CHAPTER III

DIFFERENTIAL GENE EXPRESSION BETWEEN WILD TYPE AND ANARCHIST WORKER BEES

3.1 INTRODUCTION

The European honey bee (*Apis mellifera* L.; Hymenoptera: Apidae) is a eusocial species within the insect order Hymenoptera. It is an important model system for investigating the evolution and maintenance of worker sterility. The female caste in each colony consists of several thousand sterile workers with undeveloped and reduced ovaries, and a single reproductive queen. In addition, at certain limited times male (drones) and additional queens are produced but rapidly leave the colony. A new virgin queen will mate with as many as 10-40 (average 12) drones while in her typically several mating flights (Tarpy and Nielsen, 2002). After mating, the queen will activate her ovaries within a few days and start production of fertilized eggs. Such fertilized (diploid) eggs will normally develop as females, as either sterile workers or new queens with only 2-12 or up to 180 ovariole primordia (anlagens), respectively (Hartfelder and Steinbruck, 1997; Capella and Hartfelder, 1998), depending upon the developing larval feeding regime and duration. However, if the sperm utilized was from a matched male with respect to the complimentary sex determination system, the zygote develops as a diploid male instead (Evans *et al.*, 2004).

In the normal queenright condition, the queen and the broods secrete pheromones and substances to control her offspring and suppress the development of the remaining 2-12 primordial ovarioles in workers (Free, 1987). Therefore, only a few workers (~ 0.01 %) in a queenright colony have functionally active (developed) ovaries and are capable of laying unfertilized (haploid) eggs that will develop, if allowed by the colony, as uniparental sons (Page and Erickson, 1988; Visscher, 1989; Oldroyd and Osborne, 1999). An important exception is a rare behavioral syndrome, "anarchy", in which substantial worker production of males occurs in queenright colonies. The level of worker reproduction in these anarchic colonies is far greater than in a normal queenright honey bee colony. Usually, worker policing and the pheromonal systems maintain the reproductive division of labor in queenright colonies, but in "anarchic" colonies, these systems break down and worker reproduction is more common (Barron *et al.*, 2001).

Anarchist bees are unusual in at least two ways. First, whereas ovary development is extremely rare among queenright wild type workers (Visscher, 1996), it is relatively common among queenright anarchist workers (Oldroyd *et al.*, 1999; Barron and Oldroyd, 2001). Second, their eggs are policed less (Oldroyd and Ratnieks, 2000). The characteristics of the anarchic syndrome are summarized in the Chapter I (1.7). All these characters indicate a general breakdown of the pheromonal system that normally inhibits worker reproduction in queenright colonies, possibly involving changes in the production of pheromones, perception of pheromones, or both (Barron *et al.*, 2001). That means it could be differentially genes expressed between wild-type and anarchist worker bees.

This mutant line demonstrates that worker sterility has a genetic basis (Oldroyd *et al.*, 1994). Furthermore, the patterns of inheritance of the anarchistic

phenotype show that there are a small number of genes that regulate worker ovary activation in response to social cues (Oldroyd and Osborne, 1999). Given the strong genetic component to the regulation of ovary activation in honeybees, the gene expression profiles of wild-type and anarchist workers should reveal genes associated with the regulatory control of worker sterility.

Northern blotting is often used to estimate the expression level of a gene by visualizing the abundance of its mRNA transcript in a sample. In this method, purified RNA is separated by agarose gel electrophoresis, transferred to a solid matrix (such as a nylon membrane), and probed with a specific DNA probe that is complementary to the gene of interest. Although this technique is still used to assess gene expression, it requires relatively large amounts of RNA and provides only qualitative or semi-quantitative information of mRNA levels.

In the term of ovary activation, anarchist workers had ovarian development much more than wild-type workers. The anarchist workers that were fostered into queenright wild-type host, were quantified (by real-time PCR) the level of genes expression in the abdomens against group of queenright wild-type workers. The genes selected for transcriptional evaluation in this study encode transcripts known, or suspected, to be involved in ovarian development (profilin, transferrin, vitellogenin) or in signaling pathways critical for cellular differentiation (flotillin).

The objective of this study is comparing rate of ovary activation between wild-type and anarchist workers and primarily investigating the genes that have differentially expressed in those workers using northern blot analysis. Then, quantify the level of genes expression between groups of those workers using quantitative real-time PCR.

3.2 MATERIALS AND METHODS

3.2.1 Honey bee samples

The anarchist bees were obtained from the School of Biological Sciences, University of Sydney, Australia. The experiment was performed by cross-fostering the anarchist bees to the wild-type host colony, and the control group was wild-type bees in the same wild-type host colony. The bee samples were collected 2 times in 2 different season periods for examining by 2 different techniques (northern blot hybridization and quantitative real-time PCR).

3.2.2 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 emerging sealed brood from wild type colonies and anarchist colonies were incubated at 35°C overnight. The following morning, newly-emerged workers were marked at the thorax (with difference colors) and then transferred into the wild-type host colony (Appendix A).

3.2.3 Collection and selection the samples

3.2.3.1 *Sampling techniques*

Both anarchist and wild-type workers were reared in the wild-type host colony for 15 days. After that, 50 bees of each anarchist and wild-type workers were collected from the host colony by snap-freezing them in dried ice (Appendix A).

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

3.2.3.2 *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. Ovarian development will be divided in 4 stages (Appendix A).

- stage 0 = ovary thin and lacking defined ova
- stage 1 = ovary slightly thickened
- stage 2 = ovary thick with clearly defined ova
- stage 3 = ovary thick with at least one fully mature ova (at this stage many eggs could be potentially lay with in 1 day)

After scoring the stage of ovarian development of each anarchist bee, the group of anarchist worker bees and those of wild-type worker bees were selected to extract the total RNA from the abdomen and head.

3.2.4 Total RNA extraction

For northern blot analysis, the total RNA was extracted using the combined TriZol /Qaigen protocol, the frozen abdominal tissues from single bees were individually ground with liquid nitrogen in 1.5 ml Eppendorf tubes (for head part: 2 heads per tube), and homogenized with 100 µl of TriZol reagent (Gibco BRL) using disposable pestles attached to a hand-held engraving device (Super Tool, Arlec). Following the homogenization step the volume was adjusted to 1,000 µl with TriZol reagent. The homogenate was incubated at room temperature for 5 min and 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, mixed with an equal volume of 70%

(v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water and applied on a Qaigen RNeasy column. Discarded the flow through by centrifuging at 10,000 ×g for 15 seconds, then washed the column with 700 µl of buffer RW1 and centrifuged same as above. Five hundreds microliters of buffer RPE was added to wash the column for two times. After the second wash, the column was centrifuged for 2 min to dry the RNeasy silica-gel membrane. The pure intact RNA was eluted by adding 50 µl of RNase-free water directly onto the RNeasy silica-gel membrane and leaved the column for 1 min before centrifuged at 10,000 ×g for 1 min. The RNA preps were stored at -80 °C until used.

For quantitative real-time PCR analysis, Agilent mini kit was used to extract RNA following Agilent's protocol. Frozen abdominal tissue of individual bee was homogenized in 1.5 ml Eppendorf tubes, containing 100 µl of lysis solution/β-mercaptoethanol mixture (10 µl β-mercaptoethanol/ml lysis solution; 20 µl of lysis solution/mg of tissues) using disposable pestles attached to a hand-held engraving device (Super Tool, Arlec). Following the homogenization step the volume was adjusted to 500 µl with lysis solution/β-mercaptoethanol mixture. The filtrate was recovered after spinning for 3 min at 16,000 ×g, mixed with an equal volume of 70% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water and applied on a mini isolation column. The flow through of the column was discarded by centrifuging at 16,000 ×g for 30 seconds, following by washing the column twice with 500 µl of wash solution. To completely dry, the column was centrifuged for 2 min, then the pure intact RNA was eluted by adding 50 µl of nuclease-free water and leaved the column for 1 min before centrifuged at 16,000 ×g for 1 min. The RNA preps were stored at -80 °C until used.

