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Importance of proline and other amino acids during honeybee flight

(*Apis mellifera carnica* POLLMANN)

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Summary. The levels of proline and other amino acids in the haemolymph and other body parts of honeybee foragers were investigated by HPLC analysis. The concentrations of proline in the blood of glucose-fed or -injected bees finishing their exhaustive tethered flights on a roundabout were significantly reduced compared to bees that were fed and rested for one hour. This indicates some utilization of proline during flight metabolism. The levels of essential amino acids and of the sum of all amino acids except proline remained roughly constant, indicating that the decrease of proline did not result from a changed haemolymph volume. ^{14}C-labelled proline was injected into bees either shortly before starting their flight or before a resting period of equal duration in an incubator at the same temperature. Bees that rested had incorporated more proline into thorax body protein, and less of the labelled substance was unrecovered ("missing") and considered to be respired or less probably defecated. If the entire amount of missing ^{14}C-proline is regarded as exhaled, the oxidative breakdown of proline reached higher levels after flight than in rested bees. This is another hint that proline is utilized during flight. Usually the exhaled amount did not exceed 10μg proline in half an hour of flight. Although our data indicate involvement of proline in flight metabolism, the amount metabolized is low compared to the utilization of carbohydrates.

Keywords: Amino acids – *Apis mellifera* – Proline – Foragers – Flight metabolism – Haemolymph

Introduction

The period of a worker honeybee's life devoted to in-hive tasks is generally followed by a foraging period characterized by a high energy turnover rate. The duration of each of these two periods varies and depends on the requirements of the colony (von Frisch, 1923; Lindauer, 1952). Foragers have higher

accumulations of glycogen reserves in their flight muscles compared to younger bees (Neukirch, 1981; Panzenböck and Crailsheim, 1996). Honeybees in the early stages of adult life are unable to fly, even though their wings appear to be functional. This might be due to the limited capacity of the flight muscle mitochondria to produce flight energy (Balboni, 1967).

Although it is known that honeybees utilize mainly carbohydrates for flight energy, there are hints in the literature that metabolism of the amino acid proline might also be of some importance. Proline is predominant in the haemolymph of the honeybee and many other insects, and it is known to be important for the flight metabolism of the tsetse fly *Glossina pallidipes* (Hargrove, 1975; Bursell, 1963), the African fruit beetle *Pachnoda sinuata* (Zebe and Gäde, 1993; Auerswald et al., 1998), the blowfly *Phormia regina* (Sacktor and Childress, 1966), the cockchafer *Melolontha melolontha* (Crabtree and Newsholme, 1970), the Colorado potato beetle *Leptinotarsa decemlineata* (Weeda, 1981), the Japanese beetle *Popillia japonica* (Hansford and Johnson, 1975), the cockroach *Periplaneta americana* (Crabtree and Newsholme, 1970) and many more. Beenakkers et al. (1983) provide a comprehensive summary of the studies on insect flight metabolism. In the African fruit beetle three distinct phases of energy metabolism can be distinguished in active flight muscle: (a) during the first minutes proline is used as the main substrate, (b) afterwards the huge glycogen reserves decrease and (c) after approximately 8 minutes the flight performance and the metabolite levels stabilize (Zebe and Gäde, 1993). In less than 60 minutes the preflight proline concentration is re-established. During the flight of the blowfly there is an increase in alanine and a comparable decrease in proline; then, after a few more minutes of flight, pyruvate, alanine and proline reach steady-state levels. In this case proline provides intermediates of the citric acid cycle (Sacktor and Childress, 1966). Very high levels of activity of proline-dehydrogenase and alanine-oxoglutarate-aminotransferase are found in the flight muscles of the cockchafer and the tsetse fly, and this supports the proposed pathway for proline oxidation. Proline metabolism during and after flight in certain insects, especially beetles, might be controlled by special neuropeptides which regulate substrate mobilisation in carbohydrate and lipid metabolism in insects (Gäde and Auerswald, 1998).

Among the amino acids of honeybees (Crailsheim and Leonhard, 1997; Leonhard and Crailsheim, in press) the non-essential amino acid proline is special. In drones, for example, proline is present in the haemolymph in higher concentrations than other amino acids, and it is used to a much greater extent in oxidative metabolism under non-flight conditions (Berger et al., 1997). Honeybees after flight were compared with bees that had flown, and indeed there was slightly less proline in the thorax after flight (Barker and Lehner, 1972b). Moreover, the interstitial fluid in the retina of the honeybee drone contains four amino acids (proline, glutamine, alanine and β-alanine) at concentrations higher than in the haemolymph. This indicates the existence of a blood-retina barrier, and thus these substrates must be transported actively (Cardinaud et al., 1994). Furthermore, there is a clear effect of photostimulation on several amino acids in the honeybee drone retina.

Tsacopoulos et al. (1994) propose proline as a second substrate for the Krebs cycle; light stimulation caused a strong decrease of both proline and glutamate, and the authors showed that ^{14}C-proline is oxidized by the mitochondria of the drone retina.

Bees are asynchronous flyers and it is commonly agreed that carbohydrates are the most important energy source for them. Nevertheless, there are hints that the honeybee might use other fuels as well. Rothe and Nachtigall (1989) reported that the RQ was significantly smaller than 1 during the final 5% of long-lasting flights. Finally, forager bees collected after flight had significantly lower proline concentrations than similar aged bees taken from the hive (Crailsheim and Leonhard, 1997). The present study was designed to test to what extent proline is involved in metabolism during long honeybee flights.

Materials and methods

Bees

Foragers (*Apis mellifera carnica* POLLMANN) from two different colonies in 2-frame observation hives at the University in Graz were used in five series of trials. Series A experiments were performed with bees from colony 1, and series B, C, and D with bees from colony 2. Bees carrying pollen from urban gardens and parks were collected from the entrance of the hive as they returned from their foraging flights. This was done with a catching-tube similar to that used by deGroot (1953). The foragers were then transferred to the laboratory, which took no more than 5 minutes. The bees' exact ages were not known, but it is unusual for foragers to be younger than 16–18 days (Rösch, 1925; Lindauer, 1952; Hrassnigg and Crailsheim, 1998).

