CHAPTER V

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

5.1 INTRODUCTION

The honey bee is the only organism for which identities have been proposed for any pheromone that regulates reproduction. Honey bee queens are endowed with caste-specific pheromones, including the mandibular glands (Slessor *et al.*, 1988), Dufour's gland (Katzav-Gozansky *et al.*, 1997), tergal glands (Wossler and Crewe, 1999a) and queen faeces (Page *et al.*, 1988). (E)-9-oxodec-2-enoic acid (9-ODA), one component of the queen mandibular gland pheromone (QMP), was first suggested to be a major ovary-regulating primer pheromone produced by the queen (Butler and Fairey, 1963). Partial inhibition of worker ovary development by queen heads or mandibular gland components has been demonstrated to varying degrees (de Groot and Voogd, 1954; Verheijen-Voogd, 1959; Butler, 1959; Butler and Fairey, 1963; Velthuis, 1970a; Lin, 1999). In addition, a queen abdomen alone or a queen without mandibular glands can also inhibit worker ovary development (Velthuis and van Es, 1964; Velthuis, 1970b). Extracts of whole body washes of queens have produced the most complete inhibition (Butler, 1957; Verheijen-Voogd, 1959), suggesting a second queen source of inhibitory pheromones (Winston and Slessor, 1998). Hoover *et al.*

(2003) demonstrated that QMP inhibits ovary development in worker bees, and are significant in resolving a long-standing controversy concerning the identity of the queen pheromone components involved in regulating this important process. They also found no significant difference between QMP and queen-extract treatments. QMP alone is responsible for the queen component of worker ovary inhibition, there is no additive or synergistic effects of QMP with other queen compounds to inhibit ovary development in European honey bees.

Workers that remained in their natal colony had less developed ovaries than did workers in all caged treatments, including queen extract. This difference is likely to be due to a combination of factors, including the larger group size (Lin *et al.*, 1999), exposure to ovary-inhibiting larval esters (Mohammedi *et al.*, 1998), and probable poorer nutrition of bees in colonies. The esters ethyl palmitate and methyl linolenate produced by worker larvae also regulate ovary development (Mohammedi *et al.*, 1998; Lin, 1999) when fed to workers.

While the regulation of worker honey bee ovary development may seem overly complex, involving both queen and brood, redundancy built into pheromone-based signaling systems may be an important underlying principle in sociochemical systems. In case of the honey bee, queen pheromones that regulate ovary development would be essential when no brood was present in the colony, such as during natural periods of dearth, winter, or queen replacement (Hoover *et al.*, 2003). Their study demonstrated the large amount of variability among colonies, even within a single apiary. There also is a large amount of genetic variation in the worker retinue response to different compounds of QMP, and this is probably also true for its ovary inhibiting effect. QMP plays an important role in regulating worker reproduction in honey bee colonies. But from all of these studies it is clear that QMP is not as

effective as a living queen. Moreover, another queen-specific pheromone produced by the tergal glands and inhibiting worker reproduction in small worker groups was recently discovered in *Apis mellifera capensis* (Wossler and Crewe, 1999b). Although it has not been tested in full-scale colonies, this suggests, along with the incomplete effect of QMP, that the queen may possess additional pheromones that act in concert to regulate worker physiology and behavior. It also suggests that the queen advertises her presence in additional ways to pheromone emission (Katzav-Gozansky *et al.*, 2004).

Brood pheromone is a blend of ten fatty-acid esters found on the cuticles of honey bee larvae. It was first identified as a kairomone that attracts the parasitic mite, *Varroa jacobsoni* (Le Conte *et al.*, 1989). These ten fatty-acid esters were found on bees during the fourth and fifth days of larval development (Trouiller *et al.*, 1992): methyl palmitate, methyl oleate, methyl stearate, methyl linoleate, methyl linolenate, ethyl palmitate, ethyl oleate, ethyl stearate, ethyl linoleate, ethyl linolenate. Later, it was found that some components of this blend have releaser-like effects on various aspects of brood care (Le Conte *et al.*, 1990, 1994, 1995). Some components are more active than others, but all ten individual compounds show some releaser activity, leading to their being called, collectively, brood pheromone. Brood pheromone also inhibits ovary development in worker honey bees, indicating a primer effect, which may be involved in the regulation of reproductive division of labor (Mohammedi *et al.*, 1998).

Two components of brood pheromone, methyl palmitate and ethyl oleate, were shown in laboratory studies (Mohammedi *et al.*, 1996) to increase the activity of the hypopharyngeal glands, which produce proteinaceous material that is fed by nurse bees to larvae. Differences in the activity of the hypopharyngeal glands are associated

with honey bee division of labor (Robinson, 1992) suggesting that brood pheromone might also act as a primer pheromone in division of labor among worker bees.