3.2.5 mRNA purification

Dynabeads Oligo $(dT)_{25}$ (DYNAL® Simply Magnetic) was used to purify the mRNA. The extracted total RNA was adjusted the volume to 100 µl with distilled DEPC-treated water. Then prepared an equal volume of Dynabead Oligo $(dT)_{25}$ in Binding Buffer (20 mM Tris-HCl, pH 7.5; 1.0 M LiCl; 2 mM EDTA). The total RNA solution was heated to 65 °C for 2 min to disrupt secondary structures. In the meantime, 200 µl of resuspended Dynabead Oligo $(dT)_{25}$ was removed from the stock tube suspension. Dispensed the Dynabead Oligo $(dT)_{25}$ into a 1.5 ml Eppendorf tube and placed the vial on the Dynal MPC (Magnetic Particle Concentrator). After 30 seconds, the supernatant was pipetted off. Then the tube was removed from the magnet and washed the Dynabead Oligo $(dT)_{25}$ by resuspending in 100 µl Binding Buffer and placed the tube back on the magnet. After 30 seconds, the supernatant was pipetted off and the tube was removed from the magnet. Added 100 µl of Binding Buffer to the Dynabead Oligo $(dT)_{25}$ and added the prepared total RNA into the Dynabead Oligo $(dT)_{25}$ in Binding Buffer. Mixed thoroughly and annealed by rotating on a roller or mixer for 3-5 min at room temperature. The tube was placed on the magnet for at least 30 seconds or until solution is clear and then removed the supernatant. Removed the tube from the magnet and washed twice with 200 µl Washing Buffer B (10 mM Tris-HCl, pH 7.5; 0.15 M LiCl; 1 mM EDTA) using the magnet. Completely removed the supernatant before eluted with 20 µl of 10 mM Tris-HCl and heated to 80-90 °C for 2 min. Then the tube was immediately placed on the magnet and transferred the eluted mRNA to a new RNase-free tube. The mRNA preps were stored at -80 °C until used.

3.2.6 Determination of the quantity and quality of RNA samples

The quantity of RNA was spectrophotometrically measured at 260 nm. The concentration of total RNA could be determined in ng/µl using the following formular; [RNA] = A_{260} x dilution factor x 40†.

† An OD unit at 260 nm corresponds to approximately 40 ng/µl of RNA (Sambrook *et al.*, 1989). The relative purity of RNA samples was examined by measuring the ratio of $A_{260/280}$. The maximum absorption of protein is at the wavelength of 280 nm. The good quality of RNA sample should have an $A_{260/280}$ ratio above 1.7.

The quality of the extracted RNA was analyzed by formaldehyde-agarose gel electrophoresis as following described. A 1.2% (w/v) formaldehyde agarose gel was prepared in 1x MOPS buffer (final concentration of 0.2 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0). The gel slurry was heated until completely dissolving and placed to cool down at room temp before formaldehyde (0.66 M final concentration) was added. Then, the melted formaldehyde-agarose gel was poured into a chamber set and applied the comb. The RNA samples were prepared under the denaturing condition. The RNA sample in DEPC-treated water, 7.4 M of formamide, 1.64 M of formaldehyde, 1x MOPS and DEPC-treated water to a final volume of 12 µl were heated at 70 °C for 10 min and the mixtures were immediately chilled on ice. After that, 3 µl of the 5x RNA loading dye buffer containing 50% (v/v) glycerol, 1mM EDTA, pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 0.025% (w/v) ethidium bromide was added to each sample and loaded to formaldehyde-agarose gel. Electrophoresis was run in 1x MOPS buffer at 100 volts for 50 min. RNAs were visualized under a UV transiluminator.

3.2.7 Northern blot analysis

3.2.7.1 *RNA electrophoresis*

RNA samples (3.2.4 and 3.2.5) were denatured by mixing with an equal volume of formamide, containing 0.05% bromophenol blue and 0.01% SybrGreen II, incubated at 90 °C for 7 min and immediately chilled on ice. Electrophoresis was performed in small horizontal tanks (Hoeffer HE33) using 1.5% agarose gels submerged in TBE buffere (50 mM Tris-borate, 1 mM EDTA, pH 8.2) at 20 V/cm. Alternatively, the glyoxal based system (Ambion, www.ambion.com) was used for RNA separation. Some of ice was put around the electrophoresis set to keep cool all the time. Following electrophoretic resolution, the band of RNA was seen using the Vistra FluoroImager (Amersham, www.amershambiosciences.com).

3.2.7.2 *Blotting*

The gel was soaked in 1 M ammonium acetate, 0.02 M sodium hydroxide and blotted on to Hybond N+ nylon membranes (Amersham, www.amershambiosciences.com) overnight by capillary transfer. RNA was cross-linked to the membrane by UV irradiation for 1 minute. Then, the membrane was briefly washed in 2x SSC, 0.2% SDS.

3.2.7.3 *Prepare probes for hybridization*

Based on the primary experiment from the microarray (our research group) and the information from Honey bee genomes project, six candidate genes (Transferrin [Trf], Vitellogenin [Vit], Take-out-like [JHBP], Nitric oxide synthase [NOS], Profilin [Prf], Flotillin [Flt]) were interested to primary tested the expression in the anarchist and wild-type workers.

Diluted the DNA of interested genes to be labeled to a concentration of 25 ng in 45 µl of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA), denatured the DNA by heating to 95-100 ˚C for 5 minutes in a boiling water bath and snap cool the DNA by placing on ice for 5 minutes after denaturation. Centrifuge briefly to bring the contents to the bottom of the tube. Add the denatured DNA to the reaction tube. Add 5 µl of Redivue [^{32}P] dCTP (Rediprime^{TM} II, Amersham Pharmacia Biotech, specific activity of [^{32}P] dCTP = 3,000 Ci/mmol) and mix by pipetting up and down about 12 times, moving the pipette tip around in the solution. Incubate at 37 ˚C for 10-30 minutes. Stop the reaction by adding 5 µl of o.2 M EDTA. For use in hybridization, denatured the labeled DNA by heating to 95-100 ˚C for 5 minutes, then snap cool on ice for 5 minutes. Centrifuged the tube briefly and mix the contents of the tube well.

3.2.7.4 *Pre-hybridization*

Warm 5 µl of the ExpressHyb solution (Clontech, www.clontech.com) in the hybridization tube at 68 ˚C. Rolled the membrane and placed in the hybridization tube with the side that had RNA inside and rotated tube to soak the membrane with ExpressHyb solution and avoiding the bubble. The blots were pre-hybridized at 68 ˚C for 30-60 minutes.

3.2.7.5 *Hybridization*

Added the denatured probes to the bottom of hybridization tube that had already pre-hybridize, mixed with the ExpressHyb solution (Clontech) at the bottom (avoid touching the membrane directly). Rotated the hybridization tube and allowed solution to attach with all of the membrane. Hybridization was carried out at 68 ˚C for 16 hours.

3.2.7.6 *Washing*

Blots were washed three or four times in 2x SSC, 0.2% SDS at 50 °C. Removed the excess of washing buffer and placed on plastic wrap to avoid drying.

3.2.7.7 *Data analysis*

Exposed the membrane to a phosphorstorage screen (Molecular Dynamics, www.moleculardynamics.com) without drying. To see the result, used the Phosphor-Imager 400S from Molecular Dynamics.

The membrane can be reused with the other probes about 3 times by boiling in 2x SSC, 0.2% SDS for 3 minutes and also of probes can be reused by boiling the hybridization tube for 2-3 min.

3.2.8 Quantitative Real-Time PCR (qRT-PCR)

Principle of quantitative Real-Time PCR (qRT-PCR) is based on amplify and simultaneously quantify a targeted DNA. The key feature is that the quantity of amplified DNA after each amplification cycle was detected as it accumulates in the reaction in real-time. The basic method of quantification is the use of fluorescent dye labeled nucleotide that intercalate with double-stranded DNA.

3.2.8.1 *DNase treatment of total RNA samples*

The extracted total RNA (3.2.4) was further treated with RNase Free DNase I (Promega, 1 units/5 μg of the total RNA) at 37 °C for 30 min 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. Briefly, the reaction volume was

adjusted to 40 µl with DEPC-treated water, 250 µl of TRIzol reagent were added and vortex for 10 seconds. Two hundred microliters of chloroform was then added and vigorously shaken for 15 seconds. The resulting mixture was stored at room temperature for 2-5 min and centrifuged at 12,000xg for 15 min at 4 °C. The RNA in upper phase was precipitated by isopropanol and washed 70% (v/v) ethanol. After that, RNA pellet was briefly air-dried and dissolved with an appropriate amount of DEPC-treated water. The concentration of DNA-free total RNA was determined as described above.

