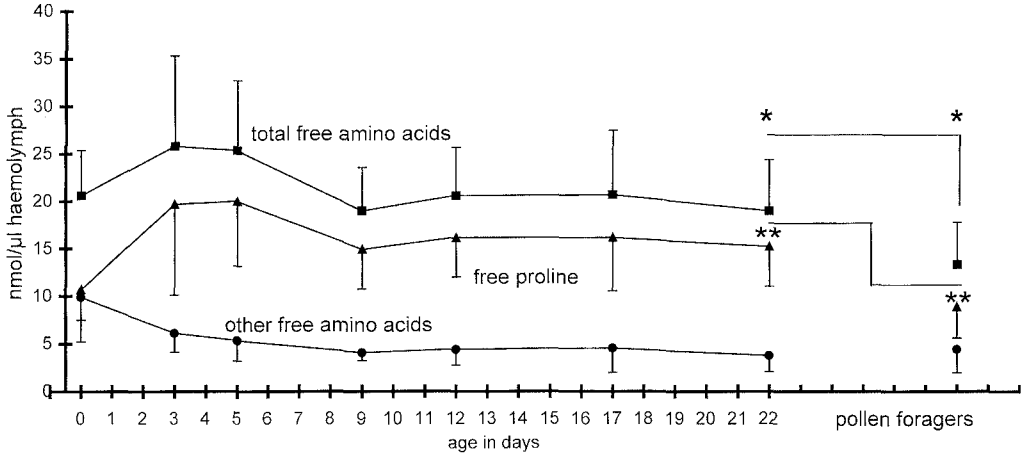

Fig. 2. Concentrations of total free amino acids, free proline and the bulk of other free amino acids in the haemolymph of honeybees of different ages and of pollen foragers. Given are the means of individuals from 6 colonies and the standard deviations (n = 34–37). Asterisks (* and **) for the total free amino acids and free proline for 22-day-old bees and pollen foragers indicate significant differences as calculated by Mann Whitney U-test

period (Table 1). Even pollen foragers, having significantly lower overall amino acid concentrations did not, in respect to essential amino acids, differ significantly from the concentration of all other age classes except newly emerged bees.

Individual extremes in total free amino acid contents were found on the lower end in one pollen forager with a total of 5.14 nmol/μl total free amino acids, (4.1 nmole/μl proline) and at the high end in one 5 day old honeybee with a total of 62 nmole/μl free amino acids (54 nmole/μl proline). In the age class with the highest content of free amino acids, the 3 day old bees, the concentration in individuals from the 6 colonies ranged from 17.8 nmol/μl to 43.6 nmol/μl total free amino acids (data not shown), and reached such the concentration range of Wang and Möller (1970) (Table 1).

In Fig. 2 the means of total free amino acids, proline and the bulk of other free amino acids are shown for bees from 6 colonies collected at emergence and at 3, 5, 9, 12, 17 and 22 days. Included are data for pollen foragers which were not age defined and were collected after return from flight. A significantly lower concentration of free amino acids was observed in pollen foragers returning from flight. This decrease was due to a significant decrease in proline content, whereas the content of other amino acids did not change significantly.

Table 2. Test for significance of differences in the overall content of free amino acids in the haemolymph of honeybee workers of the same age from different colonies

Differences in the concentration of free amino acids in honey bee workers from different colonies: test for significance									
	0 days	1 day	3 days	5 days	9 days	12 day	17 day	22 day	P.F.
C1–C2	s	s	s	ns	ns	s	ns	s	ns
C1–C3	ns	s	s	s	s	s	s	s	s
C1–C4	ns	ns	s	s	ns	ns	s		ns
C1–C5	s	s	s	s	s	s	s	ns	ns
C1–C6	ns	ns	ns	ns		ns	s		ns
C2–C3	s	ns	s	ns	s	s	s	s	s
C2–C4	s	s	s	ns	s	s	s		ns
C2–C5	ns	ns	s	ns	s	s	s	s	ns
C2–C6	ns	s	s	ns		s	s		ns
C3–C4	ns	ns	s	s	s	s	ns		s
C3–C5	s	ns	s	s	ns	ns	ns	s	s
C3–C6	ns	s	s	s		s	ns		s
C4–C5	s	ns	ns	s	s	ns	s		s
C4–C6	s	ns	ns	ns		ns	ns		s
C5–C6	ns	s	ns	s		ns	s		ns

0–22 days age of honeybees after emergence; *P.F.* pollen foragers collected on arrival at the hive; *C1–C6* colonies 1–6; *s* significant; *ns* nonsignificant in Man Whitney U-test. Absence of s/ns indicates insufficient data for this pair of age classes. C1, C2 and C3, C4 are pairs of colonies with sister queens inseminated by sibling drones. C5 and C6 are colonies with naturally inseminated queens which are not related to each other and to the pairs of colonies with single drone inseminated sister queens.

