CHAPTER IV

EFFECT OF CARBON DIOXIDE NARCOSIS ON OVARY ACTIVATION AND GENE EXPRESSION IN WORKER HONEY BEES (*Apis mellifera* L.)

4.1 INTRODUCTION

Like many other species of social insect, the honey bee *Apis mellifera* L., is characterized by extreme reproductive division of labor. The vast majority of workers are functionally sterile, and never activate their ovaries throughout their life (Barron *et al.*, 2001), leaving the production of eggs to the queen.

Carbon dioxide has been shown to greatly influence insect behavior and physiology, insects are frequently immobilized with carbon dioxide during routine studies by biologists. Treatment with carbon dioxide has been shown to affect insect reproduction (Engels *et al.*, 1976), development (Woodring *et al.*, 1978) and other behavior (Ralph, 1959; Whisenant and Brady, 1965; Mardan and Rinderer, 1980; Schneider and Gary, 1984). Woodring *et al.* (1978) showed that in crickets (*Acheta domesticus*) the effects of short term exposures to carbon dioxide are associated with a true anaesthetic effect on nervous tissue, while longer exposures cause asphyxiation that leads to anoxia of tissues. The anaesthetic effect results from the direct diffusion of carbon dioxide gas to nerve cells followed by a reduction in neuroplasmic pH resulting from the conversion of carbon dioxide to carbonic acid (Edwards and Patton,

1965) and not by the drop in haemolymph pH associated with exposure to carbon dioxide (Woodring *et al.*, 1978). Carbon dioxide narcosis of honey bees shortens their hive-period and their lifespan (Ribbands, 1950). Furthermore, it decreases the age-dependent temperature preference and influences the development of hypopharyngeal glands and fat body. For *Locusta* it has been shown that daily narcosis with carbon dioxide results in an increased juvenile hormone titer (Fuzeau-Braesch *et al.*, 1982).

In the honey bee, mating triggers the start of egg production. Unmated queens start oviposition at unpredictable times, but at an average age of more than 40 days. This is true also of instrumentally inseminated females. Mackensen's (1947) discovery that (double) carbon dioxide narcosis results in young unmated queens laying eggs 5-6 days afterwards has provided a highly reliable method that became a basis of instrumental insemination techniques and therefore of bee genetics.

This phenomenon is routinely exploited to induce oviposition in unmated but artificially inseminated queens (Laidlaw and Page, 1997). This idiosyncratic response has facilitated the physiological study of queen egg production (Engels *et al.*, 1976; Engels and Ramamurty, 1976; Engels, 1987). However, few studies have applied this technique to the study of worker egg production (Harris and Harbo, 1990; Harris *et al.*, 1996), and no study has yet examined locus-specific changes in gene expression associated with ovary activation, in queens or workers. The effects of carbon dioxide narcosis in queen honey bees indicate that the treatment triggers the up-regulation of genes that in turn activate the queen's ovaries. This particularly evident in the case of vitellogenin, an egg protein, whose synthesis is dramatically stimulated in queen abdomens following carbon dioxide treatment (Engels *et al.*, 1976). Genes directly associated with ovary activation in queens, like vitellogenin for example, are prime candidates for genes associated with ovary activation in normally sterile workers.

We speculated that the genes associated with ovary activation in queens in response to carbon dioxide narcosis might also be the genes associated with ovary activation in workers. To test this hypothesis, groups of caged workers were exposed to (double) carbon dioxide narcosis and compared rates of ovary activation and levels of gene expression against groups of control (un-narcotized) workers. Differential ovary activation and gene expression in response to carbon dioxide exposure occurred. These findings are discussed in the context of the regulation of ovary activation in the workers.

Moreover, the head of the control and treated CO_2 workers were tested the levels of genes expression with some of eight candidate genes (profillin, flotillin, transferrin, vitellogenin). The abdomen is the place where the ovary of worker bee is located. The brain, hypopharyngeal glands, mandibular glands and so on are located in the head part of the bees. We knew that vitellogenin (one of the candidate genes) is a conserved yolk precursor protein and it involves in brood food production by the hypopharyngeal glands of nurse bees and it is important in regulating honey bee lifespan. Moreover, from the northern blot analysis of the anarchist and wild-type workers (Chapter III), the expression of transferrin and vitellogenin genes seem to be differentially expressed in the heads and abdomens of those samples. So, transferrin and vitellogenin were the best candidate genes to investigate the expression levels in the heads and abdomens of activated and non-activated ovaries wild-type worker bees exposed to CO_2.

4.2   MATERIALS AND METHODS

4.2.1   Honey bee samples

The European honey bees (*Apis mellifera* L.) that were used in this study kindly provided from Professor Dr. Ben Oldroyd's Lab, School of Biological Sciences, University of Sydney, Australia (Appendix A)

4.2.2   Preparation of worker bees with different stages of ovarian development

This experiment was done in cages that was the out-of-hive rearing conditions. The carbon dioxide treated experiment was done two times with different in number of carbon dioxide treatment, the day of carbon dioxide treatment and collecting time. Various day-old worker bee samples were obtained by removing worker brood from a single wild type colony of *Apis mellifera* L. The combs of emerging sealed brood were enclosed in wood box and incubated overnight at 35°C and high relative humidity (Appendix A). The following morning, newly emerged worker honey bees were collected and groups of 35 bees (first experiment)/ 30 bees (second experiment) were transferred into steel cages (approx. 14 x 10 x 7 cm) fitted with bees wax, water and food (45 % honey; 10 % water; 45 % royal jelly, Lifetime Health Products, Australia). Food and water were changed every two days.

The first experiment

Sixteen cages were divided into 4 sets that are difference in day of carbon dioxide treatment (day 2&3, day 4&5, day 6&7 and not treat for control). In each three sets that treated with carbon dioxide have 4 cages that are difference in

collecting time (4 hrs after 1st treatment, 4 hrs after 2nd treatment, 48 hrs after 1st treatment and the others were collected when bees were 8-9 days old; the cages that were marked as 1st treatment were exposed with carbon dioxide 1 time for 10 minutes while the cages that were marked as 2nd treatment were exposed with carbon dioxide 2 times for 10 and 3 minutes in consecutive day). The bees in control group were collected at day 3, 5, 7 and 9 after emergence (Appendix A).

The second experiment

Sixteen cages were divided into 4 sets that are difference in day of carbon dioxide treatment (day 2&3, day 3&4, day 4&5 and not treat for control). In each set have 4 cages that are difference in collecting time (4 hrs, 24 hrs, 48 hrs after double carbon dioxide narcosis treatment and the others were collected when bees were 7 days old). This experiment, all workers in treated group were exposed with carbon dioxide for 2 times.

In the step of double carbon dioxide narcosis in consecutive days, all cages (except control cages) were narcotized at the specific day (The first experiment: day 2&3, day 4&5, day 6&7; and the second experiment: day 2&3, day 3&4, day 4&5) at room temperature by placing whole cages into a large plastic container and flushing it with compressed CO_2 for 10 minutes. The following day, workers were narcotized again, for 3 minutes (Engels *et al.*, 1976). In each case, workers were completely anaesthetized (Appendix A).

4.2.3 Collection and selection the samples

4.2.3.1 *Sampling techniques*

For carbon dioxide treatment, at various time intervals following the double carbon dioxide narcosis treatment (4 hrs, 24 hrs, 48 hrs and when honey bees were 7 days old), workers were collected from incubation by snap-freezing them in liquid N_2 or dried ice (Appendix A).

These frozen worker bees were subjected to observe the ovarian development by observation under a stereoscopic microscope (Appendix A).

4.2.3.2 *Dissect the ovaries to observe the ovarian development*

Each collected workers were dissected on wax plate which is putting on ice and ovarian development will be observed under a stereoscopic microscope (Appendix A). Ovarian development will be divided in 4 stages (Appendix A).

The scoring results from this experiment were plot into 3D bar graph. Eight bees from carbon dioxide treated workers and eight bees from control workers were selected for RNA extraction step.

After scoring the stage of ovarian development of each bee, the group of worker bees that received carbon dioxide and group of control workers were selected to extract the total RNA. The second experiment of carbon dioxide treatment was selected (had enough all stages of ovarian development worker bee samples). The selected worker bees had the same age of 7 days old. Eight bees from day 4&5 carbon dioxide treated workers (stage 0 and 1 of ovarian development) were used as the samples and eight bees (two each from all stages of ovarian development) from control group at the same age were used as the control.

Before dissecting the abdomen, the heads of all workers were separated kept at -80 °C in the other tubes.