3.2.8.2 *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 the SuperScript™ II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's protocol. The reactions consisting of 1 µg of total RNA, 0.5 µg of oligo (dT_{18}) primer, 1 µl of dNTP Mix (10 mM each) and adjusted the volume with distilled water to 12 µl were heated at 75 °C for 5 min and immediately chilled on ice for 5 min. After that, 4 µl of 5x First-Strand buffer, 2 µl of 0.1M DTT, 20 units of Ribonuclease inhibitor and 1 µl (200 units) of SuperScript™ II Reverse Transcriptase were added and mixed by pipetting gently up and down. The reactions were annealed by incubating at 25 °C for 10 min and

extension at 42 °C for 50 min. Then, the reverse transcriptase activity was inactivated by heating at 70 °C for 15 min. This cDNAs were stored at –20 °C until use.

3.2.8.3 *Genes selection*

Based on the primary experiment from the microarray (our research group), the information from Honey bee genomes project and the results from northern blot analysis, four genes (Profilin [Prf], Flotillin [Flt], Transferrin [Trf], Vitellogenin [Vit]) were interested to examine the expression between wild-type and anarchist workers. The genes used for transcriptional evaluation in this study encode transcripts known or suspected to be involved either in ovary/egg development (vitellogenin, transferrin, profilin) or in signalling pathways critical for cellular growth and/or differentiation (flotillin) and chose ribosomal protein (RpS8) as internal control. The candidate genes are transferrin (Trf), an iron binding protein with multiple functions; 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.

Selection of these candidate markers was based on:-

1) Their functional gene descriptions at Interactive Fly (www.sdbonline.org) or Online Mendelian Inheritance in Man (www.ncbi.nlm.nih.gov).

2) The availability of an annotated gene sequence in the honey bee (http://www.hgsc.bcm.tmc.edu/projects/honeybee/).

3.2.8.4 *Primer design*

Target sequences for PCR were selected from previously characterized cDNAs and ESTs using the genomic scaffolds at the Baylor College of Medicine

(http://www.hgsc.bcm.tmc.edu/projects/honeybee/). To eliminate genomic DNA amplification we manually designed primers that either spaned intron/exon junctions or amplified exon sequences separated by long introns. Real-time PCR primers for each gene were designed with a melting temperature (T_m) higher than the T_m of any of the predicted template secondary structures and maintain T_m between 50 °C and 65 °C. Moreover, primers should have a GC content of 50-60% and place with G's and C's on the ends. The ribosomal protein S8 (RpS8) was used as an internal control for real-time PCR.

For each gene, two sets of forward and reverse primers were designed, for example of profilin gene as shown in Figure 3.1. An electrophoretic comparison of target amplicons against a molecular size standard (100 base pair DNA Ladder, Promega Co., U.S.A.) was performed to find the set of primers that amplified a single band of expected size. Other set of primers that did not amplify as expected were discarded. The pair of primers that gave the best amplification as single band of expected size was selected to use in qRT-PCR.

3.2.8.5 *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. Amplification reaction was carried out in a 20 µl reaction volume containing 20 ng of first-stranded cDNA template, 1x Mg^{2+}-free buffer (10 mM Tris-HCl; pH 8.8, 50 mM potassium chloride, and 0.1% Triton X-100), 2 mM $MgCl_2$, 300 nM of each forward and reverse primer, 200 µM of each dNTP, and 1 unit of DyNazyme^{TM} II DNA Polymerase (Finnzymes, Finland). The temperature profiles

>profilin

Prf-F1 *Prf-F2*

TCATTGGGGC GAGAACGATT **GAATTGAGCC GCTTGTGTTA** AAAAATGAAG CTGC**CAGGAT**

TACGTTGACA AGCAGCTGCT CGGCGTCTCG GGTGTGTTAC CAAAGCTGCG ATTGCAGGAC

ACGATGGCAA TCTTTGGGCA AAGTCTGAAG GCTTCGAAGT AAGTAAAGAG GAGTTGACGA

AATTGGTCCA GGGATTTGAA GAACAAGATA TTTTGACGTC GTCGGGTGTC ACCTTGGCCG

Prf-R2

GCAACAGGTA CATT**TACCTG TCAGGTACGG ATCG**AGTGAT AAGGGCAAAA CTTGGAAAAG

TCGGCGTCCA CTGCATGAAG ACGACGCAAG CAGTAGTTGT CTCTCTTTAC GAAGATCCTA

Prf-R1

TACAACCACA GCAAGCCGCA TCGGTCGTTG AAAAACTTGG GGACTACCTT G**TGTCCTGCG**

GCTATTAGGT TAGTATATAC TAACCTCTGG GACCCAACAT AAATATCTAA TATTAATATT

AAACGATCGA TTATTTTAAT GAACAGCGAA TGGAATGCGT CATGCCGCAC GAGTCGCGCC

GGAACTGCCG CAAGCTTGCC GCGCGACATT CGATGAAAAG CAACAACTAC ATCAGCAACA

ACACCAGCAA CACAGCTATC ATATTCCAGT CGTCGTTCTC TCCTCTTCGA TTCGGCTGGT

CGACGTTCGA GGAACAGACG CAAGCGCTCA TACGTGTGCG CAATACTTTT TGAAAAAAAA

AAAAAAAAAA GCAAC

Primers	**Product sizes**
Prf-F1 : GAATTGAGCCGCTTGTGTTA	Prf-F1/R1 = 413 bp
Prf-F2 : CAGGATTACGTTGACAAGCA	Prf-F1/R2 = 254 bp
Prf-R1 : CTAACCTAATAGCCGCAGGACA	Prf-F2/R1 = 379 bp
Prf-R2 : CGATCCGTACCTGACAGGTA	Prf-F2/R2 = 220 bp

Figure 3.1 Primer designed of profilin gene.

were predenatured at 94 °C for 2 minutes followed by 37 cycles of denaturation at 94 °C for 25 seconds, annealing at 53±3/56±4 °C for 30 seconds, extension at 72 °C for 35 seconds and postextension at 72 °C for 2 minutes. After amplification, an aliquot of 5 µl of PCR products were electrophoretically analyzed on 1.0% agarose gel.

3.2.8.6 ***Agarose gel electrophoresis***

Agarose gel electrophoresis was used to size-fractionate the amplification products. Different concentrations of agarose gel were prepared depending on sizes of DNA fragments. An appropriate amount of agarose was weighted out and dissolved in the appropriate volume of 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). The gel slurry was heated until completely solubilized. The agarose gel solution was cool down at 55 °C before poured into electrophoretic gel mould and applied the well comb. After agarose was completely set, the comb was carefully removed. The gel was placed in the chamber. An enough volume of 1x TBE buffer was poured to cover the gel for 2-3 mm. Before loading into the gel, the PCR products were mixed with 1/5 volumes of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 15% Ficoll 400 in water). The 100 base pair DNA Ladder (Promega Co., U.S.A.) was used for identifying of the DNA size. The agarose gel electrophoresis was performed in 1x TBE buffer at 100 volts until the lower bromophenol blue dye migrated about 3/4 of the gel length. After that, the gel was stained with a 2.5 µg/ml ethidium bromide (EtBr) solution for 30 seconds and destained by submerged in distilled water for 10 min. Ethidium bromide-stained PCR products were visualized under the UV transilluminator and photographed with Gel Documentation System (GeneCam FLEX1, SynGene).

3.2.8.7 ***Analysis of the selected genes expression in the abdomens of anarchist and wild-type worker honey bees using qRT-PCR***

The relative amount of specific cDNA templates between different samples was quantified using real-time PCR (qRT-PCR). This technique gives the ability to quantify the amplified DNA during the exponential phase of the PCR, when none of the components of the reaction are limiting, results in an improved precision in the quantitation of target sequences. Real-time PCR provides immediate information about the kinetics of the PCR and the software can calculate the concentration of target sequences in the initial reaction mixture. This experiment was quantified with the Rotorgene 3000 Thermal Cycler (Corbett Research, Sydney, Australia) (Appendix B).

Optimization reaction components and amplification conditions will be required to obtain the maximum efficiency and specificity. *Taq* polymerase from Promega was used in 20 µl reactions containing 2 mM $MgCl_2$, 200 µM each of dATP, dTTP, dCTP, and dGTP, 0.25 µM each of forward and reverse primer, 0.2-0.5 unit *Taq* polymerase, and cDNA template equivalent to approximately 25 ng of total RNA. Every PCR reaction, individual RNA sample without reverse transcriptase was included as the control for genomic DNA contamination. Product formation was monitored by the inclusion of SYBR Green I at a final dilution of 1:40,000 (Fisher Biotech, Springfield, NJ). Thermocycling was conducted in a Rotorgene 3000 Thermal Cycler (Corbett Research, Sydney, Australia) for 35 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 30 seconds, and fluorescence acquisition at 84 °C for 15 seconds. Cycling was preceded by a 15 min 95 °C activation step. Specificity of

amplification was confirmed through a melt curve analysis of final PCR products by ramping the rotor temperature from 55 °C to 99 °C at 0.2 °C/seconds with fluorescence acquired after every 1 °C increase.