Testing the differences in overall amino acid content in the haemolymph of differently related honeybees of the same age from different colonies for significance (Table 2) showed that more of the evaluated pairs were significant (71) than not significant (50). In comparing the ratio of significantly differing pairs to nonsignificantly differing pairs of age classes from colonies which were closely related to each other ($C_1 + C_2$ and $C_3 + C_4$) and colonies that were not closely related, no difference were seen in the distribution of significances. In related colonies 60% of the tested pairs were significantly different, in not closely related colonies 62% differed significantly. Because it has been demonstrated above that pollen foragers after flight differ in their amino acid titres from older bees collected from the colony, data from pollen foragers were excluded from the interpretation of these statistics. Honeybees kept for one day on isolated combs were included into these statistic.

Discussion

A high titre of free amino acids is a characteristic of insect haemolymph. It appears however, that the importance of free amino acids increases within the most evolved groups of insects (Florkin and Jeuniaux, 1974). Hemimetabolous insects generally have not only lower overall amino acid titres than holometabolous insects but also lack in several amino acids (Pant and Agrawal, 1964).

Ten amino acids are described as essential for the growth of honeybees in diet experiments (arginine, histidine, lysine, tryptophane, phenylalanine, methionine, threonine, leucine, isoleucine, and valine) (DeGroot, 1953). We were able to quantify all but tryptophane, which was present as a trace element. The predominant free amino acid in all our samples was proline. Its concentration in individual honeybees deviated by more than the factor 10. Sinitzky and Lewtschenko (1971) reported values for proline about one order of magnitude below the concentrations we found, but generally higher concentrations of the other amino acids. This low proline concentration is possibly due to limitations in the detection of this amino acid in thin layer chromatography. Values given by Wang and Möller (1970) from a pool of 10 day old honeybees correlate dimensionally with our values. As we observed drastic differences in the concentrations of free amino acids in bees collected from different colonies, the differences between our measurements and the values given by Wang and Möller (1970) are not considered contradictory.

Emerged bees that were caged on a comb with sufficient food available showed a reduction of total amount of amino acids in their haemolymph caused mainly by the reduction of amino acids other than proline. Although these amino acids were reduced also (but to a smaller extent) when the bees were introduced into a colony immediately after emergence, the total amount did not decrease. This indicates the necessity of the natural environment – in this case we assume it to be the nurse bees. These nurse bees are known to feed all members of the colony, but especially larvae, the queen and young

workers (Crailsheim et al., 1992; Lass and Crailsheim, 1996) with protein-rich jelly. Especially the young bees need this feeding with easily digestible protein as the equipment of their gut with proteolytic enzymes is not yet developed (Moritz and Crailsheim, 1987). After 3 days in the colony bees have levels of amino acids comparable to those used in the series of experiments determining age dependency where all bees were kept up to 1 day on the combs and were then introduced into the colony. This demonstrated the ability of bees to make up periods of faults (demonstrated also for other physiological parameters by Crailsheim and Stolberg, 1989).

When the freshly emerged bees are nursed with jelly and start to consume pollen (Crailsheim et al., 1992) they reach the peak of total free amino acids in their haemolymph and increase body protein (Haydak, 1934). Not all amino acids follow this pattern. The peak is caused by high proline concentrations whereas the sum of all other amino acids is decreasing (Fig. 2). The level of essential amino acids is rather constant from the third day until day 22 (the last defined age class we investigated) and even in the foragers that were not defined by age but by their function. Usually the average age of a group of foragers is above this age. This constant level of essential amino acids seems to provide the protein turnover which is high in middle aged bees mainly being in the hive as well as in foragers (Crailsheim, 1986). The role of the high levels of proline is not clear. In contrast to some other insects with high proline levels as the stable fly *Stomoxis calcitrans* (Chen and Wagner, 1992) or the fruit beetle *Pachnoda sinuata* (Zebe and Gäde, 1993) honeybees are not utilising amino acids for flight to a considerable extent (Barker and Lehner, 1972; Nachtigall et al., 1989; Rothe and Nachtigall, 1989) as their flight muscles are poor in prolin dehydrogenase (Crabtree and Newsholme, 1970). Nevertheless the decreased level of proline in our pollen foragers just returning from their flight might be a hint for proline to be involved at least in foraging metabolism. As we did not have controls in parallel to these foragers we can only speculate on this. Further we can speculate with the concept that the availability of sugar determines the amount of amino acids utilised as metabolic fuel (Brosemer and Veerabhadrappa, 1965; Sacktor and Childress, 1967; Sacktor, 1974; Beenakkers et al., 1984). Thus proline could have a booster function for flight metabolism although its contribution to the energy supply would be at a low percentage. As we know that proline can be metabolised to CO_2, that the metabolic breakdown is higher in active honeybees than in inactive animals (Berger et al., 1996) and that furthermore proline is extensively involved as an energy source in drone retina (Cardinaud et al., 1994; Tsacopoulos et al., 1994; Tsacopoulos, 1995), proline obviously provides energy in honeybees.

Our results demonstrate a great variability of haemolymph amino acids in bees from different colonies but we could not show a dependence on relatedness. Perhaps using bees of different races would generate other results. The presented data extend the knowledge about amino acids in haemolymph as they were evaluated for a broad range of ages, in different colonies and during the same season from bees collecting food in a certain area. Thus the differences between colonies reflect individuality and

different reactions to environmental conditions. In spite of the different levels in bees from different colonies the general age depended trends seem common.

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