4.2.4 Total RNA extraction

Three protocols for extracting total RNA from worker bee abdominal or head part tissue were used to find the appropriated protocol to obtain high quality and quantity of total RNA. These three protocols are 1) Agilent mini kit protocol, 2) TriZol reagent protocol, and 3) Combined TriZol/Qaigen protocol. For head part tissue, the compound eyes which contained pigments were removed before homogenization to minimize the pigment contamination. Because the amount of tissue from head part was smaller than that of abdomen, therefore Agilent mini kit protocol was selected to attract total RNA to minimize the RNA losing from the pipetting step of the other two protocols. The head part tissue that used to extract total RNA came from the same selected bees of the abdominal tissue.

For Agilent mini kit protocol, frozen abdominal tissue of individual bee or four heads tissue from the same selected bees was homogenized per tube. The following steps were previously described in Chapter III (3.2.4).

The total RNA that extracted from head part tissue still had some pigment left in the solution. Dynabeads Oligo (dT)$_{25}$ (DYNAL® Simply Magnetic) was used to purify the mRNA. This purification method was previously described in Chapter III (3.2.5).

For the TriZol reagent protocol, the frozen abdominal tissue of single bee was ground with liquid nitrogen in 1.5 ml Eppendorf tubes, and homogenized with 100 μl

of TriZol reagent (Gibco BRL) using disposable pestles attached to a hand-held engraving device (Super Tool, Arlec). After homogenization, the volume was adjusted to 1,000 μl with TriZol reagent. The homogenates were incubated at room temperature for 5 min to serve complete segregation of nucleoprotein complexes. Then, 200 μl of chloroform were added and vigorous shaken for 15 seconds. After incubation at room temperature for 5 min, the RNA-containing upper aqueous phase was recovered after spinning for 15 min at 12,000 ×g, 4 °C, then mixed with equal volume of phenol/chloroform and centrifuged at the same condition. The RNA-containing aqueous phase was transferred to a new tube, total RNA was precipitated by addition of one volume of absolute isopropanol, then incubating at -20 °C for 15 min. The supernatant was removed by centrifugation as above and the total RNA pellets were washed with 500 μl of 70% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water. The total RNA was stored in 70% (v/v) ethanol at -80 °C until used. Before using, ethanol supernatant was completely removed by centrifugation at 12,000xg for 15 min at 4 °C. The RNA pellets were dried at room temperature for 10-15 min and dissolved in an appropriate amount of DEPC-treated water.

For the combined TriZol /Qaigen protocol, the frozen abdominal tissues from single bees were ground with liquid nitrogen in 1.5 ml Eppendorf tubes and following with the extraction protocol that previously described in Chapter III (3.2.4).

4.2.5 Determination of the quantity and quality of RNA samples

The quantity and quality of total RNA was spectrophotometrically measured at 260 nm and 280 nm and also analyzed by formaldehyde-agarose gel electrophoresis as described in Chapter III (3.2.6).

The quality of the extracted RNA was analyzed by formaldehyde-agarose gel electrophoresis as previously described in 3.2.6.

4.2.6 DNase treatment of total RNA samples

The extracted total RNA was further treated with RNase Free DNase I (Promega, 1 units/5 µg of the total RNA) to remove the contaminating chromosomal DNA before using as the template for first-stranded cDNA synthesis. Then, the RNA pellets were purified by phenol/chloroform extraction following by ethanol precipitation. This protocol was shown in Chapter III (3.2.8.1).

4.2.7 First-stranded cDNA synthesis

DNA-free total RNAs from each individual bee was pooled by treatment prior to cDNA synthesis. We used pooled rather than individual samples to examine gene expression in order to minimize inter-individual variation, as described in other studies (Grozinger *et al.*, 2003; Tian *et al.*, 2004). Each pooled RNAs were used as the template for first stranded cDNA synthesis using the SuperScript[TM] II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's protocol as described in Chapter III (3.2.8.2).

4.2.8 Quantitative Real-Time PCR (qRT-PCR)

4.2.8.1 *Genes selection*

Based on the primary experiment from the microarray (our research group), the information from Honey bee genomes project and the results from the previous experiment, eight candidate genes (Profilin [Prf], Flotillin [Flt], Transferrin [Trf], Vitellogenin [Vit], Take-out-like [JHBP], Nitric oxide synthase [NOS], Arginine kinase [ArgK], Octopamine receptor [OctR]) were interested to examine the expression in the CO_2 treated workers against group of control non-CO_2 treated workers.

These genes encoded transcripts known or suspected to be involved either in ovary/egg development (vitellogenin, transferrin, profilin, octopamine receptor) or in signalling pathways critical for cellular growth and/or differentiation (flotillin, nitric oxide synthase, take-out-like). Arginine kinase has also been selected because it is an important component of energy transfer and chose ribosomal protein (RpS8) as internal control. The candidate genes are transferrin (Trf), an iron binding protein with multiple function; vitellogenin (Vit), a zinc binding protein sequestered by developing oocytes; profilin (Prf), an intercellular cytoplasm transport molecule required during *Drosophila* oogenesis; flotillin (Flt), a co-regulator of actin cytoskeleton formation; nitric oxide synthase (NOS), an enzyme responsive to oxygen related hypoxia; arginine kinase (ArgK), an enzyme implicated in energy transfer; octopamine receptor (OctR), a binder of biogenic amines and neuromodulator of many physiological process; and take out like carrier protein (JHBP), containing a juvenile hormone-binding motif.

4.2.8.2 *Primers for quantitative real-time PCR amplification of all selected genes*

The primers of each gene were designed and selected for using in quantitative real-time PCR as described in Chapter III (3.2.8.4).

4.2.8.3 *Testing the efficiency of primers by using PCR*

All primers were experimentally tested in an Eppendorf gradient cycler to determine the optimal annealing temperature same as previously mentioned in Chapter III (3.2.8.5). After amplification, an aliquot of 5 µl of PCR products were electrophoretically analyzed on 1.0% agarose gel.

4.2.8.4 *Agarose gel electrophoresis*

The method to prepare the agarose gel, electrophoretical, stained with ethidium bromide, visualized under the UV transilluminator and photographed with Gel Documentation System (GeneCam FLEX1, SynGene) was shown in Chapter III (3.2.8.6).

4.2.8.5 *Analysis of the selected genes expression in the abdomens and head of the workers following carbon treatment using qRT-PCR*

The relative expression ratios of each transcript in head and abdominal parts of the CO_2 treated workers against the control non-CO_2 treated workers were performed using quantitative real-time PCR as described in Chapter III (3.2.8.7).

4.2.8.6 *Determination of PCR efficiency*

Each gene was amplified by different specific primers, so it might be revealed the different PCR efficiency that was determined by constructing a standard curve.

The method to make the standard curve and how to get the PCR efficiency was described in Chapter III (3.2.8.8).

4.2.8.7 *Data analysis of real-time RT-PCR*

The fluorescent signals of the amplified products were analyzed by the data analysis software of the Corbett Research Detection system (Corbett Research, Sydney, Australia) using the PCR baseline subtracted curve fit method. The method to calculate the relative expression ratio and also a mathematical model were shown in Chapter III (3.2.8.9).

Relative expression ratios were converted to percent change whereby a value of 100% equals no change.

4.3   RESULTS

Ovarian development observed from the sample preparation

This experiment was done in cages that were out-of-hive rearing conditions. The carbon dioxide treated experiment was done two times with different in number of carbon dioxide treatment, the day of carbon dioxide treatment and collecting time. After treatment and rearing, some of the honey bees died before collecting.

The first experiment

Groups of 35 bees were transferred into each cage. The number of worker bees in each cage that still alive and the age of these bees when they were collecting of the first experiment were shown in the Table 4.1.

All worker bees were dissected on wax plate which was putting on ice to observe the ovarian development stage 0, 1, 2 and 3 under the stereomicroscope.

The number of worker bees in each stage of ovarian development was counted. The scoring result of these dissected worker bees of the first experiment of carbon dioxide treatment was shown in Table 4.2. Some or all of the bees in some cages died before collecting time, so they were not 35 bees in total. The data from Table 4.2 was used to plot the 3D bar graph as shown in Figure 4.1.

The second experiment

Groups of 30 emerged worker bees were transferred into each cage. The number of the bees alive and the age of them at that collecting time points were shown in Table 4.3.

Table 4.1 The number of workers in each cage of the first experiment of carbon dioxide treatment.

Workers	4 hrs after 1st treatment*	4 hrs after 2nd treatment*	48 hrs after 1st treatment	8-9 days after emergence
CO_2 day 2&3	35 (2)**	35 (3)	35 (4)	0 (8)
CO_2 day 4&5	35 (4)	35 (5)	35 (6)	1 (8)
CO_2 day 6&7	11 (6)	32 (7)	0 (8)	0 (8)
Control***	35 (3)	35 (5)	35 (7)	33 (9)

* The cages that marked with 1st treatment were treated with CO_2 1 time and the cages that marked with 2nd treatment were treated with CO_2 2 times.

** The number in "( )" determined the age of the worker bees.

*** Workers without treated with CO_2

Table 4.2 The number of worker bees in each stage of ovarian development of the first experiment of carbon dioxide treatment.

Day of CO_2 treatment	Collecting time	Number of bees in each stage of ovarian development				
		stage 0	stage 1	stage 2	stage 3	Total
2 and 3	4 hours after 1st treatment (day 2)	25	10	0	0	35
	4 hours after 2nd treatment (day 3)	24	11	0	0	35
	48 hours after 1st treatment (day 4)	23	12	0	0	35
	8 days after emergence *	0	0	0	0	0
4 and 5	4 hours after 1st treatment (day 4)	16	19	0	0	35
	4 hours after 2nd treatment (day 5)	19	16	0	0	35
	48 hours after 1st treatment (day 6)	15	20	0	0	35
	8 days after emergence *	0	1	0	0	1
6 and 7	4 hours after 1st treatment (day 6)*	5	6	0	0	11
	4 hours after 2nd treatment (day 7)*	16	16	0	0	32
	48 hours after 1st treatment (day 8)*	0	0	0	0	0
	8 days after emergence *	0	0	0	0	0
Control	3 days after emergence	29	6	0	0	35
	5 days after emergence	19	16	0	0	35
	7 days after emergence	12	15	7	1	35
	9 days after emergence *	2	12	2	14	33

* Some of the bees in this cage (row) died before collecting.
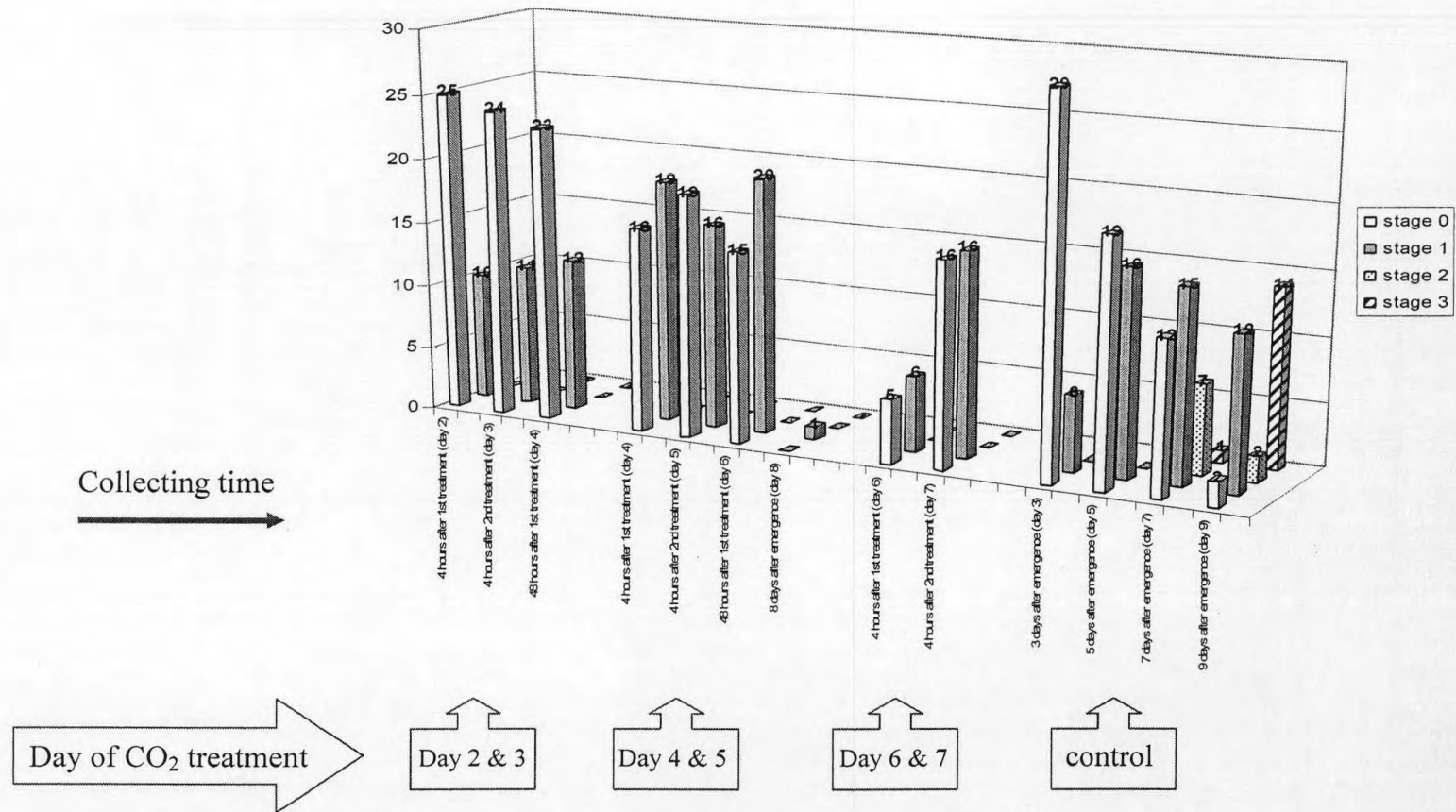
Figure 4.1 Stage of ovarian development in control and CO_2 treated workers at various times of CO_2 treatment and collecting time.

Table 4.3 The number of workers in each cage of the second experiment of carbon dioxide treatment.

	4 hrs after treatment*	24 hrs after treatment	48 hrs after treatment	7 days after emergence
CO_2 day 2&3	30 (3)**	30 (4)	30 (5)	18 (7)
CO_2 day 3&4	30 (4)	30 (5)	21 (6)	1 (7)
CO_2 day 4&5	30 (5)	30 (6)	2 (7)	28 (7)
Control***	30 (3)	30 (4)	30 (5)	30 (7)

* All treated groups of workers were treated with CO_2 for two times in consecutive day.

** The number in "( )" determined the age of the worker bees.

*** Workers without treated with CO_2

All worker bees were dissected on wax plate which was putting on ice to observe the ovarian development under the stereomicroscope same as the first experiment. The number of worker bees in each stage of ovarian development was counted and scoring result of these dissected worker bees of the second experiment of carbon dioxide treatment was shown in Table 4.4.

The 3D bar graph of ovarian development stage from the second carbon dioxide treatment experiment was shown in Figure 4.2. From the results, CO_2 treated worker bees from these two experiments were not developed their ovaries in contrast of the control workers that some of them had fully developed their ovaries at the same age.

The group of worker bees from the second experiment of carbon dioxide treatment was selected for further study because it contained enough worker bee samples of all stages of ovarian development. Eight bees from day 4&5 carbon dioxide treated workers (four each from ovarian development stage 0 and 1) at the age of 7 days old were selected as the samples. Eight bees (two each from all stages of ovarian development; stage 0 - stage3) from control group at the same age were used as the control. These selected sample and control were used to extract the RNA from both abdominal and head part tissues.

Table 4.4 The number of worker bees in each stage of ovarian development

of the second experiment of carbon dioxide treatment.

Day of CO_2 treatment	Collecting time	Number of bees in each stage of ovarian development				
		stage 0	stage 1	stage 2	stage 3	Total
2 and 3	4 hours after treatments (day 3)	17	13	0	0	30
	24 hours after treatments (day 4)	12	18	0	0	30
	48 hours after treatments (day 5)	16	14	0	0	30
	7 days after emergence (day 7)*	6	12	0	0	18
3 and 4	4 hours after treatments (day 4)	10	20	0	0	30
	24 hours after treatments (day 5)	9	21	0	0	30
	48 hours after treatments (day 6)*	4	17	0	0	21
	7 days after emergence (day 7)*	0	1	0	0	1
4 and 5	4 hours after treatments (day 5)	7	23	0	0	30
	24 hours after treatments (day 6)	16	14	0	0	30
	48 hours after treatments (day 7)*	2	0	0	0	2
	7 days after emergence (day 7)*	9	19	0	0	28
Control	3 days after emergence (day 3)	18	12	0	0	30
	4 days after emergence (day 4)	14	16	0	0	30
	5 days after emergence (day 5)	6	23	1	0	30
	7 days after emergence (day 7)	5	12	7	6	30

* Some of the bees in this cage (row) died before collecting.
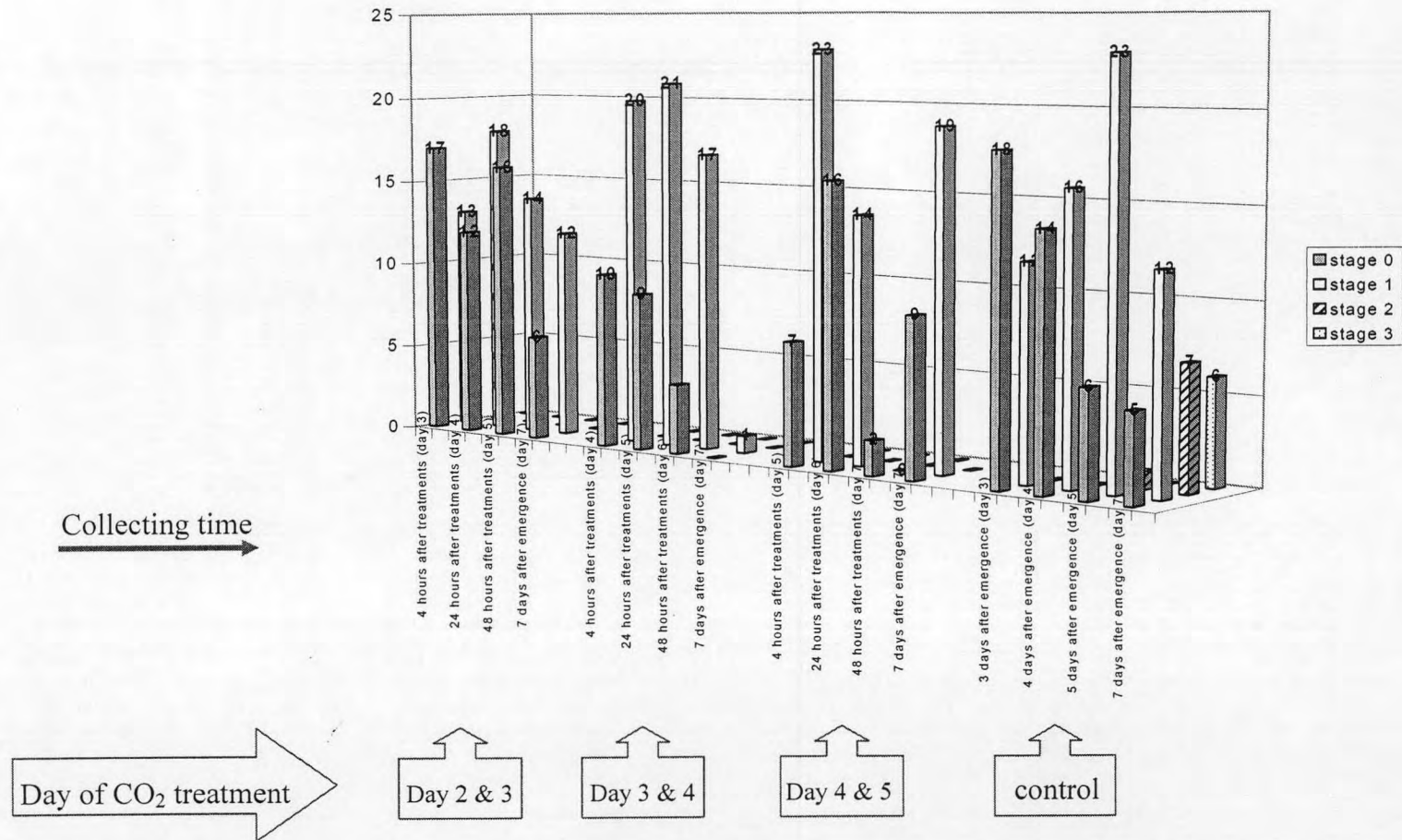
Figure 4.2 Stage of ovarian development in control and double CO_2 treated workers at various collecting time.

"Abdominal part"

Total RNA extraction

Three protocols of RNA extraction were tested. The RNA extracted from the three protocols were determined the quantity and quality of RNA samples. The amount of abdominal total RNA extracted from Agilent kit, combined TriZol/Qaigen and TriZol only were around 30 μg, 65 μg, and 12 μg, respectively. The quality of extracted RNAs were analyzed by formaldehyde-agarose gel electrophoresis (Figure 4.3) and the relative purity of each extracted RNA that examined by measuring the ratio of $A_{260/280}$ was higher than 1.7 for all extracted RNAs.

From the quality and quantity analysis of each extracted total RNA, the combined TriZol/Qaigen protocol was selected to extract the abdominal worker bee samples (high quality and quantity of extracted total RNA). The total RNA extracted from eight individual honey bees were pooled and subsequently treated with RNase Free DNase I for further first-stranded cDNA synthesis. The integrity and purity of RNA preps were analyzed by formaldehyde-agarose gel electrophoresis as shown in Figure 4.4 and the ratio of absorbance at the wavelength of 260 nm and 280 nm were measured. The OD_{260}/OD_{280} ratio of 1.78-1.90 indicated that acceptable quality of extracted RNA was obtained. The amount of pooled total RNA from CO_2 treated workers and control workers were 74 μg and 82 μg, respectively. The RNA solutions were kept at -80 °C until used.
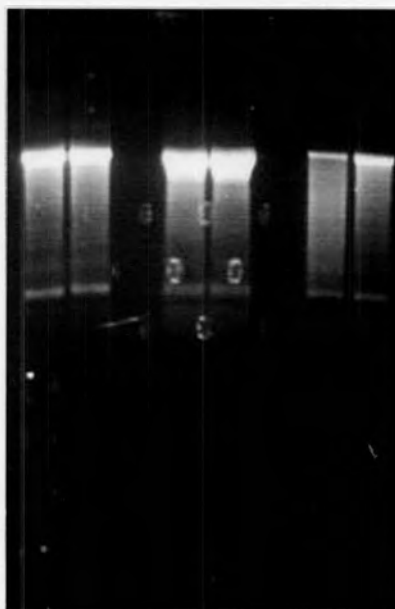
Figure 4.3 The total RNA extracted from the abdomen of worker bees using

three protocols analyzed by formaldehyde-agarose gel electrophoresis.

Lane 1 = Total RNA from treated worker extracted by Agilent kit

Lane 2 = Total RNA from control worker extracted by Agilent kit

Lane 3 = Total RNA from treated worker extracted by combined

TriZol/Qaigen

Lane 4 = Total RNA from control worker extracted by combined

TriZol/Qaigen

Lane 5 = Total RNA from treated worker extracted by TriZol reagent

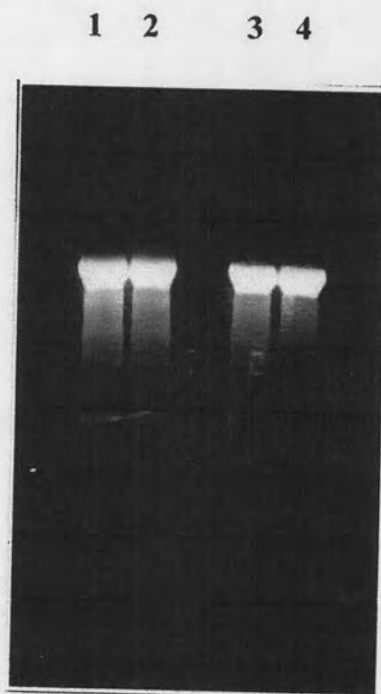Lane 6 = Total RNA from control worker extracted by TriZol reagent

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Figure 4.4 The total RNA extracted from the abdomen of selected worker bees using combined Trizol/Qaigen protocols analyzed by formaldehyde-agarose gel electrophoresis.

Lane 1-2 = Total RNA from treated workers

Lane 3-4 = Total RNA from control workers

Quantitative Real-Time PCR (qRT-PCR)

The expression level of the candidate genes transcript in the carbon dioxide treated workers were examined by quantitative real-time PCR. The cDNA of each control and carbon dioxide treated worker were prepared from the pooled DNA free total RNA of eight individuals. The ribosomal protein S8 (RpS8), the housekeeping gene, was used as a reference gene.

Selection of PCR primers for each gene

For each gene, two sets of forward and reverse primers were designed as described in Materials and Methods. The example of an electrophoretic comparison of target amplicons against a molecular size standard (100 base pair DNA Ladder, Promega Co., U.S.A.) was shown in Chapter III (Figure 3.6). The primer sequence and product size of selected four genes that used in Chapter III, were using in this experiment and those of four more selected genes were shown in Table 4.5.

Testing the efficiency of primers by using PCR

All primers were experimentally tested in an Eppendorf gradient cycler to determine the optimal annealing temperature and to ensure that only one band of correct size was produced. The example to optimize the annealing temperature of transferrin (Trf) gene was shown in Chapter III (Figure 3.7). The optimized annealing temperature of each primer was shown in Table 4.5.

Table 4.5 Primer sequences, product size and annealing temperature of each gene used in qRT-PCR assay.

Primer Name*	GenBank Acc.	Sequence 5′ to 3′	Product size (bp)	Annealing temp (°C)
RpS8 – F (Ref.) RpS8 – R (Ref.)	AF080430	ACGAGGTGCGAAACTGACTGA GCACTGTCCAGGTCTACTCGA	175	60
NOS – F NOS – R	GroupUn473	GACAAAGCTATGATAGAAGCTAA CCATAGCCAATCAGCTGGACATCCA	156	60
ArgK – F ArgK – R	AF023619	GTGCCTCGAGGAGTAACTCA GCGTAAATGCCAACGCCAGA	267	60
OctR – F OctR – R	CAD67999	AGCTCACTCAAGTCACCGAGGA GCGAATGCACAGTCGGTTCTGA	480	60
JHBP – F JHBP – R	AY736135	CGTCTTGACAACAATGTTGATGA CCAATGGCTCGATCGGCAGAA	182	60

Analysis of the selected genes expression in the worker abdomens following the carbon dioxide treatment using qRT-PCR

The expression level of the eight candidate genes transcript in the carbon dioxide treated workers was examined by quantitative real-time PCR. The cDNA of each control and carbon dioxide treated workers were prepared from the pooled total RNA of eight individuals. The ribosomal protein S8 (RpS8) gene was used as an internal control.

The quantitation curve and Ct value that obtained from qRT-PCR could be told the differentially expression between control and CO_2 treated workers. From this step, two candidate genes (tranferrin: Trf; and vitellogenin: Vit) that had differentially expressed were selected to calculate the relative expression ratio (fold changes) of control and CO_2 treated workers as mention in Chapter III.

Determination of PCR efficiency

The role and method to calculate PCR efficiency of each primer was previously described in Chapter III (3.3.4.6). The amplification of serially diluted cDNA was repeated done for 3 times (Appendix B) and the average of real-time PCR efficiency of each primer was further used to calculate the relative expression ratio (fold changes). The real-time PCR efficiency of each primer was done with the primer of reference gene (RpS8) every time of each amplification because they had the individual variation among each amplification. The melting temperatures of PCR products and real-time PCR efficiencies of each primer were represented in Table 4.6 and 4.7.

The genes that had differentially expressed between control and CO_2 treated workers were transferrin (Trf) and vitellogenin (Vit) gene. The amplification of serially diluted cDNA with Trf/RpS8 primer and Vit/RpS8 primer were done 3 times. To calculate the PCR efficiency, the graphs were plotted in log scale that were shown in Appendix B. The average PCR efficiency and the melting temperature of transferrin and vitellogenin genes were represented in Table 4.6 and Table 4.7 respectively.

The amplification of serially diluted cDNA was electrophoreticed in 1.0% agarose gel to make sure again that it was amplified the only one band of expected size (Figure 4.5).

Data analysis of real-time RT-PCR

The mRNA expression level of differentially expressed genes (transferrin: Trf, and vitellogenin: Vit) was examined by normalizing the value of the threshold cycles or Ct values of the carbon dioxide treated workers with control workers (without treated with CO_2). The method to calculate the relative expression ratio was described in Chapter III (3.2.8.9).

The Real-time PCR efficiency of each primer that previously calculated in Table 4.6 was used to calculate the relative expression ratio or fold changes of the transferrin gene in carbon dioxide treated workers relative to non-treated workers. The amplification of transferrin gene with the control and CO_2 treated workers cDNA was repeated done for 2 times and every time of amplification was carried out in triplicate.

Table 4.6 The melting temperature (T_m) of PCR product and real-time PCR efficiencies of each primer (Trf/RpS8) for carbon dioxide treated experiment (abdomen part).

Gene name	slope	PCR Efficiency	Average PCR Efficiency*	T_m (°C)
Tranferrin *(Trf)*	-3.40498	1.966472	1.935234	86.0
	-3.52124	1.923050		
	-3.54062	1.916181		
Ribosomal protein *S8 (RpS8)*	-3.07278	2.115621	1.891729	83.3
	-3.95586	1.789738		
	-4.03338	1.769829		

* The numbers in this column were used to calculate the relative expression ratios (fold changes) of transferrin gene between control and CO_2 treated workers.

Table 4.7 The melting temperature (T_m) of PCR products and real-time PCR efficiencies of each primer (Vit/RpS8) for carbon dioxide treated experiment (abdomen part).

Gene name	slope	PCR Efficiency	Average PCR Efficiency*	T_m (°C)
Vitellogenin *(Vit)*	-3.36207	1.983517	1.938798	87.5
	-3.58492	1.900844		
	-3.49633	1.932032		
Ribosomal protein *S8 (RpS8)*	-3.74547	1.849221	1.772237	83.3
	-4.39186	1.68925		
	-4.00016	1.778239		

* The numbers in this column were used to calculate the relative expression ratios (fold changes) of vitellogenin gene between control and CO_2 treated workers.
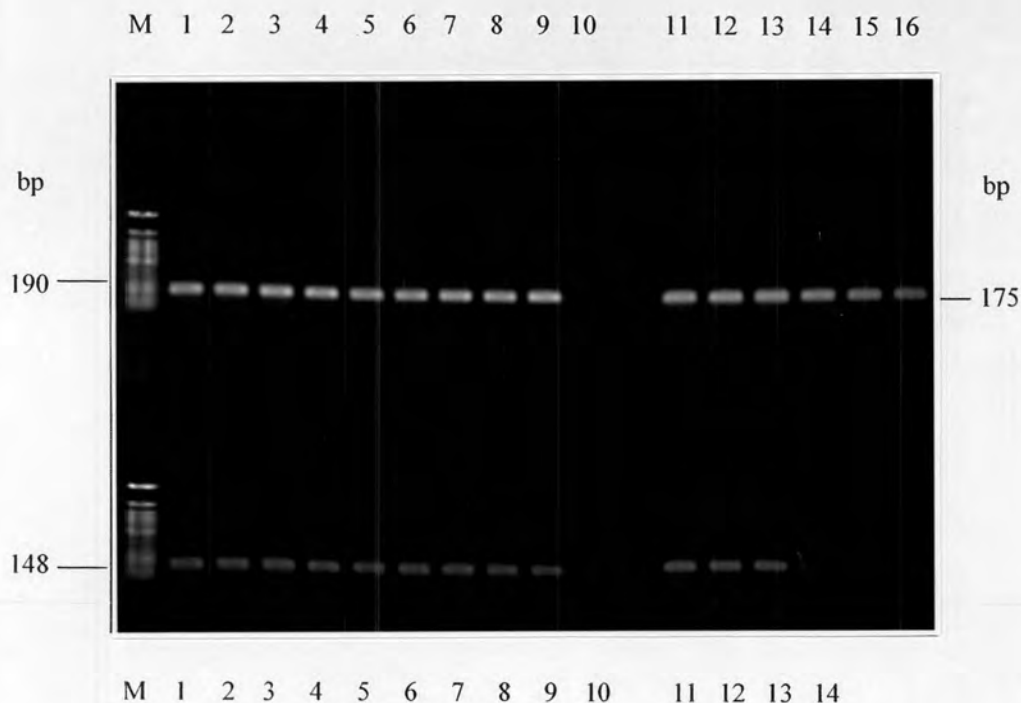
Figure 4.5 The amplification of serially diluted cDNA with candidate genes, transferring [Trf] (190 bp) and vitellogenin [Vit] (148 bp) primers and reference gene, ribosomal protein S8 [RpS8] (175 bp) primers.

Upper

Lane M = Marker 50 bp DNA Ladder

Lane 1-3 = 1X cDNA with Trf primer

Lane 4-6 = 4X cDNA with Trf primer

Lane 7-9 = 16X cDNA with Trf primer

Lane 10 = No cDNA with Trf primer

Lane 11-13 = 1X cDNA with RpS8 primer

Lane 14-16 = 4X cDNA with RpS8 primer

Lower

Lane M = Marker 50 bp DNA Ladder

Lane 1-3 = 1X cDNA with Vit primer

Lane 4-6 = 4X cDNA with Vit primer

Lane 7-9 = 16X cDNA with Vit primer

Lane 10 = No cDNA with Vit primer

Lane 11-13 = 16X cDNA with RpS8 primer

Lane 14 = No cDNA with RpS8 primer

The products of amplification were electrophoreticed in 1.0% agarose gel to make sure that they were amplified the only one band of expected size (Figure 4.6). The fold changes of transferrin gene in each amplification were shown in Table 4.8. The Real-time PCR efficiency of vitellogenin gene that shown in Table 4.7 was used to calculate the relative expression ratio or fold changes of the vitellogenin gene in carbon dioxide treated workers relative to non-treated workers. The amplification of vitellogenin gene with the control and CO_2 treated workers cDNA was repeated done for 3 times and every time of amplification was carried out in triplicate. The fold changes of vitellogenin gene in each amplification were shown in Table 4.8. The relative expression in the abdomen of CO_2 treated workers relative to control workers of eight candidate genes were represented in Table 5.9 and plotted the graph as shown in Figure 4.7. The relative expression ratios were converted to percent change whereby a value of 100% equals no change.
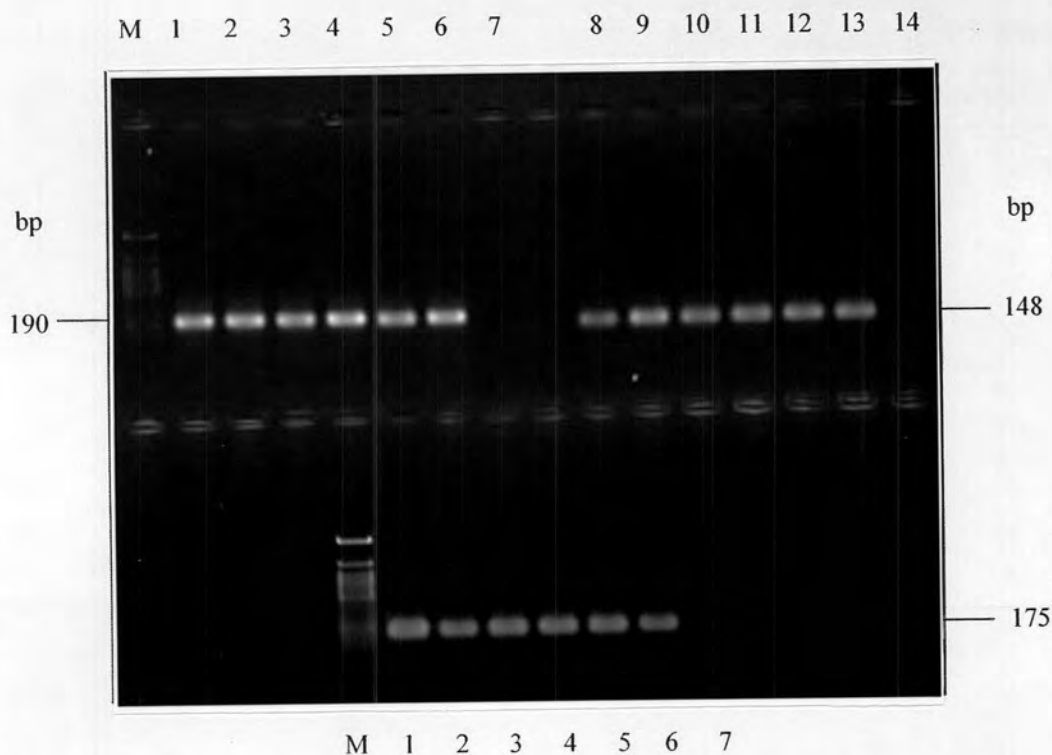
Figure 4.6 The amplification of control and CO_2 treated workers cDNA with candidate genes, Trf (190 bp) and Vit (148 bp) primers and reference gene RpS8 (175 bp) primers.

Upper

Lane M = Marker 50 bp DNA Ladder

Lane 1-3 = Treated CO_2 cDNA with Trf primer

Lane 4-6 = Control cDNA with Trf primer

Lane 7 = No cDNA with Trf primer

Lane 8-10 = Treated CO_2 cDNA with Vit primer

Lane 11-13 = Control cDNA with Vit primer

Lane 14 = No cDNA with Vit primer

Lower

Lane M = Marker 50 bp DNA Ladder

Lane 1-3 = Treated CO_2 cDNA with RpS8 primer

Lane 4-6 = Control cDNA with RpS8 primer

Lane 7 = No cDNA with RpS8 primer

Table 4.8 The expression in the abdominal tissue of Transferrin and Vitellogenin genes in CO_2 treated workers relative to control workers.

Gene name	Up or Down	Relative expression factor	Average expression factor
Transferrin (Trf)	Down	4.924272	4.599496
		4.274720	
Vitellogenin (Vit)	Down	4.412494	3.994936
		3.457670	
		4.114643	

Table 4.9 The expression in the workers' abdomen of eight candidate genes in CO_2 treated workers relative to control workers.

Gene name	Up or Down	Expression factor	% of expression*
Profilin (Prf)	–	–	100
Flotillin (Flt)	–	–	100
Transferrin (Trf)	Down	4.599496	21.74
Vitellogenin (Vit)	Down	3.994936	25.03
Nitric oxide synthase (NOS)	–	–	100
Arginine kinase (ArgK)	–	–	100
Octopamine receptor (OctR)	–	–	100
Take-out-like carrier protein (JHBP)	–	–	100

* Relative expression ratios (expression factor) were converted to percent change whereby a value of 100% equals not significantly changes.
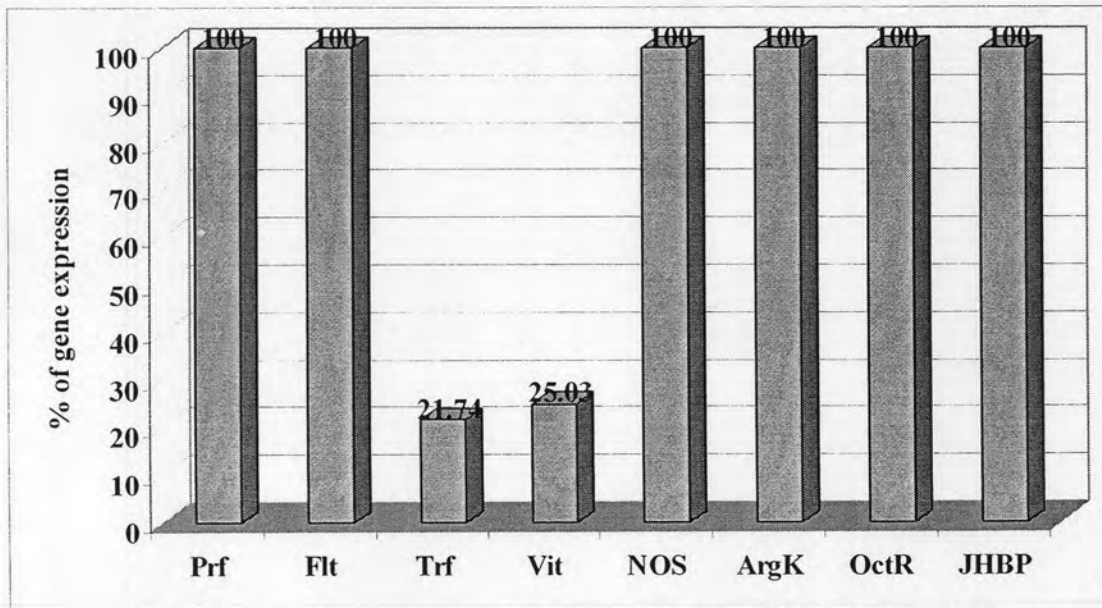
Figure 4.7 Relative expression in the abdominal tissue of CO_2 treated workers

relative to control workers.

Prf = profilin

Flt = flotillin

Trf = transferrin

Vit = vitellogenin

NOS = nitric oxide synthase

ArgK = arginine kinase

OctR = octopamine receptor

JHBP = take-out-like (juvenile hormone-binding protein)

" Head Part "

Total RNA and mRNA extraction

The head part of the same workers that selected for testing in the abdomen part were extracted the total RNA following the Agilent mini kit protocol. The integrity and purity of RNA preps were analyzed by formaldehyde-agarose gel electrophoresis as shown in Figure 4.8 and the ratio of absorbance at the wavelength of 260 nm to 280 nm were measured. The amount of pooled total RNA from 8 CO_2 treated workers and 8 control workers were 35 μg and 44 μg, respectively. Then, the pooled total RNA were purified with the Dynabeads Oligo (dT)$_{25}$ and subsequently treated with RNase Free DNase I for further first-stranded cDNA synthesis. The RNA solutions were kept at -80 °C until used.

Quantitative Real-Time PCR (qRT-PCR)

Analysis of the selected genes expression in the worker heads following the carbon dioxide treatment using qRT-PCR

This carbon dioxide treated experiment in the head part was quantified with the Rotorgene 3000 Thermal Cycler (Corbett Research, Sydney, Australia). The expression level of the four candidate genes profilin (Prf), flotillin (Flt), transferrin (Trf), and vitellogenin (Vit) transcript in the carbon dioxide treated workers was examined by quantitative real-time PCR as those described above for worker abdomens. The cDNA of each control and CO_2 treated workers were prepared from the pooled mRNA of eight heads. The ribosomal protein S8 gene (RpS8) was used as an internal control.

Figure 4.8 The total RNA extracted from the head of selected worker bees

using Agilent mini kit analyzed by formaldehyde-agarose gel electrophoresis.

Lane 1 = Total RNA from CO_2 treated workers

Lane 2 = Total RNA from control workers

The quantitation curve and Ct value that obtained from qRT-PCR showed that only two candidate genes (tranferrin: Trf; and vitellogenin: Vit) had differentially expression between head of control and CO_2 treated workers.

Determination of PCR efficiency

The role and method to calculate PCR efficiency of each primer was previously described in Chapter III (3.3.4.6). The amplification of serially diluted cDNA was repeated done for 2 times for both Trf/RpS8 and Vit/RpS8 as showed in Appendix B. The average real-time PCR efficiency was further used to calculate the relative expression ratio (fold changes). The melting temperatures and real-time PCR efficiencies of transferrin [Trf] and vitellogenin [Vit] primers were represented in Table 4.10 and Table 4.11, respectively.

The amplification of serially diluted cDNA was electrophoreticed in 1.0% agarose gel with 100 volts to make sure again that it was amplified the only one band of expected size same as in the abdomen part.

Data analysis of real-time RT-PCR

The Real-time PCR efficiency of each primer that previously calculated in Table 4.10 and Table 4.11 were used to calculate the relative expression ratio or fold changes of the transferrin and vitellogenin gene in carbon dioxide treated workers relative to non-treated workers. The amplification of these two genes with the control and CO_2 treated workers cDNA was repeated done for 2 times and every time of amplification was carried out in triplicate. The products of amplification were electrophoreticed in 1.0% agarose gel to make sure that they were amplified

the only one band of expected size same as in abdomen part. The result of fold changes of transferrin and vitellogenin genes in each amplification were shown in Table 4.12. The relative expression in the head of CO_2 treated workers relative to control workers of four candidate genes were represented in Table 4.13 and Figure 4.9. The relative expression ratios were converted to percent change whereby a value of 100% equals no change. Both transferrin and vitellogenin genes were up-regulated (~3 fold) in the head part of CO_2 treated workers relative to those of control workers.

Table 4.10 The melting temperature (T_m) and real-time PCR efficiencies of each primer (Trf/RpS8) for carbon dioxide treated experiment (head part).

Gene name	slope	PCR Efficiency	Average PCR Efficiency*	T_m (°C)
Tranferrin	-3.94756	1.791931		
(Trf)	-3.51847	1.924042	1.857986	86.0
Ribosomal protein	-3.69565	1.864613		
S8 (RpS8)	-3.56878	1.906373	1.885493	83.3

* The numbers in this column were used to calculate the relative expression ratio (fold changes) of transferrin gene between control and CO_2 treated workers.

Table 4.11 The melting temperature (T_m) and real-time PCR efficiencies of each primer (Vit/RpS8) for carbon dioxide treated experiment (head part).

Gene name	slope	PCR Efficiency	Average PCR Efficiency*	T_m (°C)
Vitellogenin	-2.99804	2.155516		
(Vit)	-3.77039	1.841725	1.998620	87.5
Ribosomal protein	-3.05895	2.122804		
S8 (RpS8)	1.841725	1.869866	1.996335	83.3

* The numbers in this column were used to calculate the relative expression ratio (fold changes) of vitellogenin gene between control and CO_2 treated workers.

Table 4.12 The expression in the head of Transferrin and Vitellogenin genes in CO_2 treated workers relative to control workers.

Gene name	Up or Down	Relative expression factor	Average expression factor
Transferrin (Trf)	Up	3.107164	3.070791
		3.034418	
Vitellogenin (Vit)	Up	3.534040	3.649049
		3.764058	

Table 4.13 The expression in the workers' head of four candidate genes in CO_2 treated workers relative to control workers.

Gene name	Up or Down	Expression factor	% of expression*
Profilin (Prf)	–	–	100
Flotillin (Flt)	–	–	100
Transferrin (Trf)	Up	3.070791	307
Vitellogenin (Vit)	Up	3.649049	365

* Relative expression ratios (expression factor) were converted to percent change whereby a value of 100% equals not significantly changes.
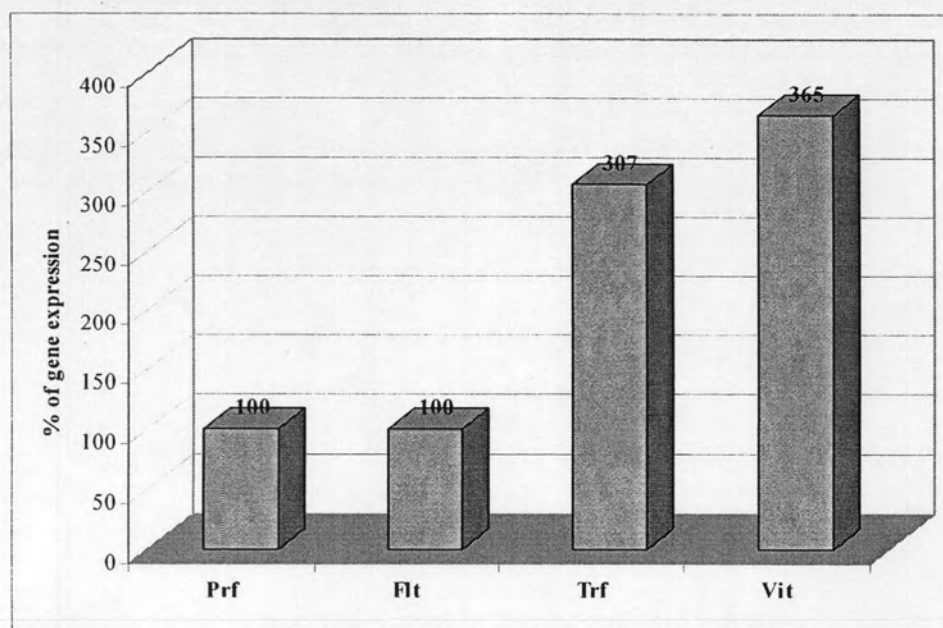
Figure 4.9 Relative expression in the head of CO_2 treated workers relative to

control workers.

Prf = profilin

Flt = flotillin

Trf = transferrin

Vit = vitellogenin

DISCUSSION

In an effort to uncover genes associated with ovary activation in worker honey bees, we examined the extent to which eight candidate genes co-varied in their expression with experimentally induced changes in worker reproductive state. We show that, as with virgin queens, workers respond to double CO_2 exposure. However, unlike queens, carbon dioxide narcosis appears to retard rather than accelerate worker ovary activation. We also demonstrate downward, not upward, expression of selected genes which are implicated in ovary activation in queens. Namely, vitellogenin and transferrin are significantly down-regulated in carbon dioxide treated workers. These results suggested constrasting effects of carbon dioxide on queen versus worker reproductive regulation, and further suggested that both transferrin and vitellogenin are integral to ovary activation in workers, and thus are potentially important to the regulatory control of functional sterility in honey bees.

Our cage based experiments are motivated by Mackensen's (1947) discovery that double CO_2 narcosis of caged virgin queens results in ovary activation and egg laying. In the absence of mating or carbon dioxide narcosis, queen honey bees will not activate their ovaries or initiate oviposition for many weeks, if ever (personal observations of Oldroyd, B.P.). Because the double carbon dioxide treatment affects ovary activation in queens (Engels *et al.*, 1976; Engels and Ramamurty, 1976), we speculated that it might also affect ovary activation in workers. We showed via control groups that queenless caged workers do activate their ovaries, as in queenless colonies, whereas caged groups exposed to double carbon dioxide narcosis do not. This was true regardless of whether workers were narcotized early (on days 2 and 3) or slightly later (on days 4 and 5) (Figure 5.1 and 5.2) in adult life. Thus, the present

study confirms that carbon dioxide narcosis does (negatively) affect ovary activation in workers (Biedermann, 1964; Harris and Harbo, 1990; Harris et al., 1996; Kropácová et al., 1968) and suggests that it is an experimentally useful technique for genetic studies on worker sterility. However, our studied had a different result of ovarian suppression. Harris and Harbo (1990) found that egg production of workers treated with a 15 minutes exposure to CO_2 was significantly lower than control group when bees were queenless ≤ 3 days before narcosis and concluded that CO_2 had no apparent effect after the workers had been queenless for 4 days. But we found that egg production of workers treated with double CO_2 for 10 and 3 minutes in consecutive days was significantly lower than control group even though the bees were queenless at day 4 and 5.

How carbon dioxide affects worker reproduction is not known but there is some evidence to suggest that honey bees are generally sensitive to carbon dioxide and that it is an important exogenous factor regulating several different aspects of social life. Firstly, honey bees are equiped with sensitive carbon dioxide receptors on their antennae (Strange and Diesendorf, 1973), and carbon dioxide is tightly regulated within honey bee colonies (Seeley, 1974). Second, carbon dioxide narcosis is known to affect honey bee foraging behavior (Ebadi et al., 1980), hoarding behavior (Mardan and Rinderer, 1980), fanning behavior (Seeley, 1974), sound production (Schneider and Gary, 1984), and some age-related polyethisms (Heran, 1952; Ribbands, 1950), suggesting that carbon dioxide may infact be an important factor in the modulation of honey bee task specialization. Third, note that honey bee queens are kin-selected for extremely high fecundity and react to narcosis by accelerating reproductive development (Engels et al., 1976; Engels and Ramamurty, 1976; Mackensen, 1947), whereas workers are selected for low fecundity or sterility and react to narcosis by

retarding reproductive development (Biedermann 1964: Harris and Harbo, 1990; Harris *et al.*, 1996; Kropácová *et al.*, 1968; present study). This contrasting effect of carbon dioxide on queen versus worker reproduction suggests a caste-specific response to carbon dioxide and, more generally, it suggests that carbon dioxide directly stimulates the regulatory mechanism underpinning honey bee reproductive division of labor.

One possibility is that exposure to carbon dioxide affects the level of neurosecretions in worker brains (Harris *et al.*, 1996). Levels of dopamine and seratonin, for example, co-vary with changes in worker ovarian development (Harris and Woodring, 1995) and even with carbon dioxide induced changes in ovarian development (Harris *et al.*, 1996). It is possible therefore that carbon dioxide triggers the regulatory mechanism that controls worker sterility by affecting, for example, the level of dopamine or seratonin, which are putative up-stream components in the regulatory pathway that controls ovary activation in workers. A second possibility is that carbon dioxide affects the titer of juvenile hormone in the haemolymph (Bühler *et al.*, 1983), which in turn affects age related behavior in workers, possibly including reproduction (Robinson *et al.*, 1991). Note that, unlike for insects generally, juvenile hormone has an inverse relationship with vitellogenin in honey bee workers: high juvenile hormone titer turns off vitellogenin synthesis (Pinto *et al.*, 2000). Thus, juvenile hormone and vitellogenin are causally linked, and carbon dioxide may stimulate the regulatory mechanism that normally controls worker sterility by causing an increase in juvenile hormone, which causes a decrease in vitellogenin, which presumably retards ovary activation as observed.

Whatever the initial changes narcosis causes to the nervous system that ultimately gives rise to observed differences in ovary activation, we can infer down

stream regulatory components via their differential expression on ovary activation or deactivation, in particular as a consequence of carbon dioxide treatment. This will give a first indication of molecules important to the maintenance of worker sterility.

Hundreds of genes with often poorly understood functions participate in building the ovaries, an organ required for both production and delivery of eggs. In addition, this process involves a hierarchy of complex events, some of which are not directly associated with the synthesis of ovarian tissue *per se*. For example, oocyte development depends on the synthetic activities of other, nonoocytic cells. Indeed, for most animals yolk is synthesized outside of the ovary and secondarily imported into the oocyte. Within the ovaries, oocytes take up the yolk proteins, such as vitellogenin, from the extracellular fluid by receptor mediated endocytosis. Similarly, transferrin mediates iron-uptake into developing eggs. By contrast, genes encoding proteins that are essential for the regulation of the actin cytoskeleton (i.e. flotillin) or linking the cytoskeleton with major signalling pathways (i.e. profilin) are expressed in the ovarian tissue and are tightly regulated during development (Cooley *et al.*, 1992).

Of eight candidate genes examined from selected worker bees, six did not change their expression with changes in reproductive state. Genes indifferentially expressed encoded profilin, an intercellular cytoplasm transport molecule required during *Drosophila* oogenesis (Cooley *et al.*, 1992), flotillin, a co-regulator of actin cytoskeleton formation (Haglund *et al.*, 2004), nitric oxide synthase, an enzyme responsive to oxygen related hypoxia (Nilsson *et al.*, 2004), arginine kinase, an enzyme implicated in energy transfer (Kucharski and Maleszka, 1998), octopamine receptor, a binder of biogenic amines and neuromodulator of many physiological processes (Grohmann *et al.*, 2003), and take-out-like carrier protein *(Kolodziejczyk et al.*, 2003), containing a juvenile hormone binding motif. These genes clearly fall into

the category of developmental regulators. Either these genes are not differentially expressed between carbon dioxide treated and control worker groups, or their differential expression was not detected by our assay, conducted 48 hours following the day 4/5 treatment.

Two genes, vitellogenin and transferrin, did show significantly differences in expression (~ 4 fold; Table 5.8, Figure 5.11) within two days of the double CO_2 treatment. For vitellogenin this response is temporally consistent with previous observations on like-treated virgin queens (Engels *et al.*, 1976). Unlike queens, however, the change in worker vitellogenin expression was not conditional on bees being re-introduced into nuclear colonies (Engels *et al.*, 1976). Instead, workers responded to carbon dioxide as they matured within cages, albeit as a group member rather than as lone individuals.

More notably, the direction of the transcriptional response is opposite to that known for queens: workers decrease, rather than increase, vitellogenin synthesis following carbon dioxide narcosis. This contrasting pattern of transcription in queens versus workers in response to carbon dioxide parallels the physiological pattern concerning ovary activation, and again accentuates the kin-selected difference in fecundity between these two castes.

Vitellogenin, a zinc-binding protein sequestered by developing oocytes, is in honey bees itself under social and hormonal control (Pinto *et al.*, 2000). Besides its proximate importance to egg production, however, vitellogenin may have played a more ultimate role in the social evolution of honey bees (Amdam *et al.*, 2004). Vitellogenin is therefore expected to be closely associated with colony level traits such as alloparental care or reproductive division of labor (Amdam *et al.*, 2003). Our finding that vitellogenin is down-regulated in ovary deactivated workers implies that

this molecule is actively linked to the reproductive status of individual workers and, together with available knowledge from queens, suggests that there is a general link between carbon dioxide, vitellogenin, and ovary activation in honey bees. Vitellogenin is therefore a new candidate component in the regulatory pathway that controls functional sterility in workers.

The relationship between transferrin and ovary activation is less clear because transferrin is multifunctional in the honey bee (Kucharski and Maleszka, 2003). However, its co-regulation with vitellogenin in the current study, and it's selective incorporation into eggs during oogenesis of *Sarcophaga* (Kurama *et al.*, 1995) and *Riptortus* (Hirai *et al.*, 2000), suggests that transferrin can have a vitellogenic role. Transferrins likely function to provide essential iron ions to developing oocytes and embryos (Hirai *et al.*, 2000), and may also play a defensive role by sequestering iron away from pathogens that have entered the egg (Weinberg, 1984). Our finding that transferrin is down-regulated in ovary deactivated workers implies that, like vitellogenin, it is intimately associated with ovary activation in honey bee workers. If transferrin is vital for ovary activation and egg development in honey bees, then transferrin may also be important to the regulation of worker sterility.

In contrast of the abdomen part, the head part of carbon dioxide treated workers had more expression of transferrin and vitellogenin genes (~ 3-4 fold) when compared with the control group. The hypopharyngeal glands, which known to be the organ where royal jelly brood food are produced, locate in the head part of the honey bees. Amdam and colleagues (2003) suggested that vitellogenin is involved in brood food (royal jelly) production. From this evidences, the expression of vitellogenin gene in the head part of nurse bee (5-15 days old worker bee) is not surprisingly. As the incorporation of vitellogenin to the hypopharyngeal glands almost reduce as the

worker becomes a forager (Crailsheim, 1991; Amdam and Omholt, 2002) and the control worker (had the ovarian activation) tend to no longer had the nurse tasks (tend to be a forager behavior), the synthesize of vitellogenin also the vitellogenin expression in the head part of CO_2 treated worker was much more than those of non-treated worker (control).

Another possibility is the necessity of the vitellogenin consumption in to the abdomen part for ovarian development may be the result of the decrease in vitellogenin synthesis (expression of vitellogenin) in the head of ovary developed workers.

As described in the abdomen part, transferrin had multifunctional roles in insects: as an iron binding protein, an antibiotic agent, a vitellogenic protein, and a protein repressible by juvenile hormone (Nichol et al., 2002). Iron is one of the essential elements required by all organisms, but it is also a potent toxin because of its ability to produce free radicals in the presence of oxygen (Crichton et al., 2002; Nichol et al., 2002). In some tissues, such as brain or retina, anti-oxidative defenses are relatively low and oxygen consumption is very high (Crichton et al., 2002). Our results show that the CO_2 treated workers had more expressed of transferrin gene than in group of non-treated. The worker bees that had not expose with CO_2 (control group) had more expose to O_2, so they might be decreased the expression of transferrin (an iron-binding protein) to reduce the uptake or transport of iron that may be toxin to the cells (produce radicals) if it has much more than necessity.

Both vitellogenin and transferrin had multifunctional roles in the honey bees, so they can be flow to any part of the body in any period of age to function in the right organ and right time.