3.2.8.8 *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 standard curve of each gene was determined by the slope of the curve generated by amplification of serially diluted cDNA over at least 3 orders of magnitude. Each amplified reactions were executed in triplicate including a negative control. After finishing the amplification, the data were analyzed the amplification plots and the standard curve by software of the Corbett Research detection system. The standard curve graphs of reference and target genes were plotted the Ct values against copy number of product (log scale). The linear graph should be obtained the correlation coefficient certainly more than 0.99 and PCR efficiency equal to $10^{-1/slope}$ in each graph. These PCR efficiency values were employed to calculate in relative expression ratio.

3.2.8.9 *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 relative expression ratio was calculated using the value of the threshold cycles or Ct value. The threshold was a reference line that used for distinguish the gene amplified signal

from background. The relative expression ratio analyses the amount of target transcript relative to an internal standard (RpS8) in the same cDNA. A mathematical model in previous reported by Pfaffl (2001) was used for determine the relative expression ratio following the formula:

$$\text{Relative expression ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta Ct_{\text{ref}}(\text{control-sample})}}$$

E_{target} is the real-time PCR efficiency of target gene transcript;

E_{ref} is the real-time PCR efficiency of reference gene transcript;

ΔCt_{target} is the Ct deviation of control - sample of the target gene transcript;

ΔCt_{ref} is the Ct deviation of control - sample of the reference gene transcript.

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

3.3 RESULTS

3.3.1 Ovarian development observed from the sample preparation

Fifty bees of each anarchist and wild-type were collected from the wild-type host colony at 15 days of age. For northern blot analysis, anarchist worker bees were dissected on the wax plate that putting on ice and observed the ovarian development under the stereomicroscope. The number of anarchist worker bees in each stage of ovarian development was counted and scoring result of these dissected anarchist worker bees was shown in Table 3.1.

For quantitative real-time PCR analysis, all 15 days old workers (fifty bees from each anarchist and wild-type) were dissected to observe the ovarian development (Table 3.2). The data from Table 3.2 was used to plot the bar graph as shown in Figure 3.2. From the result, the wild-type workers had not developed their ovaries (stage 0 and stage 1 of ovarian development), while some of the anarchist workers had developed their ovaries (stage 2 and stage 3 of ovarian development) at the same age of 15 days old.

3.3.2 RNA extraction

For northern blot analysis, the wild-type worker bees were randomly selected (stage of ovarian development was not determined) to extract the total RNA and anarchist worker bees were selected by stage of ovarian development using the combined TriZol /Qaigen protocol. Some of total RNAs were purified the mRNA with Dynabeads Oligo $(dT)_{25}$ (DYNAL® Simply Magnetic) before using in northern blot analysis (some of northern blot hybridizations were performed with total RNA).

Table 3.1 The number of 15 days old anarchist worker bees in each stage of ovarian development for using in northern blot analysis.

Type of worker bee	Number of bees in each stage of ovarian development*				
	Stage 0	Stage 1	Stage 2	Stage 3	Total
Anarchist	17	18	12	3	50

* The stages of ovarian development of wild-type worker bees were not determined. For this northern blot analysis experiment, all wild-type worker bees were assumed to have non-developed ovaries.

Table 3.2 The number of 15 days old wild-type and anarchist worker bees in each stage of ovarian development.

Type of worker bee	Number of bees in each stage of ovarian development				
	Stage 0	Stage 1	Stage 2	Stage 3	Total
Wild-type	21	29	0	0	50
Anarchist	8	20	14	8	50

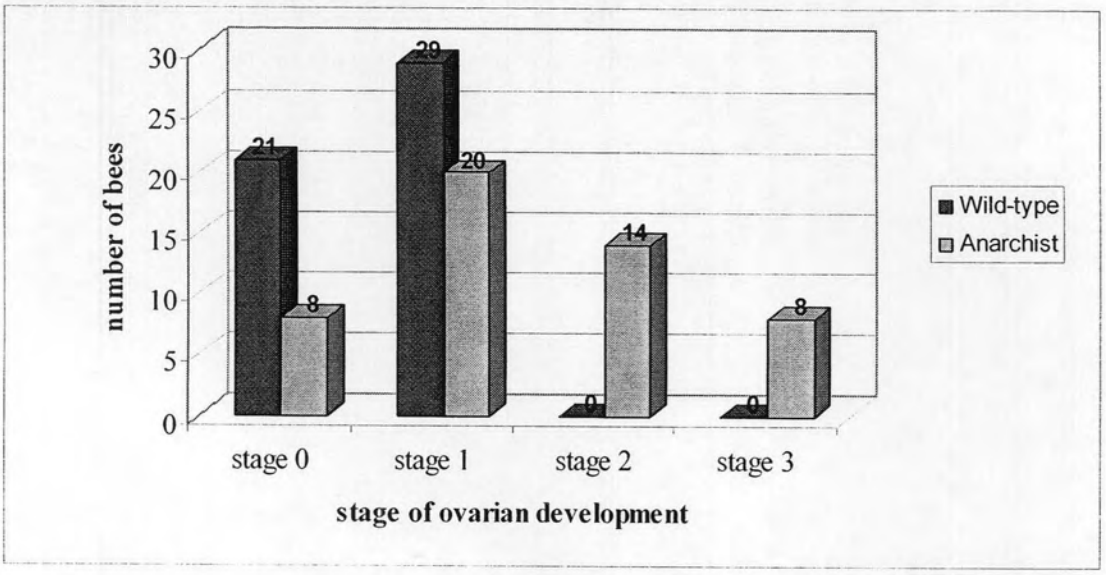


Figure 3.2 Stage of ovarian development in wild-type and anarchist workers.

Stage 0 and stage 1 were count to non-developed ovaries.

Stage 2 and stage 3 were count to developed ovaries.

3.3.3 Northern blot analysis

The northern blot hybridizations were performed with both total RNA and mRNA in the abdomen part and the head part of anarchist and wild-type workers. The RNAs in each lane of the gel were corrected the concentration before loading. Each gel was loaded with one lane of wild-type worker's RNA and another lane of anarchist worker's RNA (at stage 2 or stage 3 of ovarian development).

3.3.3.1 Abdomen part

1) *RNA electrophoresis*

Two micrograms of total RNA and 500 ng of mRNA from the abdomen part of anarchist and wild-type workers were subjected to 1.5% agarose gel with glyoxal based system electrophoresis. This gel was blotted on to Hybond N+ nylon membrane (Amersham) followed with the hybridization step.

2) *Hybridization*

The [^{32}P] dCTP labeled of six genes (Transferrin [Trf], Vitellogenin [Vit], Take-out-like [JHBP], Nitric oxide synthase [NOS], Profilin [Prf], Flotillin [Flt]) were used in hybridization. The results were observed with the Phosphor-Imager 400S from Molecular Dynamics (Figure 3.3).

