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APPENDICES

APPENDIX A

Preparation of honey bee samples

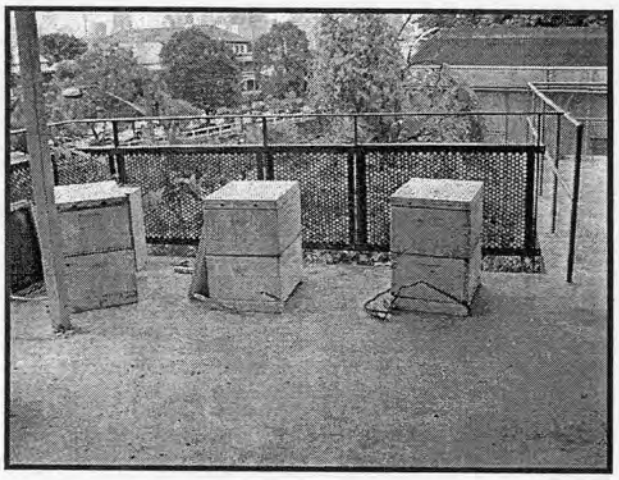


Figure A.1 The honey bee colonies reared at University of Sydney, Australia.

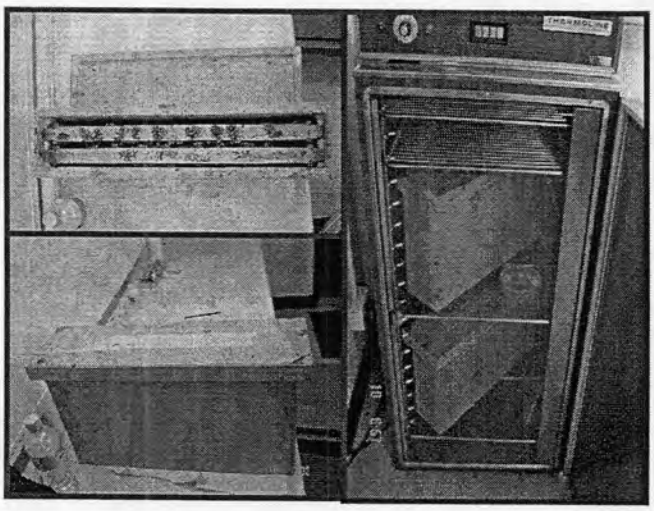


Figure A.2 Preparing of emerging sealed brood from wild type colonies.

The emerging sealed brood from wild-type colony was put in the wood box with a comb that contains honey. Incubated this box at 35°C, overnight. The following morning newly emerged workers were collected to use in the next steps.

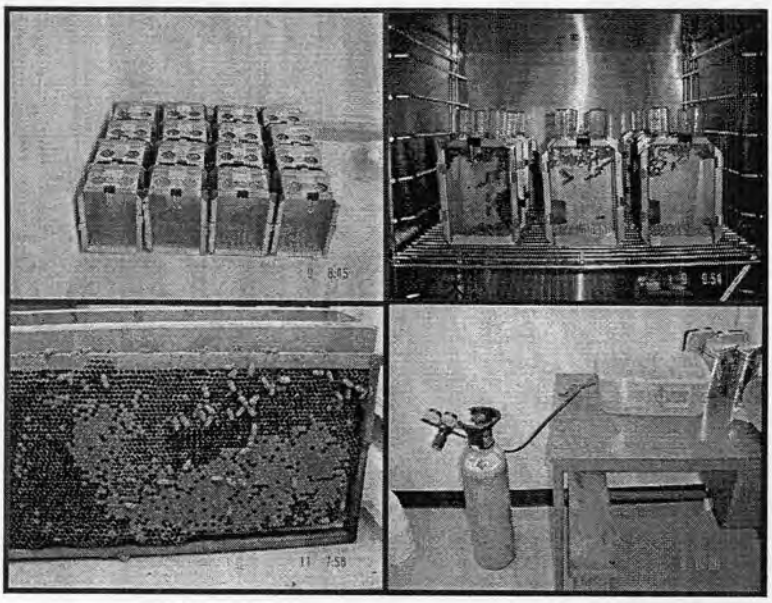


Figure A.3 The preparation of carbon dioxide treatment.

Thirty newly emerged workers were transferred to the cages. All bees were grown in the incubator at 35˚C and treated with CO_2 at the exact day by putting the cage in the plastic box, flushed with compress CO_2 until the bees were immobilized, stop flushing CO_2 and left the bees in this box for ten minutes for the first treatment and three minutes for the second treatment.

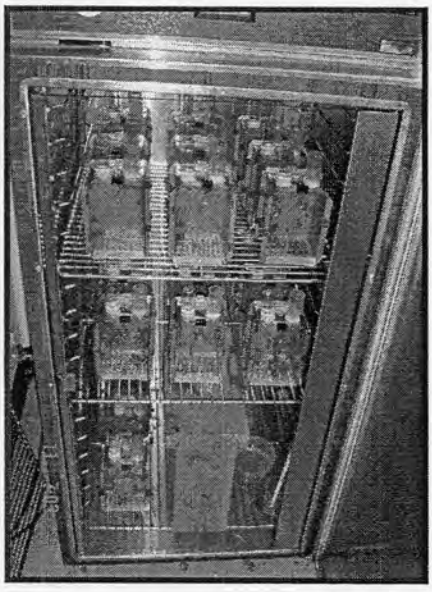


Figure A.4 All cages were incubated at 35˚C along the experiment. They were fed with royal jelly mix with honey and water.

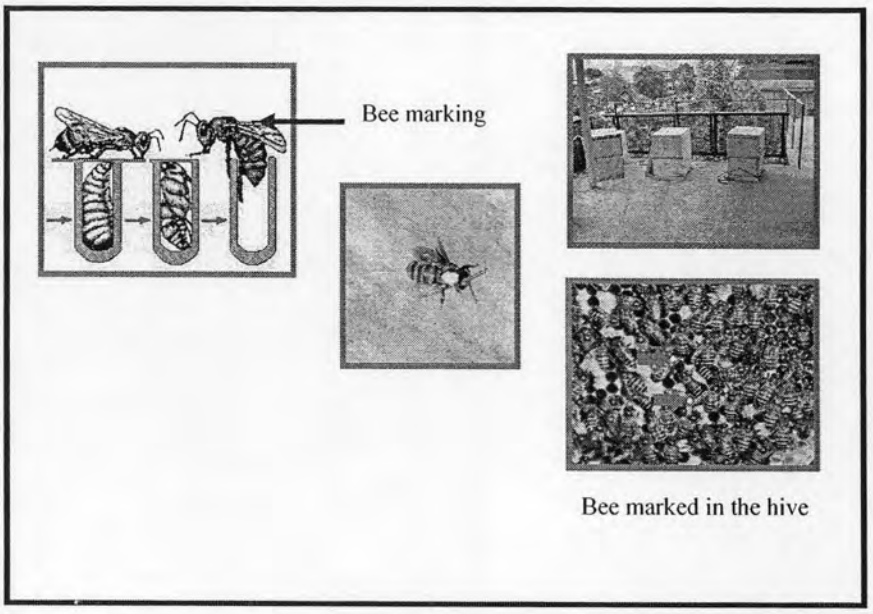


Figure A.5 The preparation of worker bees for pheromone treatment.

The newly emerged workers from wild-type colony were marked at the thorax with different colors and transferred to the queenright and queenless colonies.

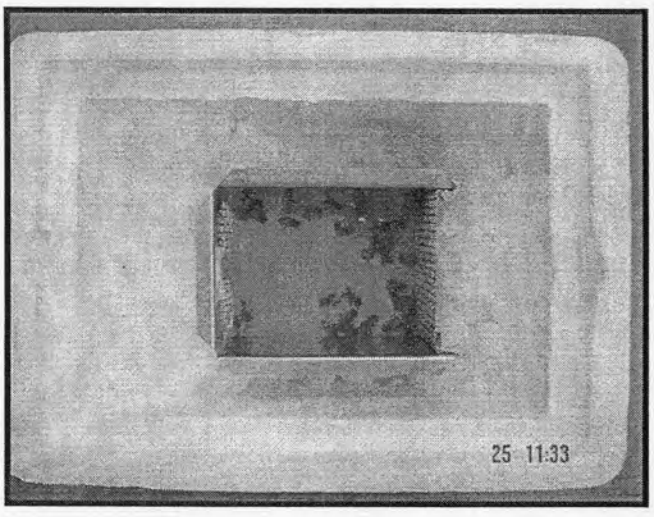


Figure A.6 The bee samples were snap-freezing in dried ice.