Additionally, quantitative real-time PCR analysis of the transcript expression levels of a selected set of eight genes associated with the activation of ovaries in the abdomen of 15 day old queenless worker bees with developed (including oocytes) and non-developed ovaries due to carbon dioxide narcosis, revealed an increased expression level of vitellogenin (Vit) and transferrin (Trf) transcripts, but not profilin, flotillin, nitric oxide synthase, arginine kinase, octopamine receptor and take-out-like carrier protein in workers with activated ovary development (Chapter V). This current study extends and tests the observation of increased Trf and Vit gene transcript levels by analyzing the same set of eight genes (Chapter V) and ten more candidate genes, to another group of sterile worker bees without activated ovaries avoiding carbon dioxide narcosis by analyzing workers from queenright colonies (workers without activated ovaries) compared them to those from a queenless colony with different stages of ovarian development.

5.2 MATERIALS AND METHODS

5.2.1 Honey bee samples

The European honey bees (*Apis mellifera* L.) that were used in this study came from two places:-

- School of Biological Sciences, University of Sydney, Australia (Appendix A)
- Purchased from commercial farm (Farm Supa), Chiang Mai, Thailand

5.2.2 Preparation of worker bees with different stages of ovarian development

This experiment was done in the colonies that are the natural in-hive conditions. The queenright colonies (colonies with queen which produced queen pheromones) represented the bees with pheromone treatment. Whereas, the queenless colonies (colonies without queen and queen pheromones) represented the bees with non-treated pheromone.

The preparation of queenless colony was prepared by removing of the queen from the hive. After two weeks, the trace of queen pheromones disappeared (Robinson *et al.*, 1990), then this hive was ready for setting as the queenless colony.

The emerging sealed brood from wild type colonies was incubated at 35°C overnight (Appendix A). The following morning, newly-emerged workers were marked at the thorax (with difference colors) and then transferred into queenright (with pheromone) and queenless (without pheromone) colonies (Appendix A).

5.2.3 Collection and selection the samples

5.2.3.1 *Sampling techniques*

For queenright and queenless workers, after 2, 4, 6, 10, 15, and 21 days, groups of 20 bees were collected from the colonies 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).

5.2.3.2 *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 queenright and queenless workers were selected for RNA extraction step.

After scoring the stage of ovarian development of each bees, the group of queenright worker bees (exposed with pheromone) and those of queenless worker bees (not exposed with pheromone) were selected to extract the total RNA. Eight workers from queenright (stage 0 and 1 of ovarian development) and queenless (two each from all stages of ovarian development) colonies at the same age (15 days old) were selected for extracting total RNA from the abdomens.

5.2.4 Total RNA extraction

This experiment used a Trireagent protocol to extract RNA under standard conditions. The frozen abdominal tissues of individual bees were ground with liquid nitrogen in 1.5 ml Eppendorf tubes, and homogenized with 100 µl of Trireagent (Molecular Research Center, Inc., www.mrcgene.com), using the sterilized disposable pestles attached to a hand-held engraving device (Super Tool, Arlec). One abdomen was used per tube. Following the homogenization step the volume was adjusted to 1,000 µl with Trireagent. The homogenates were incubated at room temperature for 5 min to serve complete segregation of nucleoprotein complexes. After incubation, 200 µl chloroform was added and tightly covered, then vigorously shake for 15 seconds. The mixture was then incubated at room temperature for 15 min and centrifuged at 12,000 ×g for 15 min at 4 °C. After centrifugation, the collected upper aqueous phase containing total RNA was transferred to a new tube. RNA in the aqueous layer was precipitated with 500 µl isopropanol. The mixture was stored at room temperature for 10 min and centrifuged at 12,000 ×g for 8 min at 4 °C. The supernatant was removed and a white RNA pellet was washed twice with 1 ml of 70% (v/v) ethanol ethanol in diethyl pyrocarbonate (DEPC)-treated water and subsequently centrifuged at 7,500 ×g for 5 min at 4 °C. The total RNA was stored in 70% (v/v) ethanol at -80 °C until used.

When total RNA was required, the ethanol supernatant was completely removed by centrifugation at 12,000x*g* 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 sterile water then incubated the solution for 10-15 min at 60 °C to completely dissolve the RNA pellet.

5.2.5 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 the same part (3.2.6).

5.2.6 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).

5.2.7 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 strand cDNA synthesis. The first-strand cDNAs were synthesized using an Omniscript RT (Qaigen, Germany) (Chapter III and IV using SuperScript™ II Reverse Transcriptase kit (Invitrogen)). Template RNA, the primers solution, 10x Buffer RT, dNTP Mix and RNase-free

water was thawed and immediately stored on ice after thawing. A fresh master mix in a final reaction volume of 20 µl containing 1x RT buffer (25 mM Tris-HCl, pH 8.3, 37.2 mM KCl, 1.5 mM $MgCl_2$, 5mM DTT) and 0.5 mM 0f each dNTP, 1 µM Oligo-dT primer, 10 units of RNase inhibitor, and 4 units of Omniscript Reverse Transcriptase was prepared and aliquot into individual reaction tubes on ice. Next, 1 µl of RNA template (2.0 µg) that was diluted from extracted total RNA in DEPC water was added to each reaction tubes. The mixture was mixed thoroughly and carefully by vortexing for no more than 5 seconds. After centrifuged briefly to collect residual liquid from the wall of the tube, reactions were incubated at 37 °C for 60 min following by 93 °C for 5 min. The synthesized first-stranded cDNA was stored at -20 °C until use.