Pre-flight preparations

Usually at the beginning of roundabout flight experiments (Gmeinbauer and Crailsheim, 1993; Panzenböck and Crailsheim, 1996) bees undergo one "emptying flight" without being fed previously. This flight forces the bee to utilize its food reserves, and the following flights are fueled by the substrates fed to them after this first flight. The pollen foragers used in our experiments had just returned from their foraging flights and usually had almost empty crops. In fact, our bees had to be fed immediately after they were collected, otherwise they would have died within a short period (Sotavalta, 1954). Accordingly, we fed each bee a measured volume of glucose before its first exhaustive flight. We defined an exhaustive flight as a flight in which all the offered substrate was utilized, as indicated by an empty crop. For a small minority of the foragers we collected, the first exhaustive flights lasted noticeably longer than those of the other bees; these bees had presumably gathered not only pollen but also nectar before we collected them, and they were excluded from the experiments.

For administering substrates by feeding or injection, bees were fixed in place on a piece of wood with two crossed needles above the petiolus. In all experiments the fuel for the first exhaustive flight was 10 μl glucose solution fed with an automatic pipette. Immediately after the end of the feeding, several foragers were caged together in Liebefelder boxes (volume 1,000 ml) for a resting period of at least 30 minutes. The fuel administered before the 2nd exhaustive flight varied with the experimental protocol, and the bees were kept isolated after feeding; they were kept in Liebefelder boxes either for 20 minutes (series B and C) or 30 minutes (series A and D) before their flight. All the bees were kept at room temperature.

In addition, as part of series A, homing foragers caught before entering their hive were immediately used for taking haemolymph samples without being fed.

Supply of substrates for the 2nd flight

In all experimental series, the glucose that was administered was an 0.8 M glucose solutions (D(+) – glucose – monohydrate, Art. 8342, MERCK). When proline was administered, a 1.5 M proline solution (L – proline, No P-0380, SIGMA) was used.

When substrates were injected, the injection was made between the 5th or 6th abdominal segment, slightly left of dorsal, with a microsyringe (Hamilton-Bonaduz-Switzerland, 10 μl) shortly after the end of the 1st exhaustive flight. The method described by Crailsheim (1992) was used, except that the wound was not sealed, because our experiments lasted such a short time. If more than one-third of the injected volume leaked out of the bee, the bee was rejected. The loss of fluid was estimated by soaking up the droplets pressed out of the wound with round pieces of filter paper (Schleicher & Schuell, 595 roundfilter, Ø 45 mm, Ref.Nr.: 311604, Lot.: AG0902-1). The size of the wet part of the filter paper was estimated and compared with reference pieces of filter paper.

Series A

This series of trials were performed to investigate changes in the amino acid concentrations in the haemolymph under different conditions of energy demand.

There were five treatment groups:

(1) bees fed 10 μl 0.8 M glucose solution, then rested for 1 hour, then sampled.
(2) foragers collected as they returned to the hive, and immediately used for haemolymph samples.
(3) bees 10 μl 0.8 M glucose solution for the 1st flight, then sampled immediately.
(4) bees fed 10 μl 0.8 M glucose solution for the 1st flight, then fed 10 μl 0.8 M glucose solution again and given a 2nd flight, but forced to stop after flying for half of the average flight time, then sampled.
(5) bees fed 10 μl 0.8 M glucose solution for the 1st flight, then fed 10 μl 0.8 M glucose solution again and given a full 2nd flight, then sampled.

The succession of the experimental steps are shown for all categories in Fig. 1.

Series C

This series of trials examined whether bees can utilize proline for flight energy, and compared flight performances of bees fed glucose only and those fed both glucose and proline.

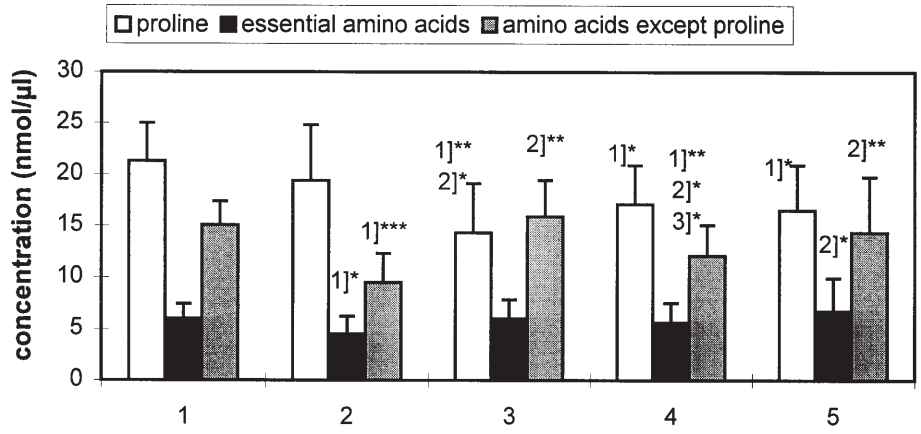
There were three treatment groups:

(1) bees fed 10 μl 0.8 M glucose solution for both flights.
(2) bees fed 10 μl 0.8 M glucose solution for the 1st flight, 6 μl 0.8 M glucose solution for the 2nd flight.
(3) bees fed 10 μl 0.8 M glucose solution for the 1st flight, a mixture of 6 μl 0.8 M glucose solution and 4 μl 1.5 M proline solution for the 2nd flight.

After the second flight, haemolymph samples were collected, and gut samples were taken to evaluate how the proline was distributed in the bee's intestine and to what extent proline reached the haemolymph.

Series D – Proline metabolism experiments

In this series of trials, bees were injected with 1 μl [U]-^{14}C-labelled proline (10.6 GBq/mmol; 1.85 MBq/0.5 ml of 0.01 N HCl from NEN) by the method described below, to



10 μl gluc fed	yes	yes	yes	yes	yes
rest 1 (min)	60	5–10	30	30	30
1 flight	no	no	yes	yes	yes
10 μl gluc fed	no	no	no	no	yes
rest 2 (min)	no	no	no	no	30
1.5 flights	no	no	no	yes	no
2 flights	no	no	no	no	yes

Fig. 1. Free amino acids in honeybee haemolymph from bees subjected to different treatments (series A, 26–28°C). The concentrations of free proline, essential amino acids and all amino acids except proline are given as nmol/μl haemolymph. The treatment conditions of groups 1–5 of bees are described directly below the colums. Values shown are means ± standard deviations; asterisks and a number in brackets indicate significances between these groups (* 0,05 ≥ p > 0,001, ** 0,001 ≥ p > 0,0001, *** p ≤ 0,0001). Shadowed cells in the below table state when the haemolymph was sampled. (n = 12–14)

determine the distribution of the administered proline in the various parts of the bees' bodies. The decrease in radioactivity in bees forced to fly was compared to the decrease in bees that rested for a period approximately as long as the flight time.

There were two treatment groups:

(1) bees fed 10μl 0.8 M glucose solution before the 1st flight, and injected with ^{14}C-labelled proline shortly before the 2nd flight. Haemolymph samples were taken after the second flight.
(2) bees fed 10μl 0.8 M glucose solution before and after the 1st flight, then injected with ^{14}C-labelled proline and rested for the average flight time in an incubator at 28.4°C. Haemolymph samples were taken after the resting period.

In addition, for some bees a droplet of haemolymph was pressed out after the injection of the ^{14}C-labelled proline. The droplet was collected on a standardized strip of filter paper (as described above) and analyzed for radioactivity to calculate the exact amount of label received per bee. The radioactivity actually injected but not recovered in the bee was assumed to have been exhaled or less probably excreted.

Series B

This series of trials, like series C, investigated whether proline can provide flight energy. In series B, however, the proline was injected directly into the haemolymph, thus bypassing the possible gut-haemolymph barrier. Furthermore, different groups of bees were injected with different substances, and we compared flight performances of the various groups. As in series C, all bees were fed 10 μl 0.8 M glucose solution before the 1st exhaustive flight.

There were six treatment groups:

(1) bees injected with 10 μl 0.8 M glucose solution after the 1st flight, then rested for 5 min, then sampled.
(2) bees injected with 10 μl 0.8 M glucose solution after the 1st flight, then rested for 20 min, then sampled.
(3) bees injected with 10 μl 0.8 M glucose solution after the 1st flight, given a 2nd flight, then sampled.
(4) bees injected with 10 μl 0.8 M glucose solution after the 1st flight, given a 2nd flight, then sampled.
(5) bees injected with a mixture of 6 μl 0.8 M glucose solution and 4 μl water after the 1st flight, given a 2nd flight, then sampled.
(6) bees injected with a mixture of 6 μl 0.8 M glucose solution and 4 μl 1.5 M proline solution after the 1st flight, then given a 2nd flight, then sampled.

These and other details are listed in the tables below Fig. 2 and in Table 2.

Flights on the roundabout

The bee was fixed on the spoke of a roundabout (length 14 cm) with a plastic tube (length 3–5 mm, diameter 2–3 mm) which was attached to the dorsal side of the thorax of bees; one rotation covered 0.88 m. The bee held a ball of paper with its legs. To induce flight, the ball of paper was removed. Bees that did not start flying within 15 minutes after the first attempt to initiate flight were rejected.

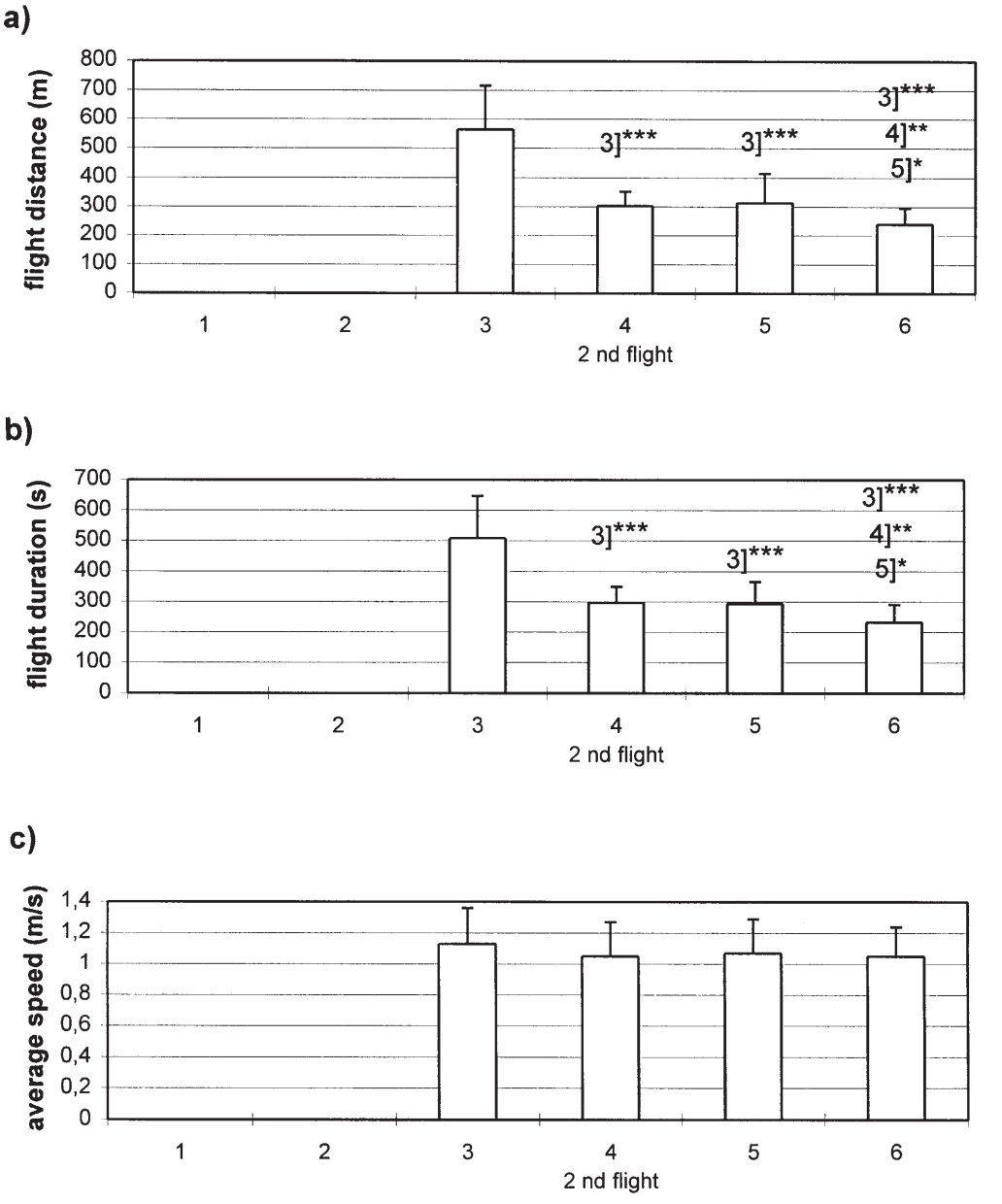
Two roundabouts were used. They were illuminated by two 60 W lamps and surrounded by a light and dark striped paperwall. The temperature was held between 25° and 31 °C but the changes within one series of trials did not exceed the range of 3 degrees. The individual temperature range of each series is specified in the figures and tables.

