CHAPTER 4 NUTRIENTS IN HONEY BEE EGGS

4:0. Introduction

The nutrients in honey bee eggs are carbohydrates, lipids, proteins, minerals, vitamins and water. All the nutrients are derived from food sources obtained in the bees' environment (Moritz 1992). Dow (1986) categorized honey bees as plant feeders, because they consume nectar (liquid) and pollen (solid).

When honey bees ingest nectar and pollen, sucrose in nectar is hydrolyzed in the crop by invertase (sucrase) from hypopharyngeal glands, while pollen is digested by proteinases in the ventriculus (Klungness and Peng 1987). Crailsheim (1988) identified the absorption site for glucose and leucine in *A. mellifera* as the anterior two-thirds of the ventriculus. Berridge (1970) reported that carbohydrates, lipids and proteins are mainly absorbed from the midgut as monosaccharides, fatty acids and amino acids, respectively. Water, inorganic ions and amino acids are also absorbed in the hindgut or rectum. Turunen (1985) described the absorption mechanisms of nutrients in the alimentary canals of insects, identifying the midgut as the main absorption site. The fat body plays an important role in regulating the nutrient equilibrium in the body. The nutrients absorbed are finally used for maintenance and reproduction (Chapman 1982).

Oogenesis plays an essential role of providing nutrients for embryogenesis (Postlethwait and Giogi 1985). Eggs which are ready for fertilization have been stocked with a variety of substances including sugars, lipoproteins, and amino acids, stored as lipid droplets, lipoprotein yolk and glycogen. Larger pools of inorganic phosphates, ATP, and nucleotides are required after fertilization, when rapid cell divisions take place (Richter and Schwarz 1991). Incorporation of these substances into the oocytes involves different transport mechanisms which are thought to include passive and active transport systems, as well as endocytosis. Carbohydrates are mainly absorbed from the hemolymph into the ova by simple diffusion, even though Woodring et al. (1993) argued that it involved active transport. Lipids are sequestered from the hemolymph by carrier proteins, known as lipophorins (Chino et al. 1981, Engler et al. 1998). The nurse cells actively assist the rapidly growing oocytes in building adequate stores of nutrients.

The biochemical changes in the level of nutrients in an egg during embryogenesis depends on its age. Premkumar et al. (1991) observed gradual depletion of carbohydrates and lipids as embryonic development proceeded in the eggs of *Laccotrephes griseus* (Hemiptera: Belostomatidae). In most insect eggs, carbohydrates are a source of energy in the early stages of embryonic development, while lipids become the main energy suppliers in later stages of embryogenesis. This has been confirmed by determination of respiratory quotient in incubated eggs. Carbohydrates function as structural components used in the lining of cellular organelles. Lipids have versatile roles in insects and occur in many forms. They form constituents of pheromones and are necessary in the synthesis of hormones. These substances have direct or indirect influence on reproduction through regulation of information transfer which helps in bringing mates together during courtship and mating as well as in the process of oogenesis (Chapman 1982, Winston 1987, Moritz and Southwick 1992). Phospholipids and steroids have structural roles in membrane systems. Neutral lipids occur in fat bodies as triacylglycerols commonly used as stores of metabolic energy. Meanwhile proteins are utilized as enzymes, hormones, and singly as structural components which mediate in cellular communications. Proteins also combine with either carbohydrates or lipids or both, to produce compounds used to build cuticular structures (Chapman 1982). The three macromolecules are utilized through glycolytic pathways to produce pyruvate which is further degraded in the tricyclic acid cycle and electron transfer systems to yield large amounts of adenosine triphosphate (ATP). Vitamins and minerals are used as cofactors in cellular reactions. Water forms the main solvent and transporter of reactants as well as other products of metabolism. It is also needed for maintenance of cell turgidity and hydrostatic skeleton among other functions (Campbell 1993).

4:1. Carbohydrates

4:1:1. Introduction

Carbohydrates are described as polyhydroxyaldeydes, ketones, alcohols, acids, amines (glucosamine), or their condensation products. They are classified according to the number of monomeric units present in the macromolecule (Lehninger 1975).

Sucrose is a major sugar in the nectar(s) collected by honey bees. After its breakdown by sucrase (invertase) from the crop of the honey bee, and a ripening process in the cells on combs, glucose and fructose become the predominant sugars in honey (Dade 1977). A small amount of sucrose remains unconverted. Other sugars of nutritional importance to honey bees, present in honey, include melezitose, trehalose, maltose, α-methyl glucoside (Winston 1987).

Honey, a supersaturated sugar solution, forms the main food consumed by workers. The queen is fed predominantly on royal jelly which has a lower sugar concentration than honey. It is therefore interesting to investigate the quantity and quality of sugars incorporated in the eggs of each caste (queens and laying workers) during oogenesis and the pattern of utilization of these nutrients during embryogenesis. Fell (1990) confirmed the presence of fructose, glucose and trehalose in the hemolymph of honey bees in appreciable quantities. Woodring et al. (1993) measured blood sugar homeostasis in honey bee workers and confirmed the presence of fructose, glucose and trehalose in the workers at different ages and under different feeding regimes. They further argued that translocation of sugars from the midgut to the hemolymph may not involve simple diffusion. However, the concentrations of carbohydrates in honey bee eggs are relatively low. This makes identification and quantification of the sugars in the eggs a great challenge (Fell and Tignor; personal communications 1996). At the same time, Fell (1990) described a quick and simple method of quantifying small volumes of insect tissue extracts. This method can be used to analyze low quantities of nutrients such as those present in honey bee eggs, since it detects nanogram to milligram quantities of sugar concentrations in insect tissues and extracts.