5.2.8 Quantitative Real-Time PCR (qRT-PCR)

5.2.8.1 *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]) that used to examine the expression following carbon dioxide treatment were interested to examine the expression in the queenright and queenless workers again. These eight candidate genes were same as used in Chapter IV (4.2.8.1).

Recently, there were many reports about the genes that may function involved in ovary activation in insects and mammalian. Ten more candidate genes were selected to observe the differential expression between queenright and queenless workers. These ten more candidates are : major royal jelly proteins-1, 3, 5 (MRJP-1, MRJP-3, MRJP-5), which are integral to honey bee reproduction and division of labour (Drapeau *et al.*, 2006); Niemann-Pick type C2 protein (Npc2), a putative component in the gene network that regulates worker sterility through ovary activation (Thompson *et al.*, 2006); ribosomal protein (RpL26), which up-regulated in ovary inactive workers relative to anarchists (Thompson *et al.*, 2006); cGMP-dependent protein kinase (foraging), which is functionally associated with foraging division of labor in workers (Ben-Shahar *et al.*, 2002, Rueppell *et al.*, 2004); phosphoinositolglycan-peptide (PIG-P); tyramine receptor (TyrR); phosphatidylinositol phosphate kinase (PIP5K); and phosphoinositide-3-kinase 68D (PI3K) are primary positional candidates for variation in foraging behavior in honey bee workers (Hunt *et al.*, 2007).

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

The roles of primer design were mentioned in Chapter III (3.2.8.4). The method for selecting the primers to use in quantitative real-time PCR was described in the same part.

5.2.8.3 *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.

5.2.8.4 ***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).

5.2.8.5 ***Analysis of the selected genes expression in the worker abdomens following pheromone treatment using qRT-PCR***

This experiment was conducted in the iCycler iQ™ Real-Time PCR detection system (Bio-Rad, U.S.A.) In Chapter III and IV were quantified with the Rotorgene 3000 Thermal Cycler (Corbett Research, Sydney, Australia) that was difference in machine and the reaction component.

Optimization reaction components and amplification conditions will be required to obtain the maximum efficiency and specificity. Real-time PCR assay was accomplished by SYBR Green I dye detection using the iCycler iQ™ Real-Time PCR detection system (Bio-Rad, U.S.A.). The amplified reactions were done in a 96-well plate containing 10 µl of 2xSYBR Green supermix (Bio-Rad), 1 µl of cDNA, 300 nM of each forward and reverse primer and adjusted the 20 µl final volume with sterile water. The SYBR Green I real-time PCR condition was 94 °C for 8 min followed by 40 cycles of denaturation at 94 °C for 25 seconds, annealing at appropriated temperature of each pair of primers that were shown in Table 5.2 for 30 seconds, and

extension at 72 °C for 30 seconds. Each experiment was carried out in triplicate and fluorescent data were monitored at the end of each extension step. The reactions containing sterile water instead of cDNA template were used as the negative control. At the end of amplification, the specificity of each primer pair was verified by assessing the melting curve of the PCR product. The reactions were analyzed at 95 °C for 1 min and afterwards 50 °C for 1 min, followed by heating 80 cycles of staring at 50 °C with 0.5°C increments for 10 seconds each cycle.

5.2.8.6 ***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).

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

The fluorescent signals of the amplified products were analyzed by the data analysis software of the iCycler iQ™ Real-Time Detection system (Bio-Rad) 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.

5.3 RESULTS

Ovarian development observed from the sample preparation

This experiment was done in the colonies that were the natural in-hive conditions. Groups of 20 bees were collected from both queenright and queenless colonies at different time points (2, 4, 6, 10, 15, and 21 days after emergence). All worker bees were dissected on wax plate that putting on ice to observe the ovarian development under the stereomicroscope. The number of worker bees in each stage of ovarian development was counted and scoring result of these dissected worker bees was shown in Table 5.1.

The data from Table 5.1 was used to plot the 3D bar graph as shown in Figure 5.1. From the result, the workers in queenright colonies (with pheromone) had not developed their ovaries, while some of the workers in queenless colonies (without pheromone) had developed their ovaries at the same age.

Total RNA extraction

After scoring and plotting the graph, the group of queenright worker bees (exposed with pheromone) and group of queenless worker bees (not exposed with pheromone) were selected to extract the total RNA using Trireagent protocol. Eight workers from queenright (stage 0 and 1 of ovarian development) and queenless (two each from all stages of ovarian development) colonies at the same age (15 days old) were selected for extracting total RNA from the abdomens.

Table 5.1 The number of worker bees in each stage of ovarian development of pheromone treatment.

