CHAPTER - 3 RESPIRATION RATES IN HONEY BEE EGGS

3:1. Introduction

Respiration is a cellular process that enables living systems to carry out metabolic activities (catabolism and anabolism) necessary for maintenance and growth. This process is characterized by exchange of oxygen, carbon dioxide and energy between a living organism and its environment. Hoffmann (1985) observed that temperature affects cellular processes by increasing or decreasing reaction rates in conformity with the laws of thermodynamics. The rates of chemical reactions and physiological processes are dependent on temperature, a relationship described by temperature coefficient, Q_{10}. It is a ratio used to measure changes in the rate of chemical reaction due to a 10^0C change in temperature. For a normal biological range in insects, Q_{10}s of 2-3 are common (Keister and Buck 1974). Alexandrov (1977) noted changes in the structures of proteins, phase transition of lipids, and changes in the structure of water molecules when cells experience adverse temperature fluctuations. From these observations, it is possible to infer that temperature exerts profound effects on living organisms in a way that may interfere with cellular processes.

Honey bees struggle to maintain the temperature in the brood area around the optimum range of 34.8 ± 0.2^0C (Seeley and Morse 1976). The optimum temperature range enhances embryonic development and survival as well as maintain a suitable environment for metabolic and social activities in adults. Workers detect temperature differences as low as 0.25^0C and make adjustments to maintain thermal homeostasis (Heran 1952). This prolonged exposure to narrow temperature range makes *A. mellifera* eggs poorly adapted to temperature changes outside the optimal range. Due to poor compensatory adjustments to temperature changes, any deviations in environmental temperatures affecting the brood area can lead to prolonged developmental time, malformations or impaired development, or premature embryonic deaths (Schnetter 1934). Any impaired development such as reduced spermathecal volume of queens, will greatly lower their reproductive success (Corbella and Goncalves 1982). In drones, any diminished flight capability will jeopardize aggressiveness during competitions to mate with virgin queens (Henderson 1993).

Temperature variations can and do occur in hives (Seeley 1985). These can occur because of seasonal changes, location of a hive, or population size of bees in a hive. Levin and Collison (1990) reported variations in temperatures found in the brood area during the summer, ranging from 25^0C in broodless cells to 34.1 ± 0.8 ^{0}C at the center of the broodnest, in cells with brood. They further observed thermal variations between larval and pupal cells. These temperature variations have a bearing on the number of reproductives or workers being raised. Seeley and Morse (1976) also found that drone cells and drone brood are normally found at the periphery of the broodnest, hence their production may be affected. Most queen cells also occur at the periphery (Fell and Morse 1984). Drescher (1968) determined the effect of a temperature gradient on development of brood in relation to its position in the broodnest and observed that temperature will affect the rate of development.

The maintenance of colony strength depends on efficient reproduction of offspring, a factor commonly used to judge the reproductive efficiency of queens. Harbo and Bolten (1981) recorded low hatchability in honey bee eggs incubated at low temperatures. Embryonic development was highly sensitive to small temperature variations and only 1% of the eggs kept at 29.8^0C realized full development but 67% developed at 31.3^0C. Milne et al. (1988) confirmed that even small decreases in temperature greatly increased developmental times. Higher temperatures not only affect developing embryos, but may have an effect on the volume and the number of eggs that can be produced as reported by Avelar (1993) in *Drosophila*. Richards (1957) observed an increase in developmental rate in the eggs of *Oncopeltus*, as temperature increased, while the development duration decreased. Temperature has the greatest effect on the survival of honey bee colonies, since it affects metabolic processes of all members of the colony, especially brood.

The objectives of this study were to measure respiratory rates in eggs incubated at 28^0C, 31^0C, 34^0C and 36^0C in order to establish the mean respiratory rate in honey bee eggs, as well as compare respiration rates in fertilized and unfertilized eggs from queens and laying workers. It was also to establish the effects of temperature on embryonic development by determining the number of embryos reaching pre-emergence stage when CO_2 analyzed eggs are re-incubated.

3:2. Materials and Methods

Eight colonies were set up in nucleus hives at the Price's Fork Bee Research Unit of Virginia Polytechnic Institute and State University. The research was done during the summers of 1997 and 1998. The first group of four hives were each headed by a mated queen, selected from a group of closely related sister queens and the second group consisted of four hives of laying workers. The workers were selected from the same stock of bees as queens. The mated queens were instrumentally inseminated twice using a random pool of semen extracted from drones taken from traps at the hive entrances of existing colonies. This was to ensure the queens were mated before the start of oviposition. The queen-right hives were checked at regular intervals to ensure there was sufficient brood to maintain strong worker populations during the project. They were given frames of honey and pollen to maintain oviposition in the queens. The workers-only hives, however, were placed without any form of brood but with adequate supply of honey and pollen to stimulate ovary development and oviposition in laying workers (Jay 1968; Jay 1970, Jay and Nelson 1973). Sampling started as soon as egg laying commenced among queens and laying workers.

Collection of egg samples.

Eggs were collected by removing them from the cells of comb (Figure A: Appendix A) on which the queen and laying workers had been confined using queen excluders (queens) and screens (laying workers) for at least 6 hours but not more than 10 hours. The caging was done in order to ensure an adequate supply of eggs of approximately the same age. The queens and laying workers were released from isolated frames before sampling began on the first day and were not re-admitted onto the isolated frames until the end of a sampling cycle. The frames were then reset in the hives for subsequent sampling at intervals outlined under each experiment. Maximum care was exercised to avoid destroying the eggs during transfer into the temperature treatment vials and the microcentrifuge tubes used for laboratory analysis of nutrients. A specially designed grafting tool was used to scoop and transfer individual viable eggs into the 2000 µL vials which were then sealed and placed in the incubators set at appropriate temperatures. Previously, the incubators had been set at correct temperatures 24 hours before the

beginning of temperature treatment to ensure no temperature fluctuations occurred during the incubation of eggs. Occasionally sampling intervals were lengthened or shortened to test the validity of experimental procedures.

Determination of wet and dry egg weights.

An analytical balance (Mettler AC 100) was used to weigh individual glass slide cover slips. A group of twenty eggs were then weighed on each tared glass slide cover slip and the eggs' weights recorded. The slips with the eggs were then transferred to a 60^0C oven for 48 hours. The slips and the contents were weighed after oven drying until at least two constant weights were recorded at consecutive weighings. The dry weight per egg was calculated by subtracting the weight of the glass slide cover slip from the final weight and dividing the result by the number of eggs weighed.

Determination of viability of eggs.

Three separate methods were used to measure the viability and hatchability of eggs in order to determine the probability of sampling live eggs for respiration experiments.

In each colony, a section of comb containing twenty cells, each having a newly laid egg was randomly selected and marked. After 3 days the cells were checked to determine the number of eggs that hatched. A total of 16 sections were examined in the hives headed by queens, while a total of 8 sections were similarly marked in laying workers' hives. The number of eggs which hatched from each section was expressed as a percent of the total number of eggs counted from the section.

A total of twelve eggs from each temperature treatment were returned to the hive after day one, day two and day three following respiratory gas determination of CO_2 in order to measure acceptance of eggs by the workers in the hives. Egg acceptance was tested by attaching the comb containing the temperature treated eggs to the cells close to undisturbed eggs on the combs. The eggs were assumed to have been accepted, and thus alive, if they were still in the cells after 24 hours. The number accepted was expressed as a percent of the total number of eggs returned to the hive in the same batch from the same temperature treatment.

Eight eggs from each temperature treatment were artificially re-incubated at 34^0C (65-80% RH) at the start of day two and day three until the eggs were assumed to have reached the hatching stage. They were then examined under a microscope to determine if they had reached a final developmental stage. Fully developed embryos were observed through the transparent chorion just before emergence (pre-emergence stage). The number reaching the pre-emergence stage was expressed as a percent of the total number of eggs from the same temperature treatment set for re-incubation.

Respiration Studies.

The procedure used in this experiment is similar to that described by Gray et al. (1991, 1995), with modifications. In the initial stages of the project, 1000 µL vials were tested as described in Gray's experiments with eggs being incubated over longer periods up to 72 hours. The results showed that rapid accumulation of CO_2 in the small vials quickly shut down respiration in honey bee eggs causing severe reduction in respiration beyond 48 hours of incubation. From these trials, larger vials of 2000 µL having wider openings to allow quick manipulation of eggs were substituted and the eggs incubated for no more than 14 hours. In addition, the short interval also made it possible to temperature treat eggs and still estimate the age of eggs at 24 hours for day one, 48 hours on day two and 72 hours for day three. The eggs were sampled at the same time each day during the sampling cycle(s) in order to maintain consistency in timing. Both fertilized and unfertilized eggs were sampled.

A group of at least five eggs were randomly sampled per queen and each laying worker colony per temperature treatment. These samples were taken at days one, two and three, respectively. The eggs were transferred by cutting comb at the base of each cell, followed by careful lifting of the eggs and comb using the grafting tool. The procedure was used in order to avoid dislodging eggs from the base of cells. Caron (1995) observed that honey bee eggs not properly deposited in the cells show lowered hatchability. Individual eggs were placed in 2000 µL glass auto-sampler vials (Fisher) which were sealed using rubber ("Teflon") septa (Fisher 03-377-5A) and screwed down with hollow plastic caps. During sealing, extraneous CO_2 was excluded as shown in Figure B: Appendix A. Five empty vials were sealed as controls for each temperature

treatment to determine ambient CO_2 levels. A second set of five vials containing comb material from the same frames but without eggs and a third set of five vials containing eggs previously frozen overnight, were also set for each temperature treatment in order to determine the difference between viable and non-viable eggs as well as observe any microbial respiration that may occur during incubation. The vials were placed in incubators (respirometer chambers) with temperatures set at 28^0C, 31^0C, 34^0C, and 36^0C ($\pm$ 0.5^0C). The duration of incubation varied between 12-14 hours before the air in each vial was analyzed to measure the CO_2 output per egg.

The actual duration the eggs were allowed to respire on combs after oviposition and before being sealed in the vials were 8 hours on day one; 32 hours by end of day two; and 56 hours by end of day three. The total incubation period (natural and artificial) before CO_2 analysis from the vials were 20-22 hours, 44-46 hours, and 68-70 hours, for day one, day two and day three, respectively. The results of respiration rates, however, show only the CO_2 output during artificial incubation.

Analysis of carbon dioxide and quantification of respiration rates.

At the end of each treatment period, the vials were removed from the chambers (incubators) and air drawn from each vial using a 250 µL gas-tight syringe (Hamilton). The air was injected into the septum of an infrared gas analyzer (IRGA) (LI-6251: Li-Cor Inc., Lincoln, NE., CO_2 analyzer). The standard sample of 250 µL CO_2 at 1868 ppm was injected first, followed by injections from vials for ambient control, then vials containing comb material, followed by vials for frozen eggs and finally the vials with room temperature eggs from the same temperature treatment (Figure C: Appendix A). The gas was removed from the analyzer by an aquarium pump pulling atmospheric air though a calcium carbonate cannister, bringing CO_2 free air into the analyzer chamber in order to replace the injected gas in readiness for subsequent injection(s). Using preset parameters, the CO_2 analyzer converted the concentration of CO_2 in each sample of gas to parts per million (ppm) by comparing the values of CO_2 levels in the samples to the value of the standard. The data logger connected to the analyzer recorded the amount of CO_2 in ppm for each 250 µL of gas analyzed. The data were transferred to a computer for recording and analysis. Respiration rate in µL CO_2/hr/egg was calculated for each egg sampled by

subtracting the mean value of five readings of ambient controls from each reading recorded for each egg sample from the same temperature treatment. The result obtained was multiplied by 8 to calculate the amount of CO_2 in each 2000 µL vial, hence obtain the total amount of CO_2 produced by each egg during the incubation period. This was then divided by 2000 to calculate the amount of CO_2 in µL/µL of gas in each vial. The result was finally divided by respective time periods for incubation to obtain the amount of CO_2 as output in µL CO_2/hr. Since wet weights were taken on day one only, all respiration rates were reported in µL CO_2/hr/egg instead of µL CO_2/hr/ µg egg.

Statistical Analysis

In order to realize the research objectives, a split-plot design was used to compare respiration rates in fertilized eggs from queens and unfertilized eggs from laying workers. For respiration experiments, the colonies were treated as blocks, while the age of eggs, represented by the three days of embryonic development were applied to batches of eggs as whole plots. Temperature treatments were applied to sub-batches of eggs as sub-plots. Different levels of statistical comparisons were then computed. The mean values of CO_2 output from fertilized eggs were compared to observe any variations among the fertilized eggs from queens. The second comparison involved the CO_2 output from fertilized eggs and unfertilized eggs from the queens at 34^0C. Finally, due to unavailability of a sufficient number of unfertilized eggs from queens, the unfertilized eggs of queens and drone eggs of laying workers were only compared at 34^0C. The temperature at 34^0C was chosen as an ideal point for comparing the effects of other temperature treatments since it is an optimum temperature range for honey bee egg development. The data obtained were compared using split plot ANOVA and regression analysis (REG procedures). Finally a test for normality on all class variables was done using the UNIVARIATE and GLM procedures (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.

3:3. Results

Establishing viability and respiration of the eggs sampled

Determination of respiration by measuring the CO_2 output from honey bee eggs confined inside the vials, then incubated artificially, depended on sampling viable eggs that would stay alive over a number of hours in order to produce detectable amounts of CO_2. It was therefore, imperative that a number of measurements were made to establish the viability of eggs during incubation.

The results of a series of tests to find a suitable duration of incubation before injection of gas into the CO_2 analyzer showed that longer incubation times and CO_2 build up in the vials exhibited a linear relationship up to 48 hours as in Figure 1. Beyond 48 hours, there was a non-linear response shown by a decline in CO_2 output. Results from eggs incubated continuously for 72 hours showed a leveling off in CO_2 output producing a similar result as CO_2 output at 48 hours. Although each sample batch was different from others, the results were attributed to a decline in metabolism or egg death causing reduction in CO_2 output. From these observations, shorter incubation periods of 11 hr 15 min to 13 hr 50 min were used in the study, giving an average incubation time of 12 hr 32.5 min (13 hr approximately). The choice of shorter incubation interval therefore made it possible to measure respiration during high CO_2 output as observed from the output response, it was also possible to temperature treat eggs on day one to within 24 hours. The final results from respiration experiments, however, were calculated and reported on a per hour per egg basis, as mentioned above.

The comparison of the mean values of CO_2 concentrations in 250 µL of gas analyzed from the vials containing eggs sampled from four queens (A, B, C, and D) with CO_2 concentrations in 250 µL of gas from ambient control vials, comb material vials, and frozen egg vials showed a significant difference ($P=0.0001$; $n=20$) across all temperature treatments: 28^0C, 31^0C, 34^0C and 36^0C. This difference therefore indicated that the sampled eggs were respiring as opposed to the inert materials in the non-egg vials. The comparison of the proportion of CO_2 concentrations in each set of vials (using ambient controls as the reference) showed there was an increase of 16.4% in the level of CO_2 in the vials with comb material above the CO_2 level detected in the ambient control vials, an

increase of 27.3 % in the vials with frozen eggs, and a mean increase of 83.3% in the egg sample vials.

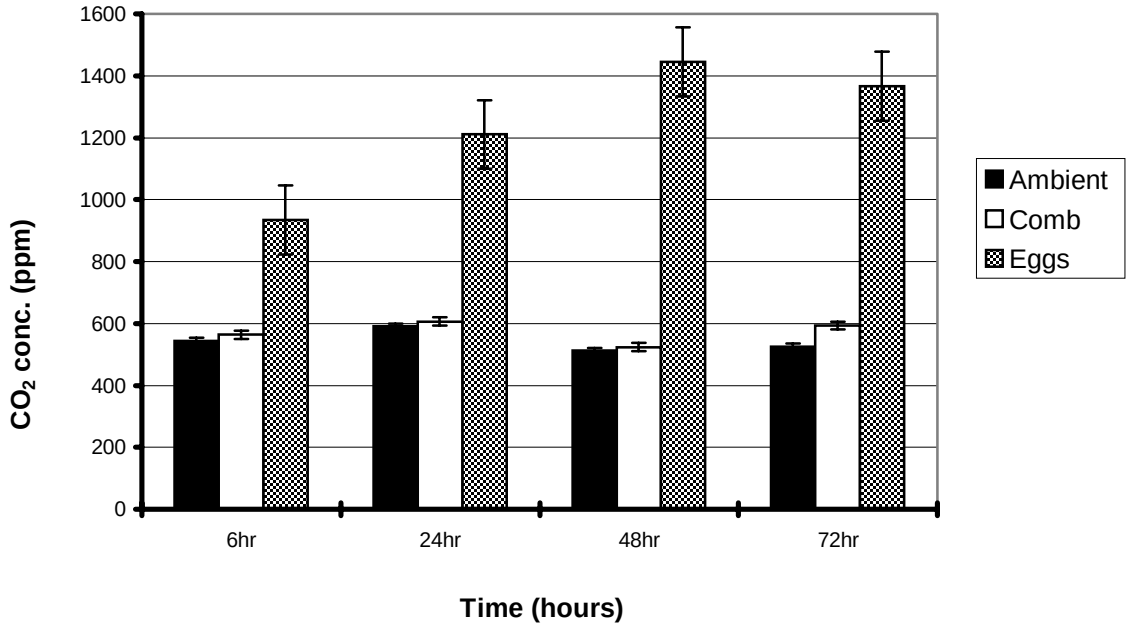

Figure 1. Comparison of the mean concentration of CO_2 (ppm ± SEM) in 250 µL of gas from queen B sample (eggs) vials along with their respective ambient control vials, and vials containing comb material. The samples (eggs) and non-egg materials were incubated at 34^0C for 6, 24, 48 hours (n=5 for each time period) and 72 hours (n=7), respectively.

The statistical comparisons of the mean values in Table 1 show a dichotomous separation between the eggs and the non-egg samples. There was no significant difference between the mean values of CO_2 concentration from eggs of queens. From the non-egg samples, the mean values of CO_2 concentration from frozen eggs and comb material also showed no significant difference. But there was a highly significant difference between the eggs and the non-egg samples (Table 1). The results also showed that some microbial activity may have taken place in frozen eggs when they were re-introduced to the temperatures used during incubation, as exhibited by the level of CO_2 produced in those vials.

a)- *Viability under natural condition*

The mean values of the number of viable eggs hatching under natural conditions in the

twenty cells per site, from sixteen randomly selected sites on the combs in the hives of queens and eight similar sites in the laying workers' hives, were 83.4 ± 0.0 % (standard error of the mean), n=16 and 60.4 ± 0.1%, n=8, respectively.

Table 1. The mean[1] concentration of CO_2 (ppm ± SEM) recorded in the gas from vials containing live eggs, frozen eggs, ambient control vials and vials with comb material[2]. All samples were incubated at 28^0C, 31^0C, 34^0C and 36^0C for approximately 13 hours. The eggs were produced by queens (A, B, C, D), and sampled on day one.

Variable	n	Mean (CO_2 ppm ± SEM)	Range
Ambient Control	20	484.7 ± 18.0b	453.0 - 533.6
Comb Material	20	564.4 ± 17.2b	530.7 - 606.4
Frozen Eggs	20	617.2 ± 30.5b	553.2 - 681.2
Queen A Eggs	20	843.6 ± 45.7a	763.0 - 949.5
Queen B Eggs	20	865.7 ± 60.8a	739.1 - 1000.4
Queen C Eggs	20	980.0 ± 61.5a	890.9 - 1155.7
Queen D Eggs	20	863.8 ± 58.2a	747.9 - 980.5

[1]Means within a column followed by the same letter are not significantly different {$p<0.05$; Tukey's Studentized Range (HSD) Test.}.
[2]The pieces of comb placed in the vials were cut using the same grafting tool used to transfer eggs, hence all the comb materials were equal size.

Although differences were detected in the viability of fertilized and unfertilized eggs from the sites selected, the combined mean value of all eggs hatching under natural conditions was calculated at 75.8 ± 0.0 %, n=24. This value was considered to represent the possibility of sampling a viable egg from all the colonies used in the study. The low value in the worker eggs could be due to the number of eggs laid in each cell. Queens place single eggs in cells, but laying workers place more than two eggs per cell which may affect hatchability.

b)- *Egg acceptance after gas analysis*

For the eggs which were returned to the hive, acceptance was determined to have occurred if an egg was present in the cell where it was attached after 24 hours because the workers had not removed it. Honey bee workers are known to inspect and remove foreign materials or objects from the hives quickly, especially dead bees and debris in the cells (Gary 1992).

The results from acceptance trials at 28^0C and 31^0C were 38.8% and 47.2% equal to 14/36 and 17/36, respectively, but was relatively low for eggs incubated at 36^0C 27.7% (10/36). At 34^0C the acceptance rate was 72.2%, equivalent to 26/36 eggs accepted. The results showed that acceptance dropped as the age of the eggs increased (Table 2). The results also showed that temperature treatment had effects on the acceptance of eggs in the hives resulting in differential acceptance levels. The results from temperatures at 28^0C and 36^0C indicated that eggs at 28^0C developed slowly while the eggs at 36^0C developed rapidly. The eggs at 31^0C developed at slower rate than the eggs at 34^0C but still faster than those at 28^0C. The eggs at 34^0C developed at the optimum temperature and were therefore most acceptable to bees when returned to the hive. It is also possible that honey bee eggs were able to handle lower temperatures and still remain viable compared to the high temperature at 36^0C. This temperature (36^0C) caused rapid embryonic mortality.

Table 2. Comparison of mean acceptance (% ± SEM) of eggs set into the hive after temperature treatment at 28^0C, 31^0C, 34^0C, and 36^0C; on day 1, day 2, and day 3.

Temperature	n	Day One	Day Two	Day Three	Total
28^0C	12	58.3 ± 0.8a[1 & 2]	33.3 ± 0.8b[2]	25.0 ± 0.0b[2]	14/36
31^0C	12	66.7 ± 0.8a[1]	41.7 ± 0.8a	33.3 ± 0.8b	17/36
34^0C	12	83.3 ± 0.8a[1]	75.0 ± 0.2a	58.3 ± 0.8a	26/36
36^0C	12	33.3 ± 0.8b[1]	25.0 ± 0.0b	16.7 ± 0.0b	10/36

[1]Mean within a column followed by the same letter are not significantly different (P<0.05: Tukey's Studentized Range (HSD) Test).
[2]Mean within a row followed by the same letter are not significantly different (P<0.05: Tukey's Studentized Range (HSD) Test).

c)- *Pre-emergence embryos*

The results from the experiments in which the eggs were initially temperature treated at 28^0C, 31^0C, 34^0C, and 36^0C for approximately 13 hours before sampling the gases for CO_2 analysis, then re-incubated artificially at 34^0C and RH 65-80% are shown in Table 3. Since the results were based on a small sample size, they were considered insignificant in demonstrating the difference between queens' and laying workers' eggs. It was also difficult to determine the exact stage of development in the embryos and therefore large time differences were possible among the different eggs sampled. However, the results still support the idea of differential development rate of eggs when subjected to different temperatures. Eggs at low temperatures of 28^0C and 31^0C took longer to develop while those at the optimum (34^0C) developed normally. The eggs at a higher temperature of 36^0C developed rapidly, but also tended to show high mortality. From these results, it is observable that there is a significant difference ($p=0.05$) between low temperature treatments of 28^0C and 31^0C compared to higher ones of 34^0C and 36^0C.

Table 3. The proportion (%) of eggs which showed the late stages of larval development as observed under the microscope after the eggs were incubated at 28^0C, 31^0C, 34^0C and 36^0C for approximately 13 hours for CO_2 analysis and re-incubated at 34^0C (RH 65-80%) up to the time the eggs were expected to hatch. The eggs were taken from day 2 and day 3 samples.

Temperature	n	%
28^0C	8	12.5
31^0C	8	50.0
34^0C	8	75.0
36^0C	8	37.5

Egg weights

The combined mean wet weight of fertilized eggs from queens and unfertilized eggs from laying workers was 124.2 ± 4.4 µg/egg (N=44) using pooled weights of 20 eggs per sample, while the overall mean dry weight for the same eggs was 22.6 ± 0.6 µg/egg (N=44). The comparison between the weights of fertilized eggs from queens and unfertilized eggs from laying workers indicated low weights for worker laid eggs. It was not possible to determine individual weights due to low sensitivity of the weighing balance, hence weights of twenty eggs per sample were measured. Since seasonal variations in weights of honey bee eggs have not been established, the sample size or genetic similarity in laying workers, arising from selection of closely related queens to raise the workers, may have affected the results of the weights obtained.

The weights of unfertilized eggs from laying workers were consistently lower than the weights of fertilized eggs from queens. Winston (1987) stated that eggs from laying workers are smaller than queen laid eggs. The possibility of sampling from a line of genetically related laying workers may have affected the variability in the weights of eggs sampled. Since it was difficult to identify individual workers producing the eggs weighed during the trials, comparing individual worker colonies was not reasonable or valid. However, for queens, individual mean weights indicated that the eggs from colony C (QC) had lower egg weights (129.0 ± 11.2 µg/egg), even though she also produced more eggs and more offspring per unit time compared to other queens. Two queens in colonies A and D (QA and QD) had similar mean weights of 136.4 ± 14.9 and 136.7 ± 12.8 µg/egg, respectively, while colony B (QB) recorded 131.6 ± 12.8 µg/egg. The summary of overall results of wet weights and dry weights are shown in Table 4.

Respiration rate

The mean respiration rate for all fertilized eggs from queens was 0.1 ± 0.0 µL CO_2/hr/egg (n=240), while the mean respiration rate for all unfertilized eggs from queens was 0.1 ± 0.0 µL CO_2/hr/egg (n=50). The mean respiration rate for unfertilized eggs from laying workers was also 0.1 ± 0.0 µL CO_2/hr/egg (n=110).

The combined mean respiration rate of all honey bee eggs tested, using fertilized and drone eggs from queens as well as drone eggs from laying workers was 0.1 ± 0.0 µL of

Table 4. Comparison of mean wet weights (µg ± SEM) and mean dry weights (µg ± SEM) of fertilized eggs from queens and unfertilized eggs from laying workers.

Caste	n[2]	Wet weight[x] Mean[1](µg/egg ± SEM)	Dry weight[y] Mean[1](µg/egg ± SEM)
Queens	28	133.0 ± 6.0a	23.7 ± 0.8a
Laying workers	16	108.0 ± 2.0b	20.6 ± 0.5b

Means within a column followed by the same letter are not significantly different {($p<0.05^x$ and $p<0.01^y$) Tukey's Studentized Range (HSD) Test}.
[1]The eggs from queens were weighed from May to November, while the eggs from laying workers were weighed from August to November. Effect of age on egg weight has not been proved.
[2]The eggs were weighed in batches of 20 per sample (n equals 20 eggs).

CO_2 per hour per egg (N=400). The eggs were incubated at 28^0C, 31^0C, 34^0C and 36^0C. The mean values of CO_2 given out by fertilized eggs on day one (0-24 hr) were high for all the queens, compared to the mean values recorded during day two, 25-48 hr. On day three (49-72 hr) the mean values were at their highest levels. The mean values of CO_2 given out by fertilized eggs from queens on individual days and temperature treatments are shown in Figure 2. The eggs incubated at 28^0C and 31^0C had similar mean values, as was also the case at 34^0C and 36^0C, but the respiration rates at 28^0C and 31^0C were lower. On day two the trend had changed showing a marked difference in the temperature treatments. The lowest respiration rate was recorded from eggs incubated at 28^0C on day two, and day three. The results for day two at 31^0C, 34^0C and 36^0C showed lower mean values compared to day three at the same temperature treatments, an indication that, on day three, substantial amount of CO_2 was produced by the eggs during incubation, especially at 34^0C and 36^0C. From these results, it is observable that there is a significant difference (p=0.05) between low temperature treatments of 28^0C and 31^0C compared to higher ones of 34^0C and 36^0C.

Respiration quotient

For fertilized eggs of queens the difference between the lowest temperature treatment and the highest was 8^0C, hence the Q_{10} values were estimated. The Q_{10} values were calculated by determining the mean value of CO_2 produced at the highest temperature treatment of 36^0C, on individual days of incubation from the queens' eggs. The values

obtained were divided by the mean values of CO_2 of eggs from the lowest temperature treatment (28^0C). The mean Q_{10} value for the whole incubation period was 1.8 ± 0.8. The results indicate that the respiration rate varied over the incubation period, but were lower on day two compared to days one and three, respectively. The results are shown in Table 5.

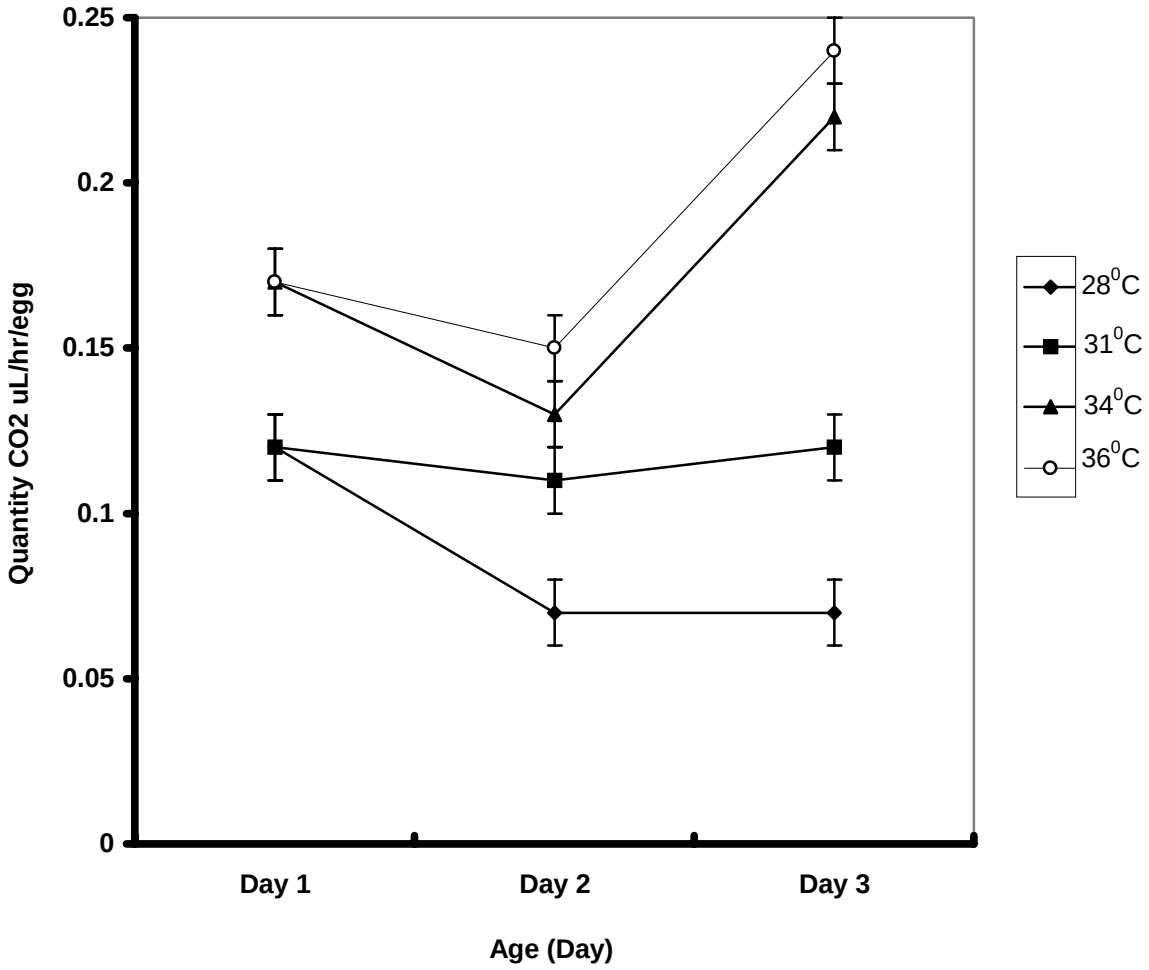

Figure 2. The trend in the mean respiration rate based on CO_2 (µL/hr/egg ± SEM) output from fertilized eggs produced by queens. The eggs were aged day 1 (≤ 24hr), day 2 (≤ 48hr), and day 3 (≤ 72 hr); and were artificially incubated at 28^0C, 31^0C, 34^0C and 36^0C, for approximately 13 hours.

The GLM procedure revealed significant quantitative difference (F=11.2, df=3, P=0.0001) in the respiration rates of the fertilized eggs from queens. Split plot ANOVA

showed a significant difference (F=9.7, df=2, 47; P=0.01) when the mean CO_2 output from fertilized eggs were compared based on age, while using the interaction of colony and age as an error term; indicating that respiration rates in honey bee eggs were affected by age.

Table 5. Q_{10} values of fertilized eggs based on CO_2 (µL/hr/egg ± SEM) output from fertilized eggs produced by queens. The eggs were aged day 1 (≤ 24hr), day 2 (≤ 48hr), and day 3 (≤ 72 hr); and were artificially incubated at 28^0C and 36^0C, respectively, for approximately 13 hours.

Temperature Range	Day One	Day Two	Day Three
28^0C - 36^0C	1.5	0.6	3.4

Highly significant differences (F=60.0, df=3, 47; P=0.0001: F=7.5, df=6, 47; P=0.0001) were observed in the mean CO_2 output from fertilized eggs when the effects of temperature treatments alone, and the effects of age and temperature treatments, respectively were considered, with colony/age/temperature interactions as error terms in both comparisons, indicating that age and temperature had effects on respiration rates. On comparing the eggs incubated at 34^0C, which is the ideal temperature for embryonic development in honey bee eggs, there was no significant difference (F=6.6; df=1, 18; P=0.1) between the fertilized eggs and unfertilized eggs from colonies headed by queens, when status of the eggs was considered, with the interaction between colony and status of the eggs as an error term.

The effect of age was highly significant (F=31.6; df=2, 18; P=0.0001), but there were no significant differences (F=0.8; df=2, 18; P=0.5), (F=1.8; df=6, 18; P=0.1), (F=0.7; df=1, 18; P=0.4) between the effects of status and age, colony and age, colony/status and age, respectively, which showed that the difference in CO_2 output depended on the age or effect of temperature treatment or both. The eggs behaved in similar manner irrespective of their source or status at the same age and temperature treatment. The results indicated that the differences in the respiration rates between the fertilized and unfertilized eggs from queens were mainly due to the age of eggs.

The regression was highly significant (F=18.2; df=1, 11; P=0.002; R^2=0.65) when all the days were compared. On comparing different temperature treatments on individual days, regression was not significant (F=9.3; df=1, 3; P=0.1: R^2=0.82) on day one, but significant differences (F=96.9; df=1, 11; P=0.01; R^2=0.98) and (F=64.8; df=1, 3; P=0.02; R^2=0.97) were observed on days two and three, respectively. The results indicated a significant relationship between temperature effects, stage of development and CO_2 output from the eggs treated (Figure 3).

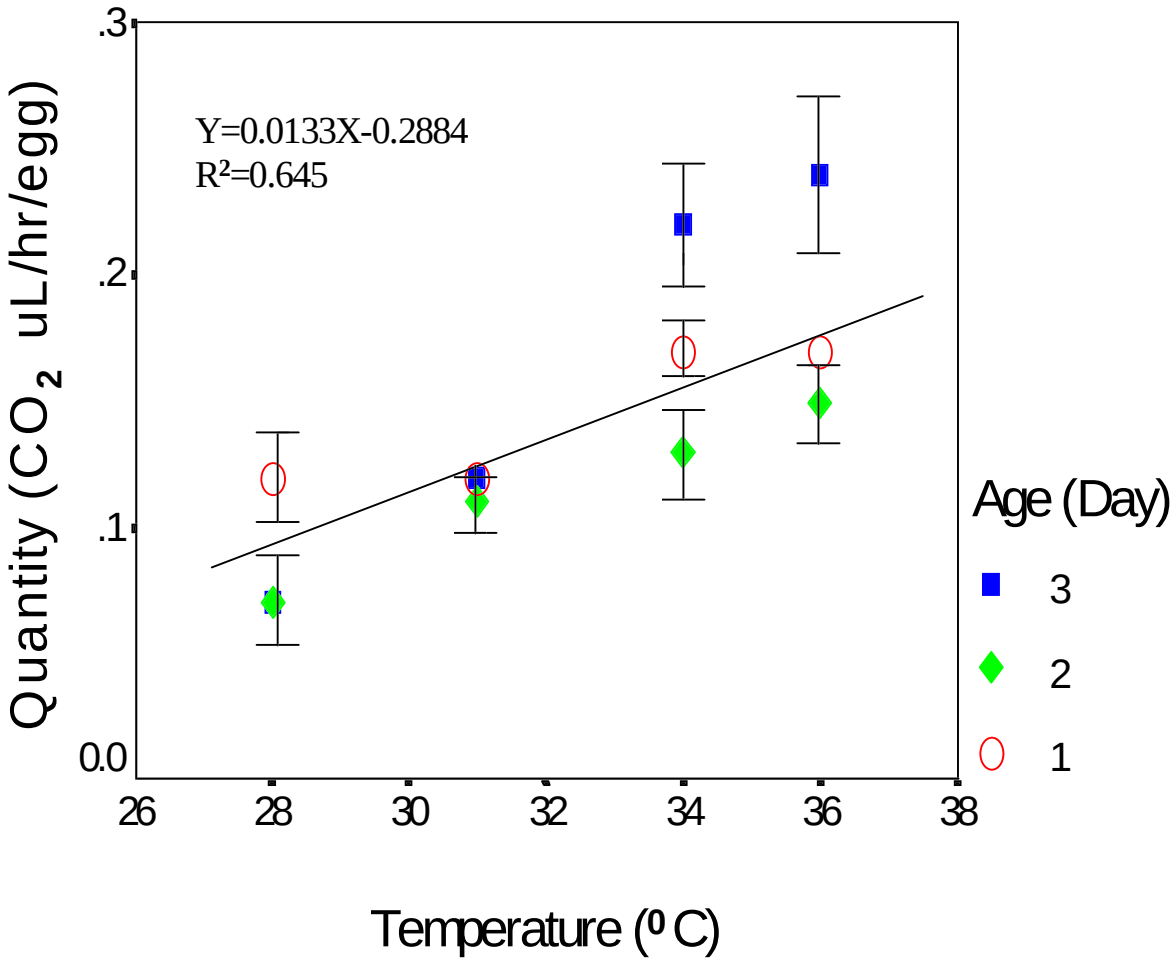

Figure 3. Regression analysis of mean respiration rate based on CO_2 (µL/hr/egg ± SEM) output from fertilized eggs produced by queens. The eggs were aged day 1 (≤ 24hr), day 2 (≤ 48hr), and day 3 (≤ 72 hr); and were artificially incubated at 28^{0}C, 31^{0}C, 34^{0}C, and 36^{0}C, for approximately 13 hours. The regression was highly significant (F=18.2; df=1, 11; P=0.002: Y=0.133X-0.2884; R^2=0.645).

The comparison of respiration rates of unfertilized eggs from queens and those from laying workers showed no significant difference (F=0.1; df=1, 11; P=0.8). Similarly, no significant difference (F=0.8; df=2, 11; P=0.4) was observed on egg type and age interactions, although the effect of age alone showed a highly significant difference (F=54.0; df=2, 11; P=0.0001). The results of comparisons between the unfertilized eggs of queens and those from laying workers also indicated that age had a significant influence on the respiration rates, but the effects of temperature and egg type were constant in both cases. The results from unfertilized eggs of queens and laying workers were not significantly different (p=0.05) at 34^0C on any individual days of incubation (Table 6).

Table 6. Comparison of mean respiration rate based on CO_2 (µL/hr/egg ± SEM) output from unfertilized eggs produced by queens and those produced by laying workers. The eggs were aged day 1 (≤ 24hr), day 2 (≤ 48hr) and day 3 (≤ 72 hr); and were artificially incubated at 34^0C, for approximately 13 hours.

Temp.	Day	n	Mean (µL/hr/egg ± SEM)	Range
			Queens	
34^0C	One	10	0.1 ± 0.2b	0.01-0.16
	Two	22	0.1 ± 0.0b	0.01-0.22
	Three	18	0.2 ± 0.1a	0.11-0.31
			Laying Workers	
34^0C	One	62	0.1 ± 0.0b	0.01-0.22
	Two	24	0.1 ± 0.0b	0.05-0.12
	Three	24	0.2 ± 0.0a	0.07-0.28

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

The overall trend in the CO_2 output from unfertilized eggs of queens and those from laying workers (Table 6) showed high levels of CO_2 on day one followed by reduced levels on day two and then higher levels on day three. Similar patterns were also observed in the fertilized eggs from queens. Gray et al. (1991) obtained three-phase respiration pattern during embryonic development in the gypsy moth eggs, but it must be emphasized that the pattern of egg development in these moths is different from honey bees.

3:4. Discussion

By using ambient control as a standard, the results from 250 µL gas analysis showed that egg sample vials had 1.8, 1.6 and 1.4 times greater CO_2 concentrations (ppm) than ambient control, comb material, and frozen eggs, respectively, after approximately 13 hours of incubation. This was an indication that honey bee eggs respired in the vials. The viability trials on the combs resulted in an overall hatchability of 75.8 ± 0.04 % for all the eggs (fertilized and unfertilized) produced by queens and workers.

The results from the temperature treatments at 28^0C, 31^0C, 34^0C, and 31^0C showed that honey bee eggs respired at different rates under different temperature conditions. Lower respiration rates were recorded at 28^0C and 31^0C while relatively high respiration rates were exhibited at 34^0C and 36^0C. Differences in respiration rates also occurred due to the age of the eggs. In addition, the results showed that slight differences exist between fertilized and unfertilized eggs from queens on day one, with fertilized eggs respiring at a higher rate of 0.2 ± 0.0 µL/hr/egg compared to unfertilized eggs (0.1 ± 0.0 µL/hr/egg). The respiration rate in laying worker's eggs was 0.1 ± 0.0 on day one. There was no difference in the respiration rate of unfertilized eggs from queens and those from the laying workers (Table 6). It was also observed that both types of eggs, fertilized and unfertilized, hatched at approximately 72 hours (three days) under natural conditions in the hives.

It was evident from the results that development in honey bee eggs was highly sensitive to slight temperature changes and any small temperature differences had severe effects on the development of honey bee eggs, which also influenced the final hatchability. Low hatchability values were recorded at extreme temperature treatments (28^0C and 36^0C).

All eggs tested exhibited respiration patterns characterized by production of CO_2 at different rates across all the temperature treatments depending on the age of the eggs. On the first day, there were rapid respiration rates (shown by high production of CO_2) followed by a decline on day two. Day three was characterized by another steep rise in CO_2 output as the eggs advanced in age toward eclosion of embryos. Gray et al. (1991) reported the existence of three phases of egg development in the gypsy moth but over a much longer period coupled with stages of diapause. They estimated increases in respiration rates of 0.14 µL CO_2/24h (25^0C) after diapause. Although the pattern of embryonic development in honey bee eggs is not the same as that in gypsy moth eggs, the results obtained in the honey bee eggs showed that respiration rates were synchronized to the stage of embryonic development at individual temperature treatments.

In fertilized eggs, the process of egg initiation and changes in the membrane potential after sperm entry into the ovum (Morrill and Kostellow 1991, Campbell 1993) have been advanced as possible explanations for the mechanism responsible for the rapid demand in oxygen (O_2) exhibited by different eggs of several animal species, including some insect eggs. Sperm entry triggers a rise in metabolic activity, which correspondingly causes an increase in CO_2 output. The plasma membrane undergoes structural changes to block excess sperm entry into an ovum (Campbell 1993). Polyspermy is common in honey bee eggs (Kerr 1956, DuPraw 1967) and is associated with impaired embryonic development. Rothenbuhler et al. (1952) observed the production of gynandromorphs from this anomaly.

Several studies (Sander et al. 1985, Milne et al. 1988) have shown that a myriad of activities take place after syngamy. First, a number of biochemical changes occur in order to promote utilization of phospholipids, protein phosphorylation, and lipid(s) turnover. Secondly, membrane permeability changes to allow movement of specific ions which triggers cell activation. This process is finally followed by cell division, and cell growth (Morrill and Kostellow 1991). The sequence of developmental processes taking place in honey bee eggs after fertilization has been mapped out, and may assist in understanding how changes in cellular activities affect respiratory patterns observed in the eggs.

Events taking place in the chronology of honey bee embryogenesis were exhaustively described by Milne et al. (1988). They observed that eggs showed apparent inactivity on day one (0-24 hr), but were uniformly filled with no space in the cytoplasm or in the periplasm. This period of 24 hours was followed by the formation of clear space at the caudal pole and an equivalent one at the cephalic end. The next prominent event noticed was the division of many nuclei at the center of the egg, then the migration of the cells to the periphery and formation of blastoderm cells. Fleig and Sander (1984) described blastoderm formation in honey bee embryogenesis as observed using the scanning electron microscope (SEM). They explained the successive changes at the cell surface of fertilized honey bee eggs, as follows: "Each change starts in the differentiation center located in the anterior egg half and from there spreads as a wave towards both poles. However, one of the waves is heterogeneous; the protrusions or caps of colemma which start enveloping the preblastoderm nuclei arise in regions of the egg cell. The events described here consumed nearly 40% of the time period between oviposition and hatching; thus, and by comparison with most other insects, blastoderm formation in the honey bee is a very lengthy process". They estimated the process to last approximately 20 hours. Meanwhile, Milne et al. (1988) indicated that at 12 hours of day one, the embryos occupied the entire egg volumes, but only small changes were visible. By the end of day one (24 hr), apart from an enlargement of cephalic pole space, no appreciable organogenesis had commenced. Milne et al. (1988) also indicated the presence of a clear space at the caudal pole by the 27th hour after fertilization. Proper organogenesis was reported on day two. Day three can be considered as the peak of embryo formation and full larval development.

From the observations made by Milne et al. (1988), Fleig and Sander (1984) and the results of respiration studies, day one of embryonic development in honey bees may be considered a preparatory and organizational stage for biosynthesis of cell components which are later converted to embryonic tissue. Grant (1978) considered the early stages of development as the stage for activation of enzyme/cofactors systems, reorganization of proteins and stimulation of metabolism products. Energy is needed for synthesis of macromolecules. Morrill and Kostellow (1991) described the events leading to oocyte

maturation, with the emphasis that the preparatory stages differ with the type of egg and species concerned, as well as the prevailing environmental conditions.

Lower temperatures (28^0C and 31^0C) caused lower respiration rates and higher mortality. Several threshold effects which contribute to the overall mortality during embryonic development at low temperatures have been proposed by many researchers. The negative effects include: (a) developmental threshold (no development occurs), (b) developmental hatching threshold (development fails to reach completion) and (c) hatching threshold (full development occurs but no hatching takes place) (Richards 1957). Equally significant are the effects at higher temperature(s) (36^0C), including acceleration of embryonic development, sometimes with inability to complete development. Even though the temperature treatment at 36^0C is not high enough to cause irreversible deformation of enzymes in honey bee eggs, which would adversely affect biochemical processes and affect embryonic development, its effect was evident from the high mortality recorded.

The respiration rates at 34^0C and the number of eggs developing after this temperature treatment confirmed it as the optimum temperature for the development of honey bee eggs. More eggs (75%) tested at 34^0C reached the pre-emergence stage compared to the lower values for other temperature treatments. Studies have also shown that the maintenance of hive temperature at 34 ± 0.8^0C, is associated with the distribution density of adult honey bees in the broodnest (Levin and Collison 1990). The significance of temperature regulation determines the efficiency of brood production, so that strong colonies are always able to produce large number of workers over a short time. The strength of a colony is a parameter that honey bee breeders can select for in queen breeding and selection programs. There is a linear relationship between population density and the efficiency of thermoregulation. Temperature maintenance involves consumption of food (honey or pollen) which needs to be gathered into the hive. Consequently, temperature maintenance is an expensive investment in the life of a colony. Thermal homeostasis involves reduction of temperature during the hot days through evaporative cooling of water effected by a stream of air blown by fanning of wings by the workers, while heat is conserved during the cold seasons by formation of strong clusters depending

on the level of temperature reduction. A large population of workers, plus a large amount of conserved food (usually in the form of honey) is needed to maintain honey bees in both circumstances.

3:5. Conclusion

Respiration rates have direct relationships with metabolic activities in developing honey bee embryos. Mortality occurs at any temperature during development, but maximum mortality is recorded as temperatures start to deviate from the optimum of 34.5^0C by more than 2^0C. Respiration rates are measurements used as determinants of the influence environmental variables, especially temperature has on eggs during embryonic development. The findings from this experiment agree with those of Harbo and Bolten (1981), and Milne et al. (1988), on the relationship between temperature and embryonic survival. The understanding of respiratory activities in honey bee eggs is far from complete, since the specific effects of temperature on development are still speculative and most of the knowledge is derived from contrasts of the developmental processes in other insects. Histological studies on embryogenesis in honey bees have been undertaken in great detail (DuPraw 1963, Fleig and Sander 1985, 1986, Milne et al. 1988), which account for specific morphological differentiation during embryonic development. The present study is intended to add another dimension to the understanding of the events taking place during embryonic development in the eggs produced by queens and laying workers. By measuring respiration rates and levels of nutrients at shorter intervals during egg development, it may become possible to understand the way temperature influences metabolic activities and how this in turn affects egg development in honey bees.