A number of studies (Gordon 1959, Dadd 1961, Chen 1966, Kuk-Meiri et al. 1966, Agrell and Lundquist 1973, Spadling 1993) have been undertaken to determine the level

of nutrients in insect eggs but there are only a few specific studies of nutrients in honey bee eggs (Engels 1990, Ute and Crailsheim 1997), especially those related to partitioning of individual sugars in the eggs from queens and laying workers. The objective of this experiment was to (a) quantify the levels of trehalose, sucrose, glucose and fructose in honey bee eggs, (b) compare the levels of these sugars in the eggs from queens and those produced by laying workers, and (c) relate what the trends observed over the three day period, may mean to the survival of developing embryos.

4:1:2. Materials and Methods

Plates for carbohydrate analysis were pretreated and activated as described by Fell (1990). Merck (10 cm x 10 cm HPTLC) silica gel 60 pre-coated non-fluorescent indicator plates were first washed in methanol (Fisher HPLC grade) in covered Camag Twin Trough Development Tank. The alcohol was allowed to migrate to the top of plates followed by drying. They were sprayed thinly (without wetting the silica gel) with citrate buffer (Sigma Chemical Company) (at 10 parts water to 1 part citrate buffer). The plates were then thinly sprayed with 0.1M sodium hydrogen sulfite buffer (at 85 parts buffer to 15 parts water). They were finally activated in the oven at 100^{o}C for at least 1 hour. At the end of activation, the plates were cooled to room temperature and stored in a desiccator.

Storage of plates for at least 48 hours before spotting samples produced good separation of sugars. Plates can be stored up to 8 weeks before use for spotting samples, as determined by Fell (1990). The plates should be reactivated for at least 5 minutes at 100^{0}C, before use. Excess moisture must be avoided from coming into contact with the plates to prevent silica gel peeling off during development and subsequent handling, especially if the plates have to be stored in a freezer before scanning. Any contaminants that may disfigure the surface of silica gel plates by coloration or scratch were avoided.

Extraction and quantification of carbohydrates

The methods of analysis, detection and quantification were similar to those described by Fell (1990) with slight modifications involving the preparation of samples. Batches of 10 honey bee eggs per sample taken from queens and laying workers, were placed in individual 1.5 ml microcentrifuge tubes (Fisher) to which 50 μL of 70% ethanol was

added and thoroughly homogenized with microcentrifuge pestles. The pestles were rinsed with additional 50 μL (70% ethanol), before the mixture was vortexed for 2 min. The tubes were centrifuged at 12 000 rpm for 5 min using a Fisher micro-centrifuge (Model 59A). From each tube, 50 μL of aqueous layer of the supernatant was removed and placed in clean 1.5 ml microcentrifuge tubes. The contents were evaporated to dryness in a Fisher tissuemat water bath under a stream of dry nitrogen. The sugars were finally re-suspended in 5 μL of 70% ethanol for spotting on precoated, pretreated, and reactivated silica gel - 60 (Merck 10 cm x 10 cm HPTLC) plates set on Camag nanomat II manual spotter. The spots were made using individual 1.0 μL Drummond micropipettes in duplicate for each sample and standard. The standards consisted of a mixture of glucose, fructose, trehalose and sucrose in 70% ethanol at concentrations of 2.0, 1.0, 0.5, 0.25, and 0.125 μg/μL.

The plates were developed in a Camag Twin Chambered development tank using acetonitrile:water (85:15) (Fisher HPLC grade). The solution was allowed to migrate up to 70 mm from the origin. The plates were dried for 1.5 min using a hair dryer and cooled for 3 - 5 min. They were developed a second time in the same separation reagent, dried for 1.5 min and cooled for 3 - 5 min. A third development was carried out using the same separation reagent, but the plates were dried for 2 min and cooled for 5 min.

The spots were visualized in 0.1N ceric sulfate (in 2N sulfuric acid) mixed with 15% sulfuric acid (at 10 parts ceric sulfate to 1 part dilute sulfuric acid). The plates were dipped in the solution contained in a Whatman dipping tank. They were dipped straight into the tank up to 90 mm from the lower end (origin of spots) of the plates and lifted out while holding firmly to avoid any damage to silica gel. Excess solution was removed by touching the end of the plates on an absorbent tissue and inclining them forward to facilitate drainage. The backs of the plates were wiped using damp tissue paper. The plates were then charred in an oven at 110^{o}C for exactly 15 min to produce good sugar spots (Figure 4). Charring period greater than 15 min produced darker or black spots with greyish brown background which interfered with the scanning process, producing errors in the results. After removal from the oven they were cooled and scanned immediately or wrapped in aluminum foil for storage in a freezer.