Type of worker honeybee	Collecting time	Number of bees in each stage of ovarian development				
		stage 0	stage 1	stage 2	stage 3	Total
Queenright worker	Day 2	13	7	0	0	20
	Day 4	17	3	0	0	20
	Day 6	14	6	0	0	20
	Day 10	17	3	0	0	20
	Day 15	16	4	0	0	20
	Day 21	18	2	0	0	20
Queenless worker	Day 2	12	8	0	0	20
	Day 4	9	11	0	0	20
	Day 6	9	10	1	0	20
	Day 10	5	12	2	1	20
	Day 15	2	7	5	6	20
	Day 21	2	3	4	11	20

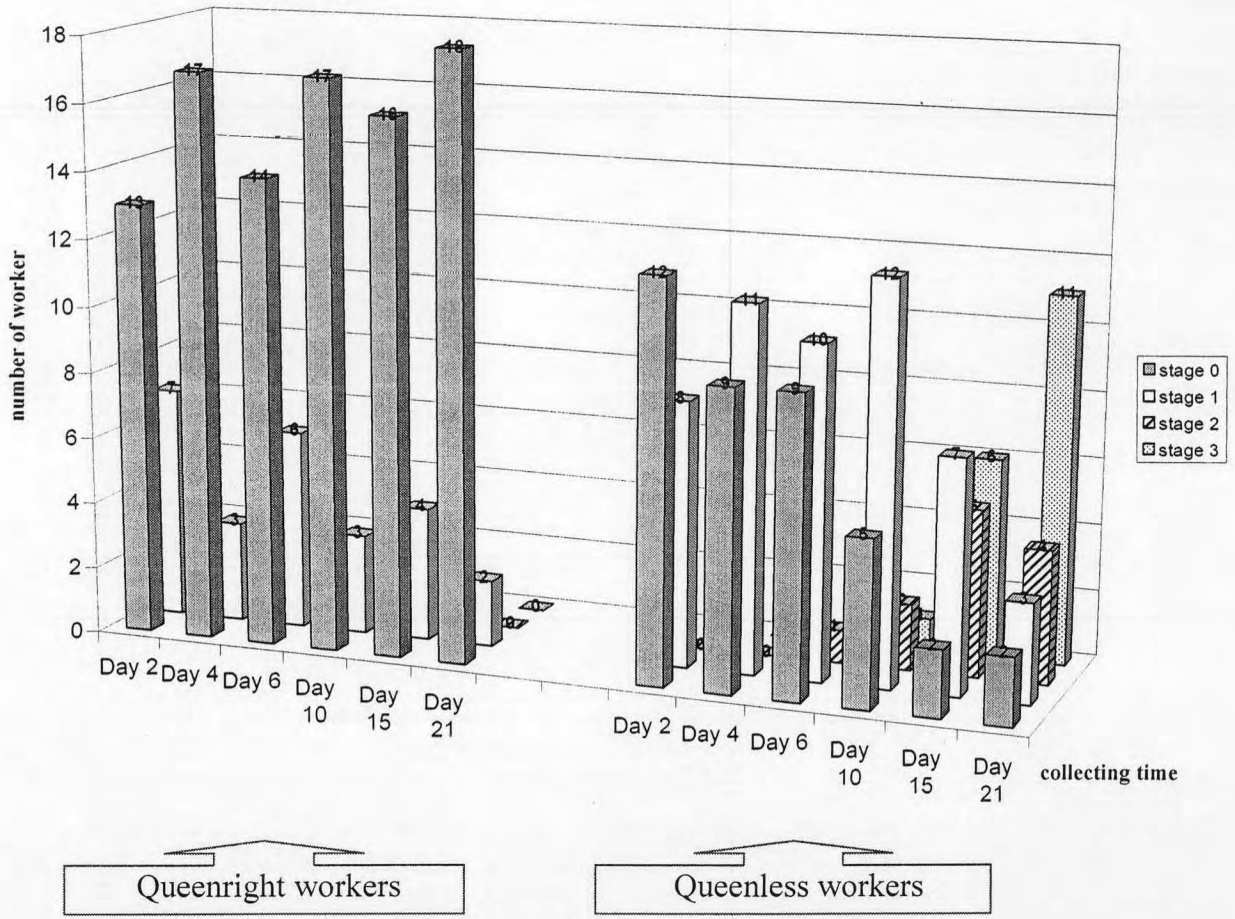

Figure 5.1 Stage of ovarian development in queenright and queenless workers at various times.

The total RNA separately extracted from each of 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 5.2 and the ratio of absorbance at the wavelength of 260 nm and 280 nm were measured. The OD_{260}/OD_{280} ratio of 1.72-1.90 indicated that acceptable quality of extracted RNA was obtained. The amount of pooled total RNA from queenright and queenless workers were 60 μg and 72 μg, respectively. The RNA solutions were kept at -80 °C until used.

Quantitative Real-Time PCR (qRT-PCR)

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

PCR primers and optimum annealing temperature for each selected gene

For each gene, two sets of forward and reverse primers were designed and selected 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 (Figure3.6). The primer sequence and product size of selected eight genes that used in Chapter IV, were using in this experiment and those of ten more selected genes were shown in Table 5.2.

Figure 5.2 The total RNA extracted from the abdomen of selected worker bees using Trireagent protocol analyzed by formaldehyde-agarose gel electrophoresis.

Lane 1 = Total RNA from queenright worker

Lane 2 = Total RNA from queenless worker

Table 5.2 Primer sequences and product size of genes used in qRT-PCR assay.

Primer Name*	ID*	Sequence 5′ to 3′	Product size (bp)	Annealing temp (°C)
RpS8 – F (Ref.)	AF080430**	ACGAGGTGCGAAACTGACTGA	175	60
RpS8 – R (Ref.)		GCACTGTCCAGGTCTACTCGA		
MRJP-1 – F	GB14888	GGCAACATTCTTCGAGGAGAGTC	188	55
MRJP-1 – R		ATCTTATCATGCCATTGGTCA		
MRJP-3 – F	GB16459	CAGCTGTGAATCATCAAAGA	187	55
MRJP-3 – R		CAAATGTCTTATCACGCCATCTGTCC		
MRJP-5 – F	GB10622	GGTATCACAAGCGTCACTGTTCGAG	202	55
MRJP-5 – R		CAAAAGTCATACCACGCCATTGATCG		
Npc2 – F	GB14261	ACGTGAAGATTTTGGGTTGC	175	55
Npc2 – R		TTGCAAGCATTTTGTTCTGG		
RpL26 – F	GB13731	TGCAGAAAAATTGGCACTGA	180	54
RpL26 – R		GCCTAATGCAGCAAGCCTAC		
Foraging – F	GB18394	CGAACTGTCGCCAGCTATTT	216	58
Foraging – R		GGAGTGAGTTGCCTGGTGAT		
PIG-P – F	GB16306	TGGGGATGCATGAATATGTTT	205	54
PIG-P – R		TGCTGGAATGAATTTTGTGC		
TyrR – F	GB17991	GGACAGCGTTTATCCTGCTC	295	55
TyrR – R		ATCAATTACGCCCAGAAACG		
PIP5K – F	GB13779	TCGTGCTGCAGTATCTCCAT	147	58
PIP5K – R		GTCCTTCACCGAGAGTTCCA		
PI3K – F	GB17429	AGAACGAAAGGGTCATGCAG	134	58
PI3K – R		TTCCGACGCTAATACCACTTG		

* The accession number from the Official Predicted Gene Set (BeeBase, http://racerx00.tamu.edu/bee_resources.html).

** GenBank accession number

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 5.2.

Analysis of the selected genes expression in the worker abdomens according to pheromone treatment using qRT-PCR

The expression level of the eighteen candidate genes transcript in the queenright (with queen pheromone) and queenless (absence of queen pheromone) workers were examined by quantitative real-time PCR. The cDNA of each queenright and queenless workers were prepared from the pooled total RNA of eight individuals. The ribosomal protein S8 (RpS8) was used as an internal control.

The quantitation curve and Ct value that obtained from qRT-PCR could be told the differentially expression between queenright and queenless workers. From this step, the candidate genes that had differentially expressed (tranferrin: Trf, vitellogenin: Vit, tyramine receptor: TyrR, and phosphoinositolglycan peptide: PIG-P) were selected to calculate the relative expression ratio (fold changes) of queenright (with queen pheromone) and queenless (absence of queen pheromone) worker bees same 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 genes that had differentially expressed between queenright workers (with queen pheromone) and queenless workers (absence of queen pheromone) were transferrin (Trf), vitellogenin (Vit), tyramine receptor (TyrR), and phosphoinositolglycan peptide (PIG-P) gene. Unfortunately, we did not have enough samples to make an amplification of serially diluted cDNA for calculating the relative expression ratio of TyrR and PIG-P genes between queenright and queenless workers. However, we did the amplification of serially diluted cDNA with Trf/RpS8 primer and Vit/RpS8 primer for 2 times. For TyrR and PIG-P genes, we can tell that they were differentially expressed between queenright and queenless workers and were down-regulated in queenright relative to queenless workers.

The standard curves plotted in log scale were done and used to calculate the Real-time PCR efficiency of transferrin (Trf) primer and vitellogenin (Vit) primer (Appendix B). The slope, melting temperatures and real-time PCR efficiencies of transferrin [Trf] and vitellogenin [Vit] primers were represented in Table 5.3 and Table 5.4, 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 as shown in Chapter IV (Figure 4.5).

Table 5.3 The melting temperature (T_m) of PCR product and real-time PCR efficiencies of each primer (Trf/RpS8) for pheromone treated experiment.

Gene name	From Figure	slope	PCR Efficiency	Average PCR Efficiency*	T_m (°C)
Transferrin (Trf)	3.13(a)	-3.40498	1.966472	1.971937	86.0
	3.13(b)	-3.37730	1.977401		
Ribosomal protein S8 (RpS8)	3.13(a)	-3.07278	2.115621	2.137870	83.3
	3.13(b)	-2.98974	2.160119		

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

Table 5.4 The melting temperature (T_m) of PCR product and real-time PCR efficiencies of each primer (Vit/RpS8) for pheromone treated experiment.

Gene name	From Figure	slope	PCR Efficiency	Average PCR Efficiency*	T_m (°C)
Vitellogenin (Vit)	3.14(a)	-3.70949	1.966472	1.954332	87.5
	3.14(b)	-3.21119	1.977401		
Ribosomal protein S8 (RpS8)	3.14(a)	-3.07278	2.115621	2.130266	83.3
	3.14(b)	-3.01742	2.144912		

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

Data analysis of real-time RT-PCR

The Real-time PCR efficiency of each primer that previously calculated in Table 5.3 and 5.4 were used to calculate the relative expression ratio or fold changes of the transferrin and vitellogenin genes in queenright workers relative to queenless workers. The amplification of transferrin and vitellogenin genes with the queenright and queenless workers cDNA was repeated done for 2 times and every time of amplification was carried out in triplicate. The fold changes of transferrin and vitellogenin genes in each amplification were shown in Table 5.5.

The relative expression in the abdomen of queenright workers relative to queenless workers of eighteen candidate genes were represented in Table 5.6 and Figure 5.3. The relative expression ratios were converted to percent change whereby a value of 100% equals no change. Four of eighteen candidate genes; transferrin, vitellogenin, phosphoinositolglycan-peptide, tyramine receptor; were down-regulated in the abdomen of queenright relative to queenless workers. Transferrin and vitellogenin were down-regulated about 9-fold and 14-fold, respectively in the abdomen of queenright relative to queenless workers.

Table 5.5 The expression in the abdonmen of Transferrin and Vitellogenin genes in queenright workers relative to queenless workers.

Gene name	Up or Down	Relative expression factor	Average Expression factor
Transferrin (trf)	Down	8.435061	9.680028
		10.92499	
Vitellogenin (Vit)	Down	14.74299	14.34948
		13.95597	

Table 5.6 The expression in the workers' abdomen of eighteen candidate genes in queenright workers relative to queenless workers.

Gene name	Up or Down	Expression factor	% of expression*
Profilin (Prf)	–	–	100
Flotillin (Flt)	–	–	100
Transferrin (Trf)	Down	9.680028	10.33
Vitellogenin (Vit)	Down	14.34948	6.97
Nitric oxide synthase (NOS)	–	–	100
Arginine kinase (ArgK)	–	–	100
Octopamine receptor (OctR)	–	–	100
Take-out-like carrier protein (JHBP)	–	–	100
Major royal jelly protein 1 (MRJP-1)	–	–	100
Major royal jelly protein 3 (MRJP-3)	–	–	100
Major royal jelly protein 5 (MRJP-5)	–	–	100
Niemann-Pick type C2 protein (Npc2)	–	–	100
Ribosomal protein (RpL26)	–	–	100
cGMP-dependent protein kinase (foraging)	–	–	100
Phosphoinositolglycan-peptide (PIG-P)	Down	–	<100
Tyramine receptor (TyrR)	Down	–	<100
Phosphatidylinositol phosphate kinase (PIP5K)	–	–	100
Phosphoinositide-3-kinase 68D (PI3K)	–	–	100

* Relative expression ratios (expression factor) were converted to percent change whereby a value of 100% equals not significantly changes.

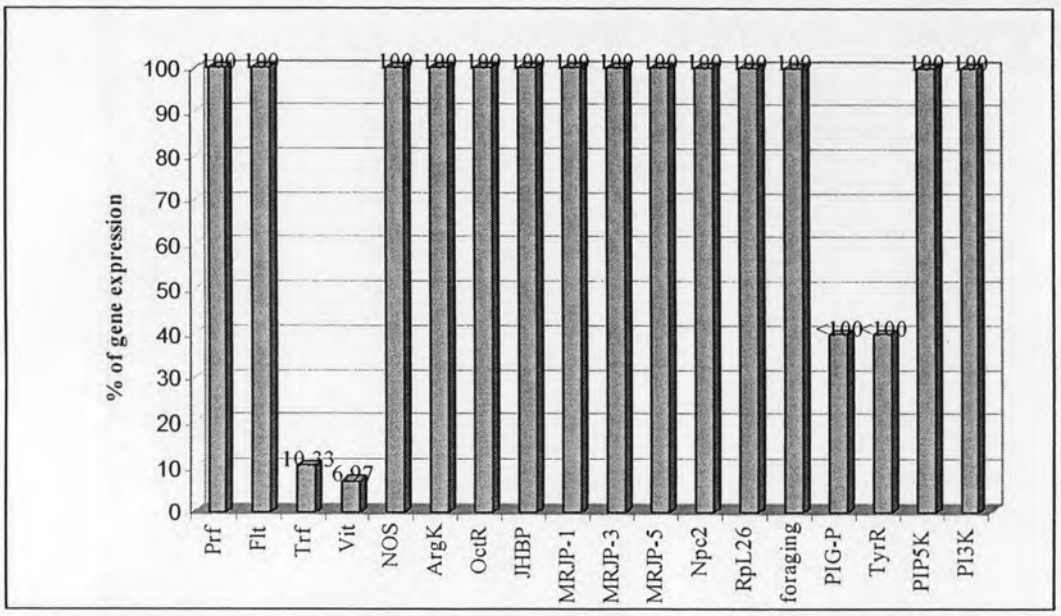

Figure 5.3 Relative expression in the abdomen of queenright workers relative to queenless workers.

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)

MRJP-1, 3, 5 = major royal jelly protein-1, 3, 5

Npc2 = Niemann-Pick type C2 protein

RpL26 = ribosomal protein L26

Foraging = cGMP-dependent protein kinase

PIG-P = Phosphoinositolglycan-peptide

TyrR = tyramine receptor

PIP5K = Phosphatidylinositol phosphate kinase

PI3K = Phosphoinositide-3-kinase 68D

DISCUSSION

In honey bees, worker sterility is a function of both the inability to mate preventing diploid egg production (female workers and queens, unless matched-mated), and more importantly, prevention of development of the few (2-12) primordial ovarioles (anlagen) which survived metamorphoses to reach maturity and so preventing all offspring production. The genes for sterility in this later case might simply encode proteins that suppress ovary activation under certain conditions, for example as potentially is the case in queenright honey bee workers (Thompson *et al.*, 2006).

It has been shown previously that workers from queenright colonies have predominantly arrested ovary development, attaining only so far as stages 0 and 1, whereas a much higher percentage of workers from queenless colonies had more developed ovaries attaining developmental stages 2 and 3 (Seehuus *et al.*, 2007). Here, queenless workers were found to show a significantly higher level of ovarian development and activation (Figure 5.1), including workers with fully active ovaries (stage 3) which first appeared in workers from 10 days post eclosure. Indeed, 5, 15, 55 and 75 % of queenless workers had developed their ovaries (stage 2 and 3) by 6, 10, 15 and 21 days of age, respectively. In contrast no well-developed and functionally active ovaries (stage 2 and 3, respectively) were found in queenright workers of all ages. This study strongly supports the notion that queen pheromones inhibit the activation of ovary development in workers (Butler and Fairey, 1963; Jay, 1968; Hartfelder and Engels, 1998; Hoover *et al.*, 2003).

The abdomens of eight queenright and eight queenless workers at 15 days old were used to compare differences in transcript expression levels of eight selected

genes in this study. At this age (15 days post eclosure), all four broad morphological categories of ovary development (stages 0, 1, 2 and 3) were found in queenless workers, whereas ovary development in queenright workers was restricted to only stages 0 and 1, consistent with earlier findings (Hoover *et al.*, 2003) (Figure 5.1).

Figure 5.3 shows the standardized relative transcript levels of the eighteen examined candidate genes displayed as the relative levels found in the abdomens by qRT-PCR in queenright relative to queenless worker bees. Thus, transcripts with no expression difference between queenright and queenless groups have a relative expression of 100%, whereas genes significantly above or below this value, are considered to be over- or under- expressed in queenright workers, respectively. For the first eight candidate genes that same as used to examine in CO_2 treatment, the abdominal expression levels for six (profilin (Prf), flotillin (Flt), take-out-like carrier protein (JHBP), nitric oxide synthase (NOS), arginine kinase (ArgK) and octopamine receptor (OctR)) did not significantly differ between queenright and queenless worker abdomens. However, two gene transcripts (transferrin (Trf) and vitellogenin (Vit)) were down-regulated about 9 and 14 fold, respectively, in queenright workers with non-developed ovaries compared to that of queenless workers with developed ovaries (Table 5.6).

This result is consistent with a previous study which suggested that vitellogenin and transferrin gene transcript levels were down-regulated in the abdomen of queenless worker bees without developed ovaries following carbon dioxide narcosis (Chapter IV, Figure 4.7). However, in contrast these studies are incongruent with the report of Thompson *et al.* (2007) where no significant change in vitellogenin transcript levels in queenless worker bees after inhibition of ovary development by carbon dioxide narcosis was detected. Moreover, the same study

revealed a some fivefold and significant increase in the transcriptional expression level of the Trf gene in 12 day old queenless workers following inhibition of ovary activation by carbon dioxide narcosis, in direct contrast to the 5-10 fold increase in Trf transcript levels seen in this study between queenless and queenright worker bees. This discrepancy might simply reflect differences in bee samples since this study involved 15 day old bees with fully developed ovaries (stage 3), whereas those used in Thompson *et al.* (2007) did not include any bees with ovaries as far developed as stage 3, and were evaluated between 8 and 12 days post eclosure and indeed within this age range showed a temporal dependence for transcriptional levels. In addition, we worked with natural in-hive conditions that are ideal for ovary development when compared with out-of-hive rearing conditions. Interestingly, the former scenario may imply a potentially biphasic response with Trf transcript levels up-regulated during initial ovary development (stages 0 to 2; Thompson *et al.*, 2007) and down-regulated in later development (stage 3; this study), but this awaits confirmation especially since whole abdomen RNA extractions were performed in both studies and thus the role of transferrin and vitellogenin from the abdominal fat body in say immunity and longevity rather than ovary development cannot be excluded. Likewise, the second scenario above implies a strong developmental sensitivity to Trf transcript levels that may not be directly related to the broad spectrum of ovarian development, and again requires further clarification.

The relationship between Trf transcript levels and ovary activation is unclear because the iron binding transferrin protein product is likely to pleotrophic in the honey bee, including an antimicrobial and anti-parasite activity (Kucharski and Maleszka, 2003), as well as sequestered uptake into developing oocytes in some insects (Kurama *et al.*, 1995; Nichol *et al.*, 2002)

The 180 kDa glycolipoprotein vitellogenin, known to be important in egg production, is synthesized in fat bodies (abdomen, thorax and head) and released into the hemolymph where it is taken up as a yolk protein by developing oocytes but it is also used to synthesize royal jelly by the hypopharyngeal glands in the head of nurse-age workers (Amdam *et al.*, 2004a). However, it is becoming clear that, at least in the honey bee, this protein has evolved pleotrophic functions not yet recorded (or examined) in other species that rely on vitellogenin for oocyte development. Rather, it appears that vitellogenin is a key and central element involved in pathways that control the life history regulation of the honey bee and including reduced longevity and immunity of foragers, thermotolerance and to coordinate worker behavior between nurse and forager bees, and within foragers between pollen and nectar foraging (Amdam *et al.*, 2003b, 2004b, 2005, 2006a, b; Guidugli *et al.*, 2005; Seehuus *et al.*, 2006; Corona *et al.*, 2007; Nelson *et al.*, 2007). Indeed, the division of foraging labor among worker honey bees is linked to the reproductive status of facultatively sterile females (Amdam *et al.*, 2006a), whilst vitellogenin gene activity influences the worker division of labor via an inhibitory effect on the shift from nest tasks to foraging (Nelson *et al.*, 2007). Taken together these data support the notion (Amdam *et al.*, 2003a, b) that a key adaptation to eusociality by honeybees was the diversion of vitellogenin from its primary role as a yolk protein to a secondary role in the production of brood food. As a consequence, vitellogenin is expected to be functionally associated with colony-level traits such as alloparental care and the reproductive division of labor (Amdam *et al.*, 2004a, b), and so favored the uncoupling of juvenile hormone control of expression levels.

This current study supports the previous result found in queenless worker bees with suppressed ovary development following carbon dioxide treatment (Chapter IV),

and thus likely removes the possibility that the altered Trf and Vit levels are the result of an artifactual altered alternative redundant pathway by carbon dioxide narcosis, and so further supports the correlation between Trf and Vit transcript down-regulation in the activation of workers ovaries. However, in contrast to most of the above effects of vitellogenin upon worker bee biology outlined above, all the current data linking Vit transcript or vitellogenin protein levels to ovary maturation or the division of reproduction is at best correlative. For example, all reports of knockdown using Vit RNAi to date have not examined, and thus reported on, the effects upon the developmental activation and maturity of worker (or queen) ovaries, but rather focused on worker behavior traits, immunity, anti-oxidative stress and longevity (e.g. Nelson *et al.*, 2007, Amdam *et al.*, 2003b, 2006, Guidugli *et al.*, 2005; Corona *et al.*, 2007). In the meantime, Trf RNAi knockdown has not been reported, we suggest that vitellogenin and transferrin are worthy of further investigation in ovary development in both worker and queen castes, and may be part of the network that regulates functional sterility in worker honey bees (*Apis mellifera*).

Regardless, it may be suggested that workers in the queenless colonies with active ovaries that have an up-regulation of Vit transcript expression levels, and thus assumed vitellogenin protein levels, will initiate foraging later in agreement with their known polyphenic biology (Amdam *et al.*, 2004b; Seehuus *et al.*, 2006) and suggested role in the division between colony level traits and reproduction (Amdam, 2003a, b, 2004a, b).

Recently, there were many reports about the genes that may function involved in ovary activation in insects and mammalian. Ten more candidate genes were selected to observe the differential expression between queenright and queenless workers. From these ten candidate genes, eight of them: Major royal jelly protein 1, 3,

5 (MRJP-1, 3, 5); Niemann-Pick type C2 protein (Npc2); Ribosomal protein (RpL26); cGMP-dependent protein kinase (foraging); Phosphatidylinositol phosphate kinase (PIP5K); and Phosphoinositide-3-kinase 68D (PI3K) show no consistent difference in expression (Table 6.5), even though differences in ovary activation were now apparent among the workers. Therefore these genes do not appear to be associated with differences in ovary activation, at least not by this assay. In contrast, the genes encoding phosphoinositolglycan peptide (PIG-P) and tyramine receptor (TyrR) were down-regulated (Table 6.5) in queenright (without ovary activation) relative to queenless worker (with ovary activation). The expression of these two genes is therefore associated with differences in functional ovary activation among workers.