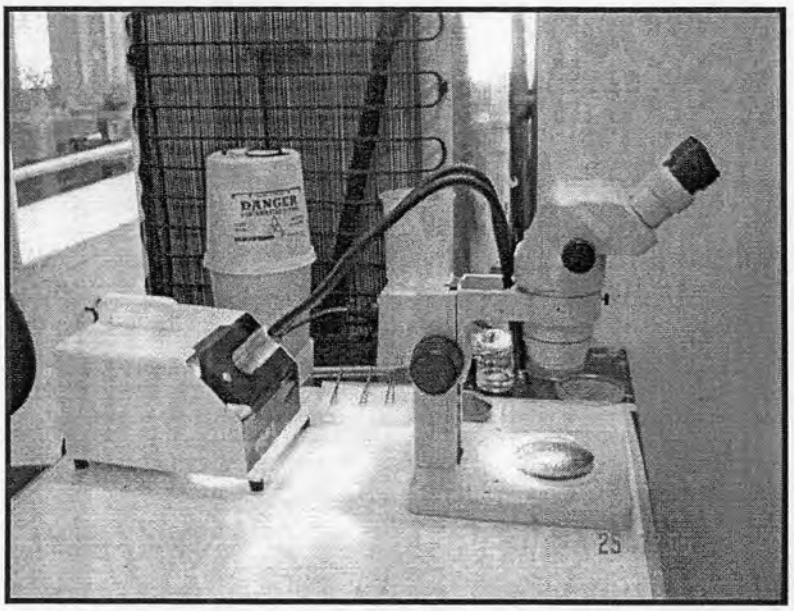


Figure A.7 The stereomicroscope is used to observe the ovarian development.

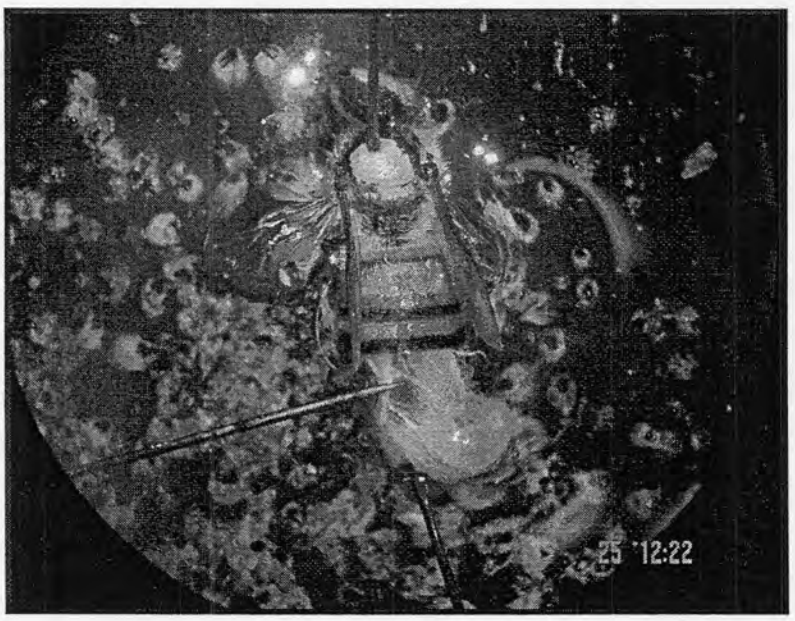


Figure A.8 The worker bee was seen from the stereomicroscope. They were dissected to observe the ovarian development.

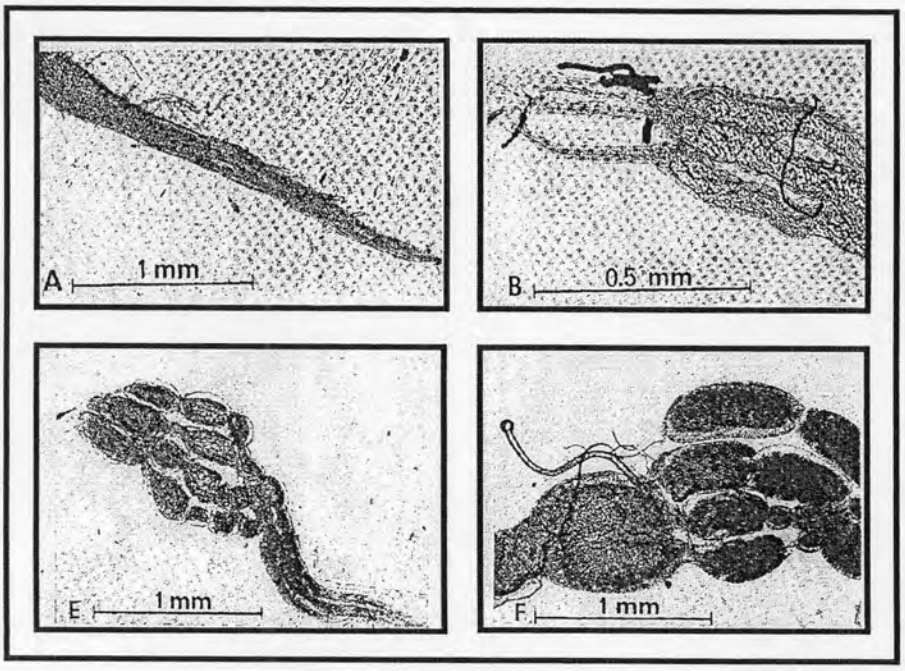


Figure A.9 The ovarian development were defined into 4 stages.

A: stage 0 - ovary thin and lacking defined ova

B: stage 1 - ovary slightly thickened

E: stage 2 - ovary thick with clearly defined ova

F: stage 3 - ovary thick with at least one fully mature ova

APPENDIX B

Quantitative Real-time PCR

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 and transferrin (candidate gene) with RpS8 (reference gene) showed a single peak at expected melting temperature, indicating that vitellogenin, transferrin and RpS8 gene were specifically amplified and there were no non-specific amplification or primer-dimer (Figure B.2). The dissociation curve of vitellogenin and RpS8 has previously shown in Chapter III (Figure 3.9).

The role and method to calculate PCR efficiency was previously described in Chapter III (3.3.4.6).

In Chapter IV, the amplification of serially diluted cDNA of the abdominal tissue with Trf/RpS8 primers and Vit/RpS8 primers were performed 3 times. The standard curves plotted in log scale were done, the slope of the graphs were used to calculate the Real-time PCR efficiency of each primer (Figure B.3, B.4 and B.5) and standard curves with a slope and PCR efficiency of Vit/RpS8 primers were shown in Figure B.6, B.7 and B.8. For the head part tissue, the slope and PCR efficiency of Trf/RpS8 primers were shown in Figure B.9 and B.10, and standard curves with the slope and PCR efficiency of Vit/RpS8 primers were shown in Figure B.11 and B.12.

The amplification of serially diluted cDNA of the abdominal tissue with Trf/RpS8 primers and Vit/RpS8 primers in Chapter V were performed 2 times. The slope and PCR efficiency of Trf/RpS8 primers were shown in Figure B.13 and B.14, and standard curves with the slope and PCR efficiency of Vit/RpS8 primers were shown in Figure B.15 and B.16.

