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Amino acids and osmolarity in honeybee drone haemolymph

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Summary. In the haemolymph of honeybee drones, concentrations of free amino acids were higher than in worker haemolymph, with different relative proportions of individual amino acids. The overall concentration of free amino acids reached its highest level at the 5th day after adult drone emergence, and after the 9th day only minor changes in the concentration and distribution of free amino acids were observed. This coincides with the age when drones reach sexual maturity and change their feeding behaviour. Levels of essential free amino acids were high during the first 3 days of life and thereafter decreased. Osmolarity was lowest at emergence (334 ± 41 mOsm), increased until the age of 3 days (423 ± 32 mOsm) and then stayed generally constant until the 16th day of life. Only 25-day-old drones had significantly higher osmolarity (532 ± 38 mOsm). The overall change in osmolarity during a drone's lifetime was about 40%.

Keywords: Amino acids – Apis mellifera – Drones – Osmolarity – Age dependency

Introduction

Honeybee drones have been investigated far less thoroughly than honeybee workers, especially with respect to their physiology. Marked differences between workers and drones have been found in glycogen content (Panzenböck and Crailsheim, 1997), pollen consumption and digestion (Szolderits and Crailsheim, 1993), and glucose utilisation during flight (Gmeinbauer and Crailsheim, 1993). This is not surprising, as the roles of workers and drones in honeybee colonies are very different.

Adult workers procure, store and distribute all the food for the larvae, the queen and the drones, as well as for themselves and each other; provide and defend the nest structure and construct the internal housing and maintain the internal climate and sanitary conditions of the colony. In contrast, drones are not known to perform any tasks inside the nest. Their only recorded social function is to mate with queens. This happens outside the nest, mostly in drone congregation areas, where they compete for queens (Ruttner, 1989;

Königer, 1988). Drones are not present in the colony at all times of the year (Free and Williams, 1975).

Most of what is known about drone physiology concerns adaptations for finding queens and mating. Drone eyes are much bigger than worker eyes and are highly sensitive to small objects (Vallet and Coles, 1993), such as a flying queen. Drones are appreciably bigger than workers, in part due to their well developed sexual organs. At emergence the spermata are still in the testes and final differentiating processes are still taking place; for example, excess cytoplasm is detaching from the sperm (Pabst and Pfeiler, 1994). Sperm motility and production of mucus increase until the 8^{th} to 9^{th} day of adult life, leading finally to sexual maturity (Mindt, 1962).

Younger drones up to 4 days of age typically do not feed themselves, although they are able to do so if the need arises (Ruttner, 1966). They are fed protein-rich jelly and pollen by worker bees (Mindt, 1962; Crailsheim, 1991; Crailsheim, 1992). Drones 4–7 days old feed themselves from reserves stored in the colony, but are also fed and cleaned by workers. Older drones feed themselves from stored food in the colony, mainly honey (Free, 1957), which is rich in carbohydrate but comparatively poor in protein and amino acids. At the time of the transition from protein-rich food to carbohydrate-rich food, the proteolytic activity in the midgut is reduced dramatically (Szolderits and Crailsheim, 1993). Because the quality of food strongly influences the content of free amino acids and protein in the haemolymph of worker bees (Crailsheim et al., in preparation) and longevity of workers also depends on the supply of pollen (Maurizio, 1954), we were interested in investigating age-related changes in the free amino acids in drone haemolymph. Furthermore, we have shown that proline is a main constituent in worker haemolymph and may play a role in flight metabolism (Crailsheim and Leonhard, 1997). As it is established that proline is oxidised readily in drones (Berger et al., 1997) and the energy supply to the drone retina is at least in part sustained through proline (Cardinaud et al., 1994; Tsacopoulos et al., 1994; Tsacopoulos, 1995), the age-related development of this amino acid in drone haemolymph was of special interest. We monitored osmolarity in the haemolymph because free amino acids constitute an appreciable fraction of osmotically active substances in honeybee haemolymph and it is known that in some insects osmolarity is in part regulated through the concentration of amino acids (Florkin and Jeuniaux, 1974; Garret and Bradley, 1987; Nakayama, 1991).

Material and methods

Animals

The drones we used came from various colonies of *Apis mellifera carnica* Pollmann kept in an urban garden. They were collected between 2^{nd} and 22^{nd} of July 1996, the time of year when drones are most readily reared by workers (Weiss, 1962; Free and Williams, 1975).

To obtain defined age classes of drones, drone brood combs with sufficient emerging brood were taken from the donor colonies and kept in an incubator at 35°C and approximately 60% relative humidity. Drones that emerged overnight were marked individually to indicate the day of emergence and the recipient colony. Marked drones were inserted into three typical queenright colonies and were collected and analysed 1, 3, 5, 7, 9, 12, 16 and 25 days after insertion.

Collection of haemolymph samples

Drones 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. This method was tested beforehand on worker bees (Crailsheim and Leonhard, 1997). Narcotised drones were punctured between the 3rd and 4th abdominal segments slightly left of mid-dorsal. The incision was made with a pair of surgical eye scissors. From the wound 1.5–4 μl haemolymph was drawn by capillary action into a calibrated 5 μl glass capillary.

Sample size for each age class was 8 individual drones from each of the 3 colonies, except that in three cases only 7 marked individuals could be recovered.

Sample preparation

In cases where the volume of haemolymph in the sample was 2 μl or more, the sample was immediately expelled into a 1.5 ml Eppendorf reaction vessel with enough distilled water to make a 10-fold volume of solution. Samples of less than 2 μl were made up into a 20-fold volume of solution. After thorough vortexing, 10 μl of the solution (representing 1 μl haemolymph resp. 0.5 μl in samples with haemolymph volumes under 2 μl) were taken for measurement of osmolarity.

The remaining haemolymph sample was supplemented with distilled water to a volume of 100 μl, mixed with an equal amount of acetonitrile and centrifuged at 8,000 g for 3 min. From this supernatant an aliquot representing 0.25 μl of drone haemolymph was transferred into a new 1.5 ml Eppendorf reaction vessel and lyophilised immediately or frozen at −70°C to be used for analysis of free amino acids.

Analysis of free amino acids

Lyophilised 0.25 μl haemolymph aliquots were redissolved in buffer and derivatized with DABS-Cl (4-(dimethylamino)-azobenzenesulfonyl chloride). Amino acid analysis was performed by reversed phase HPLC with the method described in Crailsheim and Leonhard (1997).

Measurement of osmolarity

Osmolarity in drone haemolymph was determined with a Fiske 110 Osmometer (Fiske Associates, 1,000 Highland Avenue, Needham Heights; Massachusetts 02194, USA) by measuring freeze point depression in 10 μl of diluted samples. Calibration was in the range of 0 to 850 mOsmol.

Haemolymph samples diluted 1:10 with distilled water, or diluted 1:20 in cases where less than 2 μl haemolymph could be obtained, were kept on ice between haemolymph collection and measurement of osmolarity (max 1 h).

Statistical analysis

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

Results

Free amino acids in the haemolymph

The mean values of free amino acid concentrations for individuals from three colonies at each age (n = 23–24) are given in Table 1. In addition to the amino acids listed in the table, two DABS derivatives were resolved in the chromatograms but could not be identified. Their approximate concentrations were calculated with an average response factor from the other amino acids and never exceeded 4% of the total free amino acid content. The most abundant amino acid in all samples was proline. The average content rose from 18.7 mM in 1-day-old drones to a maximum of 36.3 mM in 7-day-old drones. Thereafter the content continuously decreased with age, to 26.5 mM in 25-day-olds. In 1-day-old drones lysine was the second most concentrated amino acid at 8.5 mM, but its concentration declined to 0.35 mM by day 7. Thereafter the lysine content stayed low at 0.45 to 0.15 mM.

The maximum total concentration of all free amino acids was 90.5 mM, reached on the 5th day after adult emergence. Essential free amino acids, as categorised for workers (Groot, 1953), were at their highest level at the 1st and 3rd day at 23.9 mM. The level decreased to 8.8 mM by the 7th day and did not change significantly thereafter through the 25th day.

A comparison of the free amino acids in 5-day-old drones and 5-day-old workers showed significant differences in the levels of all amino acids except tryptophane and tyrosine (Fig. 1).

Osmolarity of the haemolymph

The mean values for haemolymph osmolarity of drones of different ages are given in Table 1. The haemolymph was measured at 334 mOsmol on the 1st day of age and increased to 423 mOsmol by day 3. Thereafter osmolarity did not change significantly until day 16, except for a significant increase from the 5th to the 7th day. Drones at the age of 25 days had a mean osmotic pressure of 532 mOsmol, significantly higher than drones of any other age. The total increase of osmolarity over the period from the 1st to the 25th day after emergence was nearly 40%. The contribution of free amino acids (expressed as the quotient of molarities) was 18% on day 1, 20% on day 3 and about 10% on day 25.

Discussion

Concentrations of selected free amino acids in honeybee drone haemolymph were measured by Cardinaud et al. (1994), but the ages of the drones were not precisely given (they were up to 3 weeks old, and kept with sucrose-fed workers). Those data are in general agreement with the range of concentrations we found (Table 2), especially in light of the fact that, in worker bees, titres of free amino acids can vary over a wide range among individuals (Crailsheim and Leonhard, 1997).

Table 1. Content of free amino acids and osmolarity in the haemolymph of drones of different age. Cited are mean values and standard deviations for 23–24 individuals from 4 colonies at each datapoint. Free amino acids were measured by reversed phase HPLC and osmolarity through freeze point depression in diluted samples. Asterisks indicate amino acids reported as essential for workers

Free amino acids and osmolarity in the haemolymph of honeybee drones

Amino acid nmol/μl	1 day mean	std	3 days mean	std	5 days mean	std	7 days mean	std	9 days mean	std	12 days mean	std	16 days mean	std	25 days mean	std
Asp	0.04	*0.07*	0.71	*0.18*	1.12	*0.61*	0.53	*0.12*	0.33	*0.08*	0.18	*0.08*	0.10	*0.06*	0.11	*0.15*
Glu	0.51	*0.79*	3.28	*0.68*	5.75	*1.85*	4.54	*0.77*	3.13	*0.60*	1.77	*0.48*	1.04	*0.42*	1.17	*0.33*
Gln	3.91	*2.51*	6.48	*0.87*	11.79	*1.88*	11.26	*2.20*	14.68	*2.83*	13.82	*3.30*	12.91	*2.05*	11.89	*3.01*
Asn	0.45	*0.20*	0.31	*0.12*	0.14	*0.12*	0.07	*0.11*	0.01	*0.03*	0.06	*0.13*	0.06	*0.09*	0.05	*0.09*
Ser	1.85	*0.57*	2.62	*0.29*	3.13	*0.62*	2.22	*0.60*	2.35	*0.47*	2.23	*0.61*	1.84	*0.36*	1.66	*0.44*
Thr*	1.44	*0.53*	2.03	*0.24*	1.98	*0.34*	0.99	*0.23*	0.80	*0.22*	0.37	*0.10*	0.16	*0.07*	0.27	*0.12*
Arg*	2.32	*1.55*	2.60	*0.31*	4.25	*1.13*	3.61	*2.42*	7.72	*3.46*	6.97	*3.63*	5.38	*4.09*	5.81	*3.73*
Gly	3.43	*1.05*	3.55	*0.42*	2.81	*0.38*	1.71	*0.36*	1.54	*0.29*	1.51	*0.40*	0.86	*0.20*	1.13	*0.23*
Ala	3.66	*1.15*	5.11	*0.43*	4.63	*0.94*	4.03	*0.83*	4.27	*0.86*	4.72	*1.05*	3.67	*0.84*	3.15	*0.98*
Pro	18.67	*3.84*	28.66	*2.39*	34.50	*5.55*	36.30	*4.55*	30.95	*4.16*	29.72	*9.06*	23.13	*4.08*	26.50	*4.70*
Val*	3.65	*1.35*	3.20	*0.99*	2.41	*0.61*	0.84	*0.24*	0.73	*1.07*	0.49	*0.25*	0.26	*0.19*	0.31	*0.37*
Met*	0.24	*0.12*	0.17	*0.03*	0.22	*0.08*	0.38	*0.10*	0.52	*0.14*	0.72	*0.22*	0.37	*0.17*	0.31	*0.16*
lle*	1.46	*0.53*	0.99	*0.13*	0.71	*0.11*	0.37	*0.09*	0.31	*0.08*	0.28	*0.07*	0.15	*0.08*	0.29	*0.23*
Leu*	1.79	*0.65*	1.89	*0.28*	1.48	*0.33*	0.65	*0.21*	0.38	*0.12*	0.32	*0.11*	0.15	*0.06*	0.31	*0.30*
Try*	0.34	*0.17*	0.25	*0.05*	0.11	*0.06*	0.00	*0.00*	0.27	*0.21*	0.09	*0.17*	0.00	*0.01*	0.08	*0.14*
Phe*	1.58	*0.53*	1.23	*0.15*	0.70	*0.26*	0.25	*0.09*	0.10	*0.05*	0.07	*0.08*	0.01	*0.02*	0.12	*0.17*
Cys	0.03	*0.05*	0.00	*0.00*	0.77	*1.55*	0.74	*0.72*	0.00	*0.00*	0.57	*0.59*	0.27	*0.36*	0.10	*0.26*
Lys*	8.46	*4.20*	7.47	*1.77*	2.52	*0.82*	0.35	*0.25*	0.32	*0.16*	0.23	*0.11*	0.15	*0.10*	0.46	*0.45*
His*	2.59	*1.10*	4.04	*0.39*	3.83	*0.52*	1.39	*0.28*	1.86	*0.44*	2.02	*0.53*	1.50	*0.26*	2.03	*0.47*
Tyr	4.34	*1.87*	3.56	*2.84*	1.09	*1.41*	0.17	*0.20*	0.19	*0.15*	0.46	*0.42*	0.04	*0.10*	0.11	*0.19*
ess. As	23.86	*10.74*	23.88	*4.33*	18.20	*4.25*	8.84	*3.91*	13.03	*5.94*	11.57	*5.28*	8.14	*5.05*	9.98	*6.13*
Sum	60.77	*12.71*	83.30	*6.04*	90.50	*10.98*	70.42	*8.91*	70.48	*9.60*	66.60	*13.61*	58.20	*9.52*	55.85	*10.37*
Osmolarity mOsm	334	*41*	423	*32*	438	*29*	470	*46*	450	*22*	471	*32*	465	*42*	532	*38*

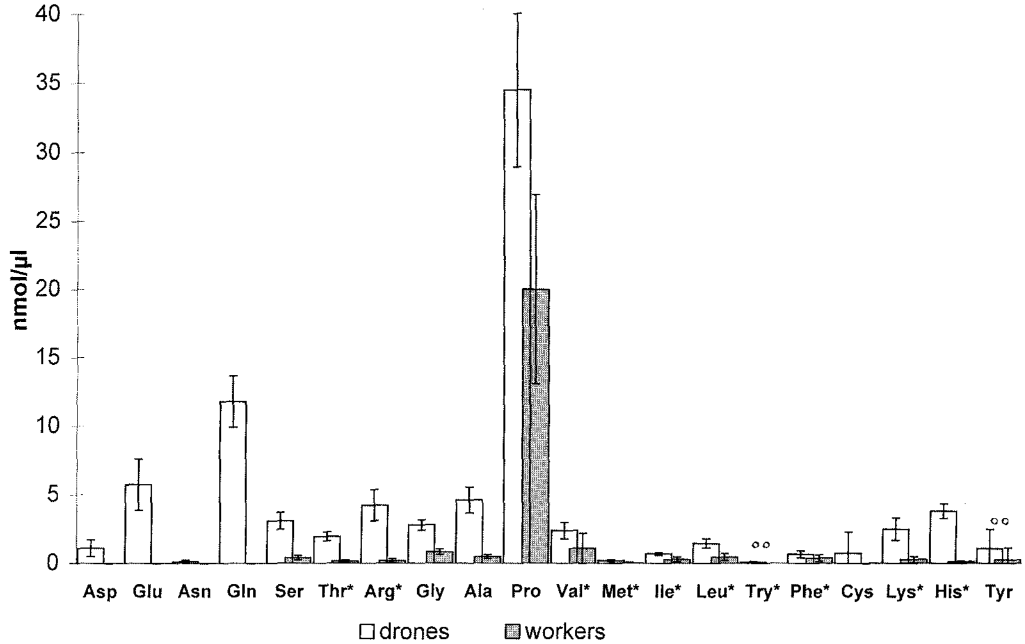

Fig. 1. Comparison of the free amino acid content in the haemolymph of 5 day old drones and 5 day old workers. Data given for workers are cited from Crailsheim and Leonhard (1997). Open circles indicate the only two amino acids that do not differ significantly (n = 34–37 for workers, n = 23–24 for drones. Asterisks indicate amino acids essential for workers

Table 2. Comparison of some free amino acids from drone haemolymph (Cardinaud et al., 1994) with data from Table 1. As the age of the drones in the cited work was not known, minimum and maximum values for the individual amino acids are given from our work

nmol/μl free aa	Cited from: Cardinaud et al. (1994)		Present data: minimum value		Present data: maximum value	
	mean	std	mean	std	mean	std
Ala	1,6	0,4	2,74	0,94	5,11	0,43
Arg	0,4	0,2	2,32	1,55	7,72	3,46
Asp	0	0	0,04	0,07	1,12	0,61
Glu	0,3	0,2	0,51	0,79	5,75	1,85
Gln	12,1	1,5	3,91	2,51	14,68	2,83
Pro	27,9	2,7	18,67	3,84	36,30	4,55

Lower values for certain amino acids found by Cardinaud et al. (1994) may be due to the fact that their drones were fed by workers given only sucrose to eat. We have shown that worker bees kept on a sucrose diet for 9 days had reduced levels of protein and amino acids, notably arginine (Crailsheim et al., in preparation). We did not measure two non-proteogenic amino acids, β-

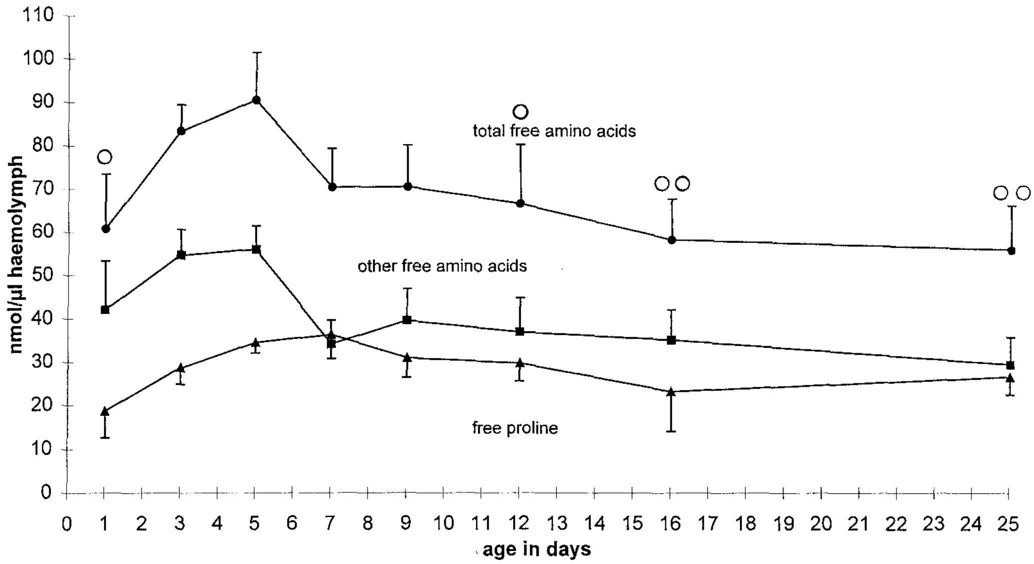

Fig. 2. Concentrations of total free amino acids, free proline and the bulk of other free amino acids in the haemolymph of honeybee drones of different ages. Given are the means of individuals from 3 colonies and the standard deviations (n = 23–24). Open circles (O) indicate no significant differences as calculated by Mann Whitney U-test

alanine and taurine, whose occurrence in drone haemolymph was reported by Cardinaud et al. (1994).

The total concentration of free amino acids in drone haemolymph rose sharply from day 1 to day 5 (Fig. 2). After a pronounced decrease on day 7, there was a slighter but continuous decline from the 9th to 12th day after emergence. The total concentration in 12-day-old drones was not significantly different from that in 1-day-old drones. Then there was a further slight decrease from the 12th to the 16th day. From the 16th to the 25th day no further change occurred. The most abundant amino acid, proline, increased from day 1 to day 7 and then showed a slight, barely significant decline over the rest of the observed period.

The concentrations of amino acids we observed in the haemolymph of drones were generally higher than the levels we have reported for workers (Crailsheim and Leonhard, 1997). Notably the very high titre of lysine in young drones and the high level of glutamine found in drones were not found in wo[illegible]lymph (Crailsheim and Leonhard, 1997). For proline the rela[illegible]newhat more complicated. Although the absolute concentrati[illegible]was higher in drones than in workers, the relative amount was [illegible]0% at each age, whereas in workers proline constituted abou[illegible]otal free amino acids in every age class, except the newly emerg[illegible]

We also considered separately the group of essential free amino acids, as determined for workers by Groot (1953). These were highest in drones on the 1st and 3rd day. On the 3rd day the concentration of essential amino acids was 7-fold higher than in workers of the same age, whereas the concentration of total amino acids differed by a factor of 3.2 (Crailsheim and Leonhard, 1997) (Table 1).

Drones reached their maximum concentration of free amino acids at the 5th day. This is consistent with previous reports that, for drones, pollen consumption and proteolytic activity in the midgut reach maximums at 4 days of age, attaining about 60% of the activity reached by 8-day-old workers (Szolderits and Crailsheim, 1993). After 4 days of age, drones showed sharp declines in proteolytic activity and pollen content in the midgut (Szolderits and Crailsheim, 1993). Drones usually start flying around the 5th to 7th day after emergence (Currie, 1987).

In workers the maximum free amino acid content in the haemolymph was measured around 3–5 days of age (Crailsheim and Leonhard, 1997), whereas the highest level of proteolytic activity in the midgut occurred at age 8 days and declined only slowly afterwards. Pollen consumption was maintained until the 16th day (Crailsheim et al., 1992; Szolderits and Crailsheim, 1993).

For drones, the rising proline content up to the 7th day can be explained, at least in part, by the continued consumption of pollen, which is generally rich in proline (Lehnherr et al., 1979). It is unlikely that the sustained proline level after the cessation of pollen consumption can be explained solely by honey and jelly consumption. Proline neogenesis has, at least to some extent, to be taken into account. A similar conclusion was reached for workers, whose free proline levels in the haemolymph were also stable well after they stopped digesting pollen (Crailsheim and Leonhard, 1997).

A comparison of the age-related patterns of free amino acids in the haemolymph of honeybees shows that the differences between workers and drones are at least as striking as those between different species (Pant and Agrawal, 1964; Chen and Wagner, 1992). Figure 1 shows a comparison of free amino acid titres in the haemolymph of honeybee workers and drones on the 5th day after emergence, the age when both genders have their highest overall content of free amino acids. Except for tryptophane and tyrosine, the concentrations of all amino acids differed significantly. Even the similarity in the concentrations of these two amino acids is doubtful, as tryptophane, although essential, was at the threshold of detectability in both genders and tyrosine was changing fast at this age.

As to the role of the high proline titres in the haemolymph, there are some indications that this amino acid plays a role in energy metabolism. Oxidative breakdown of proline in drones is about two orders of magnitude higher than that of other amino acids (Berger et al., 1997). However, the total amount of metabolised proline, as compared to consumed glucose, in sitting and walking drones amounted to only 1.2% of consumed energy (Berger et al., 1997). Other results showed that in honeybee drone retina proline is utilised as an energy source (Cardinaud et al., 1994; Tsacopoulos et al., 1994; Tsacopoulos, 1995). It is not yet clear whether all proline oxidation is related to its use in the retina; perhaps proline is also of some importance for flight energy supply. A function of proline as a stimulant for the oxidation of pyruvate in flight muscle mitochondria has been discussed (Sacktor and Childress, 1967).

Osmolarity (Table 1) increased from the 1st to the 3rd day of drone life. Then, except for a minor increase from the 5th to the 7th day, it did not change significantly between the 3rd and 16th day. A significant increase in osmolarity was observed on the 25th day. As we know that the haemolymph volume of drones after flight is significantly lower than that of drones that have not flown (Schneider and Crailsheim, 1994), this increase in osmolarity might well be related to an increased flight activity of those older drones. Yet the relative contribution of free amino acids to total osmolarity decreased from 18% on day 1 to 10% on day 25, and although there was a reduction of free amino acids after the 5th day there was no similar reduction in osmolarity.

Osmolarity in drones, ranging from 334 to 532 mOsmol, was generally lower than 573 mOsmol, the level reported for worker bees (Crailsheim, 1985). For other insects a range for osmolarity in the haemolymph from 301 mOsmol (*Dytiscus circumcinctus*, Coleoptera) to 532 mOsmol (*Acridia nasuta*, Orthoptera) is cited by Buck (1953). In honeybee drones the free amino acids in the haemolymph represent an important part of total osmolarity, amounting to up to 20%.

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