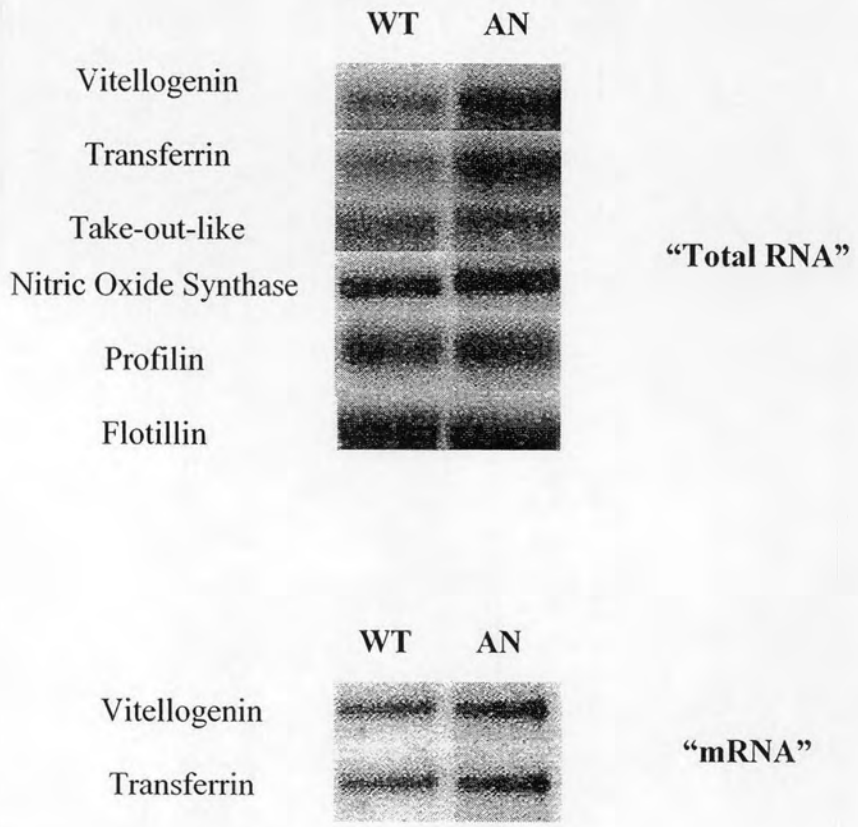


Figure 3.3 Northern blot showing the expression of six genes (Vitellogenin, Transferrin, Take-out-like, Nitric Oxide Synthase, Profilin, Flotillin) in the abdomen of wild-type (WT) and anarchist (AN) workers. The amount of loading mRNA was around 500 ng per lane, whereas 2 μg was for total RNA.

The northern blot hybridization results from total RNA and mRNA of wild-type and anarchist workers gave the same trend of expression. The hybridization of the total RNA with the six candidate genes was done first and the expression of vitellogenin and transferrin gene were significantly difference but they gave a smear broad band. The total RNAs were purified with the dynabead oligo $(dT)_{25}$, then the mRNAs were used to hybridize with the vitellogenin and transferrin genes. The blotting results from mRNA were the same trend but gave the sharper band than those of total RNA. Both vitellogenin and transferrin genes had more expressed in the abdomen of anarchist workers than in wild-type workers.

3.3.3.2 Head part

1) *RNA electrophoresis*

Two micrograms of total RNA and 500 ng of mRNA from the abdomen part of anarchist and wild-type workers were subjected to 1.5% agarose gel with glyoxal based system electrophoresis. This gel was blotted on to Hybond N+ nylon membrane (Amersham) followed with the hybridization step.

2) *Hybridization*

The [^{32}P] dCTP labeled of six candidate genes that used to examine in the abdominal tissues were used to test the differential expression in the head tissues of wild-type and anarchist workers. The blotting results were observed with the Phosphor-Imager 400S from Molecular Dynamics (Figure 3.4).

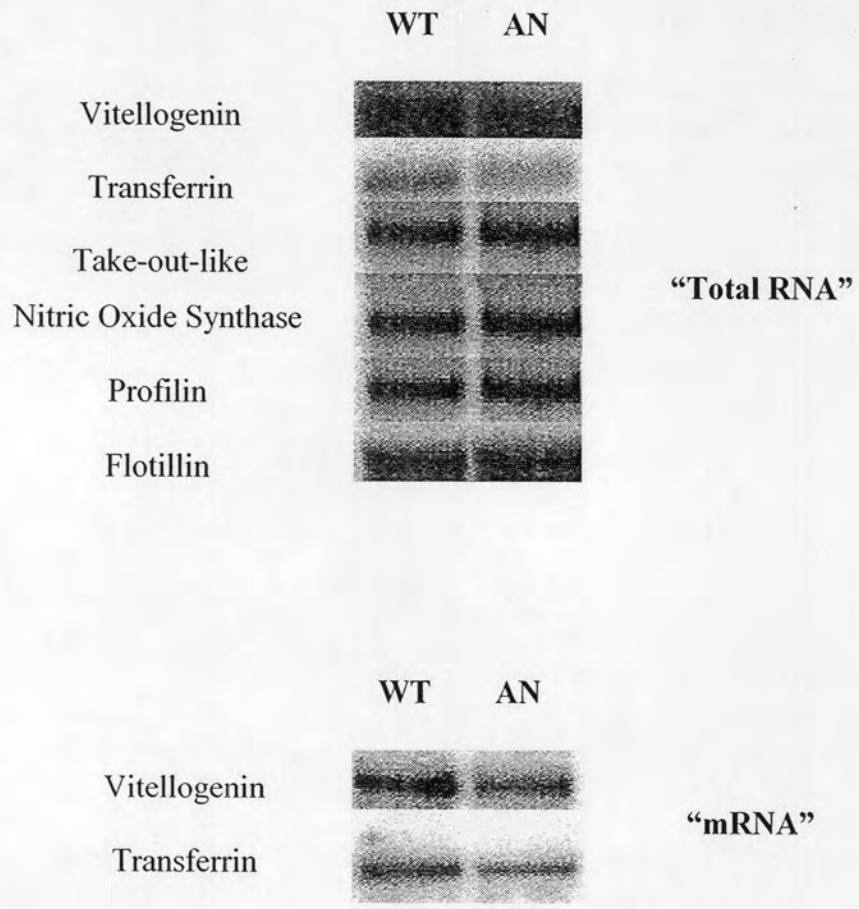


Figure 3.4 Northern blot showing the expression of six genes (Vitellogenin, Transferrin, Take-out-like, Nitric Oxide Synthase, Profilin, Flotillin) in the head of wild-type (WT) and anarchist (AN) workers. The amount of loading mRNA was around 500 ng per lane, whereas 2 μg was for total RNA.

The northern blot hybridization results from total RNA and mRNA in the head of wild-type and anarchist workers gave the same trend of expression. The same as in the abdomen, the hybridization of the total RNA with the six candidate genes was done first. Vitellogenin and transferrin gene gave the significantly differential expression and blotting results were smear broad band. So, the mRNAs were used to hybridize with the vitellogenin and transferrin genes. The blotting results from mRNA gave the same trend but the output results were sharper band than those of total RNA. In contrast with the blotting results in the abdomen, both vitellogenin and transferrin genes had less expressed in the head tissue of anarchist workers than in wild-type workers.

This northern blot hybridization technique provided the candidate genes to observe the level of genes expression between wild-type and anarchist workers using the quantitative real-time PCR. This method is valuable when amounts of RNA are low. Two genes (vitellogenin and transferrin) that had differentially expressed and two genes (profilin and flotillin) that were not significantly differential expression between wild-type and anarchist workers were selected to examine the expression level between those groups of workers.

3.3.4 Quantitative real-time PCR

To quantify the level of genes expression between wild-type and anarchist workers, the dissected honey bee samples in Table 3.2 and Figure 3.2 were selected.

3.3.4.1 *Total RNA extraction*

After scoring the stage of ovarian development and plotting the graph, the group of wild-type and anarchist worker bees were selected to extract the total RNA

using Agilent Mini Kit protocol (3.2.4). Eight workers from wild-type (stage 0 and 1 of ovarian development) and anarchist (two each from all stages of ovarian development) at the same age (15 days old) were selected for extracting total RNA from the abdomens.

The total RNA from eight individual wild-type and anarchist 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 3.5 and the ratio of absorbance at the wavelength of 260 nm and 280 nm were measured. The OD_{260}/OD_{280} ratio of 1.76-1.92 indicated that acceptable quality of extracted RNA was obtained. The amount of pooled total RNA from wild-type and anarchist workers were 70 μg and 73 μg, respectively. The RNA solutions were kept at -80 °C until used.

The expression level of the candidate genes transcript in the wild-type and anarchist workers were examined by quantitative real-time PCR. The ribosomal protein S8 (RpS8), the housekeeping gene, was used as a reference gene.

3.3.4.2 *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. An electrophoretic comparison of target amplicons against a molecular size standard (100 base pair DNA Ladder, Promega Co., U.S.A.) was performed (Figure 3.6). The set of primers that amplified a single band of expected size were selected to use in qRT-PCR (Figure 3.6, Vit_F1/R2 and Trf_F1/R2 were selected). Other sets of primer that did not amplify a single band as

expected were discarded (Figure 3.6, Flt_F1/R2 and Prf_F1/R2). The primer sequence and product size of each selected primer were shown in Table 3.3.

3.3.4.3 ***Testing the efficiency of primers by using PCR***

All primers were experimentally tested in an Eppendorf gradient cycler to determine the optimal annealing temperature and to ensure that only one band of correct size was produced. For the example of the amplification of Transferrin (Trf) gene with varied annealing temperature 60±4°C, 1.0% agarose gel electrophoresis was performed (Figure 3.7). The PCR product size of 190 bp was obtained as expected. The annealing temperature at 60.2 °C was chosen for further amplification. The optimized annealing temperature of each primer was shown in Table 3.3.

3.3.4.5 ***Analysis of the selected genes expression in the abdomens of anarchist and wild-type worker honey bees using qRT-PCR***

The expression level of the four candidate genes transcript in the wild-type and anarchist workers was examined by quantitative real-time PCR. The cDNA of each wild-type and anarchist 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 wild-type and anarchist workers. From this step, the candidate gene that had differentially expressed (vitellogenin: Vit) was selected to calculate the relative expression ratio (fold changes) of wild-type and anarchist workers. The examples of quantitation curve that had and had not differentially expressed were shown in Figure 3.8.

Figure 3.5 The total RNA extracted from the abdomen of selected worker bees using Agilent Mini Kit protocol analyzed by formaldehyde-agarose gel electrophoresis.

Lane 1 = Total RNA from wild-type workers

Lane 2 = Total RNA from anarchist workers

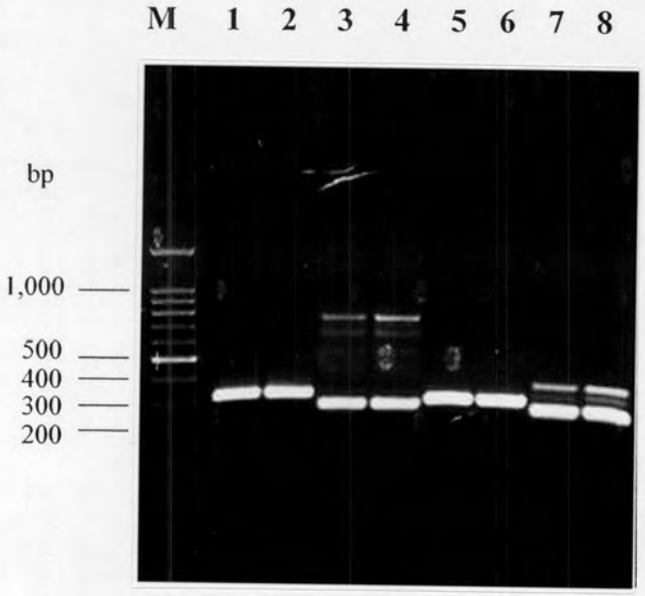


Figure 3.6 Testing the amplification of each primer.

Lane M = Marker 100 bp DNA Ladder

Lane 1 = wild-type cDNA with primer Vit_F1/R2 (330 bp)

Lane 2 = anarchist cDNA with primer Vit_F1/R2 (330 bp)

Lane 3 = wild-type cDNA with primer Flt_F1/R2 (295 bp)

Lane 4 = anarchist cDNA with primer Flt_F1/R2 (295 bp)

Lane 5 = wild-type cDNA with primer Trf_F1/R2 (324 bp)

Lane 6 = anarchist cDNA with primer Trf_F1/R2 (324 bp)

Lane 7 = wild-type cDNA with primer Prf_F1/R2 (254 bp)

Lane 8 = anarchist cDNA with primer Prf_F1/R2 (254 bp)

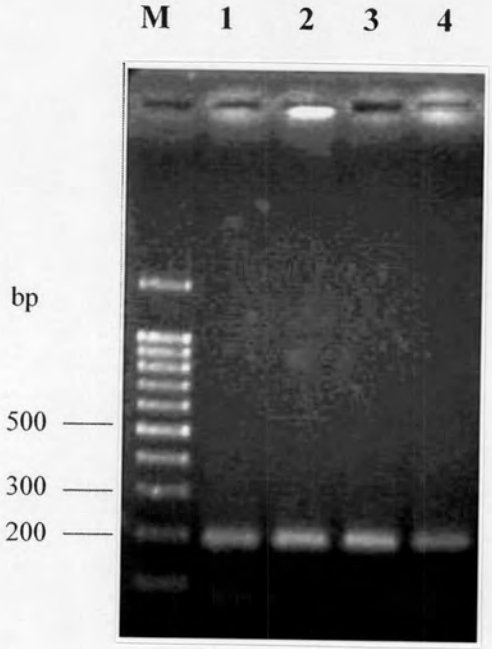


Figure 3.7 1.0% agarose gel electrophoresis showing an optimization of annealing temperature (60±4°C) with transferrin (Trf) primer (190bp).

Lane M = Marker 100 bp DNA Ladder

Lane 1 = Amplification product with annealing temp at 56.1 °C

Lane 2 = Amplification product with annealing temp at 58.4 °C

Lane 3 = Amplification product with annealing temp at 60.2 °C

Lane 4 = Amplification product with annealing temp at 62.7 °C

Table 3.3 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
Prf – F Prf – R	AY545000	CAGGATTACGTTGACAAGCA CGATCCGTACCTGACAGGTA	220	60
Flt – F Flt – R	GroupUn 10026	CCAAACGAAGCTCTCGTAGTA TACTTGTCAAGGTGTACCA	168	60
Trf – F** Trf - R	AY217097	AGCGGCATACTCCAGGGAC CGTTGAGCCTGATCCATACGA	190	60
Vit – F** Vit – R	CAD56944	CCGACGAGGACCTGTTGATTA CTAGGATACGTGGTCATGACA	148	60

* RpS8 : Ribosomal protein S8

Prf : Profilin

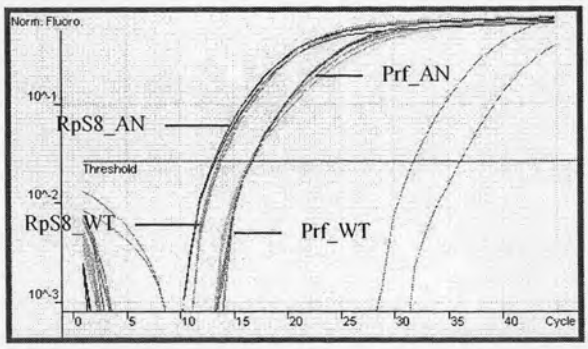
Flt : Flotillin

Trf : Transferrin

Vit : Vitellogenin

** More than one set of primers gave single band of expected size, so any set of primers can use in qRT-PCR. The pair of primers that produced smaller product size and gave more intensity were selected.

(A)



(B)

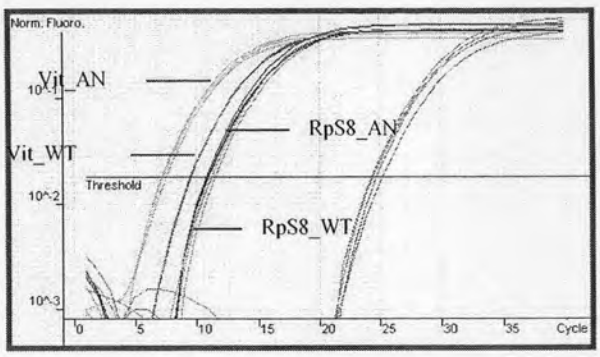


Figure 3.8 The quantitation curve of wild-type and anarchist workers from quantitative real-time PCR with different primers. (A) show the quantitation curve of profilin gene that had not differentially expressed and (B) show the quantitation curve of vitellogenin gene that had differentially expressed.

The cDNA of wild-type (WT) and anarchist (AN) workers were amplified with vitellogenin (Vit) and profilin (Prf) primers and also ribosomal protein S8 (RpS8) primer that was a reference gene.

Prf_WT = cDNA of wild-type worker with profilin primer

Prf_AN = cDNA of anarchist worker with profilin primer

RpS8_WT = cDNA of wild-type worker with RpS8 primer

RpS8_AN = cDNA of anarchist worker with RpS8 primer

Vit_WT = cDNA of wild-type worker with vitellogenin primer

Vit_AN = cDNA of anarchist worker with vitellogenin primer

3.3.4.6 *Determination of PCR efficiency*

For an accurate assessment of gene expression by real-time PCR, the PCR efficiency and the PCR specificity of gene must be taken into consideration. The specificity of the product amplified by SYBR Green I PCR was monitored by analyzing the dissociation curve of each amplicon. The dissociation curve of vitellogenin (candidate gene) and RpS8 (reference gene) showed a single peak at expected melting temperature, indicating that vitellogenin and RpS8 gene were specifically amplified and there were no non-specific amplification or primer-dimer (Figure 3.9).