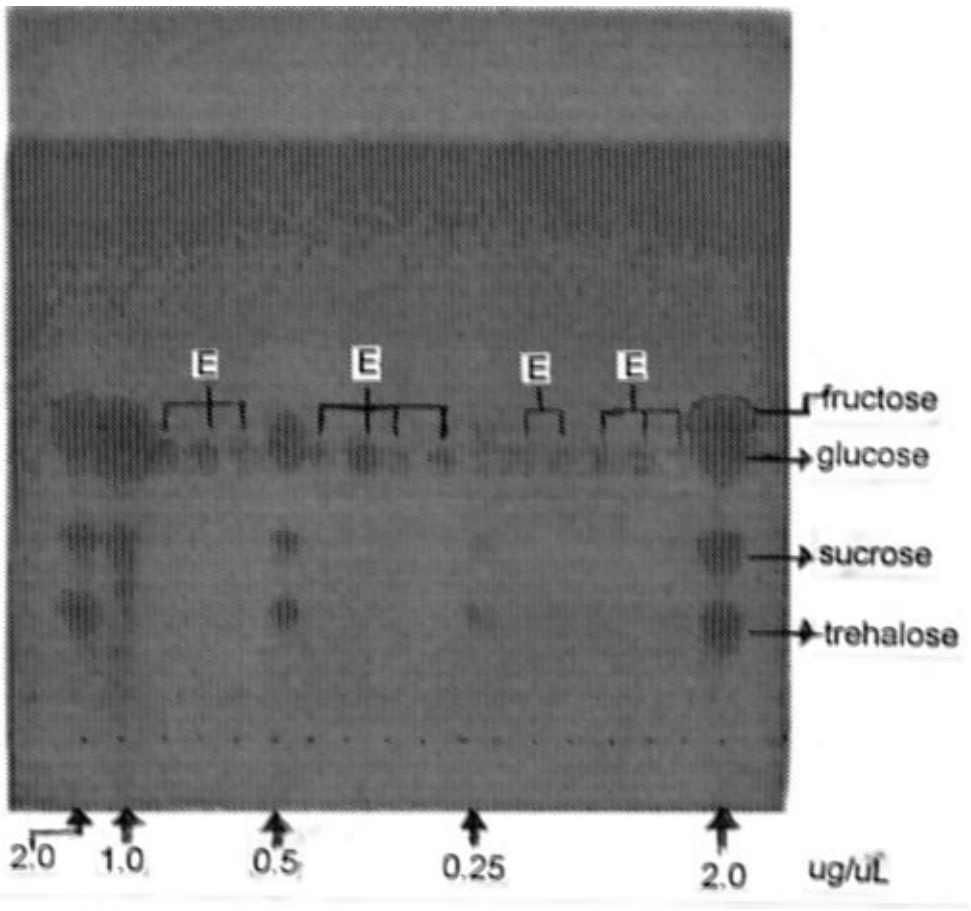

Figure 4. One dimensional HPTLC of carbohydrates from honey bee eggs (E) produced by queens, as well as fructose, glucose, trehalose and sucrose standards. Plates were developed three times in covered twin chambered Camag tanks for 10 cm x10 cm plates, with acetonitrile:water (85:15), at room temperature (RH 60%) and finally charred at 110^0C for 15 min. The size of each spot is directly proportional to the amount of sugar as shown by the standards. The highest amount of sugars in the egg samples are seen at the level of glucose spots.

The plates were scanned using Camag TLC scanner II set on absorbance mode (white light), connected to Camag Sp-4270 Spectra Physics Integrator using the parameters described by Fell (1990). The results were obtained from chromatograms by calculating linear relationships between known concentrations (standards), using corresponding retention times (RT), and the areas drawn by the integrator. From these relationships, formulas were derived for each sugar on each plate which were used to calculate the

concentrations of sugar present in the samples based on corresponding areas. The amount of sugar in each egg was then calculated using the concentration of sugar in the egg sample multiplied by the amount of sample spotted on the plate, divided by the number of eggs initially used to constitute each sample. The quantity of sugar was reported as μg/egg. Results from spikeovers made by placing weighed eggs in individual microcentrifuge tubes to which 2 μg/μL sugar standard was added and analyzed using the procedure, demonstrated 90.2 ± 3% recovery when compared to weighed eggs placed in microcentrifuge tubes and analyzed without the standard.

Statistical Analysis

Two methods were compared to determine the suitability of data for statistical analysis, due to the presence of a large number of non detectable values in the data points:

1). Categorical analysis using contingency tables (chi-square) (Zar 1996) was used to test loglinear model (H_o) against the saturated model (H_1), in order to establish the independence of *status* of the eggs (fertilized or unfertilized), the *age* of the eggs (days 1, 2, 3), and the *amount* of nutrients (μg) obtained from the eggs analyzed. The results showed that the amount of sugars detected depended on the age of eggs, and was also dependent on the status of the eggs (fertilized or unfertilized). After establishing this dependence the data were transformed for further statistical analysis.

2). The data were transformed by adding a value of 0.001 to all the data points. This made it possible to perform non-categorical statistical procedures.

The results of procedures in (1) and (2) above were compared in order to establish the validity of non-detectable values in the data. The levels of sugars and similarly the levels of lipids were compared between the colonies as well as the age of eggs. After the comparison as indicated, test of normality and analysis of variance (ANOVA) were performed on all class variables using UNIVARIATE and GLM (SAS Institute Inc. 1996; using Release 6.12 software 1989-1996). Any significant differences present at p=0.05 were located by Tukey's Studentized Range (HSD) Test.

4:1:3. Results

The HPTLC analyses showed that glucose was the most common sugar in the eggs of honey bees (Figure 4). The mean sugar level in the eggs (fertilized) from queens was 2.4

± 0.6 μg/egg (10.1%) on a dry weight basis, while the level in the drone eggs from laying workers was 1.3 ± 0.4 μg/egg (6.3%).