The number of circuits flown by each bee was recorded by an electronic counter, and the flight duration by stop-watches. After being fed, the bee was weighed before and after the flight. The difference in weight indicated how much of the flight substrate had been utilized. Depending on the experimental series, after a flight a bee either was given another flight or had its haemolymph sampled (as described below), or it was killed and its crop dissected out. An empty crop indicated an exhaustive flight.

Haemolymph samples

In the 5th or 6th abdominal segment a small incision was made in the tergite approximately 1 mm left of median by fine scissors. From there at least 1 μl haemolymph was drawn by capillary force into a calibrated 5 μl glass capillary (Blaubrand intra Mark, 5 μl) pressed lightly on the incision. Especially for the radioactive samples we tried to obtain more than 1 μl. To facilitate the collection of haemolymph, all bees that had completed their 2nd flight, including those that had been injected with radioactive proline, were fed 2 μl glucose solution and rested for 5 minutes before the incision was made.

The collected haemolymph was immediately expelled into 100 μl of ice-cold water in a 1.5 ml Eppendorf reaction vessel. We added an equal amount of acetonitrile to precipitate proteins and froze the sample at −25 °C for further analysis. Radioactive haemolymph samples were stored similarly.



10μl gluc fed	yes	yes	yes	yes	yes	yes
rest 1 (min)	30	30	30	30	30	30
1 flight	yes	yes	yes	yes	yes	yes
injection	10 gluc	10 glu	10 gluc	6 gluc	6 glu + 4 aqu	6 glu + 4 pro
rest 2 (min)	5.0	20	20	20	20	20
2 flights	no	no	yes	yes	yes	yes

Fig. 2. Flight performances of substrate injected bees in their 2nd flight (series B, 27–29°C). The treatment conditions of categories 1–6 of bees are described directly below the colums. Values shown are means ± standard deviations; asterisks and a number in brackets indicate significant differences between groups (* 0,05 ≥ p > 0,001, ** 0,001 ≥ p > 0,0001, *** p ≤ 0,0001). Shadowed cells in the table below state when the haemolymph was sampled. (**a**) Flight distance in m, (**b**) flight duration in sec and (**c**) average speed in m/sec

Gut samples

In series C, sampling included the examination of gut contents. The bees were killed and the gut was dissected into two parts: (1) the crop together with the midgut and (2) the rectum. The contents of each part were mixed with 500 μl ice-cold water and homogenized ultrasonically (Branson sonifier-B15P); 500 μl acetonitrile was added and the samples were frozen at −25°C for further analysis.

Sample preparation

Analysis of the free amino acids in the haemolymph was done by HPLC as follows. The Eppendorf reaction vessels containing the precipitated haemolymph were centrifuged at 8,000 g for 2 minutes. Then an aliquot representing 0.25 μl haemolymph was transferred into a new 1.5 Eppendorf reaction vessel and lyophilised. For greater precison, the samples of the proline-injected bees were diluted 1:20. Then the samples were dissolved in 20 μl of 100 mM $NaHCO_3$, pH 8.3, and we added 40 μl of 4 mM solution of DABS-Cl (4-Dimethylaminoazobenzene-4′-sulfonyl chloride, SIGMA D 7772) in acetonitrile. The solution was heated to 70°C in a waterbath for at least 12 minutes. Finally, after the solution cooled to room temperature, 440 μl of starting buffer (15 mM Na-citrate buffer, pH 6.4, 30% acetonitrile) were added. The gut samples were prepared in the same way, except that the amount of sample transferred to the Eppendorf reaction vessel was 250 μl, and the crop/midgut samples from proline-fed bees were diluted 1:10.

Analysis was performed on a BECKMAN System Gold chromatographic gradient system (Column: Beckman Ultrasphere XL – ODS, 4.6 × 7.0 mm, 3 μm; flow 1.2 ml/min, elution: 15 mM Na-citrate buffer, pH 6.4, 30%–100% acetonitrile, 4% DMF (Dimethylformamide), detection: photometrically at 436 nm). The separation of the free amino acids took about 20 minutes. Calculations were based on external standardization with a commercial amino acid standard (Crailsheim and Leonhard, 1997).

Radioactive samples

Each radioactive haemolymph sample was added to 100 μl ice-cold water. One half was prepared for HPLC analysis as described above. The other half was mixed with 80% ethanol, kept one hour at 4°C and centrifuged (8,000 g for 2 minutes) to test how much of the administered proline was still free in the haemolymph and how much already incorporated into protein. An aliquot of the supernatant was mixed with Hydroluma, a liquid scintillation counting medium supplied by Packard. The precipitate was solubilized with Soluene 100 (Tissue solubilizer, Packard) for at least 12 hours. The radioactivity was determined by using Hionic Flour, another liquid scintillation counting medium from Packard, counted on 1900 CA Tri-Carb-Liquid-scintillation analyzer, Packard. Samples containing ethanol were always mixed with Hydroluma, whereas the Soluene samples were mixed with Hionic Flour. Each thorax was weighed, cut into several pieces and mixed with 1 ml ice-cooled water. It was homogenized by sonification (Branson sonifier-B15P), 1 ml acetonitrile was added and the sample was centrifuged (5 minutes with 3,000 g). For HPLC analysis, 1 ml of the supernatant was separated, 0.8 μl of the solution was lyophilized and dissolved with 80% ethanol and the ^{14}C-content was determined. We estimated all ^{14}C found as being ^{14}C in proline, although some of it might have been converted already.

The remaining part of the thorax was mixed with Soluene and solubilized (and stirred) for one day, then the level of radioactivity was determined. This revealed the percentage of incorporation of proline into body protein in the thorax. The rate of incorporation into the rest of the body was determined by lumping together the dissected head and the abdomen, plus the legs and wings, and analyzing in the same way as was done for the thorax.

Statistics

In each series our sample size was between 10 and 14 bees, unless stated otherwise. In text and tables, means ± standard deviations of means are given. Differences were tested with the Mann-Whitney – U – test (Spss). The level of significance was set at $P < 0.05$, following Sachs (1972).

Results

As expected, the performances of all bees in the 1st flight were similar, as all had been fed the same amount of glucose before the flight (data not shown). A honeybee worker can fill her crop up to a weight of about 30–70 mg (Parker, 1926; Fukuda et al., 1969). In bees that were sacrificed immediately after flight, the average crop weight was recorded as 0.45 ± 0.2 mg fresh weight, of which about 0.1 mg were the crop tissue without any content. This indicated that the flights were truly exhaustive flights.

Eighteen amino acids were identified by HPLC. Proline was recorded in the highest concentrations, normally about 20 nmol/μl and artificially increased in haemolymph to about 150 nmol/μl. Most of the other free amino acids were present in much lower concentrations: a few, such as tryptophan, only in trace amounts (<0.01 nmol/μl). The data given are means ± standard deviations (nmol/μl haemolymph).

Series A – Is the proline level in haemolymph reduced during honeybee flight? (Table 1, Fig. 1)

The bees that were forced to stop halfway through the 2nd flight (1.47 ± 0.83 mg crop weight after stopping), had an average speed (1.26 ± 0.16 m/s) quite similar to that of bees finishing the 2nd flight (1.17 ± 0.25 m/s; Table 1). The temperature range for this series was between 26 and 28°C.

Bees fed 10 μl 0.8 M glucose solution and caged for one hour (treatment group A1) had a proline content of 21.3 ± 3.7 nmol/μl, which was higher than that of bees that completed their 2nd flights on the roundabout (treatment groups A3, A4 and A5). The lower proline level after flight was not due to a changed haemolymph volume, because the concentrations of all amino acids except proline and the essential amino acids did not differ in treatment group

Table 1. Flight parameters (distance, duration and speed) of the 2nd flight; series A (10 μl glucose solution *fed*, 26–28°C) compared to series B (10 μl glucose solution *injected*, 27–29°C) Asterisks indicate significant differences (* $0.001 < p \leq 0.05$; ** $0.001 < p \leq 0.001$); *n* indicates the number of bees

n	Fuel offering method	Distance (m)	Duration (s)	Speed (m/s)
12	fed	775 ± 212,4	671 ± 179,9	1.17 ± 0,25
14	injected	564 ± 152,3	508,2 ± 139	1.16 ± 0,25
		**	*	

1 compared to groups A3, A4 and A5. Bees that completed the 1st flight had lower amounts of proline in the blood than foragers just returned to the hive (group A2). Bees that were fed after returning to the hive and then rested for one hour (group A1) showed a slight increase in essential amino acids compared to group A2, and there was a difference in the essential amino acids between groups A2 and A5. The concentrations of all amino acids except proline were considerably decreased in just returned foragers (group A2), compared to bees that rested for one hour (group A1) and bees that took tethered flights (groups A3, A4, and A5) (Fig. 1). Alanine levels were never higher in bees that finished flights on the roundabout (groups A3 and A5) than in the controls (group A1) (data not shown). Just if bees were stopped after half of their flight there was a significant increase in the alanine concentration compared to the bees rested for one hour.

Series B – Does injected proline change the flight performance? (Fig. 2, Table 2)

In series B the roundabout temperatures were between 27 and 29°C. Bees that had been injected with 10μl glucose solution (group B3) covered a flight distance of 563.95 ± 152.28 m and flew for 508.21 ± 139.04 s, both figures highly significantly greater than bees in treatment groups B4, B5 and B6. The average speed was almost the same in the various groups. The flight performance of bees that were injected with a glucose-proline solution (group B6, crop weight of 0.93 ± 0.71 mg) was reduced compared to bees injected with the same amount of glucose but no proline (groups B4 and B5) (Fig. 2).

In bees that rested for five minutes following the 1st flight and an injection of 10μl glucose solution (group B1), the proline content was 11.43 ± 2.95 nmol/μl, significantly lower than bees in all other groups. The concentrations of proline, essential amino acids and all other amino acids except proline are given in Table 2. The levels of essential amino acids of those bees that were injected with glucose solution and rested for 5 or 20 minutes and did not start a 2nd flight (groups B1 and B2) were significantly lower than in all groups from series C, which were fed glucose solution and underwent 2nd flights. Furthermore, almost all injected bees had significantly reduced amounts of all amino acids except proline compared to fed bees (6 or 10μl glucose solution). This might be an effect of haemolymph dilution by injection. In group B6 bees, 138.08 ± 17.1 nmol/μl haemolymph were recovered. Assuming but slightly underestimation the total haemolymph volume in foragers as 16μl (Crailsheim, 1985), this means that not more than 37% of the injected proline was recovered in the blood.

Series C – Does fed proline change the flight performance of bees? – What happens with large amounts of proline ingested by the bee? (Table 1)

Bees that were fed the glucose-proline solution before the 2nd flight (group C3) had flights that were significantly shorter in distance (248.89 ± 61.01 m)

Table 2. Amino acids concentration (nmol) per μl haemolymph in foragers rested or given a 2nd flight on the roundabout. Flight substrates were directly injected into the blood and different among the treatment groups, as described in the row titled "injection" below (series B, 27–29°C). Values are given as means ± standard deviations Asterisks indicates significant pairwise differences between the two groups (* $0{,}001 < p \leq 0{,}05$; ** $0{,}0001 < p \leq 0{,}001$; *** $p \leq 0{,}0001$). Treatment conditions of the groups are stated below. *su ess* all essential amino acids, *su-pro* all amino acids except proline, *gluc* 0.8 M glucose solution, *aqu water*, pro 1.5 M proline solution, *n* number of bees)

Group nr.	1	2	3	4	5	6
10μl gluc fed	yes	yes	yes	yes	yes	yes
rest 1 (min)	30	30	30	30	30	30
1 flight	yes	yes	yes	yes	yes	yes
injection	10 gluc	10 gluc	10 gluc	6 gluc	6 gluc + 4 aqu	6 gluc + 4 pro
rest 2 (min)	5	20	20	20	20	20
2 flights	no	no	yes	yes	yes	yes
n	14	13	13	13	13	12
			concentration nmol/μl haemolymph			
proline	11.43 ± 2.95	16.24 ± 4.18	15.68 ± 2.98	14.63 ± 4.06	14.39 ± 3.31	138.08 ± 17.1
su ess	4.7 ± 1.76	5.0 ± 1.6	6.82 ± 2.05	5.34 ± 2.22	6.38 ± 2.05	4.06 ± 1.5
su-pro	6.74 ± 2.23	7.31 ± 2.51	9.98 ± 3.45	7.59 ± 2.75	9.38 ± 3.12	8.95 ± 6.28
significances						
proline	1–2**; 1–3**; 1–4**; 1–5*; 1–6***; 2–6***; 3–6***; 4–6***; 5–6***					
su ess	1–3*; 2–3*; 2–5*; 3–4*; 3–6**; 5–6*					
su-pro	1–3**; 1–5*; 2–3*; 3–4*; 4–5*					

than bees that were fed 10μl glucose solution (group C1, 582.3 ± 173.06 m). Also, their flights were of shorter duration (241.09 ± 58.25 s) than those of the other group of bees fed 6μl of the glucose solution, group C2 (367 ± 68.15 s), but the two groups did not differ significantly in total distance flown, because the bees in group C3 had a slightly greater average speed (1.02 ± 0.12 m/s) than those of group C2 (0.91 ± 0.23 m/s). However, the crop filling of the bees of group C3 was not as low as described above (0.45 ± 0.2 mg), but was approximately 1.5–2μl (as estimated from the crop size). The temperature during this series was between 28 and 31°C.

When bees from one colony fed glucose were compared to bees from another colony injected with the same amount of glucose (group A5 compared to group B3), the injected bees had significantly shorter flights (Table 1). But among bees from the same colony, feeding glucose (series C) did not result in significantly different flight performance than injection glucose (series B) (data for series B shown in Fig. 2, data not shown for series A).

Feeding a glucose-proline solution significantly raised the proline content in the haemolymph compared to feeding glucose alone (38.63 ± 13.14 nmol/μl

in bees fed glucose-proline compared to 13.61 ± 3.63 nmol/μl for bees fed 10 μl glucose, and 13.69 ± 4.16 nmol/μl for bees fed 6 μl glucose). Furthermore the amounts of essential amino acids and all amino acids except proline were similar (data not shown).

In bees fed 10 μl glucose solution (group C1), the proline content in the crop and midgut together was 68.85 ± 10.29 nmol, and in the rectum it was 12.01 ± 7.66 nmol. The values for the bees fed 6 μl glucose were similar in all cases (data not shown). In contrast, bees fed glucose-proline fuel (group C3) had proline levels of 2,777.28 ± 1,085.71 nmol/crop-midgut, which corresponds to up to 60% of the fed solution, and 114.20 ± 113.94 nmol proline were found in the rectum. Hence, between 50 and 80% of the fed solution was recovered in the gastrointestinum of the bee; and up to 20% was in the haemolymph. The more proline was found in the bee, the more was found in the rectum (up to 70%) (data not shown).

Series D – What is the fate of injected ^{14}C – proline in the bees? Is the proline content decreased in thorax extracts during flight? (Fig. 3, Table 3)

The temperature range for this series was between 28 and 30°C. In both groups, those given a second flight (group D1) and those rested (group D2), most of the radioactivity from the injected ^{14}C-labelled proline, about 37%, was found in the abdomen plus the wings and legs (Fig. 3). Only in the thorax was the radioactivity split up to quantify the proline incorporated into thorax protein and the proline still free. The rested bees had significantly more proline than bees that underwent a second flight. In the smallest part of the body, the caput, the lowest amounts of proline were found, 11.23% for bees with flights versus 13.24% for rested bees.

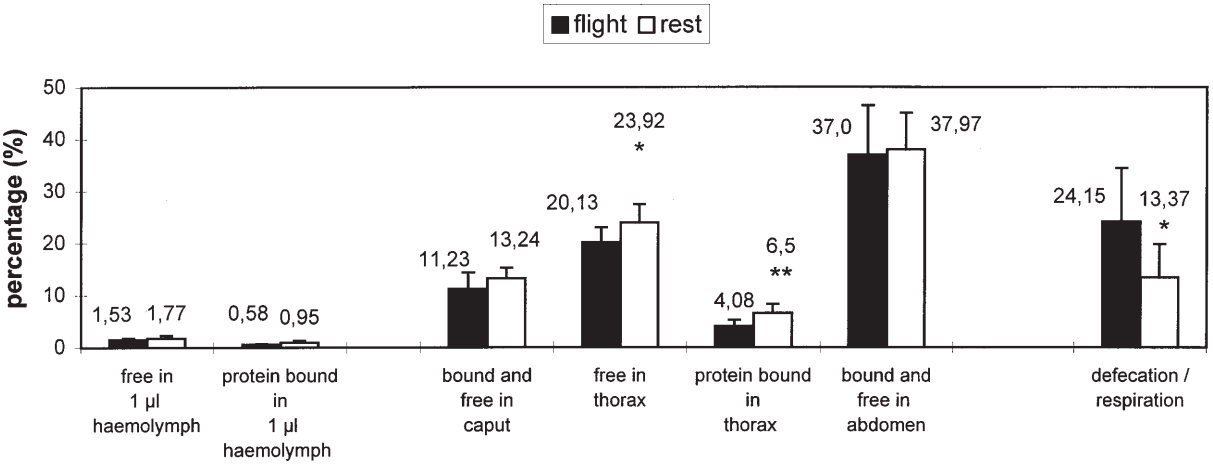


Fig. 3. Distribution of ^{14}C in bees injected with 1 μl ^{14}C-labelled proline, then given either a short flight (black columns, n = 11) or a resting period of equal duration (white columns, n = 12). Columns given are means ± standard deviations of the percentages of radioactivity; asterisks above the column indicate significant differences (* 0,05 ≥ p > 0,001, ** 0,001 ≥ p > 0,0001)

Table 3. Free amino acids in thorax extracts from honeybees injected with 1 μl ^{14}C-labelled proline shortly before a 2nd exhaustive flight (which lasted about 7 minutes) or a resting period of the same duration in an incubator at 28.4°C (series D, 28–30°C). Values are given as means ± standard deviations. Brackets indicate significances. *su ess* all essential amino acids *su-pro* all amino acids except proline, *n* number of bees

n	bees	Concentration (nmol/thorax)		
		proline	su ess	su-pro
10	flown	451,3 ± 121,8	[240,7 ± 86,6	[554,3 ± 127,3
11	rested	515,6 ± 170,7	[297,3 ± 39,6	[856,3 ± 89,8

We also investigated the percentage of ^{14}C-proline bound in haemolymph protein and the amount as free proline. The results showed the same trends as for the thorax samples. Higher amounts of ^{14}C-proline, free or protein-bound, were recovered in rested bees than in those that had flown, though the differences were not significant. The percentage of missing radioactivity, considered to be exhaled or defecated, was significantly higher in bees that had flown than in rested bees.

Unlabelled free amino acids were compared in thoraces of bees that had flown (group D1) and those that had rested (group D2), using HPLC analysis. Due to the low actual proline concentration (pmol) in 1 μl injected amino acid, this amount was in fact negligible in both groups of bees. Group D1 bees had less proline, but the difference was not significant (Table 3). But considering the total of all amino acids except proline, the bees that had flown did have significantly less than those that had rested ($p = 0.02$; Table 3) which indicates that the flight resulted in a change in the haemolymph and body fluid volume (data not shown).

The sum of all essential amino acids and the sum of all other amino acids except proline were both significantly greater in bees that had rested (Table 3). The amounts of isoleucine, tryptophane and lysine were greater in bees that took the second flight. There were no significant differences in the proline, valine, methionine, leucine and phenylalanine concentrations. Alanine and all other amino acids except those mentioned above were lower in bees that took the flight (data not shown).

Discussion

Free amino acids in the haemolymph

As an evolutionary tendency free amino acids appear in rising amounts as haemolymph constituents in most evolved groups such as Lepidoptera, Hymenoptera (to which honeybees belong) and Coleoptera (Florkin and Jeuniaux, 1974). The haemolymph of holometabolous insects contains at least 22 amino acids whereas hemimetabolous insects lack a few of them and generally have amino acids generally in lower concentrations (Pant and Agrawal, 1964). The free amino acids in the blood participate in

osmoregulation and buffering of the blood to some extent; but their main function is certainly to serve as units for protein synthesis or further metabolism.

Diet experiments of deGroot (1953) showed that ten amino acids are essential for the growth of honeybees. These are arginine, histidine, lysine, tryptophane, phenylalanine, methionine, threonine, leucine, isoleucine and valine, and the bees have largest requirements for the last three of these. Proline is synthesized from glucose and can also be formed from arginine.

In insects special intestinal transport mechanisms for amino acids have been reported, whereas sugars seem to be transported by simple diffusion (Crailsheim, 1988b). The slow movement of food due to the presence of numerous layers of peritrophic membranes within the midgut could facilitate amino acid absorption. Peritrophic membranes are produced by cells on the entire length of the midgut (Trappmann 1923; Barker and Lehner, 1972a) and generate compartments for food transport. Pollen, the main protein source for honeybees, is transported within these membranes whereas other components can be found in the ecto- or endoperitrophic space where the amount of proteolytic enzymes is age-dependent (Moritz and Crailsheim, 1987). Haszonits and Crailsheim (1990) found a rather specific carrier mechanism for leucine. Their in vitro experiments showed that the uptake of leucine involves no energy dependency (i.e. facilitated diffusion). The transport mechanisms for proline might be similar.

In our experiments bees after tethered flights had reduced levels of proline in the haemolymph compared to rested bees. This could be due to a depletion during flight. Mostly there were no remarkable changes in the essential amino acids or in the sum of all amino acids except proline; which is evidence against haemolymph volume changes. The results of trials involving the injecting of substrates tended to agree with the previously mentioned trends. After the injection of solutions the blood was somewhat diluted, as demonstrated by the significantly reduced concentrations of the 2 control parameters, in bees injected and then rested for 5 or 20 minutes compared to bees that had flights after being either injected or fed. But in bees fed the glucose-proline solution the concentrations of free amino acids in the haemolymph were similar to those in solution-injected bees. We conclude that these bees may possibly regulate their haemolymph osmolarity.

Tethered flights

For the flight speed of free foraging workers values of 8.2 m/s (v. Frisch, 1965) and 7.8 m/s (Heran and Crailsheim, 1988) have been reported. The mean average speed on the roundabout is about 2 m/s but increases directly with the concentration of the offered food (Gmeinbauer and Crailsheim, 1993).

Referring to the results of various authors Nachtigall et al. (1989) estimated higher energy need for free flight compared to tethered flight (0.4 W/s versus 0.3 W/s); probably because in tethered flight only a relatively small

vertical force is needed to compensate for body weight. However, by calculating oxygen requirements from the amount of sugar utilized by workers, Gmeinbauer and Crailsheim (1993) concluded that the two different types of flights do not demand considerably different amounts of energy. Consequently, data obtained from flights on the roundabout may be extended, at least partly, to free flight.

It is already known that proline is involved into the flight metabolism of honeybees to no greater extend than 0.1% (Barker and Lehner, 1972b) because the haemolymph amino acid concentration is that low and the availability of carbohydrates in the blood determines the amount of amino acids utilized as metabolic fuel, so that large effects on flight cannot be expected. It is commonly agreed that honeybees mainly utilize haemolymph sugars for flight energy (Jongbloed and Wiersma, 1935).

Loh and Heran (1970) investigated bees that were fed and injected a saccharose solution. They found that saccharose-nourished bees had diminished flight efficiency compared to glucose- or fructose-injected bees in tethered flight, and that the manner of fuel delivery had no influence on distance and duration in the flight mill. In contrast, in our experiments there were significant differences in flight performances of fed (series A) and injected (series B) bees. Possibly this was due to the fact that these bees were taken from 2 different colonies, and one of the colonies (series A bees) were preparing to swarming. Furthermore, comparisons between series B (injected substrates) and series C (fed substrates) results, using bees taken from the same colony, were in agreement with the findings of Loh and Heran: there were no significant differences in the flight parameters.

Bees injected with glucose-proline solution had significantly reduced flight distance and flight duration compared to bees fed the same amount of carbohydrate. So far we have no explanation for this effect. It is rather unlikely that a different blood osmolarity, usually at 500–600 mosmol (Crailsheim 1985) increased by injection of proline by about 20–30 mosmol, might be responsible for changed flight habits, because similar concentrated injections of glucose did not result in any negative impact on flight performance. Moreover bees that got the proline all seemed to be unable to fly at the onset of starting trials (20–30 minutes after fuel was injected). They were sluggish, so perhaps a substrate surplus inhibition appeared.

When the same amount of glucose-proline solution was fed, the results were similar but this time the diminished performances could possibly be explained in another way. A rise in the osmolarity and proline concentration in the haemolymph due to the transport of considerable amounts of proline from the midgut might have resulted in closing the proventriculus (Crailsheim, 1988a), so that not all of the provided fuel could pass from the crop to the midgut. Consequently not all the fed glucose could be utilized for flight activities. This corresponds quite well with the crop-filling-rates of these bees after terminating their flights (estimated at 1.5 to 2μl).

If the glucose-proline solution was fed, after a rest of 20 minutes and a flight, the majority of the fed proline was still in the entire gastrointestinum; the more proline there was in the gastrointestinum and haemolymph (about

20%), the more was found in the rectum (up to 70%). A mean of 20% of the fed proline could not be found in the bee's body. In the experiments with ^{14}C-proline (discussed below) 20% of the injected proline was missing after flight, but in those trials the proline was injected directly into the blood, so 100% of it was immediately available for metabolism and therefore the situation was not directly comparable.

Distribution of radioactive proline in the bee

The results of the experiments with [U]-^{14}C-proline also indicate proline utilization during flight. Significantly lower amounts of radioactivity were found either incorporated into thorax protein or free in the thorax extracts of bees that had flown. It appears that in the thorax, where flight muscles utilize substrates for flight energy and actively increase temperature during flight, proline is lost, presumably for energy gain. Proline is probably not extensively used in the flight metabolism, according to hints in the literature, but could be used for oxidative breakdown in foragers, as it is in drones (Berger et al., 1997). This conclusion is supported by the higher level of missing proline in bees that had flown versus those that had rested (24% versus 13%). The considerable amount of exhaled (or possibly defecated amino acid) corresponds well with results of other investigations at our institute. Our experiments showed that approximately 7μg proline for rested and 10μg proline for bees that flew were utilized by exhalation in 30 minutes, according to the calculation method of Berger et al. (1997).

Proline in general

In many insects high concentrations of proline in the haemolymph are characteristic. In their flight muscle proline is reversible transformed to glutamate by proline dehydrogenase and Δ^1-pyrroline-5-carboxylate-dehydrogenase (Brosemer and Veerabhadrappa, 1965). Glutamate is further oxidized to α-ketoglutarate by glutamate-dehydrogenase. Therefore proline can enter the tricarboxylic acid cycle. But because in bees there are low titers of proline dehydrogenase (Crabtree and Newsholme, 1970) and no glutamate dehydrogenase at all, this pathway is still uncertain and speculative.

A clear and consistent effect of photostimulation on the level of several amino acids in the drone retina has been measured. Similar declines in proline (51%) and glutamate (48%) are accompanied by an equivalent increase of alanine (56.8%); in this context proline obviously provides energy in honeybees (Tsacopoulos et al., 1994). In contrast, we did not find that a haemolymph proline decrease after finished long honeybee flights was followed by an equivalent increase in the alanine concentration. But if the bees were stopped after half of their flight (3 to 4 minutes) there was a significant increase in the alanine concentration compared to the bees that were rested for one hour. Further investigations are necessary to determine possible

alanine and proline changes in the haemolymph in the starting period of honeybee flight.

Conclusion

In our experiments proline offered in high amounts did not enhance flight performance at all. Secondly the proline concentration in the haemolymph as well as in the thorax was significantly reduced after flight (taking into account the changes in haemolymph volume) compared to the levels in rested bees, which seems to indicate some proline depletion due to flight efforts. Thirdly significantly lower amounts of labelled proline were incorporated into thorax body protein of bees that had flown, probably due to the higher concentration in the haemolymph; and in the same bees significantly lower amounts of ^{14}C not bound in proteins were recovered in the thorax extracts, which is further evidence for some utilization during flight.

Perhaps shorter flights, maybe just the early stage of flights (as for *Pachnoda sinuata*, Zebe and Gäde, 1993) or just the first few minutes when bees start heating, would cause more noticeable proline utilization. Also, proline may be utilized at the end of exhaustive flights (Rothe and Nachtigall (1989) observed a RQ below 1 during this phase). Further investigation might answer these questions, and may reveal special transport mechanisms concerning proline (and other amino acids) and define the role of proline at the mitochondrial level.

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