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. Real-time PCR efficiency was calculated from the slope, obtained from the standard curve plotted in log scale generated by amplification of serially diluted cDNA over at least 3 orders of magnitude of cDNA (Figure 3.10) and the threshold cycle (Ct), following the equation $E = 10^{[-1/slope]}$.

The real-time PCR efficiency of Vit primer was further used to calculate the relative expression ratio (fold changes). The melting temperatures and real-time PCR efficiencies of Vit primer were represented in Table 3.4.

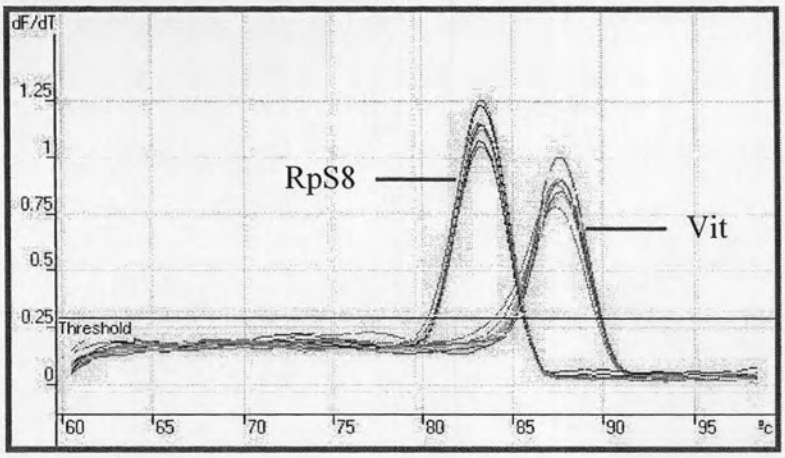
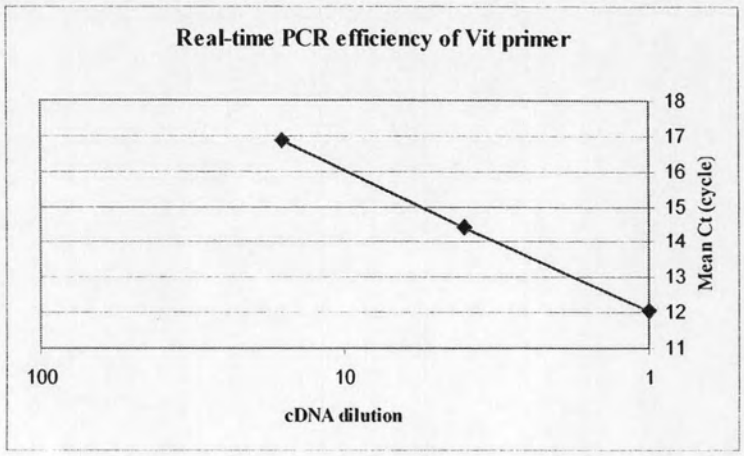


Figure 3.9 Dissociation curve analysis of the Vit & RpS8 had a different melting temperature of specific product. Dissociation curve analysis was performed after a completed PCR. Data is obtained by slowly decreasing the temperature of reaction solutions from 55°C to 94°C while continuously collecting fluorescence data. The increase in temperature causes PCR products to undergo denaturation. dF/dt indicated the Derivative Melting Curve.

Table 3.4 The melting temperature (T_m) and real-time PCR efficiencies of Vit/RpS8 primers.

Gene name	Slope	PCR Efficiency	T_m (°C)
Vitellogenin (Vit)	-4.01677	1.774011	87.5
Ribosomal protein S8 (RpS8)	-4.29637	1.709050	83.3



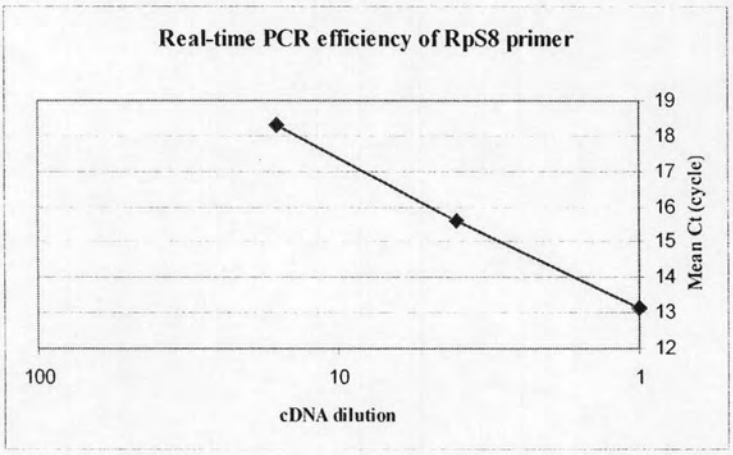


Figure 3.10 The standard curves plotted in log scale of Vit/RpS8 primer of the wild-type and anarchist workers.

Vit primer: slope = -4.01677, PCR efficiency = 1.774011

RpS8 primer: slope = -4.29637, PCR efficiency = 1.709050

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

The mRNA expression level of differentially expressed genes (vitellogenin: Vit) was examined by normalizing the value of the threshold cycles or Ct values of the anarchist workers with wild-type workers. The threshold was a reference line that used for distinguish the gene amplified signal from background. The relative expression ratio analyzes the amount of target (Vit) transcript relative to an internal standard (RpS8) in the same cDNA. A mathematical model by Pfaffl (2001) was used for determine the relative expression ratio as described in 3.2.8.9.

The Real-time PCR efficiency of each primer that previously calculated in Table 3.4 was used to calculate the relative expression ratio or fold changes of the vitellogenin gene in anarchist workers relative to wild-type workers. The amplification of vitellogenin gene with the anarchist and wild-type workers cDNA was carried out in triplicate. The relative expression in the abdomen of anarchist workers relative to wild-type workers of four candidate genes were represented in Table 3.5 and plotted the graph as shown in Figure 3.11. The relative expression ratios were converted to percent change whereby a value of 100% equals no change.

Table 3.5 The expression in the 15 days old workers' abdomen of four candidate genes in anarchist workers relative to wild-type workers.

Gene name	Up or Down	Expression factor	% of expression*
Profilin (Prf)	–	–	100
Flotillin (Flt)	–	–	100
Transferrin (Trf)	–	–	100
Vitellogenin (Vit)	Up	4.470607	447

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

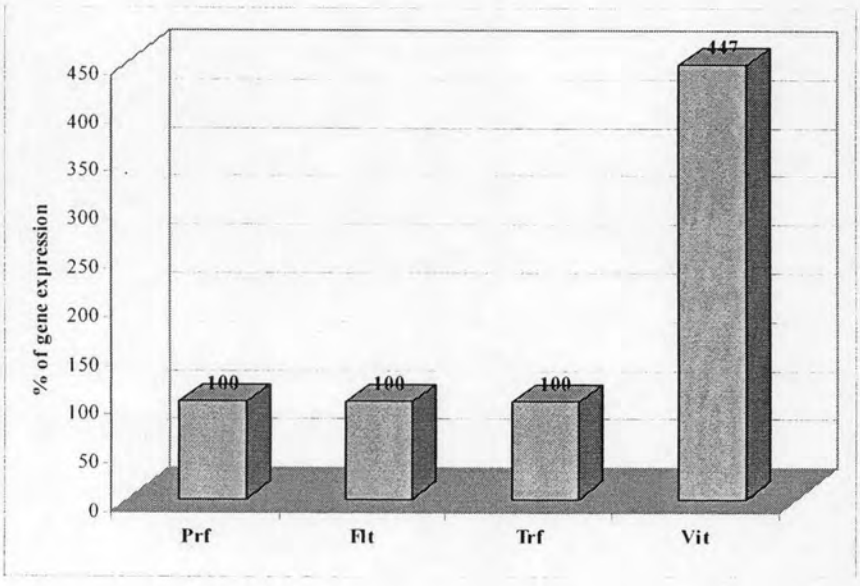


Figure 3.11 Relative expression in the 15 days old abdomen of anarchist workers relative to wild-type workers.

3.4 DISCUSSION

We did primary investigated the transcriptional expression in the abdomen and head of the six genes which had the functions in receptor/sensing molecules (take-out-like [JHBP]), signaling cascades (nitric oxide synthase [NOS], profilin [Prf], take-out-like [JHBP]), structural dynamics (profilin [Prf], flotillin [Flt]), and oocyte packaging (transferrin [Trf], vitellogenin [Vit]) between wild-type and anarchist workers.

The experiment was performed using the abdomen of the wild-type and anarchist workers because the ovary that we hypothesize to have the differential expression of any genes is located in the abdomen part. Whereas, the head part contains the brain, hypopharyngeal gland, mandibular gland, etc. that may involve in production of pheromones or perception of pheromones.

We hypothesize that differentially expressed of any genes in the head or abdomen might involve in the differential characteristics between wild-type and anarchist workers. Our northern blot result in the abdomen of wild-type and anarchist workers with the six candidate genes (Figure 3.2) showed significantly different of the vitellogenin and transferrin expression, while the others four genes (nitric oxide synthase, take-out-like, profilin, flotillin) were not significantly different between wild-type and anarchist workers. Both vitellogenin and transferrin had more expressed in the abdomen of anarchist workers than in wild-type workers.

In the head part, the expression of vitellogenin and transferrin showed significantly different between wild-type and anarchist workers but in the contrast of abdomen part, both vitellogenin and transferrin seem to be less expression in the head of anarchist workers when compared with wild-type workers. Moreover, the

expression in the head of other four genes did not show significantly different between wild-type and anarchist workers.

However, northern blot hybridization is just the semi-quantitative analysis and it is less sensitive. The northern blotting results should be re-identified with the other techniques such as quantitative real-time PCR. However, these results provided the candidate genes that may involved in the differentially characteristics expression of the wild-type and anarchist worker bees.

Vitellogenin is a prerequisite for ovary activation in honey bees (Engels *et al.*, 1990). Besides its proximate importance to egg production, however, vitellogenin is also used to synthesize proteinaceous royal jelly in the hypopharyngeal glands in the heads of workers. Nurse-age workers feed this jelly to dependent but related larvae, a behavior that constitutes a form of kin-selected alloparental care. Amdam *et al.* (2003) speculated that a key adaptation to eusociality by honey bees was the diversion of vitellogenin from its primary role in the production of brood food. Our finding that vitellogenin synthesis is up-regulated in the abdomens of ovary activated (stage 2 and 3 of ovarian development) anarchist workers implies that this molecule is actively linked to the reproductive status of individual workers.

The expression of vitellogenin in the heads of anarchist workers seems to be contrasting with in the abdomens. Our experiment was done in wild-type host colony that means the anarchist workers were cross-fostered to the wild-type host colony. This may the result why vitellogenin was less expression in the head of anarchist workers when compared with wild-type workers. It was possibly involving changes in the adaptation to the new colony, the perception of pheromones or the production of brood food. Oldroyd et al. (1999) reported that ovary activation is reduced in anarchist workers fostered into queenright wild-type hosts, this showed that the

genotype of the host colony also influences ovary activation and may be influenced any mechanisms or any genes expression in the head of worker honey bees.

The relationship between transferrin and ovary activation is less clear because transferrin is truly multifunctional in the honey bee (Kucharski and Maleszka, 2003). This multifunctional of transferrin is not involve only in the ovary or abdomen part but also in the head of the honey bees. Kucharski and Maleszka (2003) showed that the highest level of *Apis mellifera* transferrin message is found in the central brain neuropils and in the pigmented eye. By analogy to mammals, these tissues are likely to have low anti-oxidative defenses (Crichton *et al.*, 2002). Thus, transferrin may play a role in the honey bee central nervous system as a component of a protection mechanism against reactive oxygen intermediates (Kucharski and Maleszka, 2003). Lower levels of transferrin make more iron molecules available for reactions that are promoted by transition-metal ions. Iron is required for generating di-oxygen species (O_2) that are critical for many biological processes such as biosynthesis of DNA, serotonin, fatty acids and other bio-molecules (Kovacs, 2003). Iron is also an essential cofactor of a number of key enzymes needed in energy metabolism. The lower expression of transferrin in the head of anarchist workers might be the result of more needed of energy or any biosynthesis.

Transferrin may have an important role in the activation of worker ovaries. Transferrin's likely function is 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).

These higher expression of vitellogenin and transferrin in the abdomen of ovary activated anarchist workers suggesting that vitellogenin and transferrin may be the genes regulated ovary activation or at least the part of the network involved in the

regulation of ovary activation in the worker honey bees. Whatever, it has to be further investigated with more sensitive method such as real-time PCR technique.

The quantitative real-time PCR analysis showed that abdominal expression of vitellogenin had more expressed in the anarchist workers than in wild-type workers. The other three genes (transferrin, profilin and flotillin) did not show significantly differential expression in the abdomens between wild-type and anarchist workers. The expression of vitellogenin, profilin and flotillin showed the same result as the northern blot hybridization but the transferrin did not. In the northern blot analysis, transferrin showed the significantly different between wild-type and anarchist workers but in this quantitative real-time PCR analysis, transferrin did not significantly differ between wild-type and anarchist workers.

Both wild-type and anarchist workers were reared in the queenright wild-type colony making them grown in the same conditions. Moreover, the selected wild-type worker bee samples used in this experiment had already dissected the abdomens to identify the stage of ovarian development. All of the wild-type anarchist workers did not activate their ovaries (stage 0 and stage 1 of ovarian development), while some of the anarchist workers had developed their ovaries (stage 2 and stage 3 of ovarian development).

The different result of transferrin expression may be the cause of the worker bee sample collection. Both wild-type and anarchist bees that used to examine the differential genes expression in northern blot analysis and quantitative real-time PCR assay were collected in different time and different season. The level of genes expression may be difference in changes of season periods. However, transferrin is multifunctional in the honey bee and it is differentially expressed in time and space (Kucharski and Maleszka, 2003), it might be flowed or transported to the other organs

at any times that may the cause of different result between northern blot and real-time PCR or may be just the cause of sensitivity of each technique.

Because we determined the stage of ovarian development of every bee, so we can tell that the abdomens of wild-type worker bees (stage 0 and stage 1 of ovarian development) were represented the non-activated ovaries and those of the anarchist workers (stage 2 and stage 3 of ovarian development) were represented the activated ovaries. From the result that vitellogenin were up-regulated about 4-fold in the abdomens of anarchist workers when compared with wild-type workers, it was implied that vitellogenin had more expressed in the activated ovaries. We suggested that vitellogenin was the genes regulated ovary activation or at least the part of the network involved in the regulation of ovary activation in the worker honey bees.

The 180 kDa glycolipoprotein vitellogenin, known to be important in egg production, is synthesized in fat bodies and released into the hemolymph, where it is taken up as a yolk protein by developing oocytes. It appears that vitellogenin is a key and central element involved in pathways that control the life history regulation of the honey bee, including reduced longevity and immunity of foragers, thermotolerlance, and coordination of worker behavior between nurse and forager bees and within foragers between pollen and nectar foraging (Amdam *et al.*, 2003, 2004a, b, 2005; Guidugli *et al.*, 2005; Seehuus *et al.*, 2006). Nelson *et al.* (2007) reported that workers with suppressed vitellogenin levels (by knocking down) foraged earlier, preferred nectar, and lived shorter lives. Thus, vitellogenin has multiple effects on honey bee social organization, social life in bees evolved by co-opting genes involved in reproduction. Our finding that vitellogenin synthesis is up-regulated in the abdomens of ovary activated (stage 2 and stage 3 of ovarian development) anarchist workers, it

is possible that anarchist workers foraged later, preferred pollen, and lived longer lives than the wild-type workers.

Anarchist workers had at least two different characteristics compared with wild-type workers, ovary development is more common and their eggs are policed less (Oldroyd and Ratnieks, 2000; Barron and Oldroyd, 2001). These different characteristics among wild-type and anarchist workers may be the results from the differential expression of the suite of genes. Vitellogenin that had up-regulated in the abdomen, where the ovary is located, may the gene involved in ovary development of the anarchist workers or might be involved in the wild-type workers that had ovary activation. This hypothesize had to be further investigated by using the non-activated and activated ovaries wild-type workers.

However, this up-regulation of vitellogenin gene in the abdomen of anarchist workers provided the candidate gene that may regulate or at least involved in the network of worker reproduction.

Both northern blot analysis and quantitative real-time-PCR used the anarchist workers as the samples of ovary activation. Because this anarchist line is rare among honey bees, we do not want to lose this rare type so much. The experiments after this will examine the differential gene expression in the wild-type workers that were constructed to activate ovaries by using the effects of carbon dioxide and pheromones. And we will analyze with only quantitative real-time PCR because it is more sensitively and required less RNA than northern blot hybridization technique.