In fertilized eggs, trehalose levels (mean μg/egg) gradually declined from 0.4 ± 0.2 μg on day one, through 0.2 ± 0.1 μg on day two, to 0.1 ± 0.1 μg on day three. But in the drone eggs of laying workers, 0.3 ± 0.2 μg of trehalose was detected on day one, 0.1 ± 0.2 μg on day two and 0.01 ± 0.0 μg on day three. From the results showing the change in the amount of sugars on individual days, the peak levels of trehalose occurred in both queens' eggs and laying workers' eggs on day one. Similarly, peak levels of sucrose were recorded on day one from queens' and laying workers' eggs. For glucose, the peak levels were realized on day two from queens' and laying workers' eggs, while the lowest values were on day three. Peak levels of fructose were recorded on day one, with a gradual decrease on day two, and the lowest levels on day three (Figures 5 and 6). The overall individual variations indicated that eggs from queens had a higher amount of sugar compared to the eggs from laying workers. When sugar levels were compared on a one to one basis (for example, glucose in queen eggs to glucose in laying worker eggs), the results showed higher amounts of all the sugars in the eggs of queens on all the three days of embryonic development than in the eggs of laying workers.

Categorical analysis (Table 7) showed that for a fixed level of any one of the three factors, the remaining two were dependent. For example the mean glucose levels (amount) of 1.0 ± 0.2 μg/egg (queens), and 0.7 ± 0.2 μg/egg (laying workers) detected on day one, was due to the age (day 1) of the eggs, if status is held constant. The purpose of categorical analysis was to test the probability that the difference in the amounts of carbohydrates analyzed in the eggs was due to the age of the egg, or its status being either fertilized or unfertilized, and that the trend in the level of sugars observed had a natural phenomenon.

From the statistical comparisons to compute the difference in nutrient levels in the fertilized eggs of queens and those of laying workers, there was significant difference ($F=7.0$; $df=1, 11$; $P=0.01$) [N=173, with non-detectable data points represented by zero values] and no significant difference ($F=2.4$; $df=1, 11$; $P=0.1$) [N=138, when non-detectable values were omitted] when the types of eggs were compared. There were

highly significant differences (F=3.7; df=6, 11; P=0.002) and (F=3.6; df=6, 11; P=0.002) when the colonies and egg types were considered. Significant differences (F=15.4; df=2, 11; P=0.0001) and (F=5.1; df=2, 11; P=0.01) were observed when the age of the eggs were considered in comparing the nutrient levels in the eggs. No significant differences (F=0.2; df=2, 11; P=0.9) and (F=1.4; df=2, 11; P=0.3) when egg types and age were considered.

When the data were transformed by adding a constant value of 0.001 to all data points and subjecting them to statistical comparison (as above), there was a highly significant difference (P=0.0001; df=7) between the colonies used in the trials. A highly significant difference (P=0.0001; df=2) was detected on the age of eggs, while significant differences (P=0.03; df=2), and (P=0.04; df=12) were computed for status/age, and colony/age interactions, respectively. Colony/status/age interactions were not significant (Underwood 1997).

Table 7. Summary of categorical analysis of carbohydrate results, showing the level of independence in the factors (status, age, and amount).

Loglinear models[1]	df	P-value	Decision
No pair is conditionally independent (CI)[2]	2	0.1827	Fail to reject H_O
Age and *Amount* are the only pair CI	2	0.0000	Reject H_O
Status and *Age* are the only pair CI	2	0.0337	Reject H_O
Status and *Amount* are the only pair CI	1	0.0000	Reject H_O

[1]The columns contain the goodness of fit (GOF) statistics for testing each loglinear model (H_o) against the saturated model (H_1).

[2]The (No pair is conditionally independent) loglinear is the best fit is tested against the saturated model (At least one pair is conditionally independent).

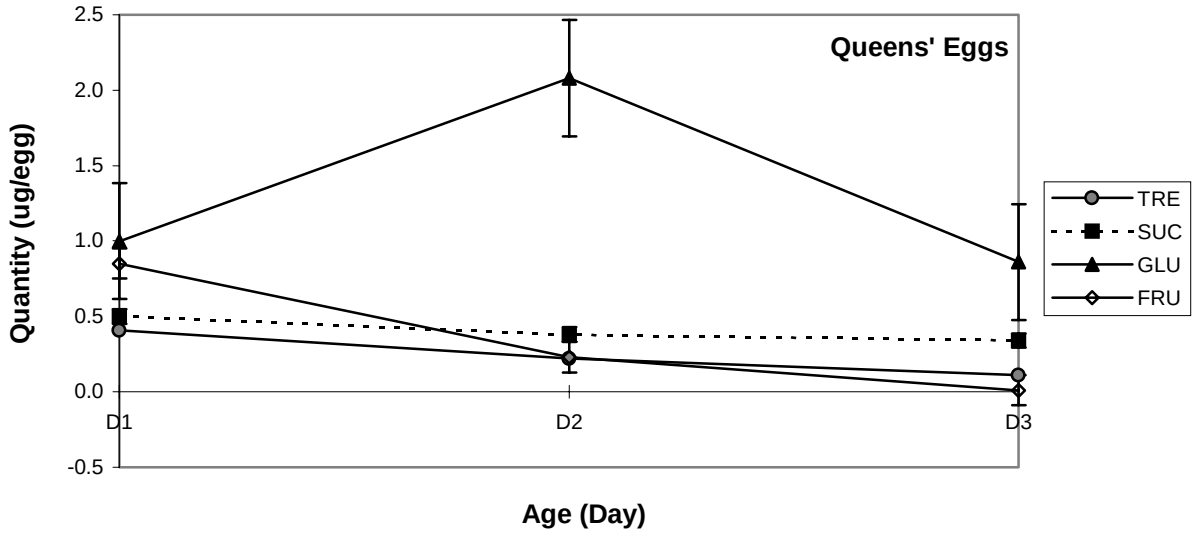

Figure 5. The trends in mean amounts (µg/egg ± SEM) of trehalose, sucrose, glucose and fructose analyzed in the eggs of queens. The eggs were sampled on day 1 (n=32), day 2 (n=32) and day 3 (n=29), respectively.

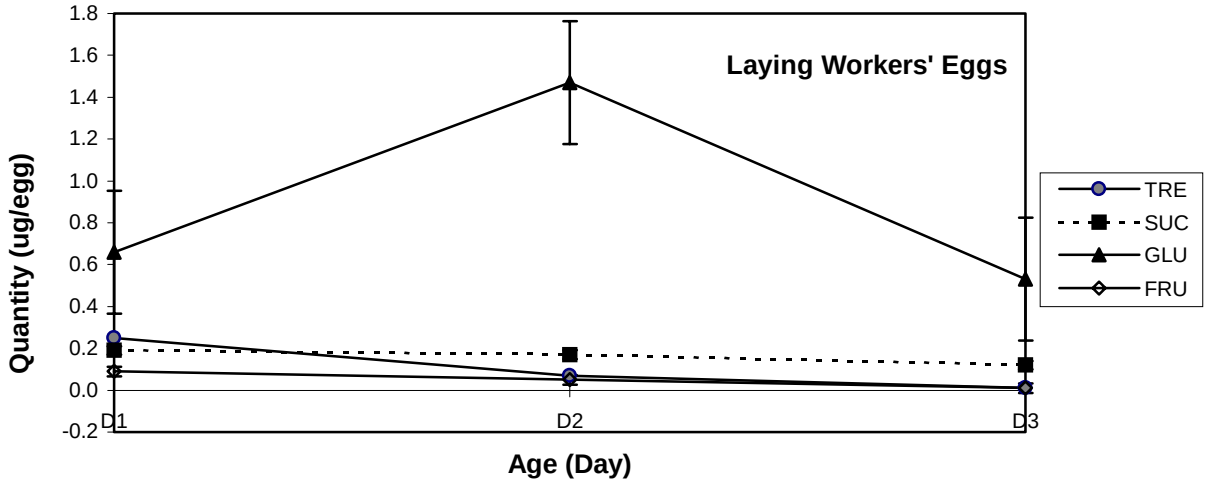

Figure 6. The trends in mean amounts (µg/egg ± SEM) of trehalose, sucrose, glucose and fructose analyzed in the eggs of laying workers. The eggs were sampled on day 1 (n=27), day 2 (n=26) and day 3 (n=24), respectively.

The results of both categorical and non-categorical statistical analyses showed similar results on the difference between the colonies, as well as the age of the eggs in relation to the amount of nutrients detected. The results indicated that there were some differences in the amount of sugars found in the fertilized eggs produced by queens and unfertilized eggs from laying workers. Table 8 gives the combined mean of sugars present in the fertilized eggs of queens and the unfertilized eggs of laying workers. Due to inadequate number of unfertilized eggs from queens, only one analysis was done to check the levels of sugars. The result obtained was not considered different from those of unfertilized eggs from the laying workers.

Table 8. Comparison of combined mean (µg/egg ± SEM) of individual sugars analyzed in the fertilized eggs of queens and unfertilized eggs from laying workers. The sugars were analyzed on day 1, 2 and 3.

Caste	n	Trehalose	Sucrose	Glucose	Fructose
L/Workers*	77	0.2 ± 0.1b	0.2 ± 0.1b	0.9 ± 0.2ab	0.1 ± 0.0c
Queens	93	0.3 ± 0.1b	0.4 ± 0.1b	1.4 ± 0.1a	0.4 ± 0.1b

Means within a column followed by the same letter are not significantly different (P=0.05; Tukey's Studentized Range (HSD) Test.
*L/Workers ≡ Laying Workers.

4:1:4. Discussion

The results of carbohydrate analysis indicate the utilization of sugars as the eggs advance in age. There is also some evidence that a conversion of trehalose and sucrose to glucose and then possibly into glycogen takes place during embryogenesis. Studies from silkworms, *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae), have shown that the ovary has a special affinity for trehalose which is then converted to glucose and may finally be converted to glycogen for incorporation into the oocytes (Shimada and Yamashita 1979). There were no differences between the fertilized eggs of queens and unfertilized eggs from laying workers in terms of the pattern of distribution of carbohydrates in the eggs. There was an overall increase in the amount of glucose on day two while the quantity of other sugars reduced. Although the pattern of mobilization of carbohydrates in queens' eggs was similar to that in the laying workers' eggs, differences

in the amounts of sugar were detectable when the age of the eggs was considered. Chippendale (1978) proposed that carbohydrates which accumulate in the eggs during vitellogenesis serve as energy reserves during embryogenesis. They are needed for the synthesis of glycosaminoglycans, and constituents of glycoproteins, as well as glycolipids. He also stated that glycogen provides glucose for chitin synthesis in early larval development. Agrell and Lundquist (1973) observed that the consumption of oxygen increases throughout development.

There was a systematic reduction of trehalose, fructose, and sucrose from day one to day three in all of the eggs analyzed. The level of glucose started low, rose, but declined gradually as opposed to the rapid decline in the other three sugars, suggesting that all energy reserves were converted to glucose for the provision of energy, and as a substrate used in the biosynthesis of substances needed for morphogenesis. Even though this study provides information on the reduction in the levels of sugars studied in honey bee eggs, the exact mechanisms of carbohydrate mobilization and utilization during embryogenesis is not properly understood (Chippendale 1978). It is therefore only possible to speculate that the sequence of mobilization of the sugars may depend on the differential affinities the sites of energy utilization and organogenesis have for a particular nutrient as embryogenesis progresses.

Stein and Fell (1990), studying the eggs of *Dolichovespula maculata* (Linnaeus) (Hymenoptera: Vespidae), showed that the mean level of sugars (dry weight) varied from 9.5% in the embryo nest eggs to 5.1% in the summer eggs, simultaneously lipid levels varied from 22.8% to 7.2% in the same eggs. The levels of these nutrients were apparently influenced by the season. Premkumar et al. (1991) found that during embryonic development, in the eggs of *Laccotrephes griseus* (Hemiptera: Belostomatidae) the levels of carbohydrates changed from 0.9% (wet weight) at oviposition to 0.40% on the eighth day (time of eclosion), and lipid changed from 26.4% to 17.2% during the same period. The results showed there was progressive depletion of nutrients as the embryos developed. Henderson (1992) studied the variation in the eggs of *A. mellifera* as well as the sizes of emerging drones and workers. She observed a high fluctuation in egg sizes (length and width), but low variability in the sizes of emerging

drones and workers. She concluded that the final sizes attained by adult bees were dependent on larval nutrition rather than the sizes of the larvae at emergence. But it is known that larval survival immediately after eclosion depends on the amount of reserve energy in the embryo. Ute and Crailsheim (1997) studied the amount of glycogen in emerging queens, workers and drones of *A. m. carnica* Pollmann. They found that the amount of glycogen in emerging drones was significantly higher than workers or queens, and concluded that it may be a strategy for surviving during the very early larval stage after the eggs hatch, especially before feeding begins.

4:1:5. Conclusion

The results of carbohydrate analysis from this study indicate that the difference between the fertilized eggs from queens and those unfertilized eggs from laying workers is observable when the amount of sugars is analyzed. Fertilized eggs showed higher levels of sugar than the unfertilized eggs. However, when the ratio of the amount of sugars and the weight of eggs were calculated for all the eggs analyzed, the difference between fertilized eggs from queens and the unfertilized eggs from laying workers was nonsignificant.

Queens produce a large number of eggs (approximately 2000 eggs per day at peak oviposition). They predominantly produce fertilized eggs throughout their life in order to maintain populations of workers in the colonies. Since they have such high investment in the eggs, part of which is sugar, it follows that queens may be utilizing high amount of energy in the production of sugar during oogenesis compared to laying workers. The queen is known to regulate the process of oviposition from a peak oviposition to complete cessation of egg production within a short period, in order to adjust to the prevailing conditions in the colony (Engels 1990).

4:2. Lipids

4:2:1. Introduction

Lipids are biosynthetic products of fatty acids and their derivatives or metabolites. They are heterogeneous group(s) of substances which are soluble in non-polar solvents (chloroform, hydrocarbons) or alcohols to some degree, but are insoluble in water (Lehninger 1975, Kates 1986). Neutral lipids constitute the major components of lipids in most insect eggs. Following the process of digestion and absorption, lipids are transported on lipophorins to the fat body (Chino et al. 1981). The demand for triacyglycerol by animals usually triggers biosynthesis into fatty acids of fat and carbohydrates which are esterified to acylglycerols (Gurr and Harwood 1991). Insects have digestive enzymes that hydrolyse triacylglycerols (triglycerides), phospholipids, glycolipids, and sulpholipids to monomeric units for absorption. The transfer of lipids from the fat body to the ovaries may be under the influence of the nervous and the endocrine systems (Girardie 1983). Storage lipids in the body are generally in the form of triacylglycerols (triglycerides) (Gunstone 1992).

Allais et al. (1964) determined the amount of lipid in *Locusta migratoria* Linnaeus (Orthoptera: Acrididae) eggs to be 26% of the dry weight. They also determined that acylglycerols account for 78.5% of total lipids at the beginning of embryogenesis, but drop rapidly due to hydrolysis of triacylglycerols (which initially form 83% of total acylglycerols). The levels of monoacylglycerols and diacylglycerols remain stable. The amount of lipid in honey bee eggs should be lower than in *L. migratoria* L. due to the difference in size and weight of the eggs, even though the level on a percent basis may be similar. The presence of lipids in honey bee eggs enhances the energy storage capacity; triacylglycerol is an important energy source and provides components required to form cellular structures in the embryos. The efficiency of energy liberation, low nitrogeneous waste emission, and high metabolic water production are the main advantages of lipid metabolism in a confined space found in the eggs (Beenakkers et al. 1981). Studies on lipids in insect eggs and their metabolism during embryogenesis (Allais et al. 1964, Stein

and Fell 1990, Premkumar et al. 1991), show that there is a steady but gradual decline in the amount of lipids as the eggs increase in age.

Sketchy information is available on the levels of nutrients in honey bee eggs and no study has been undertaken to establish the variations within and between the eggs of queens and laying workers. The objective of this study was to determine the types and amounts of lipids in the fertilized eggs of queens and the unfertilized eggs of laying workers, and observe the trends in their levels as embryonic development takes place.

4:2:2. Materials and Methods

Merck 10 cm x 10 cm HPTLC plates for lipid analysis were activated by washing them in chloroform:methanol (2:1) mixture. The mixture was allowed to migrate to the top of plates followed by oven drying at 110^0C for 1 hr. The mixture was placed in Camag Twin Trough Development Tank (for 10 cm x 10 cm HPTLC plates). Washing of plates in a development tank took about 20 min but shorter washing times of less than 8 min were achieved by using the linear developer. The plates were then stored in a desiccator until used for spotting samples. The plates were, however, reactivated for at least 2 hr before spotting, in order to produce good separations of lipids.

Extraction and quantification of lipids

The procedures for analysis, detection and quantification are those described by Judge et al. (1989) and Stein and Fell (1990). Some modification was made on the number of standards used.

Single eggs were placed in individual 1.5 ml microcentrifuge tubes and ground with microcentrifuge pestles for 1 minute before adding 400 μL of chloroform:methanol (2:1). 400 μL of double distilled water was added to each tube and the mixture vortexed for 1 minute. The tubes were centrifuged at 12 000 rpm for 5 min. The organic (lower) layers were removed from aqueous (top) layers and placed in clean microcentrifuge tubes. Additional 400 μL of chloroform:methanol (2:1) were added to aqueous layers and vortexed for 1 min. After centrifugation in similar conditions as above and obtaining aqueous layers, these were washed twice more to remove lipids. The three organic layers pulled off were combined in 1.5 microcentrifuge tubes but the aqueous layers were discarded. The organic layers were then evaporated to dryness in a Fisher tissuemat water

bath, under a stream of dry nitrogen. Lipids were removed from the dry tubes using three washings of 100 μL (chloroform) per washing which were transferred to 400 μL microcentrifuge tubes for evaporation to dryness under a stream of dry nitrogen. The lipids were resuspended in 20 μL of chloroform for spotting. The spots were made on precoated, pretreated, and reactivated silica gel - 60 (Merck 10 cm x 10 cm HPTLC) plates using 1.0 μL Drummond micropipettes in duplicate for each sample and each standard. The standards consisted of a mixture of cholesterol, monostearin, mixed isomer distearin (diacylglycerols 1,3 and 1,2) and tristearin dissolved in chloroform, at concentrations of 0.125, 0.25, 0.5, 1.0, 2.0 μg/μL. Fatty acids were not determined.

Spots were developed in a Camag linear Development tank for Merck (10 cm x 10 cm HPTLC) plates. Initial development was done using a separation reagent made of benzene:diethyl ether:ethanol:acetic acid (50:40:2.0:0.5) which was allowed to migrate up to 40 mm from the origin. The plates were dried using a hair dryer and cooled for 3 min before being developed using a second separation reagent of hexane:diethyl ether (70:30) up to 60 mm from the origin. The plates were dried using a hair drier and cooled for 5 min before visualization.

The plates were visualized in 3mM of 6-p-toluidino-naphthalene-sulfonic acid (TNS) dissolved in 95% ethanol. The plates were dipped in TNS placed in a Whatman dipping tank. They were dipped straight into the tank as opposed to spraying and lifted without damage to silica gel. They were finally dried to uniform temperatures (ambient room temperature) while protecting the plates from direct light that might damage TNS and affect the scanning results. Scanning was done immediately or plates were wrapped in aluminum foil, and stored in a freezer for subsequent scanning. The plates were scanned using Camag TLC scanner II set on fluorescence mode connected to Camag Sp-4270 Integrator. The results were obtained from the chromatograms in a similar way as in the carbohydrates analysis. The results from samples spiked over by adding 2 μg/μL lipid standard to weighed eggs individually placed in separate microcentrifuge tubes then analyzed as above, compared to weighed eggs in individual microcentrifuge tubes but analyzed without adding the lipid standard, showed a final recovery of 92.7 ± 3%.

Statistical Analysis

The presence of a few non detectable values made it necessary to transform the data by adding 0.01 to all the data points in order to allow for statistical analysis using non categorical procedures. The test of normality and analysis of variance (ANOVA) were then performed on all class variables using UNIVARIATE and GLM (SAS Institute Inc. 1996; using Release 6.12 software 1989-1996). Any significant differences present at p=0.05 were located by Tukey Studentized Range (HSD) Test.

4:2:3. Results

The results from this investigation showed that there was a mean value of 2.0 ± 0.4 μg/egg of monoacylglycerols (monoglycerides) in the eggs from queens on day one, which reduced to 0.2 ± 0.6 μg/egg on day two, but none was detected on day three. At the same time, the eggs from queens exhibited a steady decline in cholesterols, diglycerides, and triglycerides, as the eggs increased in age. From the eggs of laying workers, there was a steady decline in the levels of monoacylglycerols, cholesterol, diacylglycerols and triacylglycerols from day one to day three, but the rate of depletion of lipids in the eggs of laying workers was not as rapid as those recorded in the eggs of the queens. The total lipid content per egg on day one was computed at 10.7 μg/egg, which was 45.1% of overall mean dry weight of 23.7 μg/egg for queen produced eggs and 8.4 μg/egg for worker produced eggs which was 40.8% of overall mean dry weight of 20.6 μg/egg. Wet weights of the eggs change as embryonic development takes place, hence the dry weights change correspondingly. An approximation of the proportion of lipids based on the dry weight on day one, shows that on day two, the eggs from queens had 2.4 μg/egg, about 10.1% of mean dry weight and laying workers' eggs contained 6.8 μg/egg approximately 33.1% of mean dry weight. Finally, on day three the levels of lipids in queen produced eggs had reduced to 1.1 μg/egg, 4.6% of dry weight, but the eggs from laying workers still had 2.8 μg/egg (13.6% of mean dry weight). Gravimetric results of lipid level from twenty fertilized eggs of queens, sampled on day one and analyzed using the same procedure, as outlined above, but weighed directly after drying lipids in microcentrifuge tubes, without spotting on HPTLC plates showed 27.4% lipids. Table 9 shows the trends in the amount of lipids in the fertilized eggs from queens and the unfertilized eggs of laying workers over the three day period for embryonic development.

There was no significant difference (F=1.3; df=3, 18; P=0.2) between the colonies. But a highly significant difference (F=7.4; df=2, 18; P=0.0001) was obtained when the lipid levels were compared based on the age of the eggs, the levels of different nutrients also showed a significant difference (F=13.26; df=6, 18; P=0.0001). The results therefore demonstrated that there were no differences among queen or worker colonies. However, the levels of nutrients varied depending on the types of egg and the age at the time of analysis. The levels of individual nutrients also varied among the types of eggs.

Table 9. Comparison of the lipid levels (μg/egg ± SEM) in the eggs of queens, and those of laying workers sampled on day 1, 2, and 3.

Day	n	Mono-glycerides	Cholesterol	Diglycerides (1,3)	Diglycerides (1,2)	Triglycerides	Total
One	24	2.0 ± 0.4a	2.4 ± 0.2a	3.5 ± 0.4a	1.8 ± 0.4a	1.2 ± 0.2b	10.7
Two	24	0.2 ± 0.1d	0.5 ± 0.2c	1.2 ± 0.3b	0.4 ± 0.1c	0.2 ± 0.1d	2.4
Three	26	0.0 ± 0.0de	0.4 ±0.1c	0.3 ± 0.1c	0.4 ± 0.1c	0.0 ± 0.0de	1.1
One	24	0.7 ± 0.2bc	0.7 ± 0.2bc	2.7 ± 0.4a	1.0 ± 0.3b	3.2 ± 0.2a	8.4
Two	24	0.2 ± 0.1d	0.5 ± 0.1c	2.1 ± 0.2a	1.0 ± 0.1b	3.0 ± 0.4a	6.8
Three	24	0.2 ± 0.1d	0.1 ± 0.0d	1.2 ± 0.2b	0.5 ± 0.2c	0.8 ± 0.1b	2.8

Means within a column followed by the same letter are not significantly different (P=0.05; Tukey's Studentized Range (HSD) Test).

4:2:4. Discussion

The types of lipids detected in the eggs of laying workers were not different from those in queen-laid eggs. The results from lipid analysis showed that there were differences between the fertilized eggs of queens and the unfertilized eggs of laying

workers in terms of the amounts of lipids contained in the eggs at the end of oogenesis. However, significant differences occurred due to the age of eggs and the type of nutrient being considered. When the mean weight of eggs were taken into account, the actual amount of lipid contained in the fertilized eggs was still higher than those recorded in the unfertilized eggs of laying workers.

Substantial preparatory processes occur in the oocyte before fertilization when the organism's genes and nutrients are incorporated at oogenesis. The trend of utilization of lipids exhibited during embryogenesis in the fertilized eggs, shows the presence of a large amount of monoacylglycerol, diacylglycerol (1,3) and (1,2) and triacylglycerols on day one, followed by rapid reduction of all lipids on day two, leading to depletion of monoacylglycerol and a substantial reduction of triacylglycerol (Table 9). In the unfertilized eggs, the pattern is fairly similar but minor differences were evident. Although the patterns of lipid reduction from high to low levels were still maintained, depletion of lipids in unfertilized eggs took place at a lower rate.

The strategy for embryogenesis and post-emergence survival, may be the main factors influencing the utilization of lipids in insect eggs including honey bee eggs. The high amount of triacylglycerols on day three may also be due to accumulation of energy reserves in the body for survival during the critical stage after eclosion. Ute and Crailsheim (1997) determined elevated amounts of glycogen, which is a carbohydrate, in the drones at emergence compared to the queen or worker larvae. They concluded that the basis for this large amount of energy reserve may be a survival factor before provisioning takes place.

4:2:5. Conclusion

The results from lipid analysis showed that substantial amount of lipid is present in both the fertilized eggs of queens and the unfertilized eggs of laying workers but the quantity is gradually depleted during embryogenesis. The levels of lipids in the laying workers' eggs are relatively higher than the those in the queens' fertilized eggs. The difference between fertilized and unfertilized eggs in terms of lipid metabolism during embryogenesis may depend on the rate at which lipid types are bio-degraded to produce energy and products for incorporation into the morphogenetic moieties. However, the

amount in the workers' eggs remain relatively high at eclosion relative to those in the queens' eggs. The possible retention of nutrient in the body tissues of developing embryos to enhance survival post-emergence, may be the center piece of this pattern of nutrient utilization (Ute and Crailsheim 1997).