CHAPTER VI

CONCLUSION AND SUMMARY

Honey bees are a subset of bees, primarily distinguished by the production and storage of honey and the construction of perennial, colonial nests out of wax. Honey bees are the only extant members of the tribe Apini, all in the genus *Apis*. Engel (1999) reported that there are only seven recognized species of honey bee with a total of 44 subspecies. Honey bees represent only a small fraction of the approximately 20,000 known species of bees. Some other types of related bees produce and store honey, but only members of the genus *Apis* are true honey bees. The European honey bee (*Apis mellifera* L.) that used in this dissertation is an important model system for investigating the evolution and maintenance of worker sterility. The queen is the main reproductive in a colony. Workers can not mate, but they can lay unfertilized eggs, which develop into males if reared. Worker reproduction, while common in queenless colonies, is rare in queenright colonies. The workers that activated their ovaries could have some genetics expression different to workers that did not activate their ovaries. However, no study has yet examined locus specific changes in gene expression associated with ovary activation in workers (neither in queen). The mechanism of ovary activation in worker honey bees may involved a suite of genes that we still unclear for all of them, but at least from our study we know some genes that may involve in ovary activation in worker honey bees.

The honey bee samples that used in this study must have the differences in the ovary activation (activated and non-activated ovaries). The first honey bee samples were the anarchist and wild-type worker bees. From the characteristic of the anarchist bees, ovary development are common among queenright anarchist workers, they were the good choice for using in our experiments. The anarchist workers were represented as the activated ovaries and the wild-type workers were represented as the non-activated ovaries. We started with the primary investigation of the genes that have differentially expressed between wild-type and anarchist workers by using the northern blot analysis technique. Based on the primary experiment from the microarray (our research group) and the honey bee genomes project, the information of sequences and functions are provided. Six genes that had the functions in receptor/sensing molecules (take-out-like), signaling cascades (nitric oxide synthase, profilin, take-out-like), structural dynamics (profilin, flotillin), and oocyte packaging (transferrin, vitellogenin) were selected for this primary investigating.

We did the experiment in 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. And the head part contains the brain, hypopharyngeal gland, mandibular gland, etc. that may involve in production of pheromones or perception of pheromones.

Our northern blot result in the abdomen of wild-type and anarchist workers with the six candidate genes 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 same in the abdomen part.

Anyway, northern blot hybridization is just the semi-quantitative analysis and it is less sensitive. The northern blot results should be re-identified with the other techniques such as quantitative real-time PCR. So, the next step was quantitated the levels of differential gene expression between wild-type and anarchist workers by using quantitative real-time PCR. This experiment was confirmed the northern blot result that the expression of vitellogenin gene in the abdomen of anarchist workers was more than in those of wild-type workers. Vitellogenin was up-regulated in the abdomen of anarchist worker bees (activated ovary) about 4-fold when compared with wild-type workers. But for the other three genes (profilin, flotillin, transferrin) that we selected to quantitate did not show significantly differential expression in the abdomen between wild-type and anarchist workers.

The anarchy is a rare behavioral syndrome and the anarchic colonies are really small amount. Our experiments have to use lots of anarchist bees (workers with activated ovaries) to investigate and identify the genes that may involve in ovary activation in workers. We will lose too much the rare behavioral bees, so we have to find the other ways to make the worker bees with activated ovaries for using in our experiments instead of the anarchist bees.

Carbon dioxide has been shown to greatly influence insect behavior and physiology, insects are frequently immobilized with carbon dioxide during routine studies by biologists. Treatment with carbon dioxide has been shown to affect insect reproduction (Engels *et al.*, 1976), development (Woodring *et al.*, 1978) and other behavior (Ralph, 1959; Whisenant and Brady, 1965; Mardan and Rinderer, 1980; Schneider and Gary, 1984). Mackensen's (1947) discovery that (double) carbon dioxide narcosis results in young unmated queens laying eggs 5-6 days afterwards has provided a highly reliable method that became a basis of instrumental insemination techniques and therefore of bee genetics. Harris and Harbo (1990) found that workers treated with a 15 minutes exposure to CO_2 at 0-3 days of age produced significantly fewer eggs than controls and CO_2 had no apparent effect after the workers had been queenless for 4 days.

From this information, we exposed the groups of queenless caged workers to double CO_2 narcosis (10 minutes and 3 minutes in consecutive day) in various days of age. Our results showed via control groups (not expose to CO_2) that queenless caged workers did activate their ovaries, as in queenless colonies, whereas caged groups exposed to double CO_2 narcosis did not. This was true whether workers were narcotized early (on day 2 and 3) or slightly later (on day 4 and 5) in adult life. Thus, this study confirms that carbon dioxide narcosis does (negatively) affect of ovary activation in workers (Harris and Harbo, 1990).

In quantitative real-time PCR analysis, two more genes (arginine kinase, an enzyme implicated in energy transfer; octopamine receptor, a binder of biogenic amines and neuromodulator of many physiological processes) were selected. Six candidate genes (profilin, flotillin, transferrin, vitellogenin, take-out-like, nitric oxide synthase) from the northern blot analysis were used together.

These experimental results confirm the result of northern blot analysis. Of eight candidate genes examined from selected worker bees, six did not change their expression in the abdomen with changes in reproductive state. Genes indifferentially expressed encoded profilin, flotillin, take-out-like, nitric oxide synthase, arginine kinase, octopamine receptor. These genes clearly fall into the category of developmental regulators. Either these genes are not differentially expressed between carbon dioxide treated and control worker groups, or their differential expression was not detected by our assay, conducted 48 hours following the day 4/5 treatment. Two genes, vitellogenin and transferrin, did show significantly differences in abdomen expression (~ 4 fold) within two days of the double CO_2 treatment.

In contrast of the abdomen part, the head part of carbon dioxide treated workers (non-activated ovaries) had more expression of transferrin and vitellogenin genes (~ 3-4 fold) when compared with the control group (activated ovaries). These results were same as the northern blot analysis that transferrin and vitellogenin genes were up-regulated in the abdomen of workers that had ovary activation, while the expression of these two genes were down-regulated in the head part of the same selected worker bees.

Because carbon dioxide has been shown to be many effects to insects, the differential expression of these transferrin and vitellogenin genes may be the result from carbon dioxide, was not the result from the ovary activation of worker bees. We would like to show that these differentially expressed of transferrin and vitellogenin genes were the result of ovary activated worker bees. We made another bee sample that had activated and non-activated ovaries by using the function of pheromones.

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). In contrast, in a queenless colony, the portion of unmated workers with fully active ovaries is markedly increased with up to ~10 % of the workers being capable of laying male eggs (haploid) after seven or more days of queenlessness (Robinson 1990).

From this known information, we transferred the newly-emerged worker bees into the queenright and queenless colonies and collected them at 2, 4, 6, 10, 15, and 21 days of age. The scoring result showed that all 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.

In quantitative real-time PCR analysis, ten more genes (Major royal jelly protein 1, 3, 5, Niemann-Pick type C2 protein, Ribosomal protein L26, cGMP-dependent protein kinase (foraging), Phosphatidylinositol phosphate kinase, Phosphoinositide-3-kinase 68D, phosphoinositolglycan peptide, and tyramine receptor) were selected. Eight candidate genes (profilin, flotillin, transferrin, vitellogenin, take-out-like, nitric oxide synthase, arginine kinase, octopamine receptor) from the carbon dioxide treatment experiment were used together.

Of eighteen candidate genes examined from selected worker bees, fourteen (profilin, flotillin, take-out-like, nitric oxide synthase, arginine kinase, octopamine receptor, Major royal jelly protein 1, 3, 5, Niemann-Pick type C2 protein, Ribosomal protein L26, cGMP-dependent protein kinase (foraging), Phosphatidylinositol phosphate kinase, Phosphoinositide-3-kinase 68D) did not change their expression in the abdomen with changes in reproductive state.

Four genes, vitellogenin, transferrin, phosphoinositolglycan peptide, and tyramine, did show significantly differences in abdomen expression of 15 days old queenright and queenless worker bees. All these four genes were down-regulated in the queenright workers (non-activated ovary) compared with queenless workers (activated ovary). Transferrin and vitellogenin were down-regulated, about 9-fold and 14-fold, respectively, in the queenright (worker with non-activated ovary) relative to queenless (activated ovary) workers.

From all experiments, transferrin and vitellogenin were up-regulated in the abdominal tissue of worker that had ovary activation and down-regulated in the head tissue part of the same worker bees. For phosphoinositolglycan peptide and tyramine tend to be up-regulated in the abdominal tissue of worker that had ovary activation too, but we did not have enough samples to quantitate the level of these two genes expression. However, phosphoinositolglycan peptide and tyramine may involve in the network of ovary activation among workers, but it had to be further more studies.

Gene expression involves a cascade of events, from transcription factor transactivation to RNA processing and maturation. The candidate genes approach used in this study is based on molecules that are probably involved in relatively down-stream events and therefore may not represent molecules that initially trigger the process of ovary activation or deactivation, in particular as a consequence of CO_2/pheromone treatment and anarchic syndrome. Nonetheless, by identifying molecular components of regulatory pathways via their differential expression, we can begin to describe the molecular circuitry of reproductive regulation. Subsequent comparative studies of honey bee castes and of other insects for which regulatory mechanisms are much better understood (Bownes, 1986) will then be possible. An association between rate of vitellogenin and transferrin transcription and worker

ovary activation has not previously been demonstrated, and it is suggested that these two proteins are part of the network involved in the regulation of worker ovary functional activation, and thus involved in the regulation of functional sterility of workers.