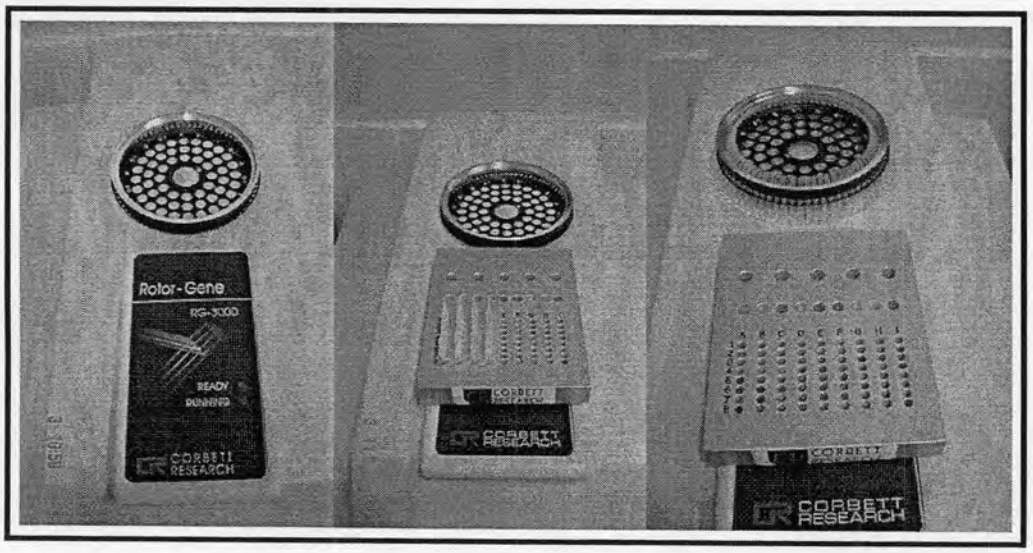


Figure B.1 Rotorgene 3000 Thermal Cycler (Corbett Research, Australia). The machine use in quantitative real-time PCR.

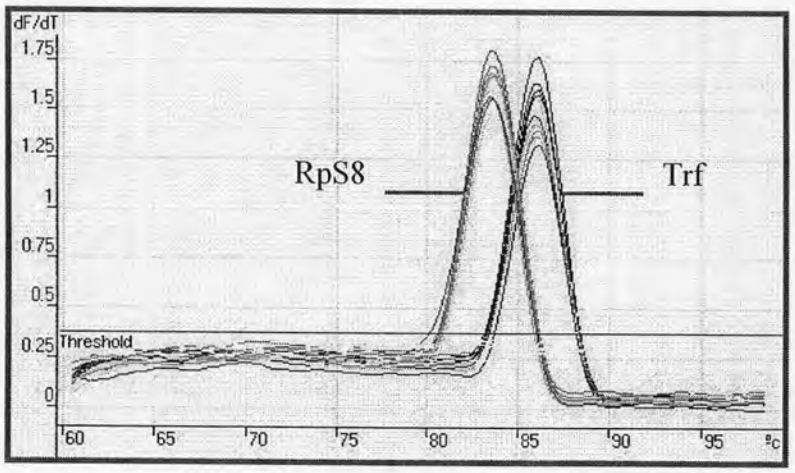
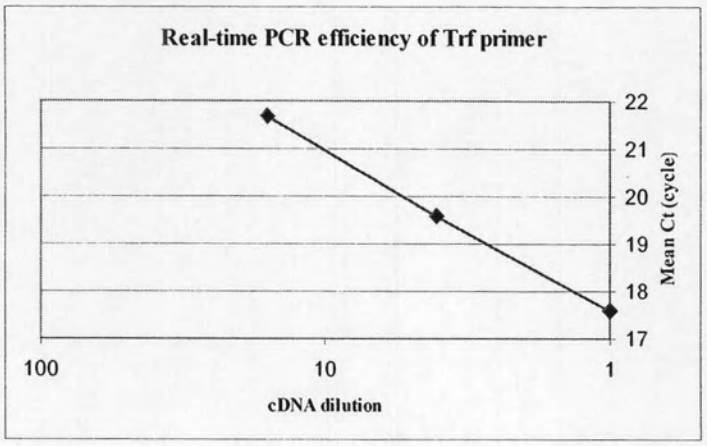


Figure B.2 Dissociation curve analysis of transferrin (Trf) & ribosomal protein S8 (RpS8) had a different melting temperature of specific product. Dissociation curve analysis was performed after a completed PCR. Data is obtained by slowly decreasing the temperature of reaction solutions from 55°C to 94°C while continuously collecting fluorescence data. The increase in temperature causes PCR products to undergo denaturation. dF/dt indicated the Derivative Melting Curve.



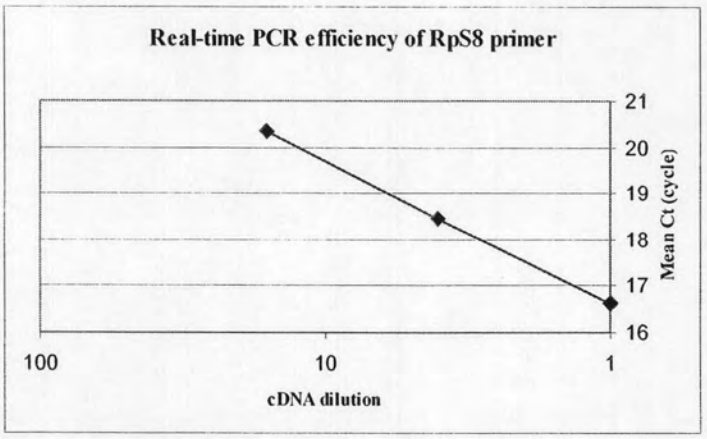
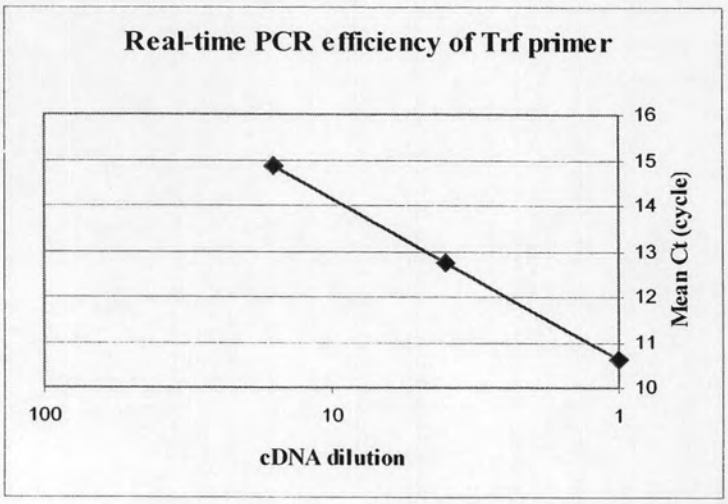


Figure B.3 The standard curves plotted in log scale of Trf/RpS8 primer of the carbon dioxide treated experiment (abdomen part).

Trf primer: slope = -3.40498, PCR efficiency = 1.966472

RpS8 primer: slope = -3.07278, PCR efficiency = 2.115621



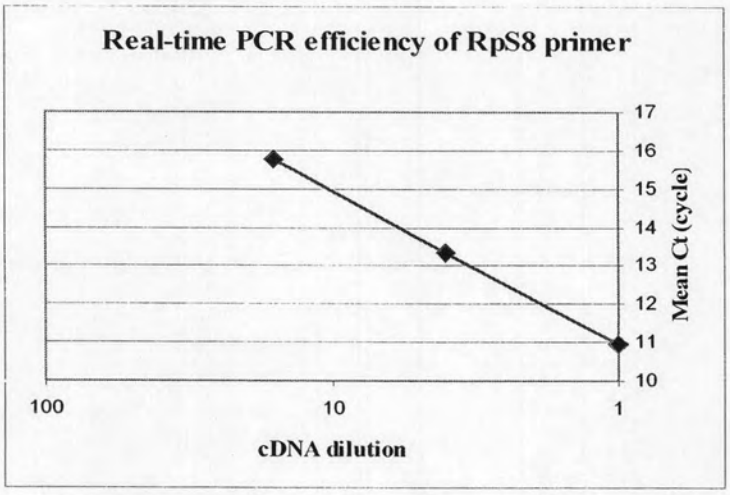
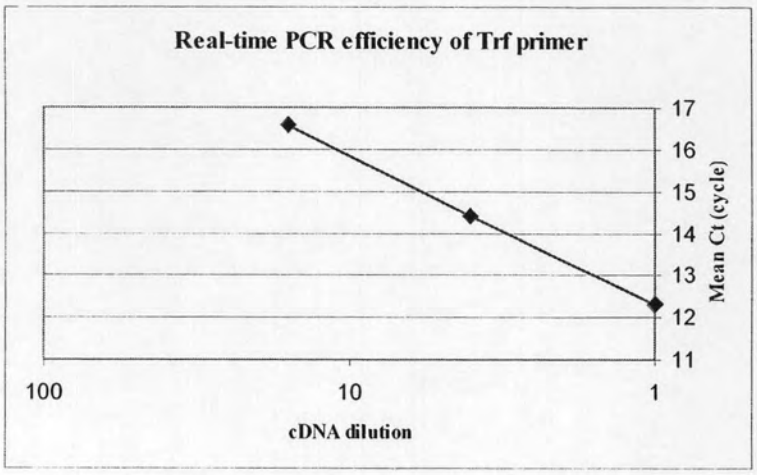


Figure B.4 The standard curves plotted in log scale of Trf/RpS8 primer of the carbon dioxide treated experiment 1st repeat (abdomen part).

Trf primer: slope = -3.52124, PCR efficiency = 1.923050

RpS8 primer: slope = -3.95586, PCR efficiency = 1.789738



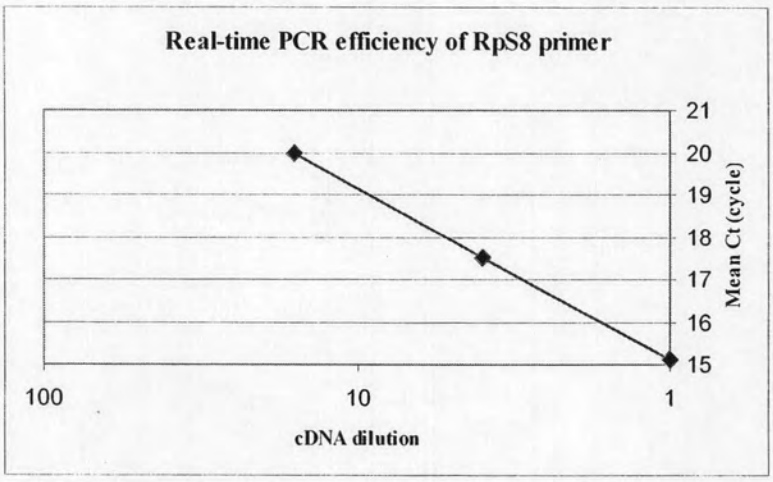
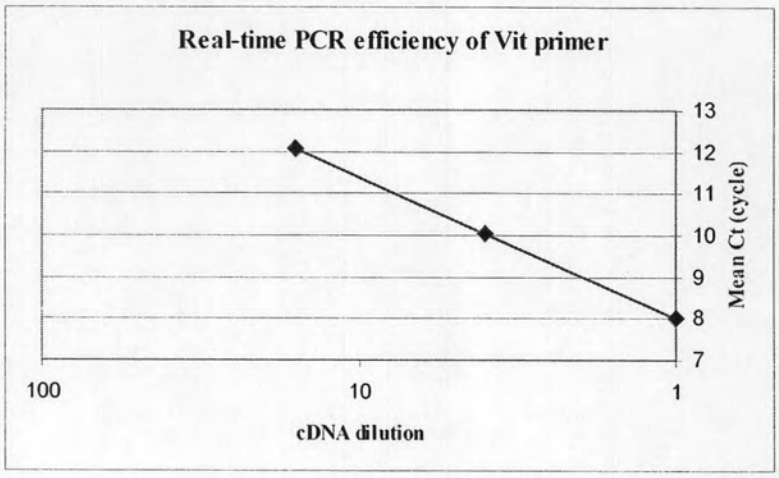


Figure B.5 The standard curves plotted in log scale of Trf/RpS8 primer of the carbon dioxide treated experiment 2nd repeat (abdomen part).

Trf primer: slope = -3.54062, PCR efficiency = 1.916181

RpS8 primer: slope = -4.03338, PCR efficiency = 1.769829



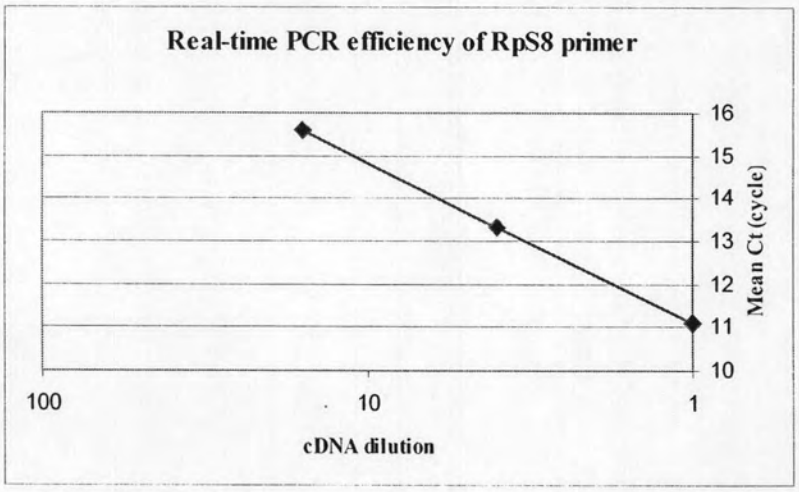
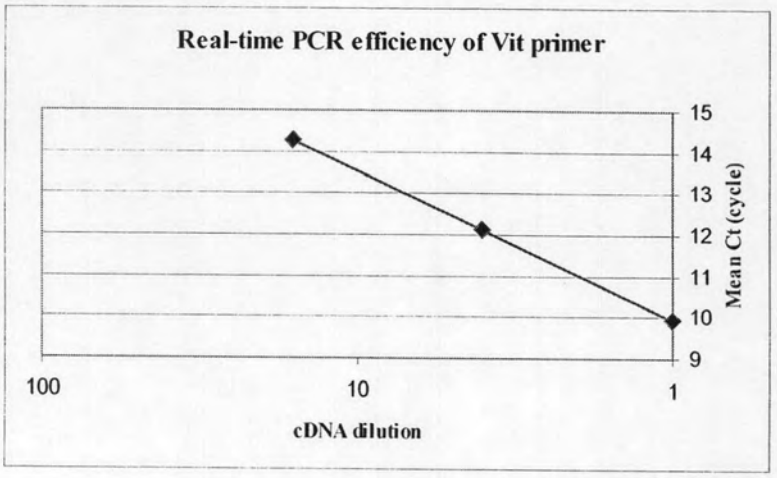


Figure B.6 The standard curves plotted in log scale of Vit/RpS8 primer of the carbon dioxide treated experiment (abdomen part).

Vit primer: slope = -3.36207, PCR efficiency = 1.983517

RpS8 primer: slope = -3.74547, PCR efficiency = 1.849221



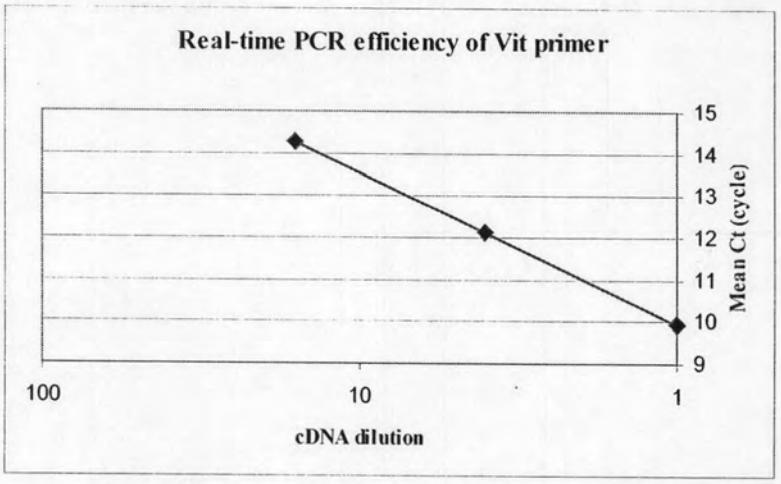
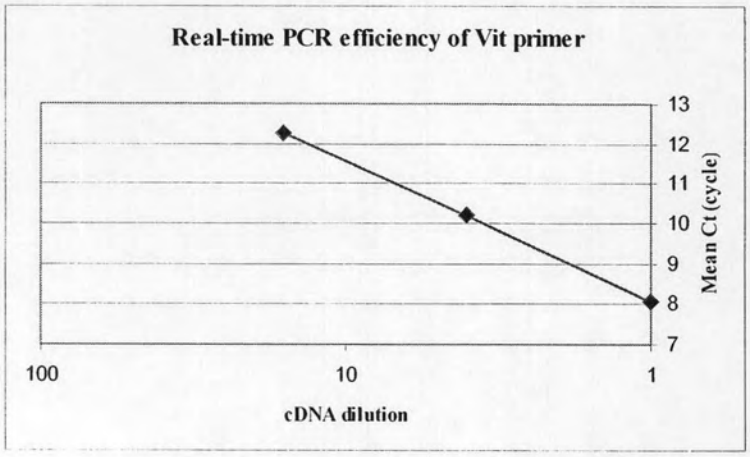


Figure B.7 The standard curves plotted in log scale of Vit/RpS8 primer of the carbon dioxide treated experiment 1st repeat (abdomen part).

Vit primer: slope = -3.58492, PCR efficiency = 1.900844

RpS8 primer: slope = -4.39186, PCR efficiency = 1.68925



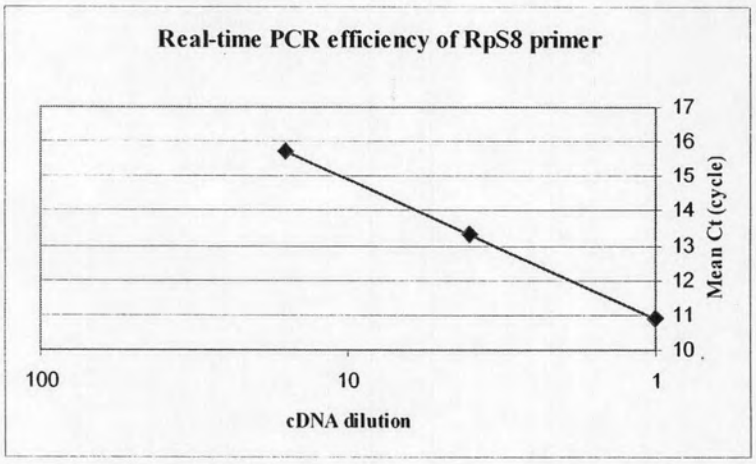
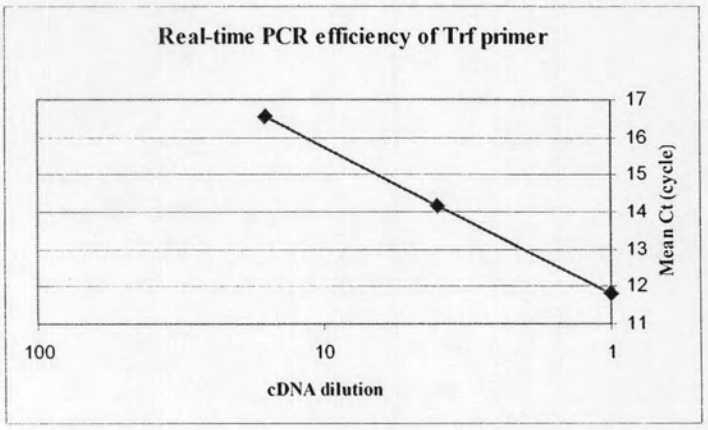


Figure B.8 The standard curves plotted in log scale of Vit/RpS8 primer of the carbon dioxide treated experiment 2nd repeat (abdomen part).

Vit primer: slope = -3.49633, PCR efficiency = 1.932032

RpS8 primer: slope = -4.00016, PCR efficiency = 1.778239



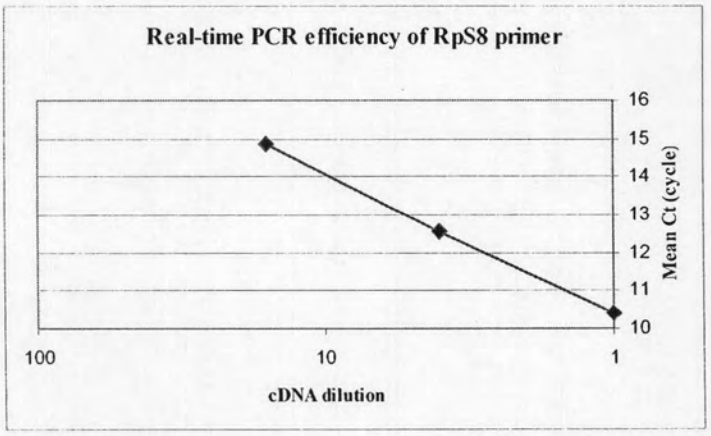
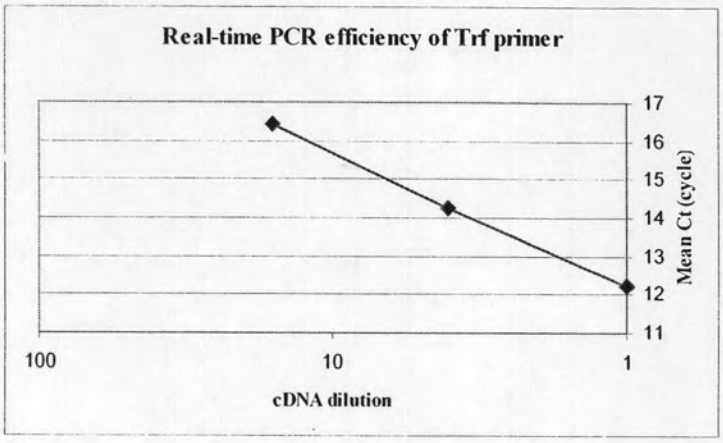


Figure B.9 The standard curves plotted in log scale of Trf/RpS8 primer of the carbon dioxide treated experiment (head part).

Trf primer: slope = -3.94756, PCR efficiency = 1.791931

RpS8 primer: slope = -3.69565, PCR efficiency = 1.864613



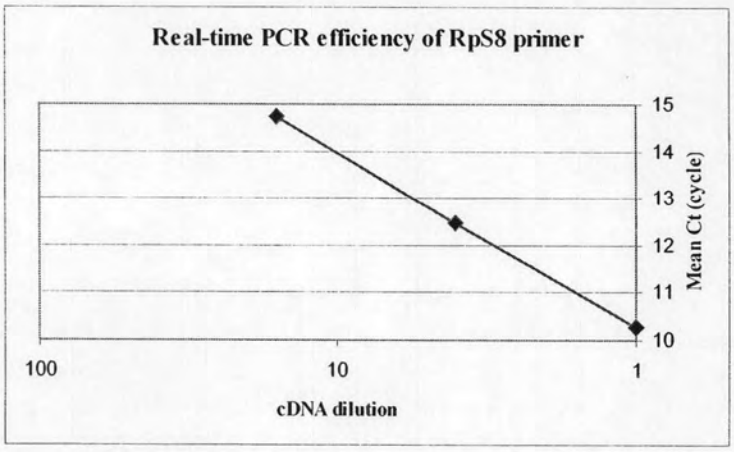
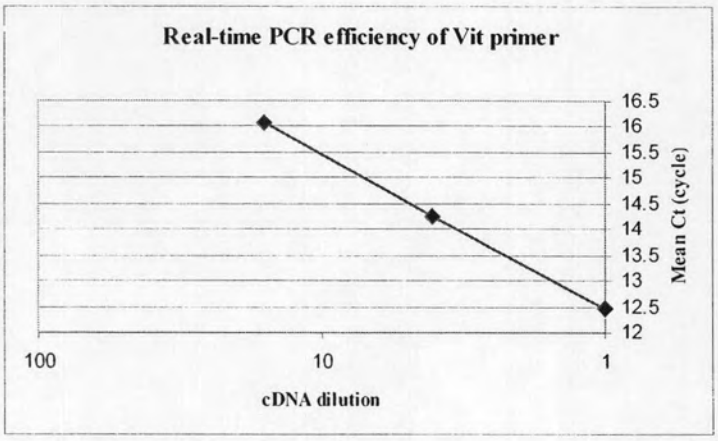


Figure B10 The standard curves plotted in log scale of Trf/RpS8 primer of the carbon dioxide treated experiment repeat (head part).

Trf primer: slope = -3.51847, PCR efficiency = 1.924042

RpS8 primer: slope = -3.56878, PCR efficiency = 1.906373



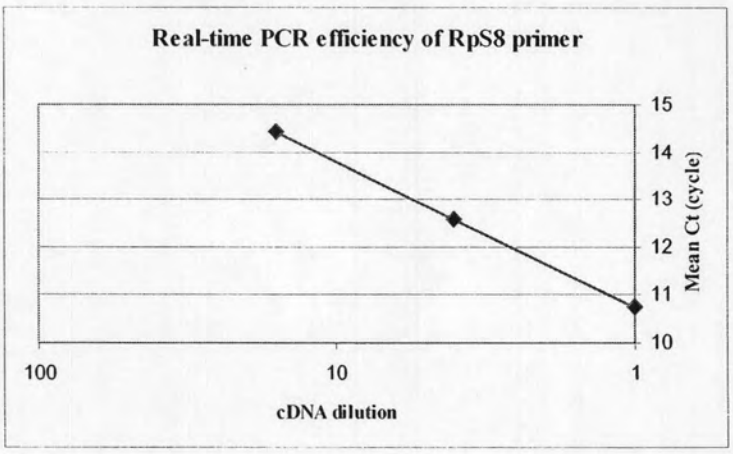
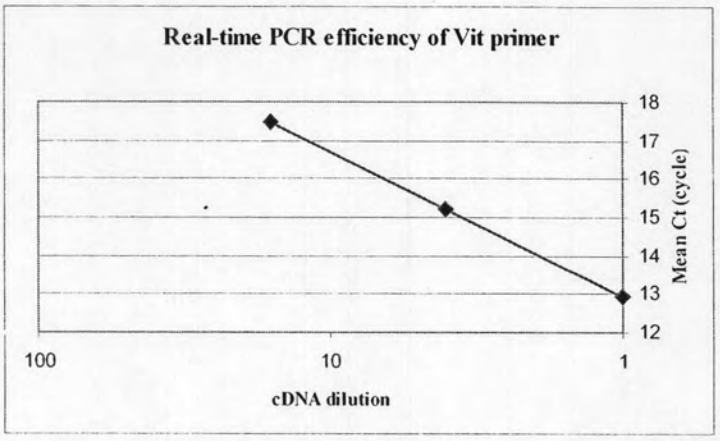


Figure B.11 The standard curves plotted in log scale of Vit/RpS8 primer of the carbon dioxide treated experiment (head part).

Vit primer: slope = -2.99804, PCR efficiency = 2.155516

RpS8 primer: slope = -3.05895, PCR efficiency = 2.122804



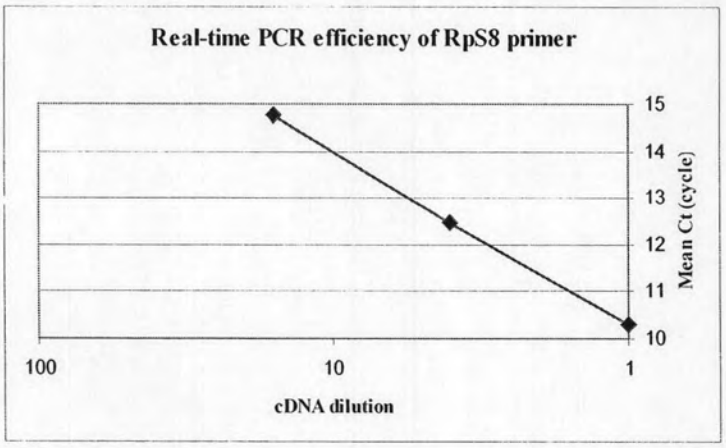
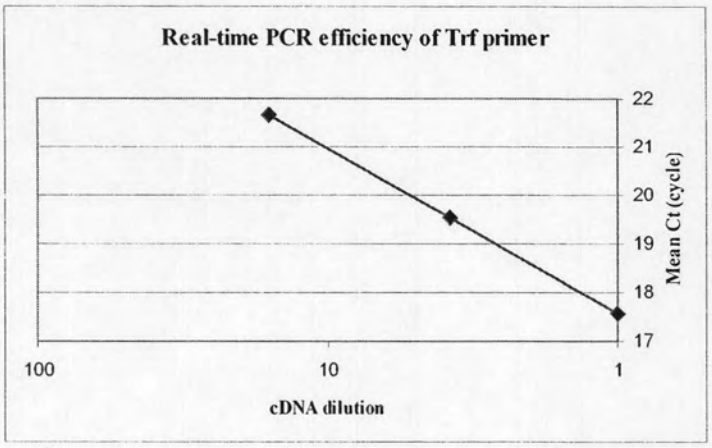


Figure B.12 The standard curves plotted in log scale of Vit/RpS8 primer of the carbon dioxide treated experiment repeat (head part).

Vit primer: slope = -3.77039, PCR efficiency = 1.841725

RpS8 primer: slope = 1.841725, PCR efficiency = 1.869866



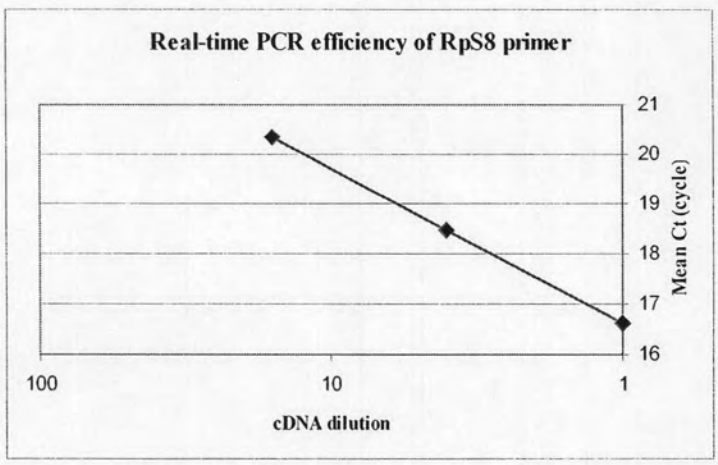
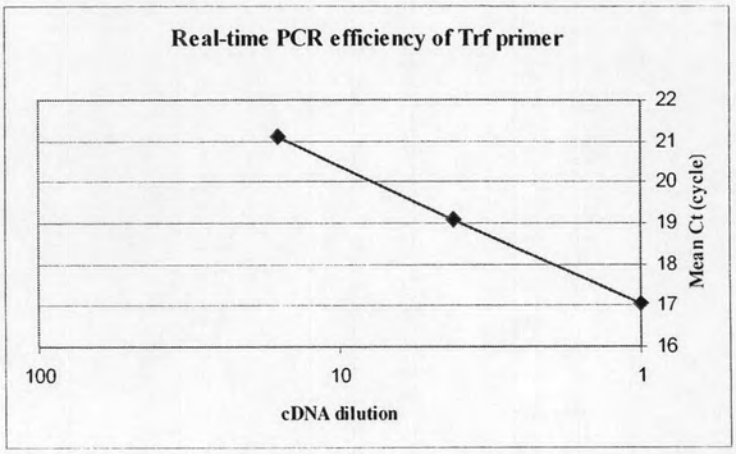


Figure B.13 The standard curves plotted in log scale of Trf/RpS8 primer of the pheromone treated experiment.

Trf primer: slope = -3.40498, PCR efficiency = 1.966472

RpS8 primer: slope = -3.07278, PCR efficiency = 2.115621



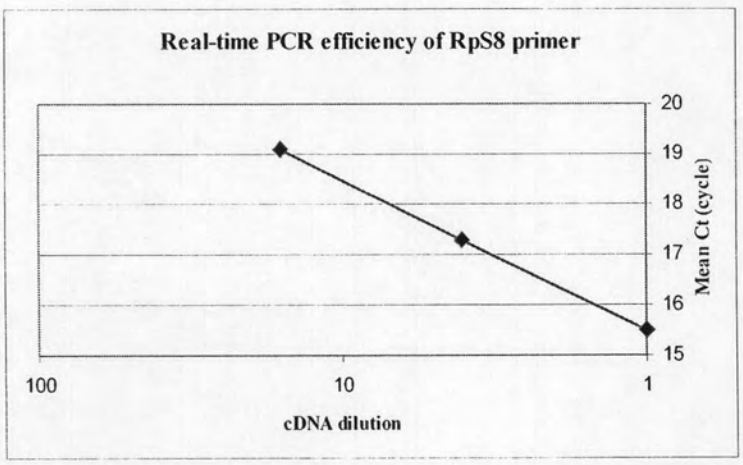
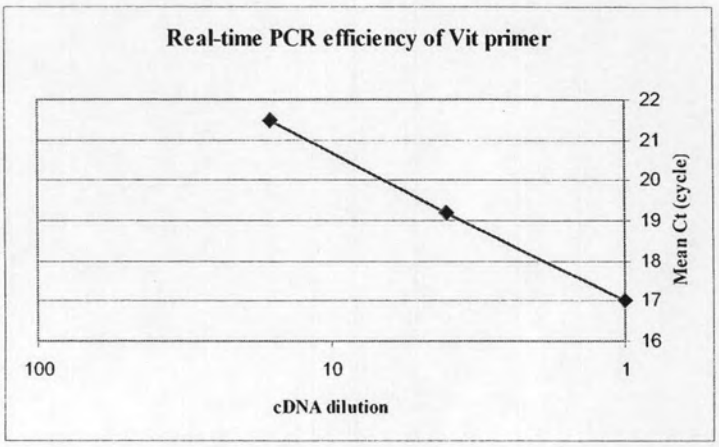


Figure B.14 The standard curves plotted in log scale of Trf/RpS8 primer of the pheromone treated experiment repeat.

Trf primer: slope = -3.37730, PCR efficiency = 1.977401

RpS8 primer: slope = -2.98974, PCR efficiency = 2.160119



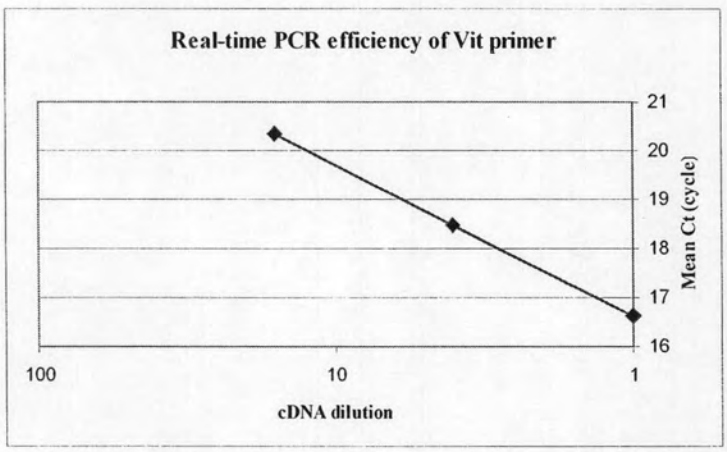
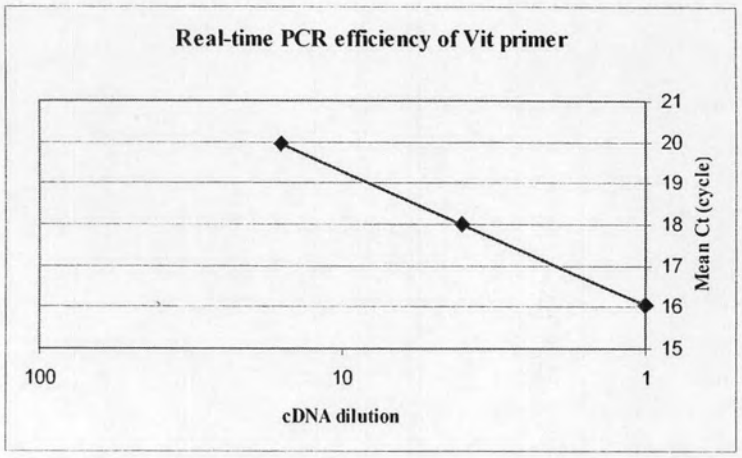


Figure B.15 The standard curves plotted in log scale of Vit/RpS8 primer of the pheromone treated experiment.

Vit primer: slope = -3.70949, PCR efficiency = 1.860282

RpS8 primer: slope = -3.07278, PCR efficiency = 2.115621



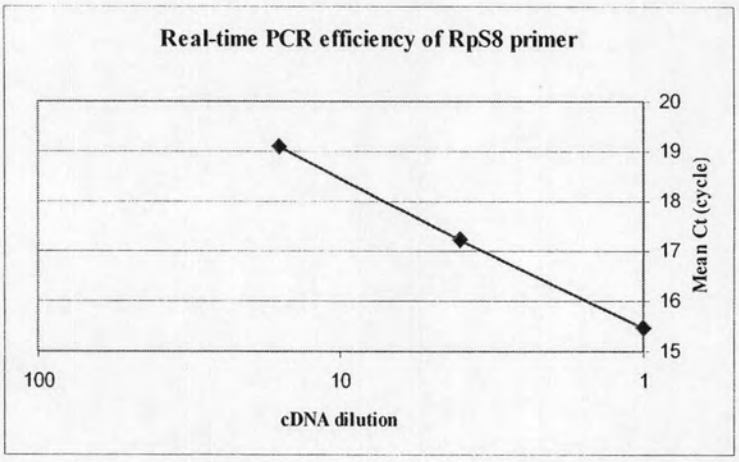


Figure B.16 The standard curves plotted in log scale of Vit/RpS8 primer of the pheromone treated experiment repeat.

Vit primer: slope = -3.21119, PCR efficiency = 2.048382

RpS8 primer: slope = -3.01742, PCR efficiency = 2.144912

APPENDIX C

Personal Information

Field of research interest

My dissertation focused on molecular biology of the honey bees *Apis mellifera* L. Methodologically, I am familiar with biochemical laboratory techniques such as Total RNA/mRNA and genomic DNA extraction, PCR, differential display PCR, quantitative real-time PCR technique, northern blot hybridization technique as well as DNA cloning.

Scholarships

2001-2008

Academic scholarship from Thailand Research Fund to the Royal Golden Jubilee Ph.D. program, Bangkok, Thailand

Academic experiences

1. The 9th Biological Sciences Graduate Congress. December 16-18, 2004. Chulalongkorn University, Bangkok, Thailand. "Effects of carbon dioxide on ovarian development of worker honey bees (*Apis mellifera*)". (Oral presentation)

2. The RGJ-Ph.D. Congress VI Congress. April 28-30, 2005. Jomtien Palm Beach Resort, Pattaya, Chonburi, Thailand. "Effects of carbon dioxide narcosis on ovary activation and gene expression in worker honey bee". (Oral presentation)

3. The 20th IUBMB – 11th FAOBMB Congress 2006 for Life: Molecular Integration and Biological Diversity. June 18-23, 2006. Kyoto International Conference Hall, Kyoto, Japan. "Effects of carbon dioxide narcosis on ovary activation and gene expression in worker honey bee". (Poster)

Publications

1. Koywiwattrakul P, Thompson GJ, Sittipraneed S, Oldroyd BP, Maleszka R. Effects of carbon dioxide narcosis on ovary activation and gene expression in worker honeybees, *Apis mellifera*. *Journal of Insect Science*. 2005; 5:36.

2. Koywiwattrakul P, and Sittipraneed S. Expression of vitellogenin and transferrin in activated ovaries of worker honey bees, *Apis mellifera*. *Biochemical Genetics*. 2009; 47(1-2):19-26.

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Effects of carbon dioxide narcosis on ovary activation and gene expression in worker honeybees, Apis mellifera

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Abstract

In an effort to uncover genes associated with ovary activation in honey bee workers, the extent to which eight candidate genes co-varied in their expression with experimentally-induced changes in worker reproductive state was examined. Groups of caged, queenless workers narcotized with CO_2 on consecutive days early in adult life showed a significantly lower level of ovary activation than did groups of untreated workers. This same experimental treatment, by contrast, is known to accelerate ovary activation and induce egg laying in virgin honey bee queens – an observation that suggests that CO_2 narcosis has contrasting effects in queen versus worker ovary activation. Experimentally-induced changes to worker reproductive state were associated with changes in gene expression. Vitellogenin, an egg yolk precursor, and transferrin, an iron transporter, were two transcripts found to be significantly down-regulated as a function of the ovary-inhibiting treatment. CO_2 narcosis did not effect the expression of six other genes selected as putative markers for processes that may underlie ovary activation. The show that the expression of vitellogenin and transferrin is correlated with ovary activation in workers, and may therefore be part of the gene network involved in the regulatory control of functional sterility in honeybees.

Cite this paper as:
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Keywords: anaesthesia, genes for sterility, reproductive division of labor, social insects, quantitative PCR, transferrin, worker sterility, vitellogenin
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Introduction

Like many other species of social insect, the honey bee *Apis mellifera* L., is characterized by extreme reproductive division of labour. The vast majority of workers are functionally sterile, and never activate their ovaries throughout their life (Barron et al., 2001), leaving the production of eggs to the queen. Uncovering the genes associated with worker sterility in social insects will provide an important step forward in our understanding of a major evolutionary transition (Maynard Smith and Szathmàry, 1995), namely, the transition from individual organisms to integrated societies, and our best example yet of kin-selected 'genes for altruism' (sensu Dawkins 1976): genes that reduce an individual's direct reproductive success while enhancing the reproductive success of relatives.

All worker honeybees develop into adults with vestigial ovaries. Although some or all wild type workers of intermediate age are able to subsequently activate their ovaries in response to a period of protracted queenlessness, and lay eggs, queenright workers almost never do so, retaining their ovaries in a vestigial state. An important exception is the selectively-bred 'anarchistic' line of honeybees maintained at The University of Sydney in which about 40% of 10 day-old workers have functionally activated ovaries and lay eggs at high frequency (Barron et al., 2001). 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 workers with and without activated ovaries should reveal genes associated with the regulatory control of worker sterility.

For reasons that are not well understood, the ovaries of unmated (virgin) queens can be experimentally activated, as if mated, by subjecting queens to double CO_2 narcosis (Mackensen, 1947). This phenomenon is routinely exploited to induce oviposition in unmated but artificially inseminated queens (Laidlaw and Page, 1997). This idiosyncratic response has facilitated the physiological study of queen egg production (Engels et al., 1976; Engels and Ramamurty, 1976; Engels, 1987). However, few studies have applied this technique to the study of worker egg production (Harris and Harbo, 1990; Harris et al., 1996), and no study has yet examined locus-specific changes in gene-expression associated with ovary activation, in queens or workers. The effects of CO_2 narcosis in queen honeybees indicate that the treatment triggers the up-regulation of genes that in turn activate the queen's ovaries. This is particularly evident in the case of vitellogenin, an egg protein, whose synthesis is dramatically stimulated in queen abdomens following CO_2 treatment (Engels et al., 1976). Genes directly associated with ovary activation in queens, like *vitellogenin* for example, are prime candidates for genes associated with ovary activation in normally sterile workers.

We speculated that the genes associated with ovary activation in queens in response to CO_2 narcosis might also be the genes associated with ovary activation in workers. To test this hypothesis we exposed groups of caged workers to double CO_2 narcosis and compared rates of ovary activation and levels of gene expression against groups of control (un-narcotized) workers. Differential ovary activation and gene expression in response to CO_2 exposure occurred. These findings are discussed in the context of the regulation of ovary activation in queens and workers.

Materials and Methods

Experimental treatment

The general approach was to use groups of caged queenless workers for comparing rates of ovary activation and levels of gene expression. To this end, we incubated emerging sealed brood from wild type colonies at 35° C overnight. The following morning, newly-emerged workers were collected and groups of 30 were transferred into wire-mesh cages (approx. 14 x 10 x 7 cm) fitted with a piece of beeswax foundation comb (6 cm x 3 cm), water and food (45% honey; 10% water; 45% royal jelly, Lifetime Health Products, Australia). After two (Experiment 1) or four (Experiment 2) days of incubation (~35° C, 40% RH), workers were narcotized at room temperature by placing whole cages into a larger plastic container and flushing the container with compressed CO_2 until the workers were immobilized. The workers were maintained in the narcotised state for 10 minutes. The following day, workers were narcotised again, for 3 minutes (after Engels et al., 1976). In each case, workers were completely anaesthetized. Then, at various time intervals following the double narcosis treatment (4-hrs, 24-hrs, 48-hrs, 96-hrs),

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workers were collected from incubation by snap-freezing them in liquid N_2. Thus, collections were single pairs of cages sampled at each collection interval.

Bee abdomens were later dissected according to Dade (1977) and assigned a numerical score reflecting the state of ovary activation: 0 (ovaries thin and lacking defined ova), 1 (ovaries slightly thickened but still lacking defined ova), 2 (ovaries thick with clearly defined ova), 3 (ovaries thick with a least one fully mature ova). For each collection, differences in levels of ovary activation between treated and control cages were evaluated using t-tests for equality of mean ovary score between cages, as well as X^2 tests of the proportions of each ovary activation class between treatments.

RNA extraction and cDNA synthesis

Total RNA was extracted from the abdomens of eight workers per treated and untreated cage. A combined Trizol/Qiagen protocol was used to extract RNA under standard conditions so that it was suitable for quantifying levels of mRNA. Briefly, frozen tissues were homogenized in 1.5 ml Eppendorf tubes, containing 50-70 µl of Trizol reagent (Invitrogen, www.invitrogen.com), using disposable pestles from Sigma (Sigma-Aldrich, www.sigmaaldrich.com) attached to a hand-held engraving device (Arlec, Super Tool, www.arlec.com.au/). One abdomen was used per tube. Following the homogenization step the volume was adjusted to 500 µl with Trizol and then mixed with 100 µl of chloroform. The RNA-containing buffer phase was recovered after spinning for 10 min at 10,000 × g, mixed with an equal volume of 70% ethanol and applied on a Qiagen (www.qiagen.com) RNeasy column. The remaining steps were carried out according to Qiagen's protocols. Standardised aliquots of individual RNA extractions were pooled by treatment prior to cDNA synthesis. We used pooled rather than individual samples to examine gene expression in order to minimize inter-individual variations, as described in other studies (Grozinger et al., 2003; Tian et al., 2004). Reverse transcription was performed according to Invitrogen's protocol using 5 µl of the pooled total RNA. The cDNA synthesis primer was $T_{(20)}MN$ (M=A,G, or C; N=A, G, C, or T).

Selection of candidate markers for ovarian activation in the honeybee

The genes selected for transcriptional evaluation in this study encode transcripts known, or that were suspected, to be involved either in ovarian development (vitellogenin, transferrin, profilin, octopamine receptor) or in signalling pathways critical for cellular growth or cellular differentiation (flotillin, nitric oxide synthase, take-out-like). Arginine kinase was also selected because it is an important component of energy transfer and its differential expression in the honey bee has already been reported (Kucharski and Maleszka, 1998). 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) and 2) the availability of an annotated gene sequence in the honey bee (http://www.hgsc.bcm.tmc.edu/projects/honeybee/). Table 1 provides more details regarding the biochemical and functional properties of these candidate genes.

Table 1. Description of genes used for transcriptional evaluation

Gene product (abbrev.)	Biochemical properties & putative function(s)	Reference
Profilin (Chickadee) (**PRF**)	An intercellular cytoplasm transport molecule required during Drosophila oogenesis. Binds actin monomers, membrane poly-phosphoinositides and poly-L-proline. Profilin may link the cytoskeleton with major signalling pathways by interacting with components of the phosphatidylinositol cycