PROTEIN AND AMINO ACID REQUIREMENTS OF THE HONEYBEE

(*APIS MELLIFICA L.*)

A. P. DE GROOT

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WIS- EN NATUURKUNDE AAN DE RIJKS-UNIVERSITEIT TE UTRECHT, OP GEZAG VAN DE RECTOR-MAGNIFICUS DR V. J. KONINGSBERGER, HOOGLERAAR IN DE FACULTEIT DER WIS- EN NATUURKUNDE, VOLGENS BESLUIT VAN DE SENAAT DER UNIVERSITEIT TEGEN DE BEDENKINGEN VAN DE FACULTEIT DER WIS- EN NATUURKUNDE TE VERDEDIGEN OP DONDERDAG 9 JULI 1953, DES NAMIDDAGS TE 3 UUR

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ANTONIUS PETRUS DE GROOT

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Aan de nagedachtenis van mijn Moeder

Aan mijn Vader

Aan mijn Vrouw

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CONTENTS [1])

[1]) 67th Communication of the Research Institute for Animal Husbandry, T.N.O.

INTRODUCTION

The study of the nutritional requirements has established that animals, except for some Protozoa, are able to maintain normal life processes only if a number of complex chemical compounds are available, being synthesized by vegetable organisms. These indispensable food components of organic origin may be distinguished in carbohydrates, amino acids, lipids and vitamins. The number of representatives of each of the groups mentioned, being required by a given animal is limited. Moreover, different non-related organisms exhibit a marked agreement with respect to their dietetic requirements. For instance the unicellular organism *Tetrahymena geleii* appeared to require the same amino acids as those being necessary for the rat. On the contrary two closely related animals, like the rat and the mouse, turned out to differ somewhat in their needs for amino acids. Hence it appears, that in this respect generalizing is not admissible.

The number of animals which has been investigated circumstantially at present, is still relatively small. Most comprehensive knowledge was obtained from the nutritional requirements of some vertebrates (chick, mouse, rat, man) and of the flagellate *Tetrahymena geleii*, while a number of insect species have been studied already rather extensively. The attention of nutritional research workers so far paid to the honeybee is not proportional to its economic significance. Even though the honeybee possesses many qualities of a suitable laboratory animal, it has been hardly used in dietetic experiments. Exact data are available only with respect to the carbohydrate component of the diet. Furthermore only the value of a number of protein containing foods has been investigated. Nothing is known regarding the requirements for amino acids, lipids, vitamins and minerals.

The natural foods of the honeybee are honey as a source of carbohydrates and pollen containing all other factors required for life. For supplying calories, the honey can be replaced by sucrose. With regard to pollen the bee colony is completely dependent on factors enabling the collection of this food, such as season, temperature, distance and amounts available. Therefore, the pollen supply is a limiting factor in beekeeping practice. For many years already search has been in progress for a food making the bee colony independent of the supply of natural pollen. However, a suitable substitute has not yet been found. The relative research is carried out rather inefficiently, because of the poor knowledge of the chemical composition of pollen and also owing to ignorance of the nutritional requirements of the honeybee. Therefore the dietetic research

on this insect is not only a matter of scientific interest, but of practical importance as well.

The Dutch ,,Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek" [1]) afforded the opportunity for studying the nutritional requirements of the honeybee under the auspices of the ,,Instituut voor Veeteeltkundig Onderzoek T.N.O." [2]). Owing to practical and technical reasons we restricted this study almost exclusively to some observations on protein metabolism of young and old bees and a more detailed investigation of the amino acid requirements of the young imago.

The experiments were conducted at the Laboratory of Comparative Physiology, University of Utrecht.

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[1]) National Council for Applied Scientific Research.
[2]) Research Institute for Animal Husbandry, T.N.O.

CHAPTER I

BIOLOGY OF THE HONEYBEE

Since some knowledge of the biology of the honeybee may contribute to a better understanding of the following chapters, a brief explanation of the structure of the bee colony and of the various activities of the different kinds of individuals shall be presented. Details are given mainly of those aspects of bee life, which played an important part in the present investigation. For further information the reader may be referred to the manuals of bees and beekeeping. (ZANDER 1921; MINDERHOUD 1948; GROUT 1946; VON FRISCH 1948, 1950; BUTLER 1949).

1. *The composition of the bee colony.*

Like all other colony building insects, with the exception only of termites, the honeybee belongs to the Hymenoptera. The bee colony is composed of three kinds of individuals:

a. *The queen.* This is one of the two female forms occurring in the bee colony. The function of the queen consists merely of laying the eggs. For this purpose she is equipped with two highly developed ovaries, taking up the major part of the abdomen. Both fertilized and unfertilized eggs are laid. The female individuals (queens and workerbees) arise from fertilized eggs; the male individuals (drones) from unfertilized eggs. The number of eggs laid by a good queen during one day may amount to 1000—2000, provided she is supplied with an abundance of a high protein food, being provided by worker bees. The mouth parts, pollen gathering apparatus and brood food glands, which have reached a high development in the worker honeybee, are poorly developed in the queen. Only one queen is present in a normal colony. In the swarming season, when the colony is about to reproduce by dividing, and further in extremely rare cases several queens may be present at the same time. No colony can exist long without the presence of a laying queen.

b. *The drones.* They are the male individuals of the colony. Their duty consists exclusively of the fecundation of the virgin queen. Like in the queen, the mouth parts, brood food glands, and pollen gathering apparatus of the drones are rudimental. Moreover the sting is absent. The drones, several hundreds in number, are present in the colony only during the summer season. In autumn they are driven out of the hive and die.

c. *The worker honeybees.* These female individuals constitute the majority of the population of the bee colony. Their number runs into

ten-thousands and may amount to 75000. Worker bees possess highly reduced sexual organs. On the other hand they have distinct anatomical-, physiological-, and instinctive specializations for the various duties being performed in succession during their life. The worker honeybee is equipped with well developed mouth parts, a complicated pollen gathering apparatus at the hind legs, consisting of hairs, bristles and combs, with two rows of wax glands situated at the ventral side of the abdomen, and two pairs of brood food glands in head and thorax. All operations necessary for the survival of the bee colony,with the exception only of laying the eggs and fecundating the virgin queen, are performed by worker bees. These highly divergent proceedings are dependent to a high degree on the age of the individual resulting in a pronounced division of labour. Roughly it holds, that after the first three weeks in which the development of the egg into the imago takes place, the young worker in a certain sequence performs all kinds of duties within the hive for about three weeks. After that, the field duties begin, likewise lasting about three weeks in the summer season, and characterized by gathering honey, pollen and water.

2. *Life cycle of the honeybee.*

A. Development of egg up to imago.

From a fertilized egg deposited in a cell by the queen, a little larva develops in three days. The larva is provided abundantly with a milky product, secreted by the brood food glands of worker bees. The older larva receives honey and pollen in addition. Growth proceeds extremely rapidly and an increasing part of the cell is occupied by the larva. Six days after hatching from the egg, the larva has attained 500 times its initial weight and is no longer fed. Worker bees close the cell with a wax cover and the larva surrounds itself with a cocoon. During the next 12 days the transformations occur which gradually give rise to the shape and structure of the imago. First of all the contours of head, thorax and abdomen become visible. At the head, antennae and mouth parts appear, while the place of the eyes can be recognized by a pink pigmentation, changing via red and purple into brown. Legs and wings appear at the thorax and the abdomen obtains its adult shape. After completion of metamorphosis the insect becomes active, gnaws through the cell capping and emerges as imago. The development from egg to imago lasts 21 days.

B. Activities of the imago honeybee.

As was already mentioned above, a pronounced division of labour occurs in the honeybee community. Each worker passes through a pattern of duties, dependent on the age of the individual (Rösch 1925, 1927, 1930; Lindauer 1952). During the first 3 weeks of imago life, the worker honeybee performs all kinds of duties within the hive. On the score of age these may be divided as follows:

a. *The first* 3 *days* the main task consists of cleaning empty brood cells, preparing them for the receipt of a new egg. Also the young bee takes part in keeping warm the developing brood at a temperature of 33—34° C. In the meantime considerable amounts of honey and pollen are digested for further growth of the young imago. Although the worker honeybee is often considered to be adult after emergence from the cell, it appears that during the first 6 days a substantial growth occurs, finding expression in an increase in body weight and total protein content of 25—50 % (HAYDAK 1934 b) and a development of the brood food glands (KRATKY 1931). The post larval growth results in a physiological condition enabling the worker to perform its nursing duties.

b. *From the* 3^d *to the* 10^{th} *day* the honeybee is in charge of feeding larvae. At first the older larvae are supplied mainly with honey and pollen. Somewhat later the younger larvae are fed exclusively with the secretory product of the brood food glands, which have been developed in the meantime. With the decline of these glands the individuals change to other duties.

c. *From the* 10^{th} *up to the* 21^{st} *day* the inner service varies widely. Nectar and pollen, being brought in by the field bees, are stored in cells and preserved as honey and bee bread respectively. The temperature is kept up to the mark, the air refreshed and excess moisture evaporated from the newly stored honey. Cleaning the hive, closing useless openings with propolis, fighting against wax moths and other enemies, likewise belongs to the inner service. If the weather allows, orientating flights are carried out near the hive to prepare for the next period, namely that of gathering. Meanwhile, the wax glands have developed to secrete the material for manufacturing new combs. After these 3 weeks of duties within the hive the last phase of bee life begins.

d. *From the* 21^{st} *day until death* the worker is a gathering bee and collects honey, pollen and water. The duration of this period varies within wide limits, depending a.o. on the intensity of activities. In the summer season, when most is required of the foragers, they perish within 3 weeks. The bees which emerged during autumn often will find no employment in several kinds of services and survive winter.

As a result of particular conditions the behaviour of worker bees may deviate from the above described sequence. Young bees may start field duties considerably before the 21^{st} day and gathering bees may again commence with the production of wax, comb building and nursing duties (NELSON 1927; RÖSCH 1930; MILOJEVIC 1939; LINDAUER 1952).

The experiments reported in chapter IV—VIII were carried out exclusively with the imago honeybee. Table 1 serves to illustrate the changes in weight and nitrogen content which occur during the free living stages as compared with those during the development from egg up to imago.

Successive stages	Duration in days	Weight in mg		Nitrogen content in mg
		fresh	dry	
egg	0—3d	0.05		
larva.	3d—9th	0.3—150	0.07—33	0.09—2.2
pupa.	9th—12th	150—117	30—18	2.2—1.8
imago: newly emerged.		80	16	1.9
nurse bee . . .	3d—14th	72	22	2.6
gathering bee .	21th—death	66	21	2.4

Table 1. *Summary of the changes in weight and nitrogen content, during the successive developmental stages of the worker honeybee.*

The data concerning the changes prior to the free living phase are derived from the work of STRAUS (1911), those for the imago from the present study. As a result of removing the alimentary tract prior to the analyses of the present work, the latter values are not quite comparable with those of STRAUS. Attention is drawn however, especially to the increase in dry weight and nitrogen content of imagines at the change to the nursing stage. In the present investigation this growth was used as an index for evaluating the significance of various dietetic components.

CHAPTER II

REVIEW OF THE LITERATURE CONCERNING THE DIETETIC REQUIREMENTS OF THE HONEYBEE AND THE AMINO ACID REQUIREMENTS OF INSECTS

The following survey of the present knowledge regarding the nutrition of the honeybee covers not only the need for definite chemically defined compounds (i.e. nutritional requirements), but also the results obtained in experiments with more complex dietetic components (i.e. foods). Like for many other invertebrates it counts also for the honeybee that much more is known about the food, than about the nutritional requirements. So far the honeybee is hardly implicated in the study of animal nutrition, except for carbohydrates. First of all the available data concerning the carbohydrate requirements are reviewed. After that, reports on the requirements for protein and protein-containing foods shall be briefly summarized. Finally a survey will be given of the recordings found in literature regarding the amino acid requirements of insects. This subject has not been completely reviewed as yet.

1. *Carbohydrates.*

The study of the dietetic requirements of the honeybee in its strict sense, remained limited so far to the carbohydrate component of the diet.

Some authors examined the availability of various carbohydrates for the honeybee using the longevity as a testing method. These results are summarized in table 2.

Carbohydrates		*Apis*			*Calliphora*
		larva BERTHOLF (1927)	adult PHILIPS (1927)	adult VOGEL (1931)	adult FRAENKEL (1939)
Pentoses	Arabinose		—	+	—
	Xylose		—	+	+
	Rhamnose		—	—	—
	Fucose			—	
Hexoses	*Glucose*	+	+	+	+
	Fructose	+	+	+	+
	Galactose	+	—	+	+
	Mannose		—	—	+
	Sorbose			—	—
Disaccharides	*Saccharose*	+	+	+	+
	Maltose	+	+	+	+
	Lactose	+	—	—	+
	Trehalose	+	+	+	+
	Melibiose			—	+
	Cellobiose			+	—
Trisaccharides	Raffinose		—	+	+
	Melezitose	+	+	+	+
Polysaccharides	Dextrin	+	—		+
	Starch	—	—		+
	Glycogen	—			+
	Inulin		—		—
Glycosides	*α-Methylglucoside*			+	+
	β-Methylglucoside				—
Sugar-alcohols	Mannitol		—	+	+
	Sorbitol			+	+
	Dulcitol			—	—
	Erythritol			—	—
	Inositol			—	—

Table 2. *Utilization of carbohydrates by the honeybee larva, the honeybee adult and the bluebottle* (*Sugars tasting sweet to bees are printed in italics*).

For comparison a number of data concerning the bluebottle *Calliphora* are recorded as well (FRAENKEL 1939). The signs + and — mean 'utilized' and 'non-utilized' respectively. In this table the carbohydrates tasting sweet to bees are printed in italics (VON FRISCH 1935). From table 2 it appears first of all, that all sugars being sweet to bees are utilized, but a number of carbohydrates is of value that are not sweet. With respect to the latter category VOGEL (1931) found more utilizable forms than did PHILIPS (1927). The cause of these discrepancies can probably be ascribed to a difference in testing method, as a result of which in the experiments of PHILIPS the sugars being not sweet have not been consumed in sufficient amounts. VOGEL warranted the ingestion of the tasteless carbohydrates

by mixing them with a 8 % (= 0.25 mol.) sucrose solution. The amount of sucrose fed daily was restricted in such a way that an eventual increase in longevity, as compared with bees fed only water, could not have been caused by the sucrose consumed. This admissible amount of sucrose was ascertained in previous experiments (0.006 ml 0.25 mol sucrose a day). Presumably in consequence of this technical precaution, VOGEL found, in contrast with PHILIPS, that the tasteless sugars arabinose, xylose, galactose, raffinose and mannitol are indeed utilized by the honeybee.

The findings of BERTHOLF (1927) have been obtained in experiments with young bee larvae, the longevity of which in solutions of various carbohydrates was compared with that in pure water. In contrast with adult honeybees, lactose seems to be utilized by the young larva.

In general, the carbohydrates being utilized by bees appear to be likewise valuable for *Calliphora*, although in the table some discrepancies will be observed. A marked deviation occurs in the case of mannose. This carbohydrate, being utilized by *Calliphora*, is not only worthless for bees, but even exerts a pronounced toxic effect. STAUDENMAYER (1939) entered more into details of the toxical symptoms of mannose in insects. He suggests as a possible cause a competitive inhibition by adsorption of the enzyme glycolase at the mannose molecule, thus inhibiting the break down of glucose.

The large number of carbohydrates being utilized by the honeybee possesses only a slight biological significance, since only a few compounds occur in the natural source of carbohydrates, the honey. The only sugars being identified in considerable amounts in nectar secreted by flowers of different plants species are sucrose, glucose and fructose (BEUTLER 1930). WYKES (1952) found by paper partition chromatography in samples from every species examined fructose, glucose and sucrose. In samples from several species maltose and in addition melibiose and raffinose were detected, though in relatively small amounts. Of these melibiose cannot be utilized by the honeybee.

Carbohydrates are likewise present in pollen. So it is possible that sugars being absent in nectar, are present in pollen. However, the carbohydrates of pollen have so far been investigated insufficiently.

2. *Protein containing foods.*

Many kinds of insects can maintain life for considerable periods in the absence of nitrogenous food. However for growth, insects like vertebrates require nitrogen containing substances. Knowledge of the protein requirements of the honeybee was obtained only in experiments with intact bee colonies and isolated imagines. No investigations have been carried out so far with isolated larvae, since it has not yet been possible to rear larvae outside the bee colony. VELICH (1930), VON RHEIN (1933, 1951), and others, only succeeded in rearing larvae in an incubator up to the adult stage, if they were nearly full grown when taken out of the comb.

A. Experiments with bee colonies.

As was already mentioned (p. 1) the amount of pollen available to bee colonies is a limiting factor in apiculture. Quite a number of nutritional investigations are recorded in literature in which complete bee colonies were tested. The aim of these experiments was mainly, to find out a satisfactory substitute for pollen, rather than to elucidate the protein requirements of the honeybee. A review of the investigations carried out prior to 1933 is given by HAYDAK (1933). In these experiments, performed under different conditions, a large number of protein containing materials have been used, a.o. meal or flour from buckwheat, oat, barley, corn, cottonseed, rye, linseed, beans, peas and soybeans, further yeast, milk-powder, casein and fishmeal. In general a stimulation of the brood rearing activity was reported. HAYDAK (1933, 1934, 1936, 1937, 1939, 1940, 1945, 1949; HAYDAK & TANQUARY 1942, 1943) investigated the pollen replacing value of different protein containing food stuffs with bee colonies, composed of just emerged bees which had never eaten pollen. They were hived on pollen free combs filled with sugar solution in isolated wire cages. A good laying queen was introduced in each of the colonies. The pollen substitute made to a candy with honey or concentrated sugar solution was placed directly on top of the frames or given in the combs. In a first experiment the changes in dry weight and nitrogen content of the bees, the number of dead bees, the amount of brood reared and the comb building activity were recorded in colonies fed with skimmed milk powder, dried yeast, fresh whole milk, egg white, egg yolk, whole egg and rye flour. The results were compared with those obtained from a colony supplied with a pollen-comb. In all the experimental colonies, except that fed with rye flour, the young bees developed their bodies quite normally and reared brood. The young bees produced were in general normally developed. However none of the substitutes employed gave as good results as pollen. The use of fresh whole milk was recommended as a satisfactory substitute for pollen. In subsequent papers (HAYDAK 1936, 1937, 1939, 1940, 1945, 1949) the author described experiments with meat scrap, blood meal, digested tankage, fish meal, casein, milkpowder and flour from oat, wheat, rye, corn, peas, cotton seeds, earth nuts, linseeds, dried yeast, whole egg, egg yolk, egg white, soybean flour, soybean meal and various combinations of these food materials. Soybean meal was chosen for more minute investigations. Results obtained with various brands of soybean flour showed considerable differences (HAYDAK & TANQUARY 1942) dependent on different processes in manufacture. Bee colonies fed with soybean flour processed by the expeller method (oil removed by pressure) reared more brood than did colonies fed with solvent extracted soybean preparations. Feeding solvent extracted soybean flour, mortality of the experimental bees decreased and the brood rearing capacity increased if the crude oil extract was restored to 5.5 %. An incorporation of dried egg yolk in the diet caused a further

improvement. The value of expeller processed soybean flour, containing about 7 % fat, could be further improved by supplementing with yeast preparations alone or with an addition of dried egg yolk (HAYDAK 1945). The best results were obtained if soybean flour was supplemented with 20 % of dried brewers' yeast. If fed on this substitute the colonies reared brood normally for four months. The inadequacy of soybean flour without the addition of yeast was ascribed to a deficiency of the vitamins niacin and riboflavin and possibly some other factors (HAYDAK 1949). CALE (1946) recommends a mixture of animal brewers' yeast and expeller processed soybean flour (1:6) moistened with sugar syrup of equal parts sugar and water as a good pollen substitute. SCHAEFER & FARRAR (1946) stating that soybean flour although it has value as a supplement to pollen is not a complete substitute, advise the use of pollen instead of yeast to be mixed with the soybean flour (FARRAR 1945, 1946).

In the United States soybean flour is widely used now in beekeeping practice. In other countries, however, results obtained with this pollen supplement are less favourable as in the above described experiments. Moreover the inexpensiveness and general availability of this material with the desired properties in the U.S.A. does not count for European countries. As a result the use of soybean flour as a pollen substitute is rarely put into practice in European beekeeping.

Recent papers in German and Swiss apicultural journals are reporting favourable results from observations with a kind of yeast preparation s.c. "Höselhefe" as a pollen substitute (MAURIZIO 1951). FEDERL (1951), however, could not detect any influence of "Höselhefe" on the brood rearing activity of bee colonies. Further more exact experiments must be awaited before definite conclusions may be drawn as to its pollen replacing value.

B. Experiments with isolated bees.

a. Brood food glands as a testing method.

The system of salivary glands of the honeybee consists of one pair of mandibular glands, one pair of pharyngeal glands (brood food glands) and two pairs of labial glands (KRATKY 1931; WIGGLESWORTH 1950). The pharyngeal glands situated in the sides of the head, consist of two long strings of small saccules. The ducts of these glands open on the floor of the mouth (for further details see SOUDEK 1927, KRATKY 1931). In newly emerged workers the saccules are small and pear-shaped. After about 6 days, due to intensive pollen consumption the saccules are greatly enlarged and round off. In this stage the glands produce the 'royal jelly' used for feeding the larvae, the queen and possibly the drones. Later in life notably when the worker has passed the nursing stage, these glands shrivel again and the secretion of royal jelly stops. Development occurs only if the newly emerged bee has the opportunity to consume pollen (SOUDEK 1927; KRATKY 1931) or other nitrogenous food. Therefore the

development of the pharyngeal glands offers an opportunity to study the nutritional requirements of the honeybee. To some extent also the ovaries and the fatbody may be used for the same purpose (PETERKA 1939; MAURIZIO 1950, 1951).

So far the development of these organs has not yet been used in studying the dietetic requirements of the honeybee, but only for the determination of the nutritional value of pollen and other proteinaceous material.

SOUDEK (1927) and KRATKY (1931) obtained an enlargement of the pharyngeal glands only if the bees were fed pollen. With casein, albumen, wheat flour, rye flour or starch no development was observed. It was suggested that the enzymes necessary for digestion were lacking. SOUDEK (1929), CURRIE (1932), PETERKA (1939), LOTMAR (1939 a), and MAURIZIO (1950, 1951) obtained development with several foods other than pollen a.o. dried yeast, egg albumen, soybean flour, skimmed milk powder and casein. SVOBODA (1940) and especially MAURIZIO (1950) used the development of the brood food glands to study the nutritional value of pollens of a great number of plant species and observed a great variety in their biological action. This knowledge of the nutritional value of pollen from different plant species is important for beekeeping practice.

b. Longevity as a testing method.

Determinations of the longevity of honeybees confined to small cages have been widely used to study the effect of environmental conditions (WOLFENBARGER 1934; WOODROW 1935, 1941, MELAMPY & MCGREGOR 1939, PHILIPS 1946), the toxic properties of various materials (MAURIZIO 1945; HILDEBRAND & PHILIPS 1935; VELTHOEN 1947; ECKERT 1949; HÄFLIGER 1949), the action of digestive enzymes (PHILIPS 1927; VOGEL 1931) and the biological value of protein containing foods (PETERKA 1939; MELAMPY & MCGREGOR 1939; WOODROW 1941; MAURIZIO 1946, 1950, 1951; BEUTLER & OPFINGER 1949). The application of the longevity method for studying the utilization of carbohydrates by PHILIPS (1927) and VOGEL (1931) has already been mentioned. However, scarcely any attempt has been made to apply this method to the evaluation of definite nitrogen containing food components. Working with young bees several authors noticed a considerable lengthening of the life span as compared with a pure carbohydrate diet, if the food was supplemented with pollen (PETERKA 1939; MELAMPY & MCGREGOR 1939; WOODROW 1941; MAURIZIO 1946, 1950, 1951; BEUTLER & OPFINGER 1949; DE GROOT 1951). So it appears that pollen exerts a longevity promoting effect. In an attempt to investigate the nutritional value of milkproducts, cereals and yeast, MELAMPY & MCGREGOR (1939) could not detect an increase in longevity if young bees were fed these materials. Since these were provided seperate from the honey it is possible that the bees did not consume the protein-foods in sufficient amounts for a perceptible effect on longevity. The same negative results were obtained by BEUTLER & OPFINGER (1949) on

feeding milkpowder, yeast or soybean flour added to sugar candy or sugar solution. In this case the absence of a favourable effect may be attributed to the high concentration of the protein food (> 10 %). This will be clear from the results presented in Chapter IV. PETERKA (1939) reported favourable results with certain soybean preparations. MAURIZIO (1950, 1951) observed an increase in longevity if soybean flour or skim milk powder was fed.

In the light of the results arrived at in the present investigation (see Chapter IV) it is likely that the poor results obtained in the above longevity experiments are mainly a consequence of feeding the protein in concentrations exerting a detrimental effect on bees in captivity. Further it may be stated that the search for pollen substitutes so far has obtained the best results with certain soybean preparations, dried yeast and milkpowder. However, a complete substitute for pollen has not yet been found.

3. *Amino acid requirements of insects.*

UVAROV (1928) was the first one to summarize the experiments on the needs of insects for definite nitrogen-containing food components. At that time however, hardly any information was available regarding the requirements of insects for amino acids. Increasing knowledge in this field after 1928 was partly reviewed by several authors (HOSKINS & CRAIG 1935; TRAGER 1941, 1947; CHAUVIN 1949; PROSSER 1950; WIGGLESWORTH 1950). Up to now no experiments have been performed on the amino acid requirements of the honeybee.

From a great number of nutritional investigations on higher animals it appeared that from the 19 amino acids generally occurring in proteins only 10 of them must be present in the diet for good growth, whereas the other 9 may be synthesized by the organism itself (ROSE 1938). The amino acids which have to be taken as such in the food to meet the needs for normal growth are called '*essential*'. The amino acids being essential for normal growth of the rat are: arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine and valine. Man needs only 8 of the amino acids required by the rat (ALBANESE 1947, 1950; ROSE 1949). In addition to the 10 essential amino acids mentioned above the chick requires for optimal growth glycine and glutamic acid as well (ALMQUIST & GRAU 1944).

The needs of invertebrates for amino acids are studied rather exhaustively on some Protozoa and Insecta in the last few years. The ciliate *Tetrahymena geleii* appeared to require the same 10 amino acids necessary for vertebrates (KIDDER & DEWEY 1945; HOGG & ELLIOTT 1951).

The first experiments with insects in which definite nitrogen compounds were fed seemed to reveal much more simple requirements for nitrogen than other heterotrophic animals. LOEB (1915) reared the fruit fly *Drosophila* from the egg up to normal imagines if only one of the following nitrogen compounds was present in the diet: alanine, glutamic acid, ammonium succinate, or ammonium tartrate. ZABINSKY (1929) likewise

obtained development of cockroaches (*Blattella germanica* and *Periplaneta orientales*) with glycine as the only nitrogen compound of the diet. Development was normal even if nitrogen was furnished in the form of gelatin, a protein known to be deficient in several amino acids. NEWSTEAD (cit. KOZHANTSHIKOV 1944) bred *Drosophila* on pure and single amino acids, alanine and glutamic acid. However, more recent investigations under aseptic conditions have shown that the nitrogen requirements of insects are much more complex than it would appear from the results of LOEB and ZABINSKY. It is clear therefore, that the synthetic action of microorganisms in the diet and in the gut accounts for the above results. Inorganic nitrates and ammonium compounds were unable to meet the needs for nitrogen in termites (ROESSLER 1932). KOZHANTSHIKOV (1944) was no more successful in rearing Calliphora larvae on single amino acids (alanine, glycine, aspartic acid, amino valerianic acid).

Feeding incomplete proteins is an easy method to study the requirements of animals for some amino acids. The incomplete proteins like gelatin, zein and gliadin are deficient in certain amino acids, essential as well as non-essential. Hence these proteins unsupplemented with the lacking amino acid are unsuitable as the sole source of nitrogen in diets of higher animals. The same appeared to apply to insects. Incomplete proteins were insufficient for *Blattella germanica* (MCCAY 1933, 1938) for the mosquito *Aëdes aegypti* (GOLDBERG & DE MEILLON 1948) for the mealworm *Tenebrio molitor* (LECLERCQ 1948) and for *Drosophila melanogaster* (LAFON 1938, 1939). Supplementing the incomplete proteins with the deficient amino acids resulted in improved growth (LAFON 1939; LECLERCQ 1948). These facts suggest that the amino acids which promote growth with incomplete proteins, are required for the insect larvae.

In an attempt to study the requirements of Drosophila for lysine, LAFON (1939) made use of the incomplete proteins gliadin and zein known to be deficient in lysine and other amino acids. However, these proteins although properly supplemented with the deficient amino acids, failed to support growth. Therefrom it was derived that these proteins are lacking an unknown amino acid essential for *Drosophila*. LECLERCQ (1948) working with the mealworm obtained a considerable improvement of growth on supplementing zein with its deficient amino acids tryptophan and lysine. Gliadin, which contains a low percentage of tryptophan could be improved by the addition of lysine only. From these experiments LECLERCQ concluded that lysine and tryptophan are both essential for Tenebrio.

Another method for studying the amino acid requirements is found in the application of chemical methods with which certain amino acids may be extracted from the diet. LAFON (1939) using the silver sulphate precipitation method removed most of the arginine and histidine from casein hydrolysates. On supplementing the deficient diets with one or both of the precipitated amino acids LAFON found histidine as well as arginine to be essential for Drosophila.

Although casein has proved to be an excellent source of amino acids in nutrition of higher animals, this seems not to apply to several insects. MICHELBACHER, HOSKINS & HERMS (1932) observed good growth of Lucilia if the protein component of the diet consisted of casein. The resulting pupae, however, were abnormal unless cystine was added. Likewise VAN 'T HOOG (1935, 1936) obtained optimal growth of *Drosophila*. only if the diet containing casein peptone, was supplemented with cystine. GAY (1938) with the scavenger beetle *Dermestes vulpinus* and recently SEDEE (1953) with *Calliphora erythrocephala* demonstrated the favourable effect of supplementing the casein with cystine. LAFON (1939) found for *Drosophila* that a diet lacking in cystine caused misshaped puparia and a very low percentage of the larvae became adults. GOLDBERG & DE MEILLON (1947, 1948) working with the mosquito fly *Aëdes aegypti* obtained a high proportion of adult mosquitoes failing to emerge if cystine was absent from the diet. From the above observations it follows that at least a number of insects are more exacting with respect to cystine than vertebrates. Although cystine seems to be non-essential for growth, the addition of this amino acid may result in a growth stimulation in insect larvae and in a higher percentage of larvae to become normal adults.

Several authors tried to replace the nitrogen component in the diet of insects by protein hydrolysates. VAN 'T HOOG (1935, 1936) with *Drosophila*, and GOLDBERG & DE MEILLON (1948) with *Aëdes* were not successful in this respect, even though the acid hydrolysates were supplemented with cystine and tryptophan, these being the two amino acids which are destroyed by prolonged hydrolysis with strong acids. On the other hand LAFON (1938, 1939), TATUM (1939), SCHULTZ et al. (1946), VILLEE & BISSELL (1948) obtained good growth of *Dosophila* larvae if the acid hydrolysate of casein was supplemented with cystine and tryptophan. If tryptophan was omitted, growth did not occur, whereas good growth was obtained on the addition of tryptophan. It was concluded that tryptophan is an essential dietary component for *Drosophila*, as it is for vertebrates.

WILSON (1941) found the addition of proline to an autoclaved diet with half the optimal amount of yeast to accelerate moulting of *Drosophila*. A similar effect was observed with arginine (WILSON & BIRCH 1944), tryptophan (WILSON 1945 a), tyrosine, phenylalanine, alanine (WILSON 1945 b), methionine, and cysteine (WILSON 1946).

As compared with higher animals, only a very few experiments have been carried out with insects whereby the nitrogen component of the diet was procured in the form of amino acid mixtures. ABDERHALDEN (1925) observing the growth of the museum weevil *Anthremus muscorum* in a non-sterilized medium, was the first one to replace the protein by a mixture of several amino acids. No details on the composition of this mixture are given, only the information that the larvae failed to grow. LAFON (1938, 1939) could not obtain any growth of *Drosophila* larvae by

replacing the protein in the medium by a mixture of 18 amino acids supplemented with the mono-amino acids from casein to provide threonine and hydroxyproline. In combination with muscle pepton, the amino acid mixture gave excellent results, thus demonstrating the absence of toxic properties. It was concluded that from the mixture a factor was missing, normally constituting a part of the protein. As he did not supply the required vitamins, it is possible that vitamin deficiencies account for the negative results. Likewise without success BUDDINGTON (1941) tried to rear *Drosophila* larvae on a mixture of 7 amino acids instead of protein.

Growth studies on diets with amino acid mixtures were carried out by HOUSE (1949 a, 1949 b) and HOUSE & PATTON (1949) using nymphs of *Blattella germanica* taken from the external sterilized ootheca. They found valine, tryptophan, histidine and probably arginine to be essential for normal growth, cystine for development and glycine and methionine non-essential. The significance of methionine differed from that in vertebrates. However, the authors recognize that intracellular symbionts may have contributed to these findings.

In the last decennium several authors reported rearing insect larvae on mixtures of amino acids as the sole source of nitrogen in the diet. MOORE (1946) obtained growth of larvae of the carpet beetle *Attagenus*, non-sterile on mixtures of 20 amino acids as well as on the 10 amino acids known to be essential for the rat (ROSE 1938). Growth on the complete series was much better than on the incomplete one. By omitting the components one by one from the diet it was found that the 10 amino acids essential for optimal growth of the rat were essential for any growth of *Attagenus*. If, on the contrary, the amino acids non-essential for rats were omitted one by one, or all at once, growth continued for a relatively long time. SCHULTZ et al. (1946) reported that growth of Drosophila is supported equally well on mixtures of amino acids as on a casein hydrolysate. However no details are given as to the composition of the amino acid mixture. Working under aseptic conditions GOLDGERG & DE MEILLON (1947, 1948) reared Aëdes larvae on a mixture of 19 amino acids substituting the protein of the diet, except for the protein present in a yeast autolysate. From the results obtained on omitting the amino acids one by one it was concluded that the following are essential for the mosquito fly: glycine, leucine, isoleucine, histidine, arginine, lysine, tryptophan, threonine, phenylalanine and methionine. The significance of valine could not be established owing to the presence of valine in the yeast fraction. RUDKIN & SCHULTZ (1947, an abstract) stated that the qualitative requirements of *Drosophila* for amino acids are the same as in higher animals. Detailed studies on growth of *Drosophila* in a chemically completely defined medium were published by HINTON et al. (1951). Nearly all the eggs developed into normal adults in the absence of microorganisms on a diet consisting of 13 to 18 amino acids, sucrose, vitamins and salts. Only the time necessary for the eggs to become adults is prolonged

for 1 or 2 days. From the experiments reported in this communication it was concluded that tryptophan, isoleucine and arginine are essential for *Drosophila*, whereas serine is not. The amount of glycine in the medium proved to be very important for normal growth. In higher concentrations glycine exerted a detoxifying effect on essential amino acids. Studies by these authors on the significance of other amino acids for Drosophila are in progress.

Table 3 summarizes the data being available at present concerning the amino acids essential for growth of several insect species.

Insects	Indispensable amino acids	Authors
Attagenus	arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, valine	MOORE (1946)
Aëdes	arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, glycine	GOLDBERG—DE MEILLON (1948)
Blattella	arginine, histidine, tryptophan, valine	HOUSE (1949b)
Drosophila	arginine, histidine, lysine, tryptophan	LAFON (1939)
Drosophila	tryptophan	TATUM (1939, 1941) SCHULTZ et al. (1946) VILLEE and BISSELL (1948)
Drosophila	arginine, tryptophan, isoleucine	HINTON et al. (1951)

Table 3. *Amino acids required for growth of different insect species.*

From the above review and from table 3 it appears that in spite of a relatively large number of papers, the knowledge regarding the amino acid requirements of insects is still only fragmentary. Obviously the list of essentials is complete only for the carpet beetle, while for the mosquito fly the importance of valine is uncertain. On the whole it may be stated that the present knowledge about the needs of insects for amino acids is in good agreement with that of higher animals.

CHAPTER III

MATERIAL AND METHODS

From the data mentioned in the previous chapter it appears that with regard to the nutritional requirements of the honeybee only the carbohydrates have been studied in detail, while other food components have so far been not investigated at all. Therefore we designed ex-

periments to obtain some knowledge about the significance for the honeybee of other dietetic constituents, notably of proteins and amino acids. The results of these experiments as recorded in the following chapters have been obtained with two different methods:

1. By comparing the average longevity of groups of caged bees kept on a basal diet of sucrose and tapwater, with that obtained on supplementing the diet with different nutrients.
2. By studying the growth of just emerged bees on different diets with the aid of weight and nitrogen determinations.

Experimental bees were supplied by the apiary at the Laboratory of Comparative Physiology except in a few cases when, owing to lack of sufficient emerging brood, several beekeepers were found willing to provide the desired material.

Experiments were conducted exclusively with the imago honeybee. Newly emerged bees were used for the growth determinations and for most of the longevity experiments. Some longevity experiments were carried out with older bees taken at the entrance of the hives or from the feed hole in the inner cover. Young bees were obtained from combs with sealed brood which were placed in incubators at 33° C. At intervals of 8 hours the emerged bees were carefully taken from the combs on a piece of filter paper and transferred into the experimental cages, mostly about 50 bees in each. It may be emphasized that the bees were not kept under aseptic conditions.

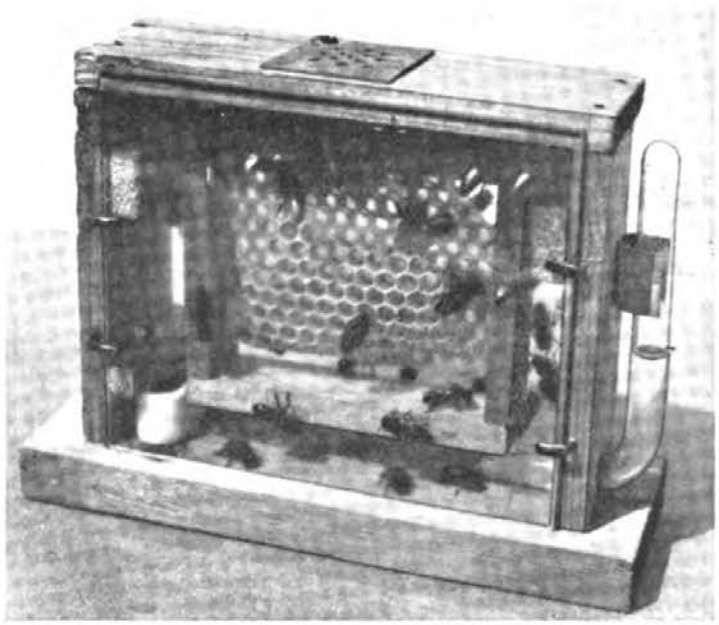

Fig. 1. Experimental cage.

The experimental cages were about equal to the "Liebefelder" experimental cages (see MAURIZIO 1945) with only some minor differences which made handling and care of the bees more convenient. These little cages (see fig. 1) consist of a rectangular little wooden frame ($12 \times 10 \times 4$ cm) closed at both sides with movable glass slides. The bottom board is somewhat broader to promote stability. For filling them with

bees as well as for taking samples the top board contains a round opening (3 cm diam.) closed with a revolving little zinc plate. Through a hole in the lower end of one of the wooden sides a rectangular bent glass tube (diam. 8 mm) is fastened to supply water. This tube, being closed at the top, has an opening within the cage provided with a cotton plug to prevent drowning the bees. The food is supplied in a porcelain tray like that used in bird cages. It is pushed into the cage through a rectangular opening ground in one of the glass slides opposite the water vial. A piece of wood above the feeder prevents contaminating the food. All cages were cleaned before use and dried at high temperature so as to prevent the transfer of bee diseases from previous inhabitants.

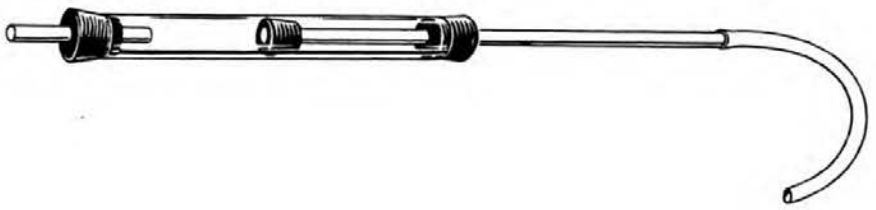

Fig. 2. Catching tube.

The catching-tube. A very useful instrument for handling active bees is the catching tube, a modified "Regina"apparatus often used in beekeeping practice for taking the queen from a comb. It consists of a cylinder of glass (20 cm long and diam. 3 cm) closed at both ends with pierced rubber stoppers. Through each of them passes a glass tube (diam. 1 cm) a short one (10 cm) and a longer one (35 cm). The longer tube is easily movable through the hole in the stopper, if necessary with the aid of some talcum powder. A rubber ring at the end of the longer tube covered with a pierced piece of felt makes it to a plunger. The plunger opening is closed with a wire screen to prevent the passage of bees. The other side of the plunger tube is lenghtened with a rubber tube (30 cm). By sucking at the rubber tube, bees can be caught in the apparatus by the air stream. This simple instrument is useful for various manipulations with living bees a.o. for catching bees from colonies, for taking samples from the experimental cages, for transferring bees from one cage to the other etc.

Food and water were provided ad libitum. Results obtained by different authors in experiments with caged honeybees demonstrated the important influence of several factors such as food, temperature humidity, and water supply on the longevity. The importance of a sufficient water supply was investigated by WOODROW (1941) and MAURIZIO (1946). They found the longevity of bees in captivity to increase up to 8 times, if besides honey or sugar candy, water was also available. Therefore in our experiments the bees were constantly supplied with water except during the first two days. Repeatedly we noted that newly emerged bees became moistened, if water was present in the vial, often resulting in a high mortality. It was prevented by furnishing water not before the third day of captivity. We employed tapwater so as to supply the bees with minerals present in it.

The purpose of our longevity experiments was mainly the study of the nutritional requirements of the honeybee. Since the use of chemically defined diets is very important in nutritional research, sucrose is a more advisable source of carbohydrates than is honey. Honeys always contain a great number of compounds other than sugars (a.o. vitamins, enzymes, minerals, dextrins, acids, pigments, aromatic compounds, pollen), in amounts varying with the plant species that supplied the nectar. Moreover, MAURIZIO (1946) obtained no appreciable difference in longevity whether honey or sucrose was fed. The homogenity of sucrose, its honey-replacing value and inexpensiveness, lead us to employ sucrose as a source of carbohydrate in the basal diet.

The basal diet consisted of sucrose and tapwater (4 : 1 by weight). The sugar is dissolved in the tapwater by boiling the mixture for a very short time (less than one minute) under reflux. After cooling at room temperature during some hours the clear colourless oversaturated sugar solution is stirred by hand with a glass rod to obtain a very finely crystallized paste. The food components to be tested may be devided homogenously in this sugar candy, thus forcing the bees to consume them in the desired concentration, even if they are insoluble. In most of the experiments with amino acid mixtures, the amino acids were added to the sugar water mixture before heating. In these cases the mixture was heated under reflux (about 5 min) until boiling on a glycerol-bath at about 145° C. It was removed from the bath and cooled when nearly all the sugar crystals had disappeared. To obtain very small sugar crystals the stirring with the glass rod must not be delayed for more than one hour.

In order to obtain a homogenous food in which pollen from dried pollen pellets is present, it proved to be desirable to leave the pellets in the candy for about 24 hours. Thereafter some stirring suffices to obtain a finely devided pollen suspension.

The temperature of the environment exerts an important influence on the longevity of caged bees. Although a temperature of about 34° C is maintained in the brood area of a bee colony, a lower temperature proved to promote the longevity (KELLER-KITZINGER 1935; MAURIZIO 1946). The latter author observing the longevity at 20, 30 and 37° C obtained a pronounced optimum at 30° C. Therefore the temperature of our incubators was regulated at 30° C. Brood combs with emerging bees, however, were always kept at 33—34° C.

The care of the bees consisted of daily counting and removing the dead ones. Each day the food was replenished or moistened with a drop of water followed by stirring with a firm dissecting needle. Once a week food and water were renewed. Longevity experiments were continued until all of the bees originally present in the experimental cage had died (1 to 4 months). A bee found dead was assumed to have died on the day it was removed. When the last bee from a cage had died, the average longevity was calculated of all the bees originally present.

Growth studies were carried out by determinations of fresh and dry weight and of the total nitrogen content of the bee bodies from which the digestive tract and the Malpighian tubus had been removed. Bees in captivity accumulate non-digestible material and excretory products in the hind gut and the Malpighian tubus, as defecation only occurs during flight. To obtain exact figures of weight and nitrogen content of the bee body it is necessary therefore to remove the alimentary tract with its content included. Data of analysis of bees are given by several authors. PARHON (1909) and STRAUS (1911) did not mention the removal of the digestive tract. So it is likely that they analyzed whole bees including the gut. KELLER-KITZINGER (1935) and LOTMAR (1939) removed hind gut and midgut, leaving the honey stomach and oesophages in the bee. In the present investigation it was found that much higher deviations of the average fresh and dry weight occur if the honey stomach is not removed. This is comprehensible since the content of the honey stomach may amount to more than 50 % of the bees weight.

The alimentary tract may easily be removed after killing the bees with chloroform. By carefully pulling the sting with a pair of tweezers the rectum comes out first, followed by the anterior intestine and the ventriculus. It is advisable to replace the tweezers proximal of the proventriculus as soon as this organ becomes visible. Otherwise the ventriculus is often separated from the proventriculus. In this way the alimentary canal including the honey stomach may be removed as a whole. The oesophagus breaks through but the contents of the honeystomach is not lost probably due to contraction of oesophagus musculature. The Malpighian tubus being attached to the proximal end of the hindgut are likewise removed. The sting with poison sac is separated from the gut and analyzed with the bee body. HAYDAK (1934 etc.) removed not only the whole gut but also the last abdominal segment. This difference with our method will cause no appreciable deviation in weight or nitrogen content.

Weight determinations were performed mostly on samples of 5 bees. They were weighed soon after killing with chloroform and again after having been dried for 3 days in a drying oven at 80° C. Since the fresh weight of different samples of the same lot of bees shows a much higher variance than the dry weight, the latter is more valuable as a testing method for recording growth. The results of the fresh weight determinations will therefore not be given in the following chapters.

Nitrogen determinations have been carried out nearly exclusively on samples of whole bees without digestive tract, mostly 5 bees being used in one sample. After drying, the destruction of the bees was performed with 3 ml concentrated sulphuric acid, 2 g potassium sulphate and 2—5 mg selenium mixture according to WIENINGER. A simple U-shaped tube of Jena glass (diam. 2 cm) was used for destilling the ammonia. The titrations were performed with 0.1 n sodium hydroxide and a mixture of methyl-red and methylene-blue in 96 % alcohol as indicator. Determinations of several nitrogen containing substances (1—26 mg N) showed a standard deviation of $\pm$ 0.013 up to $\pm$ 0,028 . $\left(s = \pm \sqrt{\frac{\Sigma d^2}{n-1}}\right)$. The maximum deviation from the average amounted to 0,05 mg N. For further details of the nitrogen determination see DE GROOT and MIGHORST (1951).

CHAPTER IV

PROTEIN CONTENT AND PROTEIN METABOLISM

1. *Growth of young worker bees.*

A considerable number of authors observed growth phenomena of worker honeybees after emergence from the cell as measured by determinations of weight and nitrogen content (PARHON 1909; STRAUS 1911; HAYDAK 1934 a; KELLER-KITZINGER 1935; LOTMAR 1939; DE GROOT 1950). It appeared that in the imago stage of the workerbee a considerable growth occurs which may amount to more than 50 % of the original dry weight and nitrogen content. HAYDAK (1934) and KELLER-KITZINGER (1935) gave a detailed picture of the changes occurring in the protein content by determining the amount of nitrogen present in the whole body (without digestive tract) or parts of it, on successive days of the imago life. An increase in nitrogen content and dry weight was found to occur in about the first five days after emergence, then a certain level is reached which is constant. These changes run parallel with the development of the brood food glands (KRATKY 1931) thus enabling the bees to secrete royal jelly, the food for young larvae.

There is no doubt that the important growth of young bees is the result of the consumption of pollen or bee bread. This may easily be verified on the base of the considerable amounts of pollen present in the ventricules and in the rectum of young bees, especially before defecation has occurred during the first orientation flight. Analysing the conditions necessary to achieve growth, KELLER-KITZINGER (1935) could obtain the normal increase in dry weight and nitrogen content only when young bees were fed honey and bee bread at a temperature between 30 and 34° C. HAYDAK

(1933, 1936, 1937), however, found a normal development of workerbees if experimental bee colonies were fed pollen substitutes instead of pollen or bee bread (cf. page 9). On account of these contradictory results we carried out some experiments, feeding caged bees sugar candy supplemented with either bee bread or casein at temperatures of 23 and 30° C (DE GROOT 1950). The results showed that neither bee bread nor honey, nor a temperature of 30° C is an indispensable requisite to obtain the normal development of young honeybees. A diet of sugar and casein at 30° C as well as at 23° C proved to be sufficient to warrant the weight and nitrogen content being normal for adult bees. These observations were repeated in 1951 with only the minor difference that pollen was used instead of bee bread.

Young bees were obtained and kept as described in chapter III in June 1950. Two groups fed sugar candy with 20 % beegathered pollen or 10 % casein respectively, were kept in an incubator at 30° C. Two other groups fed with the same diets were kept in an incubator at 23° C. The pollen was gathered with a pollen trap and dried in a vacuum dessicator over $CaCl_2$. The casein was casein-Hammarsten. In this experiment as well as in those described in the following pages the percentage of the food components supplied in the basal diet is calculated as percentage weight of the sugar in the sugar candy. Each group of caged bees consisted of 100 newly emerged individuals (0—8 hrs old). 160 Young bees were marked with a dot of paint and added to a colony in an observation hive. Analyses on samples of the experimental bees as well as of bees placed in the observation hive taken at intervals, gave a picture of the development under the different conditions of the experiment. The changes in weight and nitrogen content were followed as described in Chapter III page 20. Samples of 10 bees were analyzed at intervals from the first until the 28th day. The results are plotted in figure 3. (Irregularities in the course of the curves may be ascribed to the fact that each point is the result of only one determination).

In each experimental group a considerable growth occurs, at 30° C it surmounts that observed under the natural conditions of the bee colony. The values for dry weight and nitrogen content being reached or surpassed ultimately may be considered as normal for adult bees (viz. 21 mg and 2.5 mg respectively). These results corroborate the earlier statement (DE GROOT 1950) that a mixture of tapwater, sucrose and a suitable source of protein is an adequate diet to raise the dry weight and nitrogen content of young bees to the normal level even at a temperature about 10° below normal. The course of the curves at the lower temperature suggests that the rate of growth at 23° C is considerable lower than at 30° C. It is not justified to conclude that this retarded growth rate results from the inhibiting influence of lower temperature on digestive and metabolic processes. As may be seen in figure 3 the rate of growth is very poor during the first 2 days at 30° C and the first 6 days at 23° C. It will be shown at page 00 that the food consumption of caged bees at 30 °C during the first days of captivity is considerably less than in the following days. It is likely that the same phenomenon, perhaps to a higher degree, occurs at 23° C. Therefore one must be very careful in interpreting details in the course of these curves. In this case it has to be taken into account

that the apparent retardition in growth rate at the lower temperature may be caused to a considerable degree by the abnormal behaviour of caged bees, notably by a lower food intake.

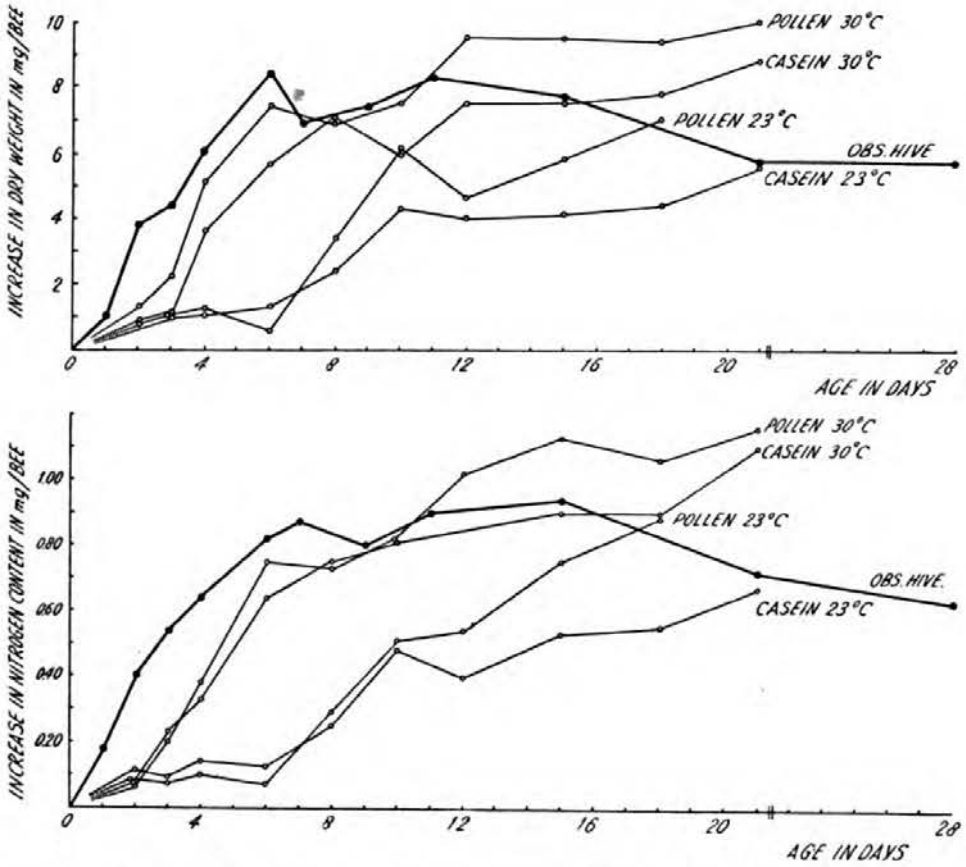

Fig. 3. Changes in dry weight and nitrogen content of bees fed on sugar candy supplemented with 20 % pollen or 10 % casein at 30° C and 23° C, as compared with those obtained under natural conditions.

2. *Growth of young drones.*

Data concerning growth phenomena during imago life of the male honeybee are given by STRAUS (1911). Values of the nitrogen content of pupal stages varied between 4.4 and 6.3 mg. In the adult a value of 7.6 mg was found. Obviously, some growth occurred after emergence.

We carried out some observations by analyzing samples of drones shortly after emergence and after a stay of 4, 8 and 12 days in a normal bee colony during June 1952. The alimentary tracts were removed before analyzing. The results of the dry weight and nitrogen determinations are plotted in figure 4.

As it will be seen from this figure, a distinct growth occurs during the

first four days of imago life amounting to 28 % of dry weight and 38 % of nitrogen content. In another experiment an increase in nitrogen content of even 62 % was observed. We found the fresh weights to decrease somewhat during imago life, which may also be deduced from the data of STRAUS. From above observations it appears that also in the drone imago, growth phenomena occur very similar to those observed in worker bees.

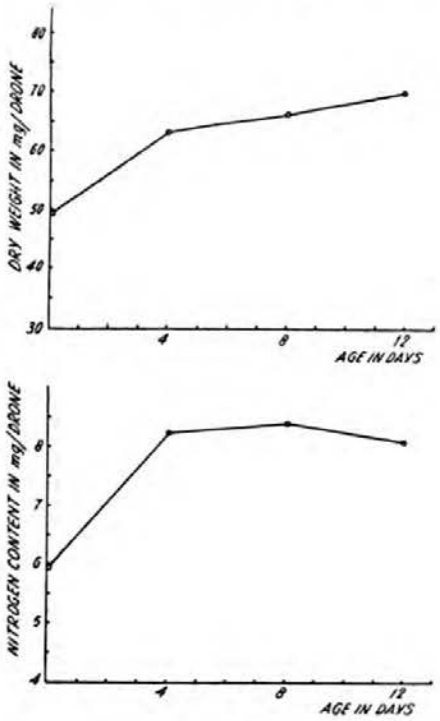

Fig. 4. Increase in dry weight and nitrogen content of young drones in a normal bee colony.

3. *Protein metabolism of bees.*

A. Decrease in body weight and nitrogen content at the change of nursing stage into gathering stage.

Following the curve for the changes in nitrogen content and dry weight of bees in a normal colony (fig. 3) it may be noted that on the 7th day the optimum level is reached. Apart from a slight irregularity (being ascribed to common variation in the testing material) the high level remains rather constant until the 15th day. This period corresponds fairly closely with the nursing duty of the young worker honeybee, as stated by RÖSCH (1925, 1930). During this time the pharyngeal glands are highly developed and secrete the royal jelly (KRATKY 1931). After the 15th day a slight decline occurs in the curve concerned, pointing to a decrease in dry weight and nitrogen content after the nursing stage. The same phenomenon was also observed in the experiment published before (DE GROOT 1950). These findings are not in agreement with the results of KELLER-KITZINGER (1935) and HAYDAK (1934b). According to these authors the total nitrogen content remains constant after having reached the maximum value. Since each point in fig. 3 was obtained from only one determination, the variation in the experimental material may have accounted for this discrepancy. To evaluate this possibility 200 young bees (less than 18 hrs old) were marked and added to a bee colony in an observation hive in Aug. 1950. 5 Samples of 10 bees were taken and analyzed after 14 days, another 5 after 28 days. The figures are given in table 4.

It will be observed from table 4 that the mean values of bees 28 days old are lower than those of bees 14 days old. According to the test of

14 days old		28 days old	
Dry weight	Nitrogen content	Dry weight	Nitrogen content
22.6	2.66	20.7	2.49
22.3	2.67	20.5	2.50
22.5	—	20.7	2.54
23.2	2.64	20.6	2.53
21.3	2.52	21.2	2.52
22.4	2.62	20.7	2.52

Table 4. *Dry weight and nitrogen content of worker honeybees (mg per bee) during and after the nursing stage.*

Wilcoxon the difference in dry weight is highly significant. In spite of one very low value in the nitrogen values after 14 days, the avęrage is significantly higher ($P = 0.04$). Moreover differences in relation to weight and nitrogen content between nurse bees and field bees appeared to exist at any time of the year (see below). Thus a decrease in body weight and nitrogen content of the worker honeybee has been established before changing into the gathering stage, apparently occurring as a result of the nursing duties.

B. Summer bees compared with winter bees.

It is a widely propagated opinion that bees in autumn consume large amounts of pollen to built up reserve stores for the winter season and that as a result the protein content of bees in winter would be higher than in summer. LOTMAR (1939, 1951) analyzing the physiological condition of bees in the winter season stated that the nitrogen content and the dry weight of winterbees is distinctly higher than that of field bees and even of nurse bees in summer. MAURIZIO (1946) studying the longevity of bees in different seasons observed an increase in the life span of bees in autumn as compared with that during the summer months.

On analyzing a great number of samples of bees taken in different months of the year at the entrance of several hives (guard bees, gathering bees) we could not detect any appreciable influence of the season either on the body weight or on the nitrogen content. On the other hand considerable differences were observed between bees from different colonies even on the same day. An illustration of these differences is given in table 5.

Bees were taken at the hive entrances at days when cold weather forced them to stay within. They were induced to come to the entrance by blowing in the hive opening and sucked up in the catching tube. It may be supposed that the bees caught in this way are guard bees or gathering bees.

Date	Colony number	Number of samples	mg Nitrogen per bee and standard deviation
18-IX-1949	3	8	2.35 ± 0.037
	6	8	2.53 ± 0.045
29-XI-1949	1	4	2.60 ± 0.072
	2	4	2.53 ± 0.074
	5	4	2.48 ± 0.040
3-XII-1949	4	4	2.52 ± 0.043
	3	3	2.43 ± 0.028
4-I-1950	4	4	2.50 ± 0.044
	3	4	2.44 ± 0.042
21-III-1953	4	8	2.51 ± 0.035
	8	8	2.57 ± 0.041

Table 5. *Nitrogen content of adult bees taken from different colonies at the same day.*

Table 2 shows considerable differences in nitrogen content between bees of different colonies. Differences being statistically significant (according to the test of WILCOXON) were obtained on 18-IX-'49, 3-XII-'49 and 21-III-'53 ($P < 0.001$, $P = 0.029$ and $P = 0.007$ respectively). These data illustrate the necessity of being extremely careful in comparing findings obtained from different colonies.

According to LOTMAR the term 'winterbees' includes those bees possessing well developed fatbodies and brood food glands in the secreting stage. Of course analyses of such bees may reveal a higher protein content and body weight than nurse bees in summer. However, there is no need to ascribe an eventual difference to additional pollen consumption in autumn. Just the suggestion that nurse bees in autumn, due to lack of larvae to be fed, remain in the stage of maximum development during the entire broodless season would suffice to explain a possible difference in nitrogen content between nurse bees released from nursing duties and those exerting their nursing duties. This idea is supported by the observed decrease in body weight and nitrogen content during the nursing stage being probably the result of feeding considerable quantities of proteinaceous material to the larvae. Moreover, MAURIZIO (1950) found pharyngeal glands and fat bodies of winterbees to be present in broodless colonies during the summer, thus establishing that they are not restricted to bees in winter. Whether nurse bees in winter attain a higher level of optimal development than do nurse bees in summer remains to be investigated. From the above considerations it seems justified to suggest that this is not the case.

C. Nurse bees compared with gathering bees.

As stated above (chapter IV, 3 A) the change from nursing duties into gathering duties is correlated with a decrease in protein content and body weight. Differences between nurse bees and field bees in relation to weight and nitrogen content appeared to exist at any time of the year. As far as is known this phenomenon was not mentioned before in literature.

Obviously one may distinguish between two kinds of worker honeybees in a bee colony, being distinctly different in weight and nitrogen content. Which kind of bees one obtains on taking samples depends mainly on the place were they are caught viz. on the alighting board or inside the hive. In general it may be said that bees with a high protein content and dry weight and well developed brood food glands (physiologically young) are present in the brood area inside the hive. These individuals are in the nursing stage. Bees with a low protein content and dry weight and degenerated brood food glands (physiologically old) may be obtained on the alighting board of bee hives, corresponding with guard bees or gathering bees. Since we were unable to distinguish between two kinds of individuals at the entrance during the inactive season we will call them gathering bees in the following pages.

Date	Colony number	Number of samples	Dry weight		Nitrogen content	
			Gathering bees	Nurse bees	Gathering bees	Nurse bees
4-III-1950	4	4	21.2	22.2	2.51	2.70
16-III-1950	1	4	20.9	21.7	2.57	2.57
21-III-1950	3	3	20.5	22.1	2.53	2.63
21-III-1950	5	4	21.0	22.4	2.55	2.71
25-III-1950	5	5	21.2	21.4	2.59	2.63
30-III-1950	4	3	21.4	22.9	2.59	2.65
15-XI-1950	2	5	20.2	22.0	2.50	2.63
17-XII-1951	1	5	19.5	22.3	2.45	2.66
12- II-1952	6	5	21.8	24.8	2.60	2.79
21-III-1953	4	8	20.8	21.2	2.51	2.58

Table 6. *Difference in dry weight and nitrogen content between nurse bees and gathering bees in the same colony.*

Table 6 comprises the average figures of analyses on 4 or 5 samples of bees taken from different colonies. Under the heading 'gathering bees', figures are given corresponding to bees being obtained by inducing them to come out of the entrance by tapping on the front of the hive or by blowing in the entrance. The figures under the heading 'nurse bees' result from bees taken from the upper part of the bee cluster via the feed opening in the inner cover.

Table 6 illustrates the occurrence of a difference in dry weight and

nitrogen content between nurse bees and gathering bees. Only in one case no difference was found in the nitrogen content (16-III-1950). Table 6 contains all the figures obtained in this way. So there was no selection, only the conditions were chosen so as to increase the possibility of obtaining gathering bees at the entrance and bees in the nursing stage from inside the hives. Even those with little knowledge of the behaviour of bees in a colony will understand, however, that it is not difficult to obtain gathering bees from the feed hole and nurse bees from the entrance of the hive. This, however, does not alter the fact of the occurrence of two kinds of bees in bee colonies differing in weight and nitrogen content. This phenomenon confirms the statement (see 3 A in this chapter) that the transition of the nursing stage into the gathering stage is accompanied by a decrease in body weight and nitrogen content.

D. Experiments on the protein metabolism of older bees.

There is much confusion in the relative literature as to the ability of older bees to digest and metabolize proteins. KRATKY (1931) stated that newly emerged bees after having been fed for 10 days a sugar solution, were not able to develop their pharyngeal glands if pollen was supplied after the 10th day. KELLER-KITZINGER (1935) could not detect any appreciable change in the nitrogen content of old bees kept for 14 days either on a diet of sucrose, or on a diet of sucrose supplemented with bee bread. She concluded that bees are not able to use proteins in catabolic reactions. HAYDAK (1937 b, 1937 c), however, keeping bees for a longer time on a protein-free diet observed a substantial decrease both in weight and nitrogen content, and concluded that young as well as older bees might catabolize their body proteins. If pollen was supplied after the bees had been kept for 18 days on a sugar diet, HAYDAK observed a development of the body. LOTMAR (1939) confirmed HAYDAK's conclusions. She observed changes in the nitrogen content of old bees kept either on a protein-free diet or on a diet supplemented with pollen. BEUTLER & OPFINGER (1948, 1949) in longevity experiments with caged bees kept on sugar solution observed a lengthening of the life span if pollen or bee bread was supplied during the first 10 days of life. On feeding pollen after the 10th day no increase in longevity was observed. From these findings the conclusion was derived that obviously after the 10th day the bee is no longer able to metabolize considerable quantities of protein. However, in these experiments the pollen was fed separated from the sugar, thus enabling the bees to consume only carbohydrates. Observations of the pollen content of the gut revealed indeed a very low pollen intake.

We kept newly emerged bees in experimental cages for a period of 10 days on a diet of sugar candy and supplied a 20% pollen-sugar candy after the 10th day, thus forcing the bees to consume the pollen. The changes in weight and nitrogen content were followed on samples of

10 bees taken at intervals from the 10th up to the 25th day of life (i.e. the 15th day after pollen feeding commenced).

The results, plotted out in figure 5, show a steady increase in dry weight and nitrogen content from the moment that the pollen candy was supplied. The values reached at the 13th day of pollen feeding (i.e. the 23th day of life) may be considered as normal for adult bees even in the nursing stage. The rate of growth however, is only about half that observed in fig. 3 between the 2nd and the 10th day on the same diet. It seems necessary therefore to modify the conclusion of BEUTLER & OPFINGER mentioned above in this way that after the 10th day of life the bee is able to metabolize considerable quantities of protein though not at the same rate as before.

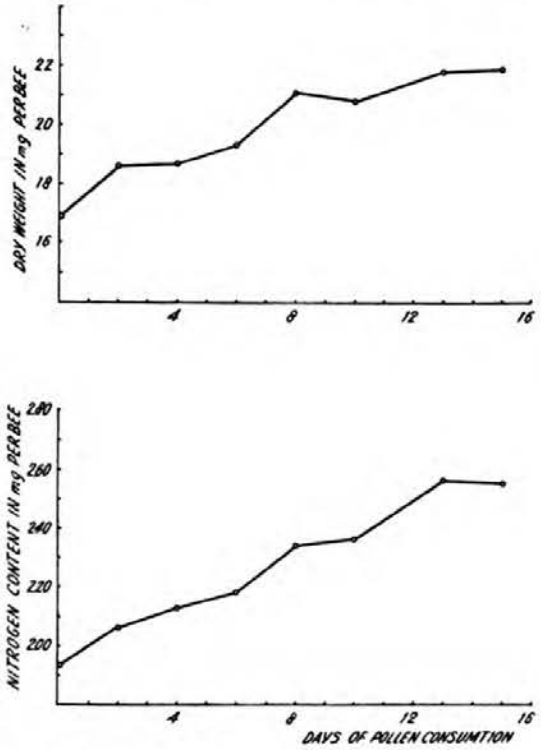

Fig. 5. Growth of young bees on a diet containing 20 % pollen after having been fed a pure carbohydrate diet for. 10 days.

HAYDAK (1937 b, 1937 c) and LOTMAR (1939) in contrast with KELLER-KITZINGER (1935) arrived at the conclusion that adult honeybees may catabolize proteins. In an attempt to verify this statement we fed 3 groups of very old bees taken at the entrance of a hive in March 1950 (i.e. gathering bees) either with sugar candy, or with sugar candy supplemented with 0.5 and 5.0 % casein respectively. By analyzing samples after 7 and 14 days (3 or 4 at a time) we recorded the changes which eventually would occur in these 3 lots of bees. The results of the nitrogen determinations are summarized below.

The figures show a decrease in nitrogen content of bees fed a pure carbohydrate diet during 14 days and an increase if supplemented with 5 % casein. Even though these data are averages of only 4 samples of 5 bees, the differences are essential according to WILCOXON ($P = 0.014$). A diet with 0.5 % casein seems to be sufficient to maintain the nitrogen content at a constant level. These results confirm the statement of HAYDAK (1937 b) that old bees can use protein in metabolic processes.

Diet	Nitrogen content in mg/bee	
	After 7 days	After 14 days
Sugar candy	2.51 ± 0.016	2.44 ± 0.018
0.5 % casein-sugar candy	2.51 ± 0.016	2.51 ± 0.041
5.0 % casein-sugar candy	2.65 ± 0.045	2.61 ± 0.034

Table 7. *Changes in nitrogen content of bees kept for 7 and 14 days on sucrose and on sucrose supplemented with casein. (Bees taken from the hive showed a value of 2.52 ± 0.033 mg).*

In a subsequent experiment performed in December 1951 the decrease in nitrogen content of adult bees on a pure carbohydrate diet was studied more accurately by analyzing 7 samples instead of 3—4 in the previous experiment. The two kinds of bees were taken as described above and kept for 30 days on a diet of sugar candy. The results of the nitrogen determinations in mg per bee may be summarized as follows.

Nurse bees : 2.66 ± 0.015. After 30 days on sugar: 2.36 ± 0.032
Gathering bees: 2.45 ± 0.051. ,, 30 ,, ,, ,, : 2.32 ± 0.051

So a distinct decrease occurred in both cases, being 11.3 % for nurse bees and 5.3 % for gathering bees .If one supposes 1 kg of bees to consist of 10.000 gathering bees and 8.000 nurse bees (the alimentary tract included) the loss of nitrogen amounts to 43 mg/kg/day for gathering bees and 80 mg/kg/day for nurse bees. LAFON (1951) working with cockroaches and meal worms on a protein free diet for 90 days observed an endogenous loss of nitrogen amounting to 84 and 159 mg/kg/day respectively. Thus the agreement of cockroaches with nurse bees is striking.

From the results of the 3 observations described above it is justified to conclude that the adult honeybee may use proteins in anabolism and catabolism. These findings emphasize the continuous need for protein of the bee colony even in the broodless winter season, a fact being often disregarded in beekeeping practice.

4. *Nitrogen content of newly emerged bees.*

Data concerning the nitrogen content of bees, newly emerged under normal conditions are scanty in literature. HAYDAK (1934b) mentioned a value of 1.74 mg nitrogen per bee, LOTMAR (1939) 1.63 mg, KELLER-KITZINGER (1935) 1.6 — 1.7 mg, STRAUS (1911) 2.2. mg. In the latter case the alimentary tract was not removed. On subtracting 0.2—0.3 mg, which is the nitrogen content usually found for alimentary tracts of just emerged bees, a value of 1.9—2.0 mg results from the work of STRAUS. It may be said that these few figures show a good agreement.

During the years 1949—1952 we analyzed more than 600 samples of 5—10 newly emerged bees being 0—8 hrs old, and taken from different colonies. The nitrogen content of these samples ranged from 1.49—2.33

and the dry weight from 13.2—18.5 mg per bee with averages of 1.95 mg and 16.0 mg respectively. It is stressed, however, that one is not justified in attaching general value to these data since it is possible to change the average simply by the performance of a larger number of analyses in a certain period of the brood rearing season. For instance in our climate the period of maximum brood rearing activity falls in April and May. The bees reared in this period emerge with a low weight and protein content. On the other hand in autumn, brood rearing decreases which involves a distinct increase in weight and nitrogen content of the emerging bees. A calculation of the mean values of 401 determinations on very young bees emerged from different colonies in 1952 reveals the following figures:

		Dry weight	Nitrogen content
26 Febr.— 1 Dec.	401 samples	16.4 ± 1.12	2.00 ± 0.173
26 Febr.—31 Aug.	204 ,,	15.7 ± 0.88	1.89 ± 0.141
1 Sept.— 1 Dec.	197 ,,	17.2 ± 0.77	2.13 ± 0.104

The higher development of newly emerged bees in autumn confirms an observation being emphasized already in a previous communication (DE GROOT 1950). The same applies to other situations in which the brood area is small as compared with the number of nurse bees, for instance when a young queen starts egg laying or if a comb containing eggs or larvae is placed into a queenless colony.

LEVIN & HAYDAK (1951) investigated the seasonal variation in weight of bees reared in 4 colonies of different strength. They found colony strength to affect the weight of the bees in such a way that the stronger colonies reared bees with higher dry weight than the weakest one. Moreover all colonies showed uniform seasonal variations with regard to the weight of the bees reared. They obtained peaks corresponding to the fluctuations in the amount of pollen gathered. Obviously in their experiment the peaks of pollen income correspond to maxima in dry weight. In contrast with these observations we found the highest values of dry weight and nitrogen content to occur in autumn when pollen income was very low or totally absent, whereas the lowest values were obtained in April and May when an abundance of pollen was brought in, being accompanied with a maximum brood rearing activity. This means that the weight and the nitrogen content of the bees reared was found to be not primarily determined by the pollen income but rather by the relative proportions of the number of larvae which have to be fed and the number of bees in charge of feeding the larvae.

Apart from differences occurring in two seasons variations may be found as well in a period of only a few days or even between bees emerged from different combs in one day. Table 8 shows examples of such extreme variation.

The differences in the nitrogen content recorded in this table are essential (according to Wilcoxon; $P < 0.04$) in those cases where more than 2

Date	Colony	Number of samples	Dry weight and standard deviation	Nitrogen content and standard deviation	Brood comb
26-II-'52	7	2	18.4 ± 0.10	2.20 ± 0.57	} the same comb
28-II-'52	7	2	15.7 ± 0.14	1.81 ± 0.781	
17-III-'52	7	2	16.8 ± 0.50	2.02 ± 0.922	} the same comb
18-III-'52	7	2	16.0 ± 0.28	1.91 ± 0.707	
19-III-'52	7	2	14.7 ± 0.22	1.76 ± 0.100	
3-IX-'52	2	4	15.0 ± 0.37	1.82 ± 0.486	} the same comb
4-IX-'52	2	4	15.6 ± 0.20	1.93 ± 0.163	
5-IX-'52	2	4	16.4 ± 0.16	2.01 ± 0.622	
26-VIII-'52	6	6	15.1 ± 0.40	1.81 ± 0.465	comb 1
26-VIII-'52	6	6	15.6 ± 0.08	1.87 ± 0.385	comb 2
24-IV-'53	4	7	15.6 ± 0.37	1.85 ± 0.252	comb 1
24-IV-'53	4	7	15.2 ± 0.46	1.79 ± 0.413	comb 2

Table 8. *Variations in dry weight and nitrogen content in mg per bee of samples of newly emerged bees from one comb on different days, or from different combs from one colony on the same day.*

samples were analyzed. This considerable variation in newly emerged bees is of high importance in bee research if homogenous material is required.

CHAPTER V

LONGEVITY EXPERIMENTS

In the survey of the literature concerning nutritional studies with caged young bees, it has been mentioned already, that the addition of pollen to a basal diet of honey or sucrose exerts a marked longevity promoting influence. Since obviously in the form of pollen, nutritional factors are supplied necessary for maintenance of life, the longevity may be used as a criterion to study the nutritional requirements of the honeybee.

1. *Significance of concentration.*

Several conflicting reports are to be found in literature concerning the effect of food stuffs other than pollen on the longevity of the honeybee. MELAMPY & MCGREGOR (1939) and BEUTLER & OPFINGER (1949) were not successful in this respect. PETERKA (1939) and recently MAURIZIO (1950, 1951) succeeded in obtaining an increase in longevity on supplementing the basal diet with protein containing materials. The effect seems to be mainly dependent on the concentration. A protein containing food component exerting a distinct increase in longevity may be deleterious if applied in a higher concentration (MAURIZIO 1950, 1951), which was ascribed to a detrimental effect of accumulation of non-digested material in the gut (MAURIZIO 1946).

In 1949 we carried out some preliminary experiments with proteinaceous food stuffs in various concentrations, the results of which are presented in table 9.

Supplement to sucrose candy	Number of series	Number of bees	Maximum longevity in days	Average longevity in days and standard deviation [1])
non-supplemented	2	99	39	29.5 ± 4.39
bee bread 10 %	1	46	98	43.6 ± 14.99
,, ,, 5 %	1	43	88	56.4 ± 19.58
brewers' yeast 10 % . . .	1	45	66	29.6 ± 7.39
,, ,, 1.25 % . .	1	51	79	58.3 ± 10.25
casein(Hammarsten)10 %	1	48	34	26.9 ± 3.69
,, ,, 5 %	1	40	36	26.2 ± 4.46
,, ,, 2.5 %	1	51	51	41.0 ± 5.81
,, ,, 1.25%	1	46	58	47.6 ± 5.60
non-supplemented	2	100	37	28.0 ± 3.87
casein (Hammarsten) 2.5 %	1	50	65	44.5 ± 7.07
,, ,, 1.25%	1	50	92	53.1 ± 10.94
,, ,, 0.63%	1	52	76	48.9 ± 12.18
non-supplemented	2	104	34	25.2 ± 3.40
soybean meal 1.25 % . .	1	52	76	42.9 ± 8.73
,, ,, 0.63 % . .	1	49	70	50.1 ± 11.91
brewers' yeast 5.00 % . .	1	50	61	47.2 ± 11.36
,, ,, 1.25 % . .	1	44	116	65.4 ± 20.39
bee bread 5.0 %	1	43	111	48.4 ± 23.02
,, ,, 2.5 %	1	54	104	67.9 ± 17.17
,, ,, 1.25 %	1	49	90	54.7 ± 15.64
casein vit.free 2.0 % . . .	1	51	65	42.0 ± 12.55
,, ,, 1.25 % . .	1	49	95	53.1 ± 13.35
,, ,, 1.0 % . . .	1	51	98	58.2 ± 16.65
non-supplemented	3	143	36	29.9 ± 4.06
pollen-mixture 3.0 % . . .	1	54	62	46.5 ± 8.66
,, 2.5 % . . .	1	51	69	52.0 ± 9.22
,, 2.0 % . . .	1	50	53	42.9 ± 6.28
,, 1.5 % . . .	1	51	45	35.2 ± 7.17
,, 1.0 % . . .	1	49	35	30.4 ± 3.85
non-supplemented	4	205	37	25.4 ± 4.25
pollen 3.5 %	1	21	85	71.2 ± 12.59
,, 3.0 % white	1	45	105	64.0 ± 13.21
,, 2.5 % Dutch	1	46	82	57.5 ± 10.62
,, 2.0 % clover	1	53	75	56.7 ± 11.13
,, 1.5 %	1	49	74	44.5 ± 14.10

Table 9. *Average longevity of young bees on diets of sucrose supplemented with various concentrations of protein or protein containing materials.*

[1]) Standard deviation $= \pm \sqrt{\frac{\Sigma d^2}{n-1}}$.

From table 9 it is evident that all the materials used as a supplement in the carbohydrate basal diet promote the longevity if supplied in a favourable concentration. The concentrations exerting the most favourable influence being less in the case of casein than in that of other protein foods, are lower in general than those employed by other authors. Obviously, the negative results of BEUTLER & OPFINGER (1949) using milkpowder, soybean meal and yeast in concentrations higher than 10 % may be ascribed to a detrimental effect of large amounts of undigested material accumulated in the gut or possibly to acidosis. On the other hand, the failure of MELAMPY & MCGREGOR (1939) to obtain an increase in longevity on feeding bees various protein containing food stuffs separated from the carbohydrate basal diet, may be a consequence of insufficient consumption of these materials. This suggestion is supported by the results of experiments carried out on occasion of the work of BEUTLER & OPFINGER (1948, 1949). These authors obtained no increase of the lifespan of bees if bee bread was fed separated from the carbohydrate diet after the 10th day of life. Upon dissection it was observed that relatively few pollen grains were present in the gut of bees fed in this way. In order to find out whether this result might be ascribed to insufficient pollen consumption, we kept just emerged bees on a sugar diet for periods of varying duration, thereafter on a diet in which pollen was supplied in the usual way (i.e. mixed with the sucrose-candy, cf. chapter III) thus forcing the bees to consume the supplement. Table 10 shows the longevities obtained on feeding a 2.5 % pollen-sugar candy after young bees had been fed a pure carbohydrate diet for periods from the 6th up to the 20th day of life.

Diet	Number of series	Number of bees	Maximum longevity	Average longevity and standard deviation
Sucrose	3	120	35	27.6 ± 5.13
Sucrose + pollen after 6 d.	1	43	114	50.7 ± 21.77
Sucrose + pollen after 8 d.	1	54	80	49.3 ± 15.04
Sucrose + pollen after 10 d.	1	51	83	48.9 ± 16.27
Sucrose + pollen after 12 d.	1	56	73	49.0 ± 11.33
Sucrose + pollen after 14 d.	1	49	94	44.9 ± 12.62
Sucrose + pollen after 16 d.	1	49	78	49.3 ± 12.98
Sucrose + pollen after 20 d.	1	50	85	45.3 ± 14.11

Table 10. *Longevity promoting effect of pollen after temporarily feeding a pure carbohydrate diet for periods of increasing duration.*

From the figures presented in table 10 it is evident that the increase in longevity as a result of feeding pollen, may occur also after the bees have been kept for a relatively long period on a pure carbohydrate diet if they are forced to consume the pollen. This observation in connection with the above mentioned negative results calls attention to the possi-

bility that the absence of a longevity-promoting action of a certain food may be due to insufficient consumption, if it is supplied separated from the carbohydrate food. So it is necessary to supply the component to be investigated mixed with the carbohydrate. Since, however, its concentration appeared to be of utmost importance, the longevity method is rather cumbersome for the evaluation of the qualitative significance of protein containing foods.

In how far the longevity promoting effect of the various supplements, as demonstrated in table 9, may be ascribed to the action of the protein may not be decided. Although casein and even a de-vitaminized casein preparation (in this case Labco casein) exerts a similar effect, this fact, however, does not give convincing evidence that only the protein accounts for the increase in longevity, for even a de-vitaminized protein always contains non-negligible quantities of several vitamins. Nevertheless it seems justified to conclude from the figures of table 9 and from the results described in the following pages that the increase in longevity on feeding protein containing materials may be ascribed at least for the major part to the protein.

2. *Simple nitrogen compounds.*

Assuming that the increase in longevity on feeding proteinaceous material is due only to the nitrogen components, we investigated some nitrogen containing compounds for their effect on the life span. These experiments will be described in a few lines, as it makes no sense to present the negative results with detailed figures.

On account of the observations of LOEB (1915), ZABINSKY (1929) and ROESSLER (1932) on rearing insect larvae on diets with nitrogen only in the form of simple compounds, we carried out longevity experiments with young honeybees, using glycine, ammonium sulphate, ammonium nitrate, or ammonium tartrate (0,1—10 %) as a source of nitrogen. Even though the bees were always kept non-sterile, as in the experiments of LOEB, ZABINSKY and ROESSLER, and so bacterial action might take place, these nitrogen compounds appeared to be unfit for bees. The effect on the longevity was either absent or even detrimental. So it appears that the honeybee is more exacting with respect to nitrogen compounds than several other insects.

3. *Pollens and pollen fractions.*

Table 11 summarizes figures on the longevity promoting value of some kinds of pollen and pollen-fractions. There is a great variety in nutritional value of pollens from different plant species (SVOBODA 1940; MAURIZIO 1950, 1951). The high value of white Dutch clover pollen (*Trifolium repens*) for longevity as compared with a mixture of several pollens has been demonstrated already in table 9.

According to SVOBODA (1940) and MAURIZIO (1950) pine pollen has no value at all, which was ascribed by the first mentioned author, to its low

protein content. Indeed we found a nitrogen content of hand-collected pine pollen (*Pinus sylvestris*) of 2.9 % of the dry weight, whereas that of a mixture of bee gathered pollen varied between 3.8 and 5.0 %. However, one can hardly imagine, that the low protein content in itself suffices to explain the low food value. A deficiency in some essential factor seems to be more likely.

Supplement to sucrose candy	Number of series	Number of bees	Maximum longevity	Average longevity in days and standard deviation
non-supplemented	4	195	36	27.1 ± 3.66
pine pollen 1.5 %	1	52	38	33.9 ± 3.36
non-supplemented	4	205	37	25.4 ± 4.24
pine pollen 4.0 %	1	53	42	31.0 ± 5..11
pine pollen 4.0 %	3	153	38	27.5 ± 4.78
,, ,, 4.0 % + Amino acids	3	147	42	27.3 ± 5.11
non-supplemented	3	155	34	26.2 ± 3.96
pollen-mixture 2.5 % . . .	3	149	75	46.5 ± 9.80
Aesculus pollen 2.5 % . .	3	152	77	53.2 ± 9.98
non-supplemented	3	145	35	25.7 ± 4.22
pollen 2.5 % fresh	3	150	74	40.9 ± 12.14
,, 2.5 % 1 year old .	3	138	67	39.4 ± 12.99
,, 2.5 % 2 years old.	3	151	77	41.4 ± 13.52
,, 2.5 % heated 24 hrs	3	148	70	36.6 ± 10.72
,, 2.5 % heated 48 hrs	3	144	46	24.0 ± 7.28
non-supplemented	3	161	32	22.6 ± 4.51
pollen 2.5 %	3	148	69	37.8 ± 9.00
,, 2.5 % ether-extracted	3	149	65	42.1 ± 8.30
non-supplemented	3	133	29	21.4 ± 2.98
albumin + globulin from pollen 1.5 %	1	53	40	30.6 ± 6.26
metaprotein from pollen 0.5 %	1	47	30	25.0 ± 2.48
pollen residue 2.0 % . . .	1	53	36	30.0 ± 3.91
pollen (intact) 2.5 % . . .	3	155	60	34.9 ± 10.37

Table 11. *Average longevity of young bees on diets of sucrose supplemented with different kinds of pollen and protein fractions from pollen.*

Table 11 comprises the figures of the longest average life span obtained in two experiments with pine pollen in several concentrations. A small increase in longevity occurred, being of doubtful significance. Kok (1952), with the aid of paper partition chromatography, made observations on the amino acids present in pine pollen and found the following

amino acids to be absent or present in very small amounts: cystine, hydroxyproline, thryptophan, and methionine. Addition of these amino acids did not improve the effect of pine pollen on longevity (see table 10).

Pollen from the horse chestnut (*Aesculus species*) was reported to be toxic to bees (MAURIZIO 1945; VELTHOEN 1947). As is demonstrated in table 10 we could not observe any toxicity on feeding 2.5 % *Aesculus* pollen obtained with a pollen trap. Several factors may account for these conflicting results. However, we will not go into this subject.

Beegathered pollen obtained with a pollen trap and preserved in a dry state for two years showed no decrease in nutritional value in longevity experiments (see table 11). Drying, however, must not occur at higher temperature since after heating for 48 hours at 80° C the pollen proved to have lost its longevity promoting properties.

Pollen exhaustively extracted with ether in a soxhlet apparatus during 16 hours exhibited no decrease in its effect on longevity (see table 11). Obviously the ether-soluble compounds present in pollen do not contribute to the lengthening of the life span.

A quantitative study of the pollen-nitrogen extractable with salt and alkaline solutions was carried out on several samples of beegathered pollen-mixtures. Some protein fractions were isolated and purified and their nitrogen content was determined. The mean values of 4 to 6 determinations are presented in table 12.

	Nitrogen extracted in % of total	Substance in % of original material	% Nitrogen in dry material
Pollen mixtures		100.00	3.8
Ether extract		3.87	
10 % NaCl extract	41.6		
Coagulable by heat (albumin, globulin)	12.7	2.82	13.63
Precipitable by trichloroacetic acid in residu	4.6	0.42	12.02
0.5 % NaOH extract	19.8		
Precipitable by acid	13.2	3.19	11.75
Pollen residu		24.04	5.20

Table 12. *Distribution of nitrogen, amounts of substances and their nitrogen content obtained on extracting several mixtures of bee collected pollen with ether, salt and alkali in succession.*

It may be seen in table 12 that a considerable amount of a protein like substance was obtained by alkaline extraction of the pollen residu after having been extracted with salt. This protein is separated from the solution as a dirty, darkbrown, flocky precipitate by slightly acidifying the medium. It dissolves again in alkaline solutions and is not heat coagulable. On studying the active principle of pollen causing hay fever, several authors obtained a similiar substance from pollens of various

grass species which was called a glutelin (HEYL 1919; HEYL & HOPKINS 1920; CSONKA, BERNTON & JONES 1925; VINSON 1927).

After repeated washings with water, alcohol and ether, the three protein fractions prepared from a pollen mixture were fed as a supplement to sugar candy in various concentrations in some tentative longevity experiments. The longevities obtained with the most favourable concentrations have been recorded in table 10. The proteins exerted a distinct influence on longevity though not in such a degree as intact pollen. For practical reasons however, the study of the nutritional requirements of the honeybee was not continued along these lines.

4. *Hydrolyzed proteins.*

After having established the considerable increase in longevity of young bees if pure protein is added to sugar candy, we found hydrolysates of proteins to constitute a means of studying the requirements for some amino acids.

On hydrolyzing proteins with strong acids cystine and tryptophan are destroyed. These amino acids were added to the diets in amounts corresponding to their original concentration in casein.

Some results of longevity experiments with protein hydrolysates are summarized in table 13.

Supplement to sucrose	Number of series	Number of bees	Maximum longevity	Average longevity and standard deviation
Non-supplemented	3	146	40	29.3 ± 5.82
HCl-hydrolyzed casein 1.3 % . .	1	51	5	3.6 ± 0.57
idib 1.0 % . .	1	34	5	3.1 ± 0.41
ibid 0.7 % . .	1	55	6	3.8 ± 0.81
H_2SO_4-hydrolyzed casein 1.3 % .	1	46	45	34.7 ± 6.97
ibid 1.0 % .	1	47	50	39.5 ± 6.86
ibid 0.7 % .	1	50	54	44.6 ± 6.96
Non-supplemented	3	147	38	27.9 ± 4.94
H_2SO_4-hydrolyzed casein 0.6 % .	3	154	79	56.0 ± 9.32
ibid 0.4 % .	3	143	80	54.7 ± 12.68
ibid 0.2 % .	3	145	78	43.0 ± 9.28
H_2SO_4-hydr. cas. 0.4% -tryptophan	3	154	54	33.0 ± 6.87
ibid + dl-tryptophan	3	145	85	54.2 ± 8.94
ibid + d-tryptophan	3	151	58	35.5 ± 3.95
Non-supplemented	3	150	35	27.5 ± 4.52
H_2SO_4-hydrolyzed zein 0.5 % . .	3	153	68	42.7 ± 8.50
ibid + lysine . . .	3	145	78	54.0 ± 9.65

Table 13. *Average longevity of young bees fed hydrolysates of casein and zein, supplemented with amino acids.*

Casein was hydrolyzed under reflux in an oil bath for 30—48 hrs either with strong hydrochloric acid or with 30 % sulphuric acid. After dilution with distilled water (1:1) the HCl was removed as far as possible by repeated evaporation under reduced pressure at 50° C, and in the case of H_2SO_4 hydrolysates, the acid was neutralized with barium hydroxide. The precipitated $BaSO_4$ was centrifuged and washed 3 times with distilled water. The combined centrifugates were concentrated in vacuo to such a point that on dissolving the sugar in the concentrated hydrolysate a sugar candy with 2.5 % amino acids could be prepared. The desired concentration of the diet was obtained by adding sugar candy.

Hydrolysates prepared with hydrochloric acid properly supplemented with tryptophan and cystine turned out to be extremely toxic to bees. Apparently the hydrolysate contained too much HCl even though it was diluted with distilled water and concentrated 10 times, 3 of which to a thick paste. The suggestion that the toxic properties of HCl-hydrolyzed casein are caused by excess HCl is supported by the absence of deleterious symptoms if the HCl hydrolysate was passed through a column of amberlite IR-4B which removed the HCl quantitatively (see chapter VI, 1 B). Hydrolysates prepared with sulphuric acid and supplemented with tryptophan and cystine increased the longevity to about the same extent as do intact proteins, the optimal concentration being about 0.5 %.

A H_2SO_4 hydrolysate supplemented with only cystine, or with cystine and d-tryptophan increased the longevity considerably less than if supplemented with either dl-, or l-tryptophan. From this it may be concluded first that tryptophan is required and secondly that only the natural configuration is utilized at a normal rate.

Zein, the protein from corn, is known to be deficient in lysine (BLOCK & BOLLING 1951). The addition of lysine to a diet with zein hydrolysate supplemented with tryptophan and cystine results in a substantial increase in longevity. Thus, as in higher animals, lysine seems to be required by the honeybee. These findings were verified in growth experiments (see chapter VI, 2 A).

5. *Mixtures of amino acids.*

In addition to tryptophan and cystine several other amino acids may be removed from protein hydrolysates by means of chemical methods. These methods, however, besides being rather troublesome and time consuming, are often not quantitative. More favourable in this respect is the chromatographic separation with the aid of ion-exchange resins, however, often with the disadvantage of insufficient capacity for feeding purposes. The most ideal method for studying the amino acid requirements is undoubtedly the application of mixtures of pure amino acids.

We used several amino acid mixtures of different composition as a supplement to sugar candy, the results, however, being not very promising. In most cases the average longevities were much shorter than those obtained with intact proteins or protein hydrolysates even if supplement-

ed with vitamins or some intact protein. Some results have been recorded in Table 14.

Amino acid mixtures added to sucrose candy	Number of series	Number of bees	Maximum longevity	Average longevity and standard deviation
Non-supplemented	3	147	36	27.6 ± 3.93
Casein „vitamin free" 0.4 % . .	3	153	69	43.1 ± 3.86
19 Amino acids similar to casein 0.4 %	3	146	44	32.5 ± 4.74
Non-supplemented	3	146	40	29.3 ± 5.82
19 Amino acids according to ROSE (1948) 0.4 %	1	50	41	32.8 ± 4.16
Non-supplemented	3	150	35	27.5 ± 4.52
17 Amino acids similar to casein glutamic acid and aspartic acid omitted 0.5 %	3	151	63	39.9 ± 9.50
Non-supplemented	3	149	36	26.0 ± 5.51
Casein „vitamin free" 0.4 % . .	3	141	88	49.9 ± 15.55
18 Amino acids similar to casein hydroxyproline omitted 0.4 % .	3	156	50	37.1 ± 6.54
Ibid + vitamins 0.4 %	3	135	60	29.6 ± 6.27

Table 14. *Average longevity of young bees on diets supplemented with several mixtures of amino acids or an equivalent amount of casein.*

From table 14 it is evident that amino acid mixtures increased the longevity, but, not to the same extent as does casein. Moreover in experiments not reported in table 14 an effect of amino acid mixtures was sometimes much less or even totally absent. The addition of the vitamins supporting good growth of *Calliphora* larvae under aseptic conditions (SEDEE 1953) was without appreciable effect. No more improvement was obtained on adding the following nutrients together: thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, pteroyl glutamic acid, cholin, biotin, vitamin B_{12}, cholesterin, nucleic acid, vitamins A, D, E, linseed oil, liver extract (pernaemon) and salt mixture (OSBORNE-MENDEL 1917).

On substituting for part of the amino acids an equivalent of intact casein, thus supplying strepogenin (WOOLLEY 1945) no beneficial effect was noted. Strepogenin appears not to be required, as acid hydrolyzed casein being devoid of stepogenin (WOOLLEY 1945), showed excellent results.

Another possible cause of the observed discrepancy between amino acid mixtures and intact proteins may be the relative concentrations of single amino acids in the mixture. It was shown in experiments with mammals that an imbalance in amino acids results in a lower growth rate

or even in toxicity (FROST 1950, ALMQUIST 1951). As the composition of our amino acid mixture corresponds to casein, an amino acid imbalance is rather unlikely.

Several conflicting reports concerning the toxic properties of racemic amino acids have appeared in literature (FROST 1950, ALMQUIST 1951). Although any toxicity of d-amino acids is not generally accepted, the lower nutritive value of amino acid mixtures as compared with intact proteins is often ascribed to the presence of unnatural isomers. Since in our experiments some amino acids were present in the racemic forms (viz. methionine, threonine and serine) the poor results in longevity experiments with bees may possibly be ascribed to the same cause as in experiments with mammals.

Furthermore it may be noted, that reactions between sucrose and amino acids (MAILLARD reactions) often impair the nutritional value of a diet. In recent years numerous reports appeared in literature regarding the reactions which take place when amino acids and sugars are autoclaved together in concentrated aqueous solutions (for reviews of the literature see SWANSON & CLARK 1950, ALMQUIST 1951, and BIGWOOD 1952). As a result the growth promoting properties of diets treated in such a way are often greatly impaired. It is suggested that heating amino acid-sugar mixtures under pressure is attended with a destruction of amino acids, while part of the sugars becomes firmly bound to amino acids which renders them resistant to digestion. Such reactions may likewise occur during the preparation of the diets in the present investigation. Since, however, the mixtures were heated only for some minutes it seems reasonable to suggest that these reactions will not greatly influence the nutritional value of our diets. This idea is supported by the results of growth experiments (see chapter VI), showing a rather high value of amino acid mixtures in diets prepared in the same way.

Finally it may be suggested that the poor value of the amino acid mixtures in longevity experiments might be attributed to the absence of an unknown factor required for maintenance of life, which is present in proteins and resistant against prolonged hydrolysis with strong acids.

Without further experiments, however, it cannot be decided which of these or other factors may be held responsible for the observed poor value of amino acid mixtures in longevity experiments.

6. *Protein requirements of old bees.*

Experiments on the life span of bees in captivity have been performed mostly on very young bees. If older bees were used, no increase in longevity could be obtained on adding pollen to the basal diet (KELLER-KITZINGER 1935; WOODROW 1941; MAURIZIO 1946; BEUTLER & OPFINGER 1949).

After having been established the very important influence of the amount of protein food on the longevity of young bees, it was suspected that too high concentrations of the supplement might possibly cause the

Supplement to sucrose candy	Number of series	Number of bees	Maximum longevity	Average longevity and standard deviation
Un-supplemented	2	102	33	24.2 ± 4.75
Casein Hammarsten 20 %	1	35	12	9.1 ± 1.24
Casein Hammersten 10 %	1	55	13	9.8 ± 2.29
Casein Hammersten 5 %	1	55	18	13.9 ± 2.08
Casein Hammersten 2.5 %	1	58	19	16.1 ± 3.40
Un-supplemented	3	138	50	32.7 ± 4.54
Casein Labco 1.0 %	1	55	49	36.6 ± 5.30
Casein Labco 0.5 %	1	66	63	45.6 ± 9.07
Un-supplemented	3	160	39	28.6 ± 4.07
Soybean meal 5.0 %	1	58	13	6.6 ± 1.40
Soybean meal 2.0 %	1	56	29	21.6 ± 4.86
Soybean meal 1.0 %	1	50	35	22.0 ± 4.19
Soybean meal 0.5 %	1	55	44	32.1 ± 7.45
Un-supplemented	3	145	41	27.3 ± 5.42
Bee bread in piece of comb	3	154	66	41.3 ± 8.13
Bee bread in sugar candy 2.0 %	3	142	88	40.2 ± 6.68
Bee bread in sugar candy 1.0 %	3	145	59	33.0 ± 7.18
Bee bread in sugar candy 0.5 %	3	145	48	30.9 ± 5.25
Skimmed milk powder 2.0 %	3	141	52	34.0 ± 5.00
Skimmed milk powder 1.5 %	3	146	49	37.7 ± 6.70
Skimmed milk powder 1.0 %	3	153	58	36.6 ± 8.75
Skimmed milk powder 0.5 %	3	149	66	42.0 ± 7.27
Un-supplemented	3	116	39	26.3 ± 8.51
Bee bread in piece of comb.	3	138	74	41.6 ± 9.42
Skimmed milk powder 1.0 %	3	120	48	32.2 ± 6.77
Skimmed milk powder 0.75 %	3	117	61	34.4 ± 7.14
Skimmed milk powder 0.50 %	3	107	51	34.2 ± 6.22
Skimmed milk powder 0.25 %	3	91	44	32.7 ± 5.68
Un-supplemented:				
gathering bees Colony 1	3	145	41	27.3 ± 5.42
nurse bees Colony 1	3	156	58	42.2 ± 7.56
Skimmed milk powder 1 %				
nurse bees Colony 1	3	146	87	53.5 ± 15.34
Un-supplemented:				
gathering bees Colony 6	3	116	39	26.3 ± 8.51
nurse bees Colony 6	3	173	60	42.2 ± 7.84
Skimmed milk powder 1 %				
nurse bees Colony 6	3	157	81	44.2 ± 10.17
Un-supplemented:				
gathering bees Colony 5	3	145	40	28.7 ± 4.23
nurse bees Colony 5	3	158	46	33.9 ± 5.27

Table 15. *Average longevity of old bees on diets whether or not supplemented with various concentrations of protein or protein-containing materials.*

failure to obtain an increase in longevity on feeding protein to older bees.

We carried out several longevity experiments on bees taken from the entrance of different hives (i.e. physiologically old bees: gathering bees), as well as on those taken from inside these hives (i.e. physiologically young bees: nurse bees). The results are recorded in table 15. Except where otherwise stated the bees used were physiologically old.

Similar to results obtained with young bees, it is evident from table 15 that the concentration of the supplement is of utmost importance for its effect on longevity. The concentrations exerting a favourable influence on longevity are lower in general than was the case with young bees. The increase in longevity with each of the supplements used, was however, less than that observed in young bees.

A considerable increase occurs on supplying bee bread in a piece of comb, so separated from the sugar candy. Even though the bees were not forced to consume the bee bread they apparently did so, thus demonstrating that even very old bees (more than 3 months) consume pollen. In this connection it is interesting to note that BEUTLER & OPFINGER (1949) observed a very low pollen consumption and absence of an increase in longevity if bee bread was supplied (separated from the carbohydrate) to bees more than 10 days old. Thus the discrepancy with our findings is very marked.

In the lower part of table 15 values are recorded on the longevity of old bees taken from different parts of the bee colony. From these data it is evident that on a pure carbohydrate diet bees being physiologically young, live substantially longer than do bees being physiologically old. The differences are essential ($P < 0.001$). This result confirms the findings reported in chapter IV, 3 C, of the occurrence of two kinds of workerbees in a bee colony differing in weight and nitrogen content. Now it appears that in addition these bees differ in life span if kept on a pure carbohydrate diet.

Likewise in the lower part of table 15, two experiments are reported in which nurse bees were fed with 1 % skimmed milk powder in the sugar candy. This supplementation resulted in a small, though significant increase in longevity ($P < 0.05$). So it appears that the addition of protein material is not only beneficial for physiologically young bees but for physiologically old bees as well.

From the above results it may be concluded, that in contrast with findings of other authors the longevity of old bees is increased by supplementing the carbohydrate diet with various protein containing foods or pure protein in a suitable concentration. Obviously in the bee colony the need for protein is not restricted to the brood rearing period but proteins are required for maintenance of adult bees as well. This finding is in striking contrast with the widespread opinion, that during the winter period the bee requires only carbohydrates. It is common use in beekeeping practice to substitute the honey for stores of pure sugar for overwintering bee colonies, which is not considered to be disadvantagous

from the nutritional point of view. However, honeys contain small amounts of protein varying in the region where we found the protein containing material to exert its longevity promoting action on old bees. Data concerning the amounts of nitrogen containing substances present in a great number of honeys obtained from different plant species varied between 0.03 and 2.67 %, with an average of 1.42 % (KÖNIG 1903). Lower mean values (0.2—0.4 %) are recorded by BARTELS (1938) though much higher figures are given as well. With respect to nitrogen compounds, sugar is an inferior substitute for honey. Especially in those regions where owing to a lack of pollen income in autumn bee colonies enter the winter without pollen reserves, the beekeeper must reckon with the possibility that the bees are unable to meet the needs for their nitrogen metabolism. Therefore it is obvious to suggest that sucrose stores enriched with a suitable amount of protein food with a high biological value may be more favourable for overwintering bee colonies. than stores of pure carbohydrate generally used at present in apiculture. Large scale experiments with bee colonies under field conditions are necessary to evaluate this suggestion.

7. *Relation between longevity and protein content.*

In the foregoing paragraph it was mentioned that the longer life span of nurse bees over that of gathering bees is accompanied by a higher nitrogen content.

According to BERTHOLF (1942) and MAURIZIO (1946, 1951) the longevity of newly emerged bees is increased if they live for some time in a normal bee colony. It is known (see chapter IV, 1) that a substantial increase in the protein content of the young bees occurs during the first few days of imago life. Therefore we attempted to demonstrate a correlation between nitrogen content and longevity.

Newly emerged bees were fed 2.5 % 'vitamin-free' Labco casein beginning at emergence for periods of varying duration, followed by feeding a pure carbohydrate diet. Four days after terminating the protein period, samples from each group were analyzed for weight and nitrogen content. The remaining bees served for determination of the longevity. The results have been recorded in table 16.

This table shows an increase in longevity with increased duration of feeding protein, both being correlated with an increase in nitrogen content of the experimental bees. It may be noted that in this experiment the rate of growth is considerably lower than in other cases on feeding 2.5 % protein (see chapter VI). This abnormality is ascribed to the fact that the casein was not dissolved but mixed with the sugar candy. It is likely that the casein particles have not been divided finely enough to enable the bees to consume them in the concentration supplied. This, however, does not alter the phenomenon of the correlation between protein content and longevity.

The interaction of protein content and longevity was observed many

times in young bees fed a pure carbohydrate diet, these being used as controls in a great number of longevity experiments. In fig. 6 the longevities obtained during 1951 (•) and 1952 (o) are plotted against dry weight and nitrogen content respectively.

Diet	Nitrogen content	Number of series	Number of bees	Maximum longevity	Average longevity and standard deviation
Sugar		3	150	42	33.0 ± 3.61
Sugar + casein for 2 d.	1.88	3	150	44	33.3 ± 4.98
Sugar + casein for 4 d.	1.88	3	148	53	35.9 ± 5.75
Sugar + casein for 8 d.	1.98	3	153	72	44.6 ± 10.54
Sugar + casein for 16 d.	2.13	3	155	89	52.9 ± 9.73
Sugar + casein for 32 d.	2.16	3	139	75	54.4 ± 11.68

Table 16. *Longevity and nitrogen content of bees fed casein for periods of varying duration.*

From fig. 6 it may be seen that with increasing dry weight and nitrogen content the longevity increases likewise. Therefore we are justified to conclude that the longevity of newly emerged bees fed a pure carbohydrate diet is dependent on their development at emergence.

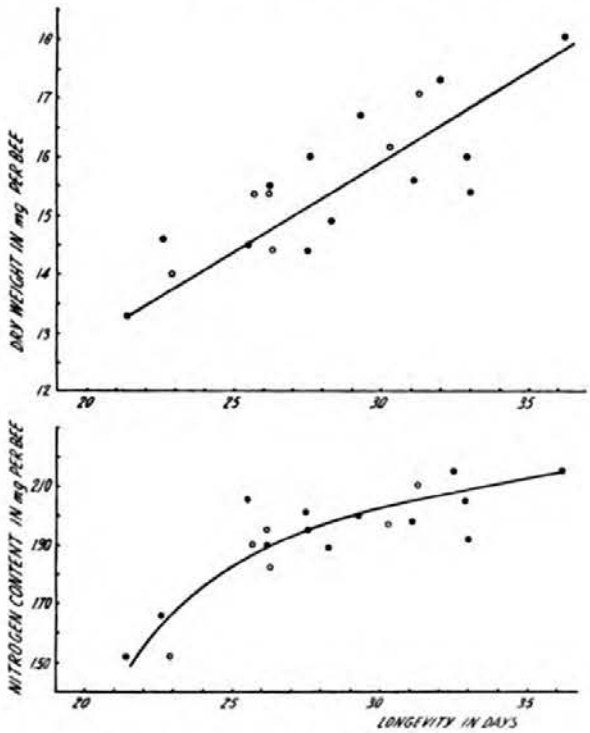

Fig. 6. Relation between development of bees at emergence and longevity on a sugar diet.

This finding in connection with the variation in development of emerging bees not only in different seasons, but also within a very short time (see chapter IV, table 8), emphasizes the occurrence of a disturbing factor in our longevity experiments with newly emerged bees. In order to avoid the influence of variations in test bees on the effect of a dietetic component it is necessary to divide the bees of each emerging period equally over all cages used in one experiment. In our experiments however, the cages were filled in succession completely with 50 bees in succession. Consequently the variation in the test bees may have influenced the longevity. Therefore one is not justified in applying Students' t-test for the statistical evaluation of the observed differences in longevity experiments with newly emerged bees.

Besides the disadvantage of the large variation in development of newly emerged bees, the longevity method is not ideal for more reasons. In experiments with higher animals it was shown that the nutritive requirements for maintenance are less than for growth. In longevity experiments it will be possible only to reveal the needs for maintenance without giving much information regarding the factors necessary for growth. Thus on studying the longevity, only part of the dietetic requirements may be explored. Moreover longevity experiments are rather cumbersome and time consuming, since the calculation of the average longevity requires the continuation of the experiments until all bees are dead. On a diet of high nutritive quality this duration may amount to four months. For these reasons we attempted other methods and found the growth of young bees to offer a much more valuable criterion for studying the nutritional requirements than longevity. The growth experiments will be reported in the next chapters.

8. *Food consumption.*

In a number of longevity- and growth experiments we determined the amounts of food consumed, by daily weighings of the feeder with its contents included. The loss in weight was diminished with the decrease by evaporation (determined in control feeders with food) and divided by the number of bees. From these figures obtained on successive days the average daily consumption per bee was calculated. Results obtained in several experiments both with old and young bees are summarized in table 17. As far as available the corresponding longevities are recorded likewise. To appreciate the significance of the figures as well as for comparison with figures from other authors, it may be noted that the foods contained 20 % water.

Table 17 shows important differences in food consumption both between old and young bees and on different diets. Old bees consume much more food than do young bees on the same diets which agrees with the findings of MAURIZIO (1946).

A close correlation occurs between longevity and amount of food consumed in such a sense, that a higher food consumption corresponds

with a longer life span. GONTARSKI (1950) observed a decrease in food consumption of adult bees if an extract of pollen was added to a diet of sugar water. In our experiments the addition of whole pollen always resulted in a marked increase in the amount of food consumed. This apparent discrepancy was not further investigated.

Supplement to sugar candy	Days of observation	Average longevity	Average food consumption mg/bee/day
Young bees			
Unsupplemented	3—17	25.1	12.2
Pollenmixture 2.5 %	3—17	39.6	25.8
,, 2.5 % 48 hrs at 80° .	3—17	28.7	15.7
Hydrolyzed casein 1.0 %	1—15	34.1	15.4
,, ,, 1.0 % + cystine	1—15	26.8	14.5
,, ,, 1.0 % + trypt..	1—15	42.4	29.4
,, ,, 1.0 % + {cystine, trypt.	1—15	44.5	24.3
Un-supplemented	3—14	—	12.7
Casein intact 2.5 %	3—14	—	27.4
Hydrol. casein 2.5 % + cystine + trypt.	3—14	—	23.9
Amino acid mixture 2.5 % complete	3—14	—	17.5
Amino acid mixture 2.5 % {arginine omitted	3—14	—	7.7
Un-supplemented	1—14	—	19.7
Pollen 9.0 % } equivalent	1—14	—	39.1
Casein intact 2.5 % } in	1—14	—	23.8
Amino acid mixt. 3.0 % } nitrogen	1—14	—	16.8
Old bees			
Un-supplemented	2—23	25.9	35.2
Skimmed milk powder.	2—30	30.2	36.2
Un-supplemented	8—26	25.0	29.2
Bee bread . . . . . 1.0 % . . .	8—26	29.5	48.7
Skimmed milk powder 1.0 % . . .	8—26	31.8	37.6

Table 17. *Average daily food consumption of caged bees fed various diets.*

The amount of food consumed on a given diet may vary greatly on successive days. An illustration is given in fig. 7. The curves show the daily food consumption per bee on a diet with 1.0 % hydrolyzed casein either supplemented with amino acids or not. The averages are recorded in table 16 (upper part).

Besides the important differences in food consumption on successive days it may be seen from this figure that each curve shows a steep rise between the first and the fourth day of life, a phenomenon being regularly

observed only in experiments with young bees. The low food consumption during the first few days explains the fact of the slight growth which was observed in young bees during the first days of captivity (see chapter IV, 1).

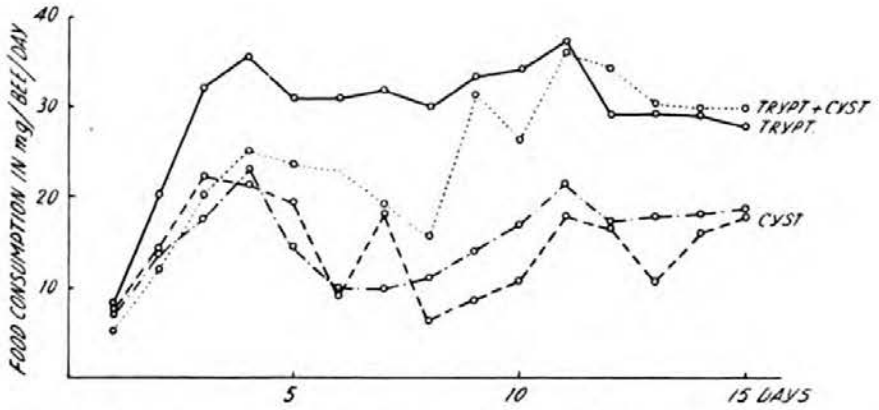

Fig. 7. Daily food consumption of young bees fed with 1.0 % hydrolyzed casein (—·—·—·—) supplemented with cystine (— — — —) with tryptophan (————), and with cystine and tryptophan (. . . . .).

The low food consumption on diets with hydrolyzed casein without tryptophan and with a mixture of amino acids lacking in arginine, is in agreement with findings of ROSE (1938), FRAZIER et al. (1947), BENDITT et al. (1950), and others. They observed a marked failure in appetite of rats when the diet was completely devoid of an essential component. The indispensable nature of arginine and tryptophan for bees will be shown furtheron.

CHAPTER VI

QUALITATIVE AMINO ACID REQUIREMENTS INVESTIGATED BY MEANS OF GROWTH DETERMINATIONS

In the foregoing chapter (see page 45) it has been mentioned, that the longevity method, though suitable to get some impression of the nutritive value of a certain diet, has several disadvantages. Other methods applied for studying the nutritive requirements of honeybees are based on the development of brood food glands and ovaries, using a subjective classification of developmental stages. The objective methods, being generally used in studies of insect nutrition and based on growth and rate of development in various stages from the egg up to imago cannot be applied to the honeybee since one has not yet succeeded in rearing honeybee larvae outside the bee colony. Searching for an objective and simple method to study the amino acid requirements of isolated bees, we

used as a starting point the growth phenomena of young honeybees (described in chapter IV, 1) and tried to obtain this growth on supplementing the sugar candy with acid-hydrolyzed casein. This method turned out to be very valuable and gave the opportunity to investigate the significance of some amino acids which may easily be eliminated from a hydrolysate. However, the number of available deficient amino acid combinations is limited due to the lack of specific reagents and suitable methods. Attempts to replace the hydrolysate by mixtures of pure crystalline amino acids were sucessful, thus enabling the evaluation for growth of any of the amino acids available.

Even though it has been shown that growth of the young worker honeybee may be measured in terms of dry weight as well as nitrogen content, we thought it better to apply both methods. As early as 1905 it was shown (FOLIN 1905) that changes in body weight are not always accompanied by changes in protein metabolism. So the combined determinations of weight and nitrogen content is more trustworthy. Moreover it may happen that nitrogen determinations get lost. In these cases weight determinations are valuable. Since however, the nitrogen content proved to be less variable than weight, illustrations of results will be given mostly in terms of nitrogen.

1. *Experiments with acid-hydrolyzed casein.*

A. Significance of tryptophan and cystine.

In longevity studies with young honeybees we obtained a very important lengthening of life span if H_2SO_4-hydrolyzed casein supplemented with tryptophan and cystine was added to a basal diet of sucrose. Now it was attempted to obtain growth on such a diet.

Hydrolysis was performed as described on page 38. The hydrolysate was incorporated in sugar candy in a concentration of 2.5 % (of the dry sugar). For that purpose the nitrogen content of the neutralized and concentrated hydrolysate was determined and adjusted to 100 mg protein per ml, supposing a nitrogen content of 15 % in casein. For each ml of hydrolysate 4 g sucrose was added and dissolved by boiling. Crystallisation was brought about in the usual way. Four diets consisting of 50 g hydrolysate-sugar candy were prepared and supplemented with amino acids in the following way: 1) 12.5 mg l-cystine 2) 25.0 mg l-tryptophan 3) 12.5 mg l-cystine and 25.0 mg l-tryptophan 4) unsupplemented. Each diet was fed to one group of newly emerged bees. A fifth group received sugar candy only. 5 Samples of 5 bees were analyzed for weight and nitrogen content after 7 and 14 days.

The increase in nitrogen content of the mean values in mg per bee are plotted in fig. 8, the supplements being mentioned next to the curve belonging to the corresponding diet. The dotted line belongs to the sucrose diet.

As it may be seen in fig. 8, growth is almost absent on a diet with non-supplemented hydrolyzed casein. The addition of cystine exerts no distinct growth stimulation. However, if tryptophan is added a very

important increase in dry weight and nitrogen content occurs resulting in values of more than 20.0 and 2.50 mg respectively, being normal for adult bees. From these findings we are justified to conclude that

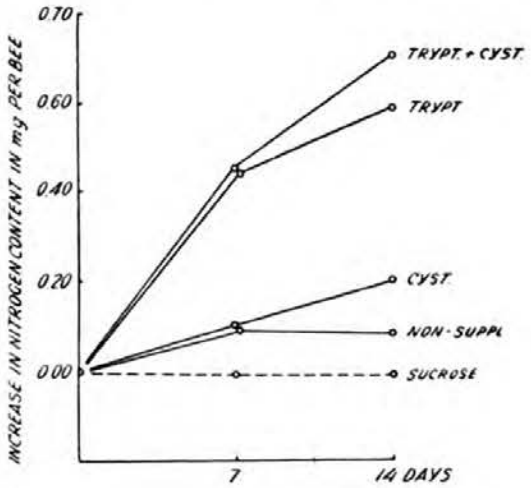

Fig. 8. Increase in nitrogen content of newly emerged bees fed 2.5 % casein hydrolysate in sugar candy supplemented with either cystine, tryptophan, cystine and tryptophan, or un-supplemented. The absence of an increase on a pure carbohydrate diet is given likewise.

tryptophan is an indispensable amino acid for growth of the young honeybee. On the other hand cystine seems to be dispensable, as the addition of tryptophan without cystine is sufficient to warrant good growth. However, the possibility may not be excluded that small amounts of cystine not destroyed by hydrolysis, account for this result.

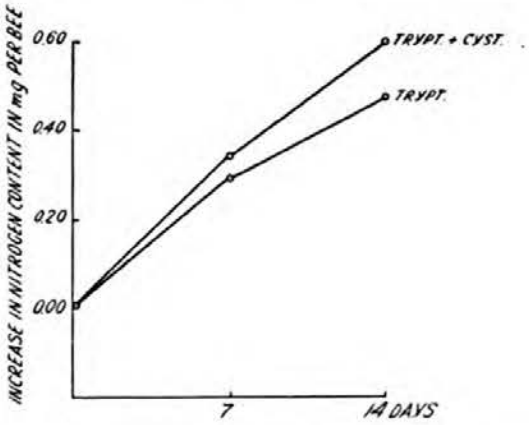

Fig. 9. Increase in nitrogen content of young honeybees fed two diets with 2.5 % cystine-free hydrolysate, one supplemented with tryptophan, the other supplemented with cystine in addition.

In order to remove the last traces of cystine eventually present in the acid hydrolyzed casein we applied the cuprous oxide method as described by VICKERY & WHITE (1933).

The acid hydrolyzed casein was treated with cuprous oxide in excess in acid medium at 45° C. After cooling, the fluid is neutralized to pH 4.5 and centrifuged of, thus removing cystine as cysteine cuprous mercaptide. Growth of newly emerged bees was determined as described above on diets with 2.5 % hydrolysate with and without cystine both supplemented with tryptophan.

The results are shown in fig. 9.

This figure shows a rather steep rise of the curves, thus demonstrating that the presence of cystine in the diet is not necessary to obtain good growth. From this result it may be concluded that cystine is dispensable for growth of the young honeybee.

B. Significance of glutamic acid and aspartic acid.

The anion-exchange resin Amberlite IR-4 B offers an excellent means of preparing diets devoid of glutamic and aspartic acids (CANNAN 1944). We employed this synthetic resin to obtain acid hydrolyzed casein from which the dicarboxylic amino acids were quantitatively removed.

130 g Air-dry Amberlite IR-4B. (40—60 mesh/inch) [1]) preserved in 2 n H_2SO_4 was stirred and washed repeatedly with 500 ml portions of distilled water and then treated with 0.1 n NaOH until the supernatant liquor was alkaline to lithmus paper.

The resin was washed 2 times with distilled water and treated with 2 n H_2SO_4 until pH = 2. Afterwards it was transferred to a glass tube (3 cm internal diameter, long 60 cm) closed at the narrowed lower end with a cock. In this tube the Amberlite formed a column of 50 cm in height. A sodium acetate solution (34 g/l) from a separation funnel was then slowly passed down the column until the pH of the eluate was 5 (2 l sodium acetate). The acetate was then washed out with distilled water until pH = 3.5 (0.5 l distilled water). At this stage the resin is ready for use.

380 ml H_2SO_4-hydrolyzed casein of pH 2.4 containing 6.6 g amino acids were transferred to the column leaving it pass down very slowly (30 ml per hour). Distilled water was used to eluate the amino acids other than dicarboxylic amino acids, the latter remaining firmly bound at the resin. The elution was continued until negative ninhydrin-test on filter paper. The eluate (about 1 l), containing the amino acids except glutamic and aspartic acids was concentrated under reduced pressure at 50° C to such a volume, that 1 ml contained 100 mg amino acids (determined as N; casein contains 15 % N). The absence of the dicarboxylic amino acids was established by two dimensional paper partition chromatography on a sheet of Whatman no. 4 filter paper, using water saturated butanol for the first run and fresh distilled, water suturated phenol for the second at right angles to the first. After drying and spraying the paper with 0.1 % of ninhydrin in 96 % alcohol and drying again, no coloration was present in the area of glutamic or aspartic acid whereas the other amino acids showed heavy spots. Thus the dicarboxylic amino acids had been removed quantitatively [2]).

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[1]) Amberlite IR-4B was kindly supplied by Dr T. J. BARENDREGT from the Laboratory of Organic Chemistry, Utrecht.

[2]) We express heartfelt thanks to Mr PH. D. J. W. SEDEE for many valuable suggestions and help in applying chemical methods and to Miss M. GROENEVELD for making the chromatograms.

The hydrolyzed casein, devoid of glumatic and aspartic acids was incorporated in sugar candy in a concentration of 2.5 % and tryptophan and cystine were added. Three out of 4 diets, each consisting of portions of 50 g, were supplemented with either 200 mg glutamic acid, 100 mg aspartic acid, or 200 mg glutamic and 100 mg aspartic acid. Growth of newly emerged bees was determined in the usual way on each of the four diets. The results are shown in figure 10.

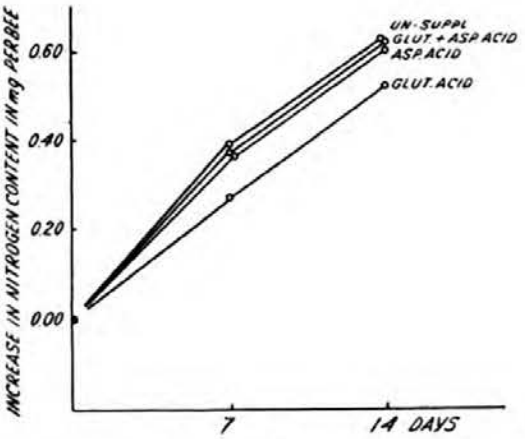

Fig. 10. Increase in nitrogen content of young honeybees fed diets containing 2.5 % hydrolyzed casein devoid of glutamic and aspartic acids.

It may be seen from figure 10 that good growth occurs without the addition of glutamic or aspartic acid while the addition of one or both does not bring about any appreciable increase in growth. It is justified to conclude from these findings that neither glutamic nor aspartic acid is essential for growth of the young honeybee.

When this experiment was repeated with HCl-hydrolyzed casein instead of H_2SO_4-hydrolyzed casein, exactly the same results were obtained. In contrast with findings in longevity experiments with HCl-hydrolyzed casein no toxic symptoms manifested itself. Since Amberlite IR-4B adsorbs the HCl quantitatively this finding supports the suggestion that the deleterious effects of HCl-hydrolysates can be ascribed to excess hydrochloric acid not being removed by repeated concentration in vacuo.

2. *Experiments with mixtures of pure amino acids.*

The study of the amino acid requirements was greatly simplified when growth of the young honeybee was found to occur on diets with a mixture of pure amino acids as the sole source of nitrogen. The difficulty of eliminating a given amino acid from the diet was completely overcome. By preparing mixtures of several combinations of the pure crystalline amino acids we were able to acquire the desired deficiency of the diet. Obviously, this finding opened the way to investigate the requirements of the young honeybee for each of the amino acids.

A. Indispensable amino acids.

Amino acid mixtures were prepared by weighing the pure compounds in the desired amounts. [1]) Since the dispensable nature of glutamic and aspartic acids had been established these amino acids were omitted. The relative amounts of the amino acids approximated those in casein, however the level of cystine (being very low in casein) was increased, that of proline (being rather high in casein) decreased. Hydroxyproline was added, though not present in casein according to BLOCK and BOLLING (1951). In view of the reported toxicity of dl-forms in experiments with higher animals the quantities of amino acids present in racemic mixtures were not doubled, except for dl-tryptophan as its level in casein is already rather low.

The "complete" amino acid mixture contained the following amounts per 1 g:

l-arginine (base)	50 mg	dl-threonine	50 mg
l-histidine (base)	40 ,,	l-leucine	100 ,,
l-lysine (= 135 mg dihydrochloride)	90 ,,	l-isoleucine	80 ,,
		l-valine	80 ,,
l-tyrosine	70 ,,	glycine	25 ,,
dl-tryptophan	40 ,,	l-alanine	40 ,,
l-phenylalanine	70 ,,	l-proline	100 ,,
l-cystine	20 ,,	l-hydroxyproline	25 ,,
dl-methionine	40 ,,		
dl-serine	80 ,,		

Each 100 g basal ration consisted of: amino acids 2.5 g, sucrose 77.5 g, tapwater 20 g. Vitamins or salts were not added since in preliminary experiments conflicting results were obtained on the addition of these ingredients.

In some experiments a somewhat better growth occurred, in others no influence was appreciable at all if the following ingredients were added together; thiamine, riboflavine, pyridoxine, nicotinic acid, calcium panthothenate, p-aminobenzoic acid, pteroyl glutamic acid, biotine, choline, inositol, vitamin B_{12}, cholesterine, yeast nucleic acid, vitamins A, D and E, linseed oil and salt mixture according to OSBORNE-MENDEL (1917). Apparently the vitamin requirements of the bees were met, either by the vitamin reserves present in the bees at emergence, or by synthetic action of micro-organisms in the gut. The presence of wood and bees wax in the experimental cages was of no importance for growth on diets with amino acid mixtures as bees kept in cages constructed of "perspex" (methyl methacrylate) instead of wood and wire screen instead of wax, showed the same growth rate as those kept in the common cages. Since a sufficient growth rate was obtained without the addition of vitamins, these components were not incorporated in the diets of subsequent experiments.

The amino acids were added to the sugar-water mixture and the whole was heated for some minutes to dissolve the sugar. After cooling, crystallization was obtained in the usual way (see chapter III).

Besides the complete diet, including 17 amino acids, 17 diets were prepared each deficient in one amino acid. On each diet growth was determined, by analyzing 5 samples of 5 bees after 7 and 14 days. The changes in dry weight and nitrogen content are presented in figure 11 and 12. The amino acid omitted is mentioned next to the corresponding curve. The curve designated with 'complete' represents the growth on a diet with 17 amino acids, viz. those present in casein except glutamic and aspartic acids.

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[1]) The amino acids used in these studies were kindly supplied by F. Hoffmann La Roche, Basel, Schweiz.

Both of the graphs show two distinctive groups of curves, the difference being most marked in their course between the 7th and the 14th day. In this period an increase in dry weight and nitrogen content is either absent or negligible in the lower group, whereas in the upper group a substantial increase occurs. The value of 21 mg and 2.5 mg for dry weight and nitrogen content respectively, being considered as minima for adult bees in a free flying colony have been attained after 14 days on any diet

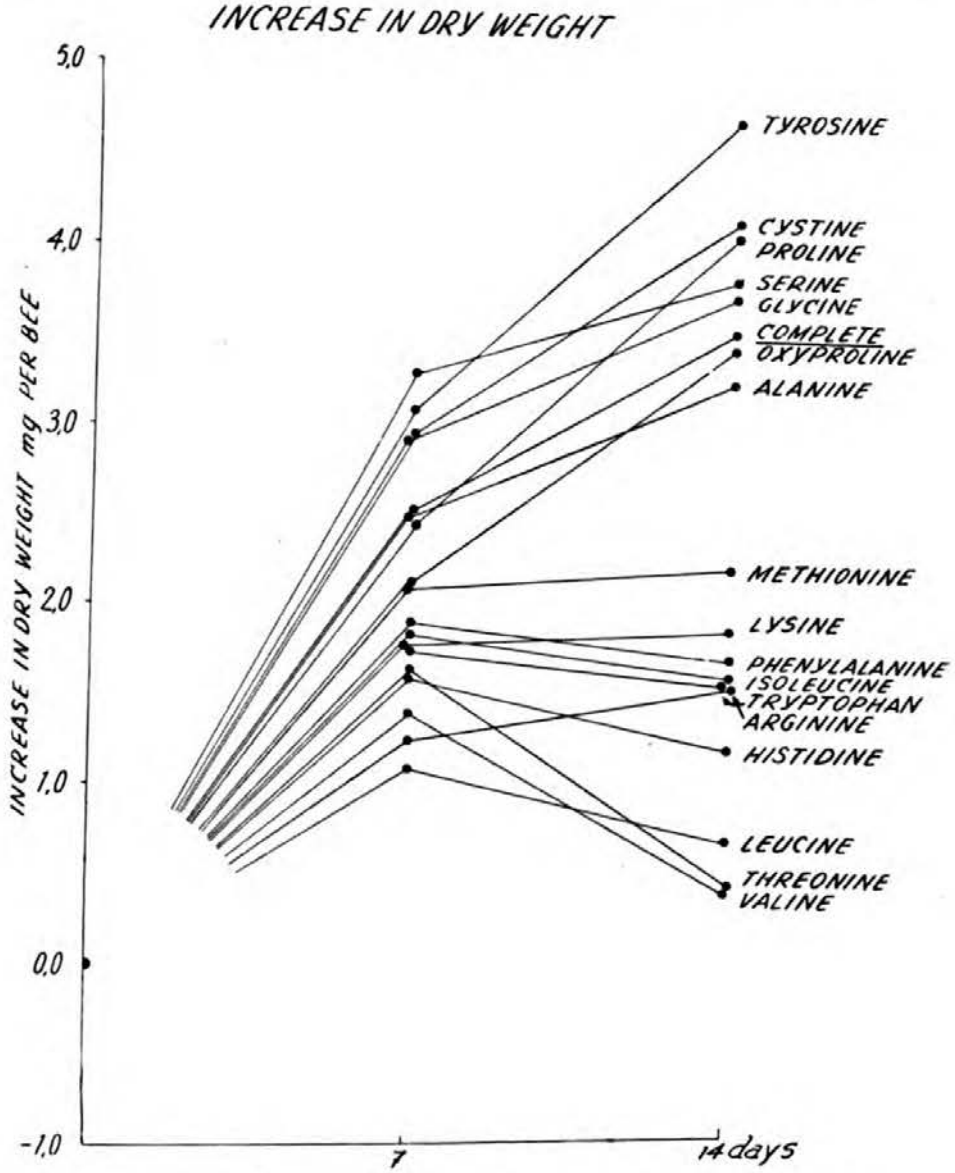

Fig. 11. Changes in dry weight of young honeybees on 18 synthetic diets one with 17 amino acids ("complete"), the other 17 devoid of one amino acid being designated next to the corresponding curve.

of the upper group. The absence of an important depression in growth in the upper group and the almost complete growth inhibition in the lower group after the 7th day, justifies the conclusion that the diets represented by the lower group of curves are deficient in an amino acid being required for good growth of the honeybee. Hence these amino acids can be

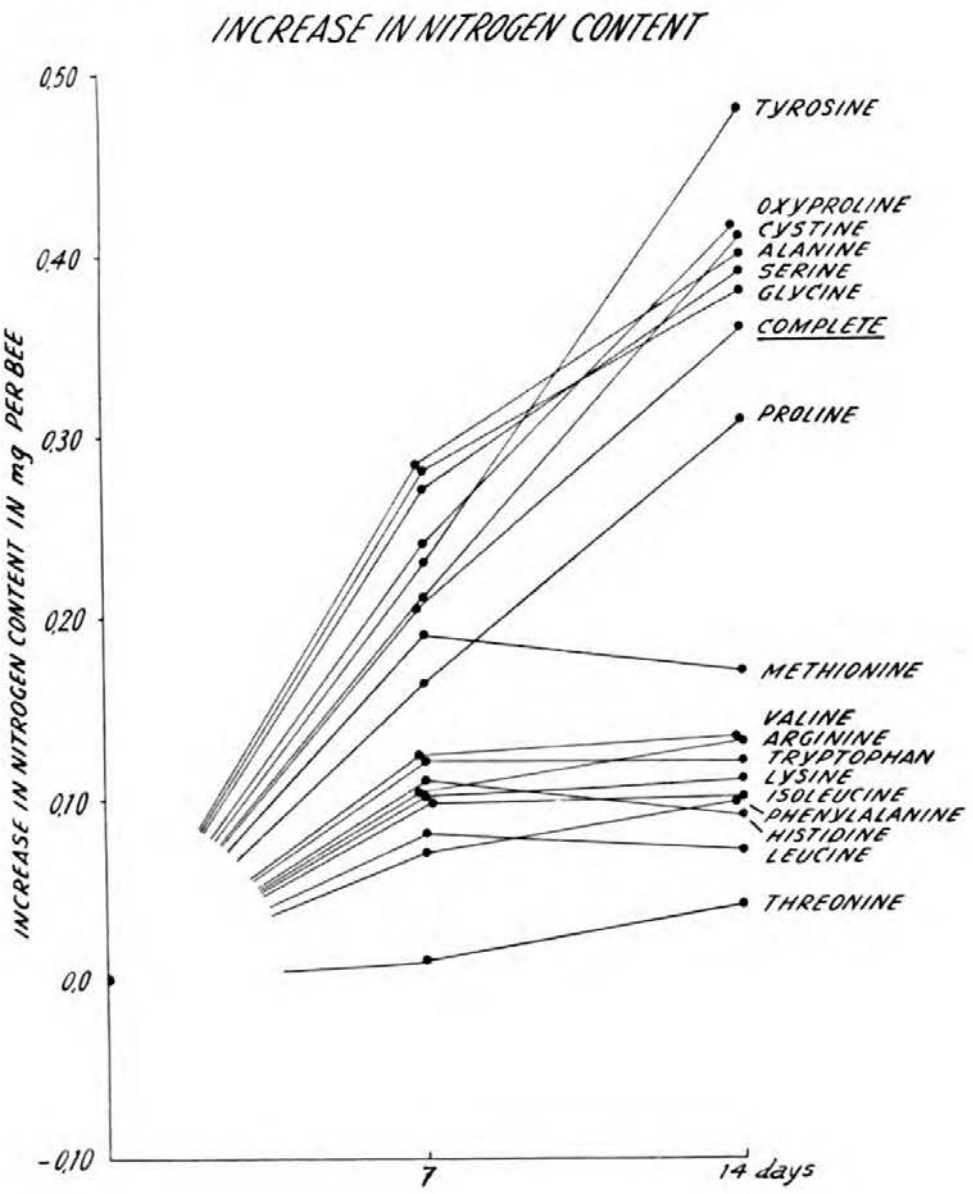

Fig. 12. Changes in nitrogen content of young honeybees on 18 synthetic diets, one with 17 amino acids ("complete"), the other 17 devoid of one amino acid being designated next to the corresponding curve.

designated with the term 'essential'. This means that the following amino acids cannot be synthesized, or not sufficiently rapid to meet fully the needs for growth: arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine and valine. It may be noted that exactly the same 10 amino acids were found to be essential for normal growth of the rat (ROSE 1938). These findings confirm the conclusion arrived at in experiments with hydrolyzed casein, notably that tryptophan is required by the honeybee, whereas glutamic acid, aspartic acid and cystine are not. Moreover evidence is given of the suggestion derived from longevity experiments with hydrolyzed zein, that lysine is an indispensable nutritional factor.

Attention may be drawn to the distinct growth which occurs during the first 7 days on diets devoid of an essential amino acid. A considerable variation may be observed as to the extent of growth during this period, reaching extreme values in the absence of methionine or threonine. In subsequent experiments it was noted, that the considerable initial growth on diets lacking in methionine and the negligible growth in the absence of threonine constitute a regularly occurring phenomenon. Obviously in the case of methionine deficiency, the honeybee is able to synthesize relatively large amounts of body proteins with the methionine present in the body at emergence, and only minor quantities in the case of a threonine deficiency.

B. Dispensable amino acids.

In addition to glutamic and aspartic acids, the significance of which was already established in experiments with hydrolyzed casein, it appears from fig. 11 and 12 that diets devoid of the following amino acids support good growth: tyrosine, cystine, serine, glycine, alanine, proline and hydroxyproline.

Under the conditions of the above described experiments, the rate of growth is relatively slow as compared with that observed under natural conditions. In chapter IV, 1 pag. 22 it was shown that growth of bees in captivity was about optimal on diets with 20 % pollen or 10 % casein. In growth experiments with amino acids, however, we fed the mixtures in a concentration of only 2.5 %, to prevent the toxic effects of higher concentrations which had been observed in longevity experiments. Moreover for technical reasons it was desirable to lengthen the growth period by using lower concentrations. SWANSON & CLARK (1950) call attention to the possibility that amino acids considered non-essential on the base of studies in which low rates of growth were obtained may become limiting when growth proceeds faster. It is recognized that in our experiments with suboptimal growth rates, one must be careful in classifying amino acids the omission of which does not result in a perceptible growth inhibition. These compounds, though not required for growth may exert stimulatory effects. To evaluate this possibility, experiments were designed with mixtures of 19 amino acids similating

casein, in a concentration of 2.0 %. The amino acids to be investigated were added in amounts supplying 50 % of the nitrogen present in the components generally regarded as non-essential. 8 Samples of 5 bees were analyzed after having been kept for 10 days on the synthetic diets. The results of the nitrogen determinations are presented in table 18.

Experiment	Un-supplemented amino acid mixture	Amino acid in excess									
		l-tyrosine	l-cystine	dl-serine	l-glutamic acid	l-glutamine	l-aspartic acid	glycine	l-alanine	l-proline	l-hydroxy-proline
82	0.22			0.42	0.24	0.26		0.44		0.34	
84	0.29										0.29
87	0.26							0.34			
90	0.39				0.39	0.38					
90	0.26			0.43				0.33		0.31	
96	0.30							0.37			
97	0.21	0.25						0.32	0.22		
107	0.30		0.29	0.39							
107	0.41						0.40				

Table 18. *Influence of various amino acids on the increase in nitrogen content of young honeybees, kept for 10 days on diets with 19 amino acids similating casein.*

This table demonstrates clearly the great variety in growth stimulation exerted by different amino acids. The important improvement of growth as a result of excess quantities of amino acids is most marked in the case of dl-serine, glycine and l-proline. Any influence of the other amino acids is doubtful. Growth improvements being statistically significant ($P < 0.001$) occurred on the addition of dl-serine (exp. 82, 90 and 107), of glycine (exp. 82, 96 and 97), and of l-proline (exp. 82). From these results it may be concluded that serine, glycine and proline, though not essential for growth, exert a stimulating effect, if amino acids replace the protein in the diet of honeybees at a level supporting suboptimal growth. It may not be decided, however, whether these amino acids are to be considered as sources of nitrogen being readily available for synthesis of other compounds or as specific growth stimulators. Further on (see table 21) it will be shown, that glycine is an effective substitute for the dispensable amino acids, which points to its suitability as a source of nitrogen. The absence of a growth improvement on the addition of tyrosine, cystine, glutamic acid, aspartic acid, alanine and hydroxyproline, justifies the classification of these amino acids as dispensable. Other amino acids which may be present in proteins such as citrulline and hydroxy glutamic acid may be considered likewise as non-essential.

The level of glycine in casein is relatively low (2.1 %, BLOCK and BOLLING 1951). Since our amino acid mixtures were patterned after

the composition of casein it seemed advisable to investigate the optimum level of glycine in the mixture. Diets were prepared with 2 % and 3 % amino acids approximating the composition of casein, but without glycine. In each experiment several different concentrations of glycine were added ranging from 0 to 10 %. Growth on each diet was ascertained by analyzing 8 samples of 5 bees after 10 days. The resultant relation between increase in nitrogen content of the bees and glycine concentration obtained in three different experiments is plotted in fig. 13.

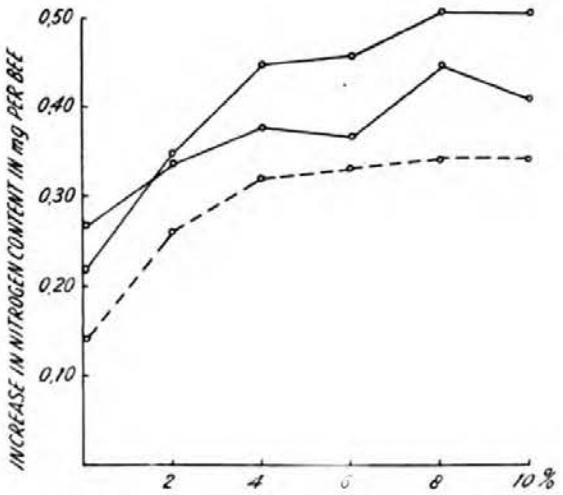

Fig. 13. Relation between increase in nitrogen content of young honeybees and level of glycine in diets containing a mixture of 17 amino acids (— — — — = 2.0%, ——— = 3.0 % amino acid mixture).

From the curves in fig. 13 it may be deduced that indeed an improvement of growth occurs if the glycine level of the diet is raised. Obviously its optimum is approximately twice the amount present in casein.

In an attempt to find out whether the need for glycine is influenced by the specific properties of the amino acid mixture, we fed intact casein at a level of 2.5 % and studied an eventually effect of added glycine. In these cases, however, no significant improvement of growth was observed pointing to the specific properties of the synthetic mixture affecting the growth stimulation exerted by glycine.

3. *Comparison of the qualitative amino acid requirements of the honeybee and of other animals.*

The significance of each of the amino acids for the young honeybee as has been established in the present study are recorded in table 19 together with data available in literature concerning growth experiments with several different organisms.

Amino acid	Vertebrates				Insects				Protozoa
	Man	Rat	Mouse	Chick	Mosquito fly	Fruit fly	Carpet beetle	Honeybee	Tetrahymena
	(Albanese 1950)	(Rose 1938)	(Bauer-Berg 1943)	(Almquist Grau 1944)	(Goldberg-DeMeillon 1948)	(Rudkin-Schultz 1947)	(Moore 1946)		(Kidder-Dewey 1945)
Tryptophan	+	+	+	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+	+	+	+
Leucine	+	+	+	+	+	+	+	+	+
Isoleucine	+	+	+	+	+	+	+	+	+
Threonine	+	+	+	+	+	+	+	+	+
Methionine	+	+	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+	+	+
Valine	+	+	+	+		+	+	+	+
Histidine	—	+	+	+	+	+	+	+	+
Arginine	—	—	—	+	+	+	+	+	—
Glycine	—	—	—	—	+		—	—	—
Glutamic acid	—	—	—	—	—		—	—	—
Cystine		—	—	—	—		—	—	—
Serine		—	—	—	—		—	—	—
Proline		—	—	—	—		—	—	—
Hydroxyprolin		—	—	—	—		—	—	—
Alanine		—	—	—	—		—	—	—
Tyrosine	—	—	—	—	—		—	—	—
Aspartic acid	—	—	—	—	—		—	—	—

Table 19. *Qualitative amino acid requirements for growth of different organisms.* (+ = *indispensable*; — = *dispensable*).

As will be observed in this table, the following seven amino acids are required for growth of all organisms investigated so far: tryptophan, phenylalanine, leucine, isoleucine, threonine, methionine and lysine. It is very probable that valine likewise belongs to this series; for the mosquito fly however, its significance could not be established with certainty since the experimental diet without added valine contained considerable amounts of this amino acid in the yeast fraction (Goldberg & de Meillon 1948). The same 8 amino acids (plus histidine) appeared to be essential also for maintenance of nitrogen equilibrium in the adult dog (Rose & Rice 1939).

The amino acids which turned out to be dispensable for growth of all animals investigated are: cystine, serine, proline, hydroxyproline, alanine tyrosine, glutamic acid and aspartic acid. The significance of serine, proline, hydroxyproline and alanine for growing children has not yet been ascertained, but these amino acids are not required for maintenance of the adult man (Rose 1949). Thus it may be stated that, as far as investigated, the 8 amino acids mentioned are dispensable for all organisms. Some of these unessentials were found to exert a stimulating effect on growth. This happens with cystine in several insects species

(MICHELBACHER et al. 1932; VAN 'T HOOG 1935, 1936; LAFON 1939; GOLDBERG & DE MEILLON 1948; SEDEE 1953), with tyrosine and alanine in *Drosophila* (WILSON 1945), and with proline (WOMACK & ROSE 1947) and tyrosine (RAMASARMA et al. 1949) in rats. In the present investigation we found glycine, proline and serine to stimulate growth of the honeybee. The growth improvement caused by dl-serine is in striking contrast with its reported toxicity in *Drosophila* (HINTON et al., 1951). In the honeybee no sign of toxicity was observed, even not if dl-serine was supplied in a concentration of 24 % of the amino acid mixture. The efect of a certain amino acid may be dependent however, on several factors such as amounts and relative concentrations of the amino acids present in the diet. As the diets employed by different authors varied greatly it is impossible to evaluate the observed discrepancies.

In contrast with the above mentioned striking similarity in essential and non-essential amino acids for various organisms, table 19 also shows differences with regard to the significance of some amino acids.

Histidine being classified as indispensable for all animals investigated, turned out not to be required by man for growth (ALBANESE 1950) or for maintenance (ALBANESE 1947; ROSE 1949). In this respect the honeybee equals the other organisms.

Arginine which occupies an intermediate position between essential and non-essential in the rat (ROSE 1938) is neither required for optimal growth of mice (BAUER & BERG 1943), nor for maintenance of man (ALBANESE 1947; ROSE 1949). Likewise it seems to be dispensable for growth of the infant (ALBANESE 1950). Its significance for the flagellate *Tetrahymena* (KIDDER & DEWEY 1945) equals that for the rat. On the other hand the presence of arginine is required for any growth of the chick (ALMQUIST & GRAU 1944), the mosquito fly (GOLDBERG & DE MEILLON 1948), the fruit fly (RUDKIN & SCHULTZ 1947) and the carpet beetle (MOORE 1946). The same applies to the honeybee.

Glycine has been classified as an essential dietary component only for *Aëdes* (GOLDBERG & DE MEILLON 1948). Its addition to the diet, greatly improved growth of the chick (ALMQUIST & GRAU 1944) of *Tetrahymena* (KIDDER & DEWEY 1945) and *Drosophila* (HINTON et al. 1951), which was likewise observed in the honeybee. According to KIDDER & DEWEY (1945) glycine acts as a detoxifying agent for one or more of the dispensable amino acids. Therefore the observed growth stimulation does not justify the classification of glycine in the group of the essentials.

Glutamic acid though dispensable for any of the organisms investigated, appeared to improve growth considerably in the chick (ALMQUIST & GRAU 1944). Stimulating effects of glutamic acid added to diets lacking in a number of dispensable amino acids have been reported by several authors (WOMACK & ROSE 1947; ROSE, OESTERLING & WOMACK 1948; ROSE et al. 1949; MADDY & ELVEHJEM 1949). As was shown by ROSE et al. (1949) glutamic acid acts as a source of nitrogen for the synthesis of non-essential amino acids. Whether the growth stimulation exerted by

glutamic is connected moreover with a specific function, notably to form part of the strepogin molecule, remains to be established. In the honeybee however the absence of glutamic acid either from a mixture of pure amino acids or from a casein hydrolysate was without influence on growth.

In general it may be stated that the striking similarity in amino acid requirements reveals a pronounced conformation in synthetic abilities of different organisms.

4. *Substitution experiments with amino acids and non-specific nitrogen compounds.*

A. Replacement of indispensable amino acids.

In experiments with mammals as well as with Protozoa it has been established that several indispensable amino acids may be replaced by compounds which are structurally or metabolically related. For instance α-keto acids and α-hydroxy acids can be converted into the corresponding α-amino acids (ROSE 1938; WOMACK & KADE 1948, review) which means that α-keto acids may replace essential amino acids in the diet if an adequate source of nitrogen is present. In chicks, citrulline is utilized in place of argine but ornithine is not (KLOSE & ALMQUIST 1940; KLOSE, STOKSTAD & ALMQUIST 1938). The same appeared to apply to the mosquito larvea (GOLDBERG & DE MEILLON 1948). Phenylpyruvate completely replaced phenylalanine (ROSE 1937) and carnosine was as effective as histidine for growth of the rat (DU VIGNEAUD et al. 1937). Norleucine replaced leucine for maintenance of N-equilibrium in the rat (BURROUGHS et al. 1940).

The above mentioned nitrogen compounds were investigated for their ability to replace essential amino acids in the diet of the young honeybee. Experiments were carried out on synthetic diets with 2.0 or 3.0 % amino acids. The compounds to be tested were added at a percentage concentration equal to the optimal concentration of the related amino acid. For comparison growth was measured on diets with the related amino acid either totally absent or present in optimal concentration. On each diet, growth was determined by analyzing 8 samples of 5 bees after 10 days. The changes in the nitrogen content are recorded in table 20.

Out of the compounds tested, only carnosine possesses important replacement value, the replacement being practically complete. Norleucine and Na-phenylpyruvate are of no value, only the first being in accordance with findings on higher animals. Growth in the presence of citrulline is very poor, though significantly better than if arginine is absent ($P = 0.003$ and < 0.001 resp.) while ornithine is of no value at all. Obviously the honeybee is unable to transform at a sufficient rate ornithine and citrulline into arginine, which is the basal reaction of the KREBS-HENSELEIT cycle for the formation of urea in mammals. These results with ornithine and citrulline are striking inasmuch as in a

No. of exp.	amino acid	test-compound	Increase in nitrogen content mg per bee		
			amino acid		com-pound
			lacking	optimal	
81	l-arginine (base)		0.09	0.29	
		l-ornithine di-HCl			0.14
		l-citrulline			0.18
91	l-arginine (base)		0.14	0.42	
		l-ornithine di-HCl			0.11
		l-citrulline			0.21
72	l-histidine (base)		0.03	0.30	
		l-carnosine			0.25
83	l-phenylalanine		0.12	0.32	
		Na-phenylpyruvate			0.18
98	l-phenylalanine			0.33	
		Na-phenylpyruvate			0.13
76	l-isoleucine		0.10	0.33	
		dl-norleucine			0.10
75	l-leucine		0.09	0.28	
		dl-norleucine			0.12

Table 20. *Replacement of essential amino acids for the young honeybee.*

related organism, the mosquito fly, growth is supported with citrulline instead of arginine, and to a certain degree also with ornithine (GOLDBERG & DE MEILLON 1948). These findings suggest that the honeybee is more exacting in its requirements for amino acids than many other animals, insects as well as vertebrates. However, one must take into account that the synthetic diet used in our experiments is much more simple than that being generally used in nutrition studies. So it is possible that certain deficiencies of the diet, finding no expression in the observed growth are connected with the observed discrepancies with other animals.

B. Replacement of dispensable amino acids.

In 1938 ROSE stated that the 10 essential amino acids when serving as the sole source of dietary nitrogen, supported growth of rats as rapidly as if all of the amino acids were supplied. Probably as a consequence of a better basal ration promoting faster weight gains, in subsequent experiments it was found that the addition of non-essential amino acids resulted in improved growth of rats and mice (ROSE, OESTERLING, WOMACK 1948; FROST & SANDY 1949; MADDY & ELVEHJEM 1949; ROSE & SMITH 1950). It was suggested that the simultaneous synthesis of all the non-essential amino acids presents too great a burden upon the chemical resources of the cells (ROSE, OESTERLING & WOMACK 1948). In recent years evidence is accumulating with regard to the substitution of other nitrogen compounds for non-essential amino acids. ROSE et al. (1949) in growth experiments with rats fed only 10 amino acids, observed

a striking acceleration in the rate of gain, if excess nitrogen was supplied in the form of ammonium salts, glutamic acid, glycine, or urea. These findings were confirmed, independently and at the same time, by LARDY & FELDOTT (1950). These authors found the same growth response even if the 10 essential amino acids were supplemented with either ammonium citrate or a mixture of non-essential amino acids containing equal amounts of nitrogen. The ability of the rat to utilize ammonium compounds agrees with the statement of FOSTER, SCHOENHEIMER & RITTENBERG (1939), deduced from experiments with isotopic nitrogen, that dietary ammonia can be utilized for amino acid synthesis.

In the light of the above findings we thought it of interest to ascertain whether the honeybee, as a representative of a totally different class of animals, would likewise show the ability to use dietary ammonia. For this purpose we kept young honeybees for 10 days on a diet supplemented with only the 10 essentials from a 2 % amino acid mixture and observed the growth responses on adding various nitrogen compounds in isonitrogenous amounts. Table 21 comprises the results. Each figure is the average of 8 analyses of 5 bees.

Exp.	10 ess. amino acids	Supplement to 10 essential amino acids					
		urea	ammonium acetate	glycine	glutamic acid	di amm. citrate	noness. amino acids
69	0.18	0.28	0.28	0.31	0.26	0.33	0.26
95	0.10	0.16	0.11	0.16	0.18		
95	0.11					0.24	0.37
108	0.21	0.23	0.29			0.32	
124	0.15	0.23	0.22	0.19	0.25	0.23	

Table 21. *Influence of various nitrogen compounds on the increase in nitrogen content of young honeybees (mg/bee) fed a diet with 10 essential amino acids.*

As will be observed, the supplemented rations induced a higher increase in nitrogen content than that which is obtained with 10 essential amino acids alone. The differences being statistically significant are underlined. The weight determinations, which are not recorded in the table, showed essentially the same results. Obviously the improved growth is due to utilization for protein synthesis of the nitrogen present in the various compounds. At any rate, we are justified in concluding that various nitrogen compounds supplemented to a diet with 10 essential amino acids induce improved growth in the young honeybee. Since however, the experimental bees are not kept sterile, it may not be decided whether and how far the utilization of the nitrogen is the result of synthetic action of microorganisms present in the gut. It is known that symbionts may supply vitamins not present in the diet of the host and

obviously in our experiments the required vitamins are obtained in this way. However, the needs for vitamins are of a different order of magnitude than those for amino acids, and therefore it is not likely that the above findings may be ascribed entirely to the action of symbionts. This suggestion is supported by the statement of ROSE (1949), that in rats the alimentary microorganisms play a much less dominant role in the formation of amino acids than they do in the synthesis of vitamins. ROSE & SMITH (1950) established that the microorganisms susceptible for sulphasuccidine have no influence upon the utilization of ammonium nitrogen in the growing rat.

Attempts to verify these results in honeybees were unsuccessful owing to a detrimental effect of sulphasuccidine on growth, even if applied in concentrations of 0.1, 0.3 and 1.0 % in a diet with 8 % pollen. Growth inhibition could not be abolished by the addition of the B-vitamins (B1, B2, nicotinic acid, B6, pantothenic acid, folic acid, choline, biotine and B12). The results of the nitrogen determinations on 8 samples of 5 bees after 10 days are recorded in table 22.

Supplement to sugar candy	Sulphasuccidine			
	0 %	0.1 %	0.3 %	1.0 %
Pollen mixture 8 %	0.39	0.27	0.24	0.10
Amino acid mixture 2.5 %	0.30	0.24	0.23	0.17
Ibid + vitamins				0.17

Table 22. *Inhibition of growth by sulphasuccidine.*

From this table the growth inhibition exerted by sulphasuccidine is evident. Hence sulphasuccidine was unsuitable to evaluate the significance of protein synthesis by microorganisms in the gut. However, with regard to essential amino acids, nothing points to a perceptible synthesis by microorganisms; the absence of one essential leads to complete inhibition of growth. It is unreasonable, therefore, to suggest that the non-essentials should be synthesized in amounts sufficient for good growth. Hence, it seems justified to ascribe the growth improvement observed on adding various nitrogen compounds, to anabolic activities of the test animal rather than to synthesis by microorganisms.

5. *Growth on diets containing amino acids, casein or pollen.*

It has frequently been reported, that the rate of growth induced by feeding amino acid mixtures to higher animals, in most cases does not equal that on intact protein (BAUER & BERG 1943; MARTIN 1944; MADDY & ELVEHJEM 1949; RAMASARMA et al. 1949; MADDY & SWIFT 1952). This inferiority of amino acid mixtures was ascribed to different causes such as imbalance of the mixture, toxicity of dl-forms, lack of strepogenin and lowered palatability of the diet.

In order to find out how closely growth in the young honeybee on amino acid mixtures approaches that obtained with intact proteins we fed diets supplemented with either pure amino acids, intact casein or pollen in isonitrogenous amounts. The unnatural isomers present in the amino acid mixture were considered to be inactive, which is nearly correct as will be shown in chapter VIII. The amino acid mixture was patterned after the composition of casein, however, with less glutamic acid and proline whereas the concentration of tryptophan was somewhat increased. Each of the three diets contained 0.30 % nitrogen, which is

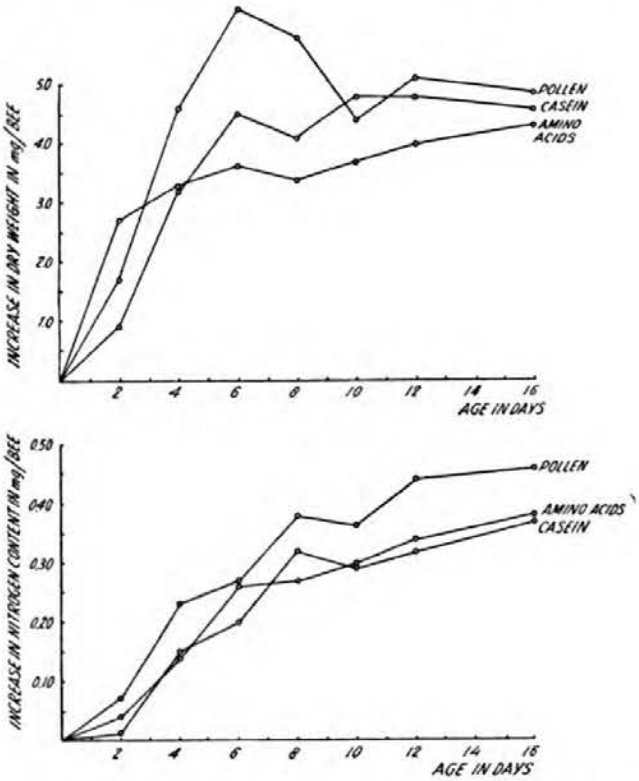

Fig. 14. Growth of young honeybees fed rations containing a mixture of 19 amino acids, "vitaminfree" casein or pollen in isonitrogenous amounts.

equivalent to about 3 % amino acids, 2.5 % casein and 9 % pollen. Daily food consumption was determined in each experimental group. Five samples of 5 bees were analyzed at intervals of 2 days from emergence up to the 16th day. The increase in the nitrogen content is plotted against time in fig. 14.

It will be seen, that the pollen diet induced a somewhat higher growth rate than did the other diets. The average daily food consumption on the former diet however, amounted to more than twice that on the amino acid diet (39.1 and 16.8 mg/bee/day respectively). No difference in

growth rate was observed between the amino acid and casein diet, even though the food consumption was substantially higher in the latter sase (16.8 and 23.8 mg/bee/day respectively). However, it may be noted, that as a result of the concentration of the nitrogen compounds in the diets employed, growth was suboptimal. Whether the same applies to conditions enabling a higher growth rate remains to be investigated. Therefore we are justified in concluding only, that under the conditions concerned, a ration containing 18 amino acids supported a growth rate equal to that obtained with a comparable amount of casein.

From the experiment reported above, we calculated biological values of the diets employed, i.e. the per cent of nitrogen from the food consumed, which was retained by the test bees. The following data were obtained: pollen 39, casein 46, amino acids 71. Since already a biological value of about 50 is low for growing rats, the value 39 for pollen in the growing honeybee is abnormally low. Thus it appears that the nitrogen in pollen is poorly utilized by the honeybee.

CHAPTER VII

QUANTITATIVE AMINO ACID REQUIREMENTS

1. *Introduction.*

For growth as well as for maintenance of the protein stores present in tissues and body fluids, the animal organism requires the consumption of essential amino acids normally taken in the form of protein. The amount of protein required is dependent a.o. on the relative concentrations of the essential amino acids. If a protein devoid of a certain essential amino acid but otherwise complete is taken as the sole protein in the diet, nitrogen equilibrium and growth is impossible, no matter how much of the protein is consumed. On supplementing the lacking component in increasing levels to the diet, the growth rate increases until another component becomes limiting, presupposed that the diet is adequate in other respects. Hence the amount of protein necessary for a certain growth rate is dependent on the quantitative amino acid composition of the protein concerned and on the requirement of the organism for each of the amino acids. It will be clear therefore, that knowledge of the quantitative requirements for amino acids is not only a matter of academic interest but of practical importance as well.

In the review of the literature on the value of different foods as substitutes for pollen in bee colonies (see chapter III) it was pointed out that a complete substitute has not been found as yet. Though soybean flour seems to be deficient in niacin, the vitamin content is not the sole cause of its failure to maintain honeybee colonies (HAYDAK 1949). It is quite possible that the poor nutritive value of various foods for bee

colonies is related to the quantitative needs of the honeybee for amino acids. Therefore a study of the quantitative amino acid requirements of the honeybee was thought to be of importance as a base in further search of a valuable substitute for pollen.

As early as 1937, ROSE published the quantitative amino acid requirements of the growing rat, however without detailed data. ALMQUIST (1947) reviewed experiments concerning the needs of the growing chick. Recently the requirements have been investigated for growth of the child (ALBANESE 1950), for tissue protein regeneration of the adult rat (STEFFEE et al. 1949, 1950) and for maintenance likewise of the adult rat (FRAZIER et al. 1949; BENDITT et al. 1950). Insects have not been studied as yet, except for the cystine requirements for normal development (see chapter III page 14).

The general procedure in these studies consisted of the construction of a series of diets kept constant in all constituents, except for the amino acid under consideration. The variable component is added at increasing levels. The amount above which no further increase in growth rate occurs, or in the case of maintenance studies, the amount necessary for maintaining nitrogen equilibrium, is considered to be the minimum level required. In this way each of the essential amino acids is studied successively. Such quantitative studies supply information not only with regard to the amounts of a given protein required, but also they give indications as to which protein or combinations of proteins supply the most favourable combination of essential amino acids required for synthesis of body proteins. In the case of the honeybee knowledge concerning the quantitative amino acid requirements may be valuable in elucidating a possible deficiency of one or more amino acids in the foods which have been used to study their replacing value for pollen.

In the present study we approximated the minimum concentration of each of the essential amino acids supporting optimal growth of the young honeybee under the experimental conditions described.

2. *Methods.*

Diets were prepared in the usual way with mixtures of 18 amino acids approximating the composition of casein, except for a lower level of glutamic acid and proline and an increased level of tryptophan. The amounts of the racemic amino acids were doubled only in the case of tryptophan and threonine. The composition of the mixture for each 20 g of diet was as follows:

l-arginine (base)	13.4 mg	dl-threonine	28.8 mg
l-histidine (base)	10.2 ,,	l-leucine	32.0 ,,
l-lysine dihydrochloric acid	40.8 ,,	l-isoleucine	24.0 ,,
l-tyrosine	20.5 ,,	l-valine	24.6 ,,
dl-tryptophan	8.4 ,,	l-glutamic acid	32.0 ,,
l-phenylalanine	20.2 ,,	l-aspartic acid	22.4 ,,
l-cystine	2.0 ,,	glycine	6.7 ,,
dl-methionine	11.2 ,,	l-alanine	10.6 ,,
dl-serine	21.8 ,,	l-proline	22.4 ,,

31.3 mg $NaHCO_3$ were added to neutralize the hydrochloric acid present in lysine di-hydrochloric acid. These amounts were incorporated in the diets with 16 g sucrose and 4 g tapwater thus supplying an amino acid level of 2.0 % of the dry sugar. In a subsequent series of experiments the amino acid mixture constituted 3.0 % of the dry sugar.

A multiple of the above amounts, dependent on the number of concentrations to be tested in one experiment, was weighed together. The amino acid examined was omitted and added in the desired amount to each portion of 20 g diet by pipetting from a soluiton. The consequences of the varying amino acid levels were measured in terms of dry weight and nitrogen content after an experimental period of 10 days. At each amino acid concentration examined, 8 samples of 5 bees were analyzed in the usual way (see chapter III, page 19).

3. *Results.*

Because of limitations of space, not all of the tables prepared can be presented. The results of only one experiment are given in detail to illustrate the variation in the figures (table 23). The relation between growth and concentration of each of the essential amino acids are illustrated in a series of curves in which the average increase in nitrogen content after a 10 days period, is plotted against the concentration (fig. 15). The concentration being expressed as a percentage of the total amount of amino acids.

Diet	Average of 8 samples of 5 bees in mg per bee			
	Dry weight and standard deviation	Increase in dry weight	Nitrogen content and standard deviation	Increase in nitrogen content
Without isoleucine	18.4 ± 0.32	1.9	2.16 ± 0.029	0.10
1 % l— ,,	19.1 ± 0.64	2.6	2.22 ± 0.028	0.16
2 % l— ,,	19.2 ± 0.37	2.7	2.27 ± 0.035	0.21
3 % l— ,,	20.3 ± 0.60	3.8	2.35 ± 0.035	0.29
4 % l— ,,	20.2 ± 0.19	3.7	2.39 ± 0.056	0.33
5 % l— ,,	20.2 ± 0.42	3.7	2.38 ± 0.043	0.32
6 % l— ,,	20.2 ± 0.53	3.7	2.39 ± 0.041	0.33
4 % dl— ,,	19.5 ± 0.22	3.0	2.27 ± 0.031	0.21
4 % d — ,,	18.1 ± 0.32	1.6	2.16 ± 0.033	0.10

Table 23. *Data obtained in an experiment with varying concentrations of l-isoleucine and one concentration of the racemic mixture and of the unnatural form.*

As will be observed from table 23, an increased amount of l-isoleucine in the diet resulted in increased growth until a maximum was obtained at a concentration of 4 %. Thus the minimum concentration of isoleucine for maximum growth under these conditions approximates 4 %. Moreover it is shown that 4 % dl-isoleucine induces an increase in dry weight and nitrogen content equal to that obtained with 2 % of the l-enantiomorph, whereas the increase with the d-isomer is equal to that obtained on a diet devoid of isoleucine.

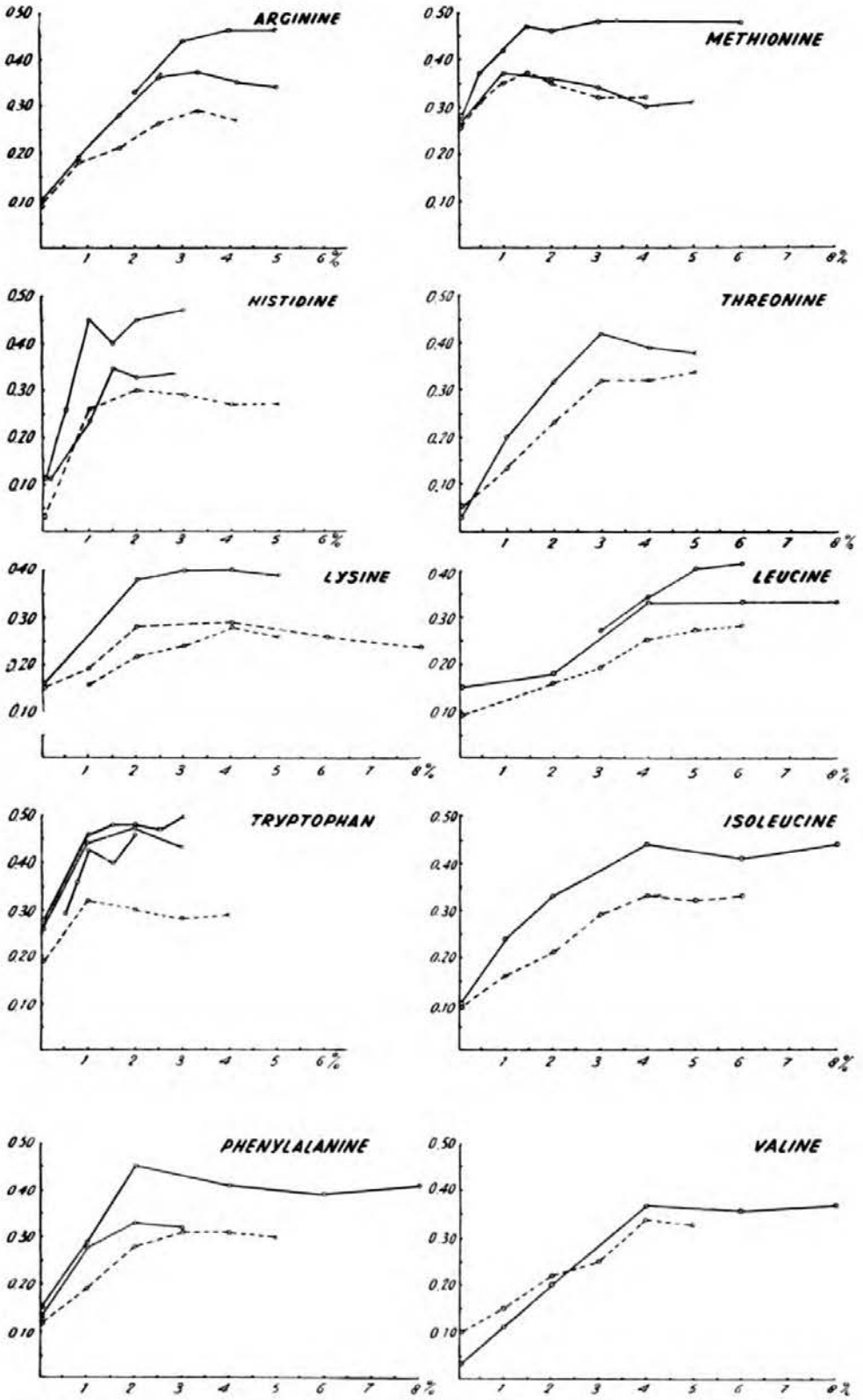

Fig. 15. Relation between growth and concentration of each of the essential amino acids expressed as percentage of the total amount of amino acids in the diets. The amino acid mixtures were fed at levels of 2 % (dotted lines) and 3 % (drawn lines).

The average increase in the nitrogen content at different levels of each of the essential amino acids is illustrated in the curves of fig. 15.

Each of the amino acids has been examined at two levels of the amino acid mixture viz. 3 % and 2 %, at least one experiment at each level. A rather regular course of the curves was obtained in nearly any case even though no smoothed lines were drawn. Each curve shows a rise with increasing concentration until a further increase is no longer accompanied by an upward trend of the curve. In the latter region the concentration is above its minimal level for maximal growth. Obviously, this minimal level is situated near the angle of the graphs. In general there is a good agreement in the minimal concentrations obtained with both levels of the amino acid mixture. This is comprehensible since the same set of proteins is synthesized by an animal at any growth rate and hence requires the same proportions of amino acids for synthesis. The differences obtained in different experiments with the same amino acid, appear to be of minor importance if evaluated with Wilcoxon's test. For instance in the phenylalanine curve at a level of 2 % amino acid mixture (dotted line) the difference in nitrogen content at 2 and 3 % phenylalanine is not essential ($P = 0.13$). In this case we suggest a 2.5 % phenylalanine concentration to approximate roughly the minimal concentration for maximal growth.

Comparing the curves for different amino acids it will be observed that the minimal concentration for maximal growth differs widely with the amino acid investigated. As all bees consumed rations identical in composition, it is obvious that the required amounts of the essential amino acids are different. Apparently the requirements for growth of the young honeybee are highest for leucine, isoleucine, and valine and lowest for tryptophan methionine, and histidine, while threonine, phenylalanine, arginine and lysine take intermediary positions.

It may be emphasized, however, that the observed minimal concentrations for maximal growth have nothing to do with the minimal required intake for maximal growth under natural conditions. They only represent the minimal amounts which, in combination with the amounts of the other amino acids, induce a growth rate maximal under the conditions of these experiments. Even though we are not justified in attaching absolute value to these minimal concentrations, they are valuable in combination with each other. From the minimal concentrations supporting maximal growth, an approximation of a proportionality pattern of essential amino acids can be deduced, supplying information regarding the relative quantities which are required for protein synthesis. From the curves in fig. 15 the following proportionality ratio may be estimated: arginine 3.0, histidine 1.5, lysine 3.0, tryptophan 1.0, phenylalanine 2.5, methionine 1.5, threonine 3.0, leucine 4.5, isoleucine 4.0 and valine 4.0. These values constitute the proportions of the essential amino acids used for anabolic reactions in the growing honeybee. Since it has been shown that in the rat non-essential amino acids (viz. tyrosine and

cystine) may spare essential ones (phenylalanine and methionine resp.) the above proportionality ratio is to be regarded as dependent on the proportions of other amino acids in the diet. As the mixture used was patterned after the composition of casein the proportions of amino acids are rather close approximations of a natural diet. Therefore it may be suggested that the above relative amounts will not differ greatly from the requirements under natural conditions.

4. *Discussion.*

The proportional relationship of the essential amino acids is of both theoretical and practical importance. It may help not only to explain poor growth in experiments with amino acid mixtures but may also give indications as to the composition of foods supplying the essential amino acids in the desired proportions for efficient utilization.

In table 24 the proportions of the minimum levels of essential amino acids for maintenance, repletion, or growth in different animals, as recorded in literature, are brought together, calculated with tryptophan as unity.

	Maintenance man ROSE 1949	Maintenance rat BENDITT et al. 1950	Repletion rat STEFFEE et al. 1950	Growth infant ALBANESE 1950	Growth rat ROSE 1937	Growth chick ALMQUIST 1947	Growth honey bee
arginine	—	—	—	—	1.0	4.8	3.0
histidine	—	1.0	1.7	—	2.0	1.2	1.5
lysine	3.2	2.1	5.4	5.6	5.0	3.6	3.0
tryptophan . . .	1.0	1.0	1.0	1.0	1.0	1.0	1.0
phenylalanine. .	4.4	2.7	4.1	5.6	3.5	3.6	2.5
methionine . . .	4.4	3.3	2.6	2.8	3.0	2.0	1.5
threonine . . .	2.0	2.4	3.2	2.9	3.0	2.4	3.0
leucine	4.4	3.6	6.1	14.0	4.5	5.6	4.5
isoleucine. . . .	2.8	6.2	3.3	3.0	2.5	2.4	4.0
valine	3.2	4.5	3.4	5.4	3.5	3.2	1.

Table 24. *Proportionality ratios of essential amino acids for maintenance, repletion, or growth of different organisms, with tryptophan expressed as unity.*

From this table a reasonable agreement is apparent between the quantitative requirements of different organisms. The requirements for methionine in the honeybee, however, are lower than in any of the other species investigated so far. This may be connected with the considerable initial growth observed in the honeybee on diets devoid of methionine, which was suggested to be caused by a reserve of this amino acid in honeybees at emergence (see page 55). The much higher requirements for arginine in the chick and the honeybee as compared with the rat is reasonable in connection with its synthesis in this animal (ROSE 1938).

One may ask how far the above requirements of the honeybee are realized in the food being consumed under natural conditions. Quantita-

tive determinations of the amino acids in pollen are scanty. VINSON (1927) claimed, that sweet corn pollen contains large amounts of β-hydroxy glutamic acid. This finding however, was not confirmed in more recent investigations. Several authors were unable to identify tryptophan in pollens (HEYL & HOPKINS 1920; AUCLAIR & JAMIESON 1948) and royal jelly (PRATT & HOUSE 1949). Since the identification of tryptophan is difficult one must be careful in drawing any conclusion from these negative results. On feeding pollen to rats, VIVINO & PALMER (1944) obtained indications of tryptophan and methionine (or cystine) deficiency in pollen. More recently, however, probably as a result of a better technique the presence of normal levels of tryptophan was ascertained in sweet corn pollen (SARKAR et al. 1949) and in pollen obtained from various plant species (WEAVER & KUIKEN 1951). The latter authors determined microbiologically the essential amino acid content of royal jelly and six pollens, and found a very similar composition. The agreement was somewhat less with respect to histidine, which ranged from 2.0 to 3.5 %. In table 25 the figures for royal jelly, the average values for pollen, and the values for soybean flour derived from the paper of WEAVER & KUIKEN 1951), are compared with the approximate minimum levels for optimal growth in the honeybee.

Amino acid	Percentage in:			Minimal level required in honeybee
	Royal jelly	Pollen	Soybean flour	
arginine	5.1	5.3	7.7	3.0
histidine	2.2	2.5	2.3	1.5
lysine	6.7	6.4	6.6	3.0
tryptophan	1.3	1.4	1.5	1.0
phenylalanine	4.1	4.1	5.1	2.5
methionine	1.9	1.9	1.4	1.5
threonine	4.0	4.1	3.9	3.0
leucine	7.7	7.1	8.0	4.5
isoleucine	5.3	5.1	5.3	4.0
valine	6.7	5.8	5.3	4.0

Table 25. *Comparison of the essential amino acid content of royal jelly, pollen, and soybean flour with the approximate minimal levels for optimal growth in the honeybee (expressed as per cent of the protein).*

It can be seen, that there are no great differences in the amino acid composition of the foods investigated. Moreover the figures demonstrate a nutritional surplus of each of the essential amino acids for maximal growth except for methionine in soybean flour.

Soybean flour seems to be somewhat deficient in methionine for growth of the honeybee, which is in agreement with the results of experiments with higher animals (HAYWARD & HAFNER 1941; ALMQUIST et al. 1942). This finding may possibly be connected with the failure of

soybean flour to maintain honeybee colonies being deprived of pollen (see page 10). Therefore it seems advisable to investigate whether the addition of some source of methionine may increase the nutritional value of soybean flour for honeybee colonies under field conditions.

CHAPTER VIII

UTILIZATION OF d-AMINO ACIDS

1. *Introduction.*

The amino acids present in the food of animals belong practically exclusively to the l-series [1]). In nutritional investigations however, part of the components used in mixtures op pure amino acids are always supplied in the racemic form. This happens because several amino acids can only be obtained in adequate quantities by syntheses which yield mixtures of the d- and l-modification in equal quantities. The procedures to separate both forms are laborious, often making the natural isomer too expensive for nutritional studies. Therefore it is not only of theoretical but also of practical importance to know if and to what extent the so called 'unnatural' d-modification may be utilized in the organism.

The numerous reports in literature concerning the availability of the d-amino acids in different animals are reviewed by several authors (ROSE 1938, 1949; BERG 1942; ALBANESE 1947; NEUBERGER 1948; ALBANESE 1950). The significance of the d-compound of almost all the essential amino acids have been determined for growth of the rat and the mouse and for maintenance of man. Further only the chick has been investigated to some extent. From the standpoint of the comparative physiologist it is interesting to know in how far the significance of the d-forms in lower animals agrees with that in mammals and birds.

In the present study we determined the significance of each of the essential amino acids for growth of the young honeybee.

2. *Methods.*

The mixture of pure amino acids used, was patterned after the composition of casein, the chief differences being a lower level of glutamic acid and proline and a higher level of tryptophan. The amount of amino acids constituted 2 or 3 % of the sucrose in the diet. Neither vitamins nor salts were added. All of the amino acids were supplied by Hoffmann la Roche except for d-lysine and d-arginine which were kindly supplied by Dr J. P. GREENSTEIN. [2])

The significance of the d-compound was tested in experiments designed primarily to determine the minimal concentration of the l-isomer for optimal growth (see chapter VII). So, for the evaluation of the growth promoting proporties of the

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[1]) The prefix "d-" or "l-" refers to configuration and not to direction of rotation.

[2]) The author gratefully acknowledges the kindness of Dr J. P. GREENSTEIN, Bethesda, Md, to supply the d-amino acids not commercially available.

unnatural compound we had at our disposal growth data obtained in the same experiment with different levels of the corresponding l-amino acid. Each of the essential amino acids was tested in at least two experiments. The d-isomer to be investigated was supplied in only one concentration in a diet devoid of this amino acid. In a subsequent experiment a higher concentration was often used. After having kept the bees for 10 days on the experimental diets, dry weight and nitrogen content were determined on 8 samples of 5 bees.

3. *Results.*

The growth data measured in terms of nitrogen content are illustrated in fig. 16. The increase in the nitrogen content after a 10-day experimental period is represented by the height of a rectangle. Each triad of rectangles is derived from one experiment. The left, middle, and right rectangle of each corresponds respectively with diets devoid of the amino acid tested, with the l-isomer in about optimal concentration and with the d-isomer at the same or at a higher level than the corresponding l-compound. Below the rectangles the concentration is mentioned expressed as a percentage of the amino acid mixture.

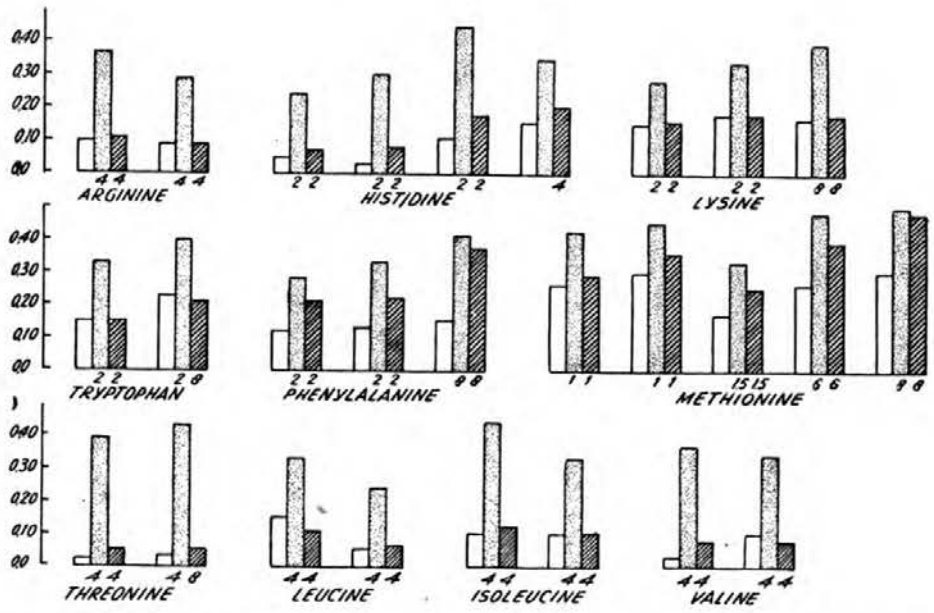

Fig. 16. Utilization of d-amino acids (hatched) as compared with the corresponding l-compound (punctuated) and with the corresponding amino acid totally lacking (open). For further explanation see text.

As it will be observed from fig. 16 the increase in the nitrogen content on diets with a d-amino acid exceeds that on diets devoid of one of the essential amino acids only in the case of histidine, phenylalanine and methionine. In no case does growth in the presence of a d-amino acid equal that occurring if the natural enantiomorph is supplied. This means

that in the honeybee only d-histidine, d-phenylalanine, and d-methionine is utilized to a certain degree but considerably less than is the corresponding l-amino acid. A tentative estimation of the degree of effectiveness as compared with the natural isomer reveals the following values: for phenylalanine about 60 %, for methionine 25—50 %, and for histidine only about 25 %. The unnatural form of the other 7 indispensable amino acids are not utilized at all.

4. *Comparison of the honeybee with other animals.*

Marked differences occur in the availability of the d-amino acids between the honeybee and other animals investigated. For comparison the data available in literature are summarized in table 26. In this table the sign + means 'utilized' and the sign — 'not utilized'. The data concern results obtained in either growth or maintenance experiments. Those for man and rat are given by ROSE (1938, 1949), for the mouse by BAUER & BERG (1943) and TOTTER & BERG (1939), for the chick by GRAU & ALMQUIST (1943) and ALMQUIST (1948).

d-Amino acid	Man	Rat	Mouse	Chick	Honeybee
valine	—	—	—	—	—
isoleucine	—	—	—	—	—
threonine	—	—	—		—
lysine	—	—	—		—
arginine					—
leucine.	—	—	—	+	—
tryptophan . . .	—	+	+	—	—
histidine		+	+		+
phenylalanine . .	+	+	+	+	+
methionine. . . .	+	+	+	+	+

Table 26. *Availability of d-amino acids for maintenance in man, and for growth in the rat, the mouse, the chick and the honeybee.*

It appears from this table, that for neither species investigated the data available in literature are complete. Especially for the chick there are many gaps. Moreover the sign + for a certain amino acid does not furnish any indication as to the degree of utilization. Since the effectiveness of a certain d-amino acid utilized by different organisms may vary greatly, the extent of agreement cannot be judged from the table.

d-Phenylalanine and d-methionine are utilized by all species investigated, the honeybee included. In man, rat, mouse, and chick they are efficiently utilized or even equivalent with the natural isomer (ROSE 1949; ROSE 1938; BAUER & BERG 1943; ALMQUIST 1948, and GRAU & ALMQUIST 1943). In the honeybee their replacement value is rather poor, especially in the case of methionine. d-Histidine, being less efficient than is l-histidine for the rat and the mouse (ROSE 1938; TOTTER & BERG

1939) is hardly utilized in the honeybee. d-Tryptophan is equivalent to l-tryptophan in promoting growth of the rat (ROSE 1938). In the mouse the d-isomer is less effective (TOTTER & BERG 1939), whereas in the honeybee any significance for growth is absent. d-Leucine so far appeared to be utilized only in the chick (GRAU & PETERSON 1946). d-Arginine being of no value in the honeybee, seems not to be investigated in other animals. Valine, isoleucine, threonine and lysine are available only in the l-configuration for any species investigated.

The availability of d-amino acids has not yet been investigated in other insects, except for tryptophan in *Drosophila*. It was found that l-tryptophan is a precursor of one of the components of the eye pigment of certain strains of *Drosophila*. In this function d-tryptophan was without effect (TATUM & HAAGEN-SMIT 1941). HINTON et al. (1951) found d-tryptophan to be of no value for growth of *Drosophila*. These findings agree with the fact that d-tryptophan is not utilized for growth of the honeybee.

From the above it appears that much conformity exists in the significance of the unnatural amino acids for different organisms. However, taking into account the quantitative difference in the utilization of some d-amino acids there is also much noncomformity. Especially the poor effectiveness of the d-isomers utilized in the honeybee may be stressed. How far the observed differences are related to biochemical abilities of the organisms examined cannot be judged with certainty, since variations in the experimental diets used by different authors may have contributed to the above findings. Judging from the quantitative differences mentioned, it seems justified to conclude that with respect to the configuration of the essential amino acids the honeybee is more specialized than other organisms investigated.

SUMMARY

1. During the first few days of the imago life of the worker honeybee a considerable growth occurs. Under experimental conditions this growth was obtained if the bees were supplied with cane sugar and a suitable source of protein.

2. The transition of the period of nursing activities into that of gathering activities is attended with a decrease in weight and protein content of the bees body. As a result two groups of worker bees can be distinguished in a bee colony: *a*. bees with high weight and protein content and well developed brood food glands (physiologically young: nurse bees); *b*. bees with low weight and protein content and degenerated brood food glands (physiologically old: gathering bees). Bees of the first group can be obtained from the inner part of the bee colony, those of the second group from the entrance of bee hives.

3. The opinion of additional pollen consumption by bees in autumn to build up reserve protein stores for the winter season has been opposed.

The absence of nursing duties in autumn is sufficient to explain the physiological condition of youth in a great part of old bees during the winter season.

4. The discrepancies met with in literature regarding the ability of old bees to digest and metabolise proteins, have been investigated. The results confirmed the more recent opinion that indeed old bees are able to do this. The protein anabolism in young bees, however, seems to proceed at a higher rate than in older bees.

5. The degree of development of just emerged bees proved to be very variable. Body weight and protein content is higher in autumn than during the summer season. In general the degree of development is dependent on the number of larvae in proportion to the number of nurse bees.

6. Young bees kept in captivity on a pure carbohydrate diet maintain life for a considerable time (25—30 days). The longevity is substantially increased by adding protein containing foods, proteins, or protein hydrolysates in adequate concentrations. This increase may amount to more than 150 %. Mixtures of amino acids similating casein were likewise effective, however, to a less extent. No improvement resulted from adding a salt mixture and the known vitamins including cholesterin, nucleic acid, linseed oil and liver extract. Simple nitrogen containing substances like ammonium compounds or glycine exerted no favourable influence on longevity.

7. In contrast with findings recorded in literature, the favourable effect of protein containing food on longevity was obtained also with old bees. The discrepancy with the results of other authors was ascribed to the detrimental effect of high protein concentrations. Contrary to current opinion it was concluded, that bees need protein not only for growth and secretion of royal jelly but also for maintenance of protein metabolism. In this connection attention was drawn to the significance of the proteins present in honey. It was suggested to investigate the value of sucrose supplemented with protein as a food for bee colonies.

8. A relation was observed between longevity and the amount of food consumed, in such a sense that a longer life span corresponds with a higher food intake.

9. The longevity both of adult- and of newly emerged bees on a protein free diet was found to be dependent on the degree of development of the bees. This relation may be of great disadvantage in longevity experiments in view of the great variation in the degree of development, not only in different seasons but even at successive days.

10. The qualitative amino acid requirements have been studied by determining the changes in weight and nitrogen content of young bees kept on diets of sugar candy supplemented with either acid hydrolyzed casein or mixtures of pure amino acids. The following 10 amino acids were classified as essential for growth of the honeybee: arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine,

isoleucine and valine. The same amino acids are required likewise for optimal growth of the rat. As non-essential were classified: tyrosine, cystine, serine, glutamic acid, aspartic acid, glycine, alanine, proline and hydroxyproline. Glycine, serine and proline exerted a stimulatory function on growth.

11. The qualitative amino acid requirements of the honeybee have been compared with those of other animals, being exhaustively studied at present. Each of them requires the same 7 and possibly 8 amino acids, whereas 6 others are required by neither of them. The significance of 5 amino acids varies with the species under consideration.

12. Some compounds have been tested for their ability to replace essential-, or non-essential amino acids. Histidine could be replaced by carnosine. Norleucine, Na-phenylpyruvate and ornithine proved to possess no replacing value for leucine, phenylalanine, or arginine respectively. Citrulline had only slight replacing value for arginine. The dispensable amino acids could be replaced to a considerable extent by each of the following nitrogen compounds: glycine, glutamic acid, urea, ammonium acetate, or ammonium citrate. This agrees with recent findings obtained with the rat. The growth improvement obtained on adding the nitrogen compounds mentioned to a diet containing the indispensable amino acids only, was ascribed to anabolic activities of the honeybee, rather than to the action of microorganisms present in the gut.

13. The quantitative requirements for each of the 10 essential amino acids have been studied in terms of gain in weight and nitrogen content of young bees kept on diets consisting of sugar candy with mixtures of 18 amino acids. The proportions of the minimal quantities of the essential amino acids required for growth were approximated from curves relating increase in nitrogen content of experimental bees and concentration of the amino acid examined. The requirements were found to be highest for leucine, isoleucine and valine, and lowest for tryptophan, histidine, and methionine. The requirements for arginine, lysine, phenylalanine and threonine are intermediary.

14. A comparison of the quantitative requirements of the honeybee with those of other animals revealed a good agreement.

15. The methionine concentration of soybean flour turned out to be limiting factor for growth of the honeybee, as is known likewise for higher animals.

16. The utilization in the honeybee of the unnatural isomers of the essential amino acids has been investigated, likewise by means of growth determinations. Growth on diets with mixtures of amino acids devoid of the component to be investigated, was compared with that obtained on supplementing either the d-, or l-isomer. The results revealed that only the d-enantiomorph of phenylalanine, methionine and histidine is utilized to an appreciable extent, the effectiveness decreasing in the order mentioned.

17. These results have been compared with reports in literature

regarding other animals. From this it appeared, that the effectiveness of the d-isomers being utilized by the honeybee, is considerably less than that in other animals investigated so far.

RÉSUMÉ

1. Pendant les premiers jours de la vie de l'abeille ouvrière comme imago, il y a une croissance considérable. En circonstances expérimentelles cette croissance a été obtenue en donnant les abeilles la disposition du sucre de canne et d'une source de protéines apte.

2. Quand les nourrices changent en butineuses, elles diminuent en poids et en teneur en protéine. Ainsi on peut distinguer deux groupes d'ouvrières dans une colonie d'abeilles: *a*. Des abeilles à poids et teneur en protéine élevés et avec des glandes à gelée royale bien développées (des abeilles physiologiquement jeunes: des nourrices); *b*. Des abeilles à poids et teneur en protéine peu élevés et avec des glandes à gelée royale réduites. (des abeilles physiologiquement vieilles: des butineuses).

Les abeilles de la première groupe peuvent être obtenues dans le centre de la colonie, ceux de la deuxième groupe près de la sortie de la ruche.

3. L'idée, que les abeilles consommeraient un surplus de pollen en automme pour faire une réserve de protéines pour l'hiver a été contestée. L'absence du nourrissage en automne explique suffisament l'état physiologiquement jeune d'une grande partie des abeilles vieilles pendant l'hiver.

4. Les contradictions qu'on trouve dans la littérature, par rapport à la possibilité des vieilles abeilles pour digérer les protéines et pour les user pour le métabolisme, sont vérifiés. Les résultats confirment l'opinion plus récente qu'en effet les vieilles abeilles possèdent cette capacité. Pourtant l'anabolisme des protéines semble d'être plus intensive chez les jeunes abeilles que chez les abeilles plus vieilles.

5. Le degré du développement des abeilles nouveau-nées parut d'être très variable. En automne le poids et la teneur en protéine du corps sont plus hauts que pendant l'été. En général l'état du développement dépend du proportion du nombre de larves et du nombre de nourrices.

6. Des jeunes abeilles en captivité restent assez longtemps en vie avec un régime d'hydrates de carbone purs (25—30 jours). La durée de la vie est prolongée considérablement quand on ajoute en concentrations aptes des espèces de nourriture protéineuses, des protéines ou des hydrolysats de protéines. Cette prolongation peut dépasser le 150 %. Des mixtures d'acides aminés, à compositions resemblant la caséine, prolongaient aussi la durée de la vie quoiqu' à moindre degré. Pas d'amélioration a été obtenu en ajoutant une mixture saline et les vitamines connues y compris la cholestérine, l'acid nucléinique, l'huile de lin et l'extrait hépatique. Des matières azotées simples, comme des composés ammoniaquales ou du glycocolle n'avaient pas d'influence favorable sur la durée de la vie.

7. Au contraire des résultats mentionnés dans la littérature l'effet

avantageux de la nourriture protéineuse sur la durée de la vie a été aussi obtenu chez les abeilles vieilles. La différence avec les résultats d'autres auteurs a été attribuée à l'effet désavantageux de hautes concentrations de protéines. Contrairement à l'opinion en vigueur il était conclu que les abeilles n'ont pas seulement besoin de protéines pour la croissance et la sécrétion de la gelée royale, mais aussi pour le maintien du métabolisme protéique. En rapport avec ce qui précède, l'attention a été fait à l'importance des protéines qui se trouvent dans le miel. La proposition a été faite de faire des recherches sur la valeur du sucre de canne complèté avec du protéine, comme nourriture pour des colonies d'abeilles.

8. Une corrélation a été observée entre la durée de la vie et la consommation de la nourriture, tel qu'une durée de la vie plus longue correspond à une plus grande consommation.

9. La durée de la vie des abeilles adultes, aussi bien que des abeilles nouveau nées avec un régime privé de protéine parut de dépendre de l'état du développement des abeilles. Ce rapport peut être très nuisible aux expériences sur la durée de la vie en considération des grandes variations du développement d'abeilles sortant des cellules, non seulement pendant diverses saisons, mais aussi aux jours successifs.

10. Les besoins qualitatifs en acides aminés ont été examinés en déterminant les changements du poids et de la teneur en azote des jeunes abeilles qui sont mis au régime de sucre en pâte complèté avec des hydrolysats acides de caséine ou avec des mixtures d'acides aminés purs. Les 10 acides aminés suivants étaient classifiés comme essentiel pour la croissance de l'abeille: arginine, histidine, lysine, tryptophane, phenylalanine, méthionine, thréonine, leucine, isoleucine et valine. Les mêmes acides aminés sont aussi nécessaire pour la croissance optimale du rat.

Les acides aminés classifiés comme non-essentiels sont: tyrosine, cystine, acide glutamique, acide aspartique, glycocolle, alanine, proline et hydroxyproline. La glycocolle, la sérine et la proline avaient une influence stimulante sur la croissance.

11. Les besoins qualitatifs de l'abeille en acides aminés ont été comparés avec ceux d'autres animaux autant qu'ils sont examinés tout au long jusqu'à présent.

Chaqu'un de ces animaux a besoin des 7 (possiblement 8) acides aminés mêmes, tandis que 6 autres ne sont nécessaires pour aucun de ces animaux. L'importance de 5 acides aminés dépend de l'animal examiné.

12. Quelques matières ont été examinées sur la capacité de remplacer des acides aminés essentiels ou non-essentiels. Histidine pouvait être remplacée par carnosine. Norleucine, phénylpyruvate de sodium et ornithine ne parûrent pas d'avoir valeur comme remplacant de respectivement leucine, phénylalanine et arginine. Les acides aminés non-essentiels pouvaient être remplacés largement par jacqu'une des matières azotées suivantes: glycocolle, acide glutamique, urée, ammonium acétate,

ou ammonium citrate. Ceci correspond avec des résultats récents obtenus avec le rat. L'amélioration du croissance par l'addition des matières azotées les dites à un régime contenant exclusivement les acides aminés essentiels était attribué aux activités anaboliques de l'abeille et pas aux micro-organismes qui se trouvent dans l'intestin.

13. Les besoins quantitatifs de chaque des 10 acides aminés essentiels ont été examinés en déterminant l'accroissement du poids et de la teneur en azote de jeunes abeilles dont le régime se composait de sucre en pâte avec des mixtures des 18 acides aminés. La proportion des quantités nécessaires minimales des acides aminés essentiels a été calculée par approximation des courbes, qui indiquent la rélation entre l'accroissement de la teneur en azote des abeilles d'essai et la concentration de l'acide aminé examiné. Les besoins parûrent d'être le plus grands en leucine, isoleucine et valine et le plus petit en histidine, méthionine et tryptophane. Les besoins en arginine, lysine, phénylalanine et thréonine sont intermédiaires.

14. Les besoins quantitatifs de l'abeille en acides aminés correspondent bien avec ceux d'autres animaux.

15. Tel qu'il est connu chez les vertébrès il parût qu'aussi pour la croissance de l'abeille le teneur en méthionine du farine de soya est le facteur restrictif.

16. L'utilité pour l'abeille des formes pas naturelles des acides aminés essentiels a été examiné, aussi au moyen de déterminations du croissance. La croissance avec un régime de mixtures d'acides aminés privées du composant examiné a été comparé avec la croissance quand on ajoute le d- ou l-isomère. Les résultats ont révélé que seulement les d-isomères de phenylalanine, méthionine et histidine sont utilisable quelque peu. Leurs efficacité diminue en succession susdite.

17. Ces résultats ont été comparés avec les données de la littérature concernant d'autre animaux. Ainsi il parut que l'efficacité des d-isomères qui sont utilisable pour l'abeille est beaucoup plus petit que chez les autres animaux examinés jusqu'à présent.

SAMENVATTING

1. Gedurende de eerste levensdagen van de werkbij als imago treedt een aanzienlijke groei op. Onder experimentele omstandigheden werd deze groei verkregen, wanneer de bijen rietsuiker en een geschikte eiwitbron beschikbaar werden gesteld.

2. De overgang van de periode van de broedverzorging naar die van het verzamelen, gaat gepaard met een afname van het gewicht en van het eiwitgehalte van het lichaam. Hierdoor zijn in een bijenvolk twee groepen van werkbijen te onderscheiden: *a*. bijen met hoog gewicht en eiwitgehalte en goed ontwikkelde voedersapklieren (physiologisch jong: broedbijen), *b*. bijen met laag gewicht en eiwitgehalte en gereduceerde voedersapklieren (physiologisch oud: verzamelbijen). Bijen van de eerste

groep kunnen verkregen worden uit het centrum van het bijenvolk, die van de tweede groep bij de vliegopening van de bijenwoning.

3. De opvatting, dat bijen in het najaar extra stuifmeel zouden opnemen om een eiwitreserve voor het winterseizoen aan te leggen werd bestreden. Het achterwege blijven van de broedverzorging in het najaar vormt een voldoende verklaring voor de physiologisch jeugdige toestand van een groot gedeelte van de oude bijen gedurende het winterseizoen.

4. De tegenstrijdigheden die in de literatuur worden aangetroffen met betrekking tot de mogelijkheid voor oude bijen om eiwitten te verteren en te gebruiken voor de stofwisseling, werden onderzocht. De resultaten bevestigden de meer recente opvatting, dat oude bijen hiertoe inderdaad in staat zijn. Het eiwitanabolisme schijnt echter in jonge bijen intensiever te verlopen dan in oudere bijen.

5. De graad van ontwikkeling van pasgeboren bijen bleek zeer variabel te zijn. In de herfst is het gewicht en het eiwitgehalte van het lichaam hoger dan gedurende het zomerseizoen. In het algemeen is de ontwikkelingstoestand afhankelijk van de verhouding tussen het aantal larven en het aantal broedbijen.

6. Jonge bijen blijven in gevangenschap een aanzienlijke tijd in leven op een zuiver koolhydraat dieet (25—30 dagen). De levensduur wordt belangrijk verlengd door toevoeging van geschikte concentraties eiwitbevattende voedselsoorten, eiwitten en eiwithydrolysaten. Deze verlenging kan meer dan 150 % bedragen. Mengsels van aminozuren, in samenstelling gelijkend op caseïne, verlengden eveneens de levensduur, hoewel in mindere mate. Er werd geen verbetering verkregen door toevoeging van een zoutmengsel en van de bekende vitamines, met inbegrip van cholesterine, nucleïnezuur, lijnolie en leverextract. Eenvoudige stikstofhoudende stoffen zoals ammoniumverbindingen of glycocol, oefenden geen gunstige invloed uit op de levensduur.

7. In tegenstelling met in de literatuur vermelde resultaten werd het gunstige effect van eiwithoudend voedsel op de levensduur ook verkregen met oude bijen. De discrepantie met de resultaten van andere auteurs werd toegeschreven aan de nadelige werking van hoge concentraties eiwit. In afwijking van de geldende mening werd geconcludeerd, dat bijen niet alleen eiwit nodig hebben voor de groei en de secretie van larvenvoedsel, maar ook voor handhaving van de eiwitstofwisseling. In verband hiermede werd de aandacht gevestigd op de betekenis van de eiwitten die in honing aanwezig zijn. Er werd voorgesteld om de waarde te onderzoeken van met eiwit aangevulde rietsuiker als voedsel voor bijenvolken.

8. Er werd een correlatie waargenomen tussen de levensduur en het voedselverbruik, in dien zin dat een langere levensduur correspondeert met een grotere voedselopname.

9. De levensduur op een eiwitvrij dieet van volwassen-, zowel als van pasgeboren bijen bleek afhankelijk te zijn van de ontwikkelingstoestand der bijen. Dit verband kan van groot nadeel zijn in levensduurexperimen-

ten met het oog op de grote variatie in de ontwikkeling van uitlopende bijen, niet alleen in verschillende seizoenen, maar zelfs op opeenvolgende dagen.

10. De kwalitatieve aminozuurbehoeften werden bestudeerd door bepalingen van de veranderingen in het gewicht en het stikstofgehalte van jonge bijen, die gehouden werden op diëten van suikerdeeg aangevuld met zuurhydrolysaten van caseïne, of met mengsels van zuivere aminozuren.

De volgende 10 aminozuren werden geclassificeerd als essentieel voor de groei van de bij: arginine, histidine, lysine, tryptophaan, phenylalanine methionine, threonine, leucine, isoleucine en valine. Dezelfde aminozuren zijn ook noodzakelijk voor optimale groei van de rat. Als niet-essentieel werden geclassificeerd: tyrosine, cystine, glutaminezuur, asparaginezuur, glycocol, alanine, proline en oxyproline. Glycocol, serine en proline oefenden een stimulerende werking uit op de groei.

11. De kwalitatieve aminozuurbehoeften van de bij, werden vergeleken met die van andere dieren welke thans uitvoerig zijn onderzocht. Elk van deze dieren heeft dezelfde 7 en mogelijk 8 aminozuren nodig, terwijl 6 andere voor geen van hen nodig zijn. De betekenis van 5 aminozuren varieert met de onderzochte diersoort.

12. Enige stoffen werden onderzocht op hun vermogen om essentiele-, of niet-essentiele aminozuren te vervangen. Histidine kon vervangen worden door carnosine. Norleucine, Na-phenylpyrodruivenzuur en ornithine bleken geen vervangingswaarde te hebben respectievelijk voor leucine, phenylalanine, of arginine. Citrulline had slechts geringe vervangingswaarde voor arginine. De niet- essentiele aminozuren konden in aanzienlijke mate vervangen worden door elk van de volgende stikstofverbindingen: glycocol, glutaminezuur, ureum, ammonium acetaat, of ammonium citraat. Dit stemt overeen met recente gegevens verkregen met de rat. De groeiverbetering door toevoeging van genoemde stikstofverbindingen aan een dieet, dat uitsluitend de essentiele aminozuren bevat werd toegeschreven aan de anabolische activiteiten van de bij en niet van in de darm aanwezige micro-organismen.

13. De kwantitatieve behoeften aan elk van de 10 essentiele aminozuren werden bestudeerd door bepalingen van de toename in gewicht en stikstofgehalte van jonge bijen op diëten bestaande uit suikerdeeg met mengsels van 18 aminozuren. De verhouding van de minimum hoeveelheden van de essentiele aminozuren benodigd voor de groei werd benaderd uit curven, die het verband aangeven tussen de toename in stikstofgehalte van de proefbijen en de concentratie van het onderzochte aminozuur. De behoeften bleken het grootst te zijn voor leucine, isoleucine en valine, en het laagst voor tryptophaan, histidine en methionine. De behoeften aan arginine, lysine, phenylalanine en threonine zijn intermediair.

14. Een vergelijking van de kwantitatieve behoeften van de bij met die van andere dieren gaf een goede overeenstemming te zien.

15. Zoals voor hogere dieren bekend is, bleek ook voor de groei van de bij de methionineconcentratie in sojameel beperkende factor te zijn.

16. De bruikbaarheid van de niet-natuurlijke vormen der essentiele aminozuren voor de bij werd onderzocht, eveneens door middel van groeibepalingen. De groei op diëten met mengsels van aminozuren die de te onderzoeken component missen, werd vergeleken met de groei verkregen na toevoeging van de d-, of de l-isomeer. De resultaten brachten aan het licht dat slechts de d-vorm van phenylalanine, methionine en histidine enigermate bruikbaar is, terwijl hun werkzaamheid in de genoemde volgorde afneemt.

17. Deze resultaten werden vergeleken met literatuurgegevens betreffende andere dieren. Daarbij bleek, dat de werkzaamheid van de d-isomeren die voor de bij bruikbaar zijn, veel geringer is dan in andere tot dusver onderzochte dieren.

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STELLINGEN

I

De langere levensduur van bijen in het winterseizoen vergeleken met die in de zomer, wordt niet veroorzaakt door extra stuifmeelconsumptie in het najaar, maar door het ontbreken van de broedverzorging.

II

De ontwikkelingstoestand van pasgeboren bijen is niet op de eerste plaats afhankelijk van de voor het bijenvolk beschikbare hoeveelheid stuifmeel, maar van de verhouding tussen het aantal broedbijen en het aantal larven.

III

De verbetering van de groei van ratten en bijen, door toevoeging van niet-aminozuurstikstof aan diëten met essentiele aminozuren als enige stikstofbron, kan niet uitsluitend worden toegeschreven aan de werking van in de darm aanwezige micro-organismen.

IV

De tegenwoordige opvattingen over de invloed van narcotica op het gedrag van bijen, steunen in aanzienlijke mate op waarnemingen die ten dele onjuist, ten dele onvolledig zijn.

V

Dat de ovarien van werkbijen in een normaal bijenvolk niet tot ontwikkeling komen, is een gevolg van direct contact tussen koningin en werkbijen.

VI

Optomotorische reacties treden zowel bij actieve, als bij passieve bewegingen op.

VII

De na toediening van groeistoffen optredende celstrekking wordt niet veroorzaakt door een verhoogde plasticiteit van de celwand.

VIII

De experimenten van DETWILER hebben aangetoond, dat de normale differentiatie van de gehoorplacode bij Amblystoma afhankelijk is van de aanwezigheid van de medulla.

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IX

Bij de normale sexuele differentiatie van het Vertebraten-embryo spelen hormonen een rol, die chemisch nauw verwant zijn aan of identiek zijn met de voortplantingshormonen van het volwassen stadium.

X

De opvatting dat volwassen bijen geen eiwitten in het voedsel nodig hebben, is onjuist. De in de bijenteelt algemeen gebruikelijke voedering van bijenvolken met uitsluitend suiker is daarom niet verantwoord.

STELLINGEN

I

De langere levensduur van bijen in het winterseizoen vergeleken met die in de zomer, wordt niet veroorzaakt door extra stuifmeelconsumptie in het najaar, maar door het ontbreken van de broedverzorging.

II

De ontwikkelingstoestand van pasgeboren bijen is niet op de eerste plaats afhankelijk van de voor het bijenvolk beschikbare hoeveelheid stuifmeel, maar van de verhouding tussen het aantal broedbijen en het aantal larven.

III

De verbetering van de groei van ratten en bijen, door toevoeging van niet-aminozuurstikstof aan diëten met essentiele aminozuren als enige stikstofbron, kan niet uitsluitend worden toegeschreven aan de werking van in de darm aanwezige micro-organismen.

IV

De tegenwoordige opvattingen over de invloed van narcotica op het gedrag van bijen, steunen in aanzienlijke mate op waarnemingen die ten dele onjuist, ten dele onvolledig zijn.

V

Dat de ovarien van werkbijen in een normaal bijenvolk niet tot ontwikkeling komen, is een gevolg van direct contact tussen koningin en werkbijen.

VI

Optomotorische reacties treden zowel bij actieve, als bij passieve bewegingen op.

VII

De na toediening van groeistoffen optredende celstrekking wordt niet veroorzaakt door een verhoogde plasticiteit van de celwand.

VIII

De experimenten van DETWILER hebben aangetoond, dat de normale differentiatie van de gehoorplacode bij Amblystoma afhankelijk is van de aanwezigheid van de medulla.

DETWILER, S. R. (1951): J. Exptl Zool. **116**: 415—430.
DETWILER, S. R. en VAN DYKE, R. H. (1951): J. Exptl Zool. **118**: 389—405.

IX

Bij de normale sexuele differentiatie van het Vertebraten-embryo spelen hormonen een rol, die chemisch nauw verwant zijn aan of identiek zijn met de voortplantingshormonen van het volwassen stadium.

X

De opvatting dat volwassen bijen geen eiwitten in het voedsel nodig hebben, is onjuist. De in de bijenteelt algemeen gebruikelijke voedering van bijenvolken met uitsluitend suiker is daarom niet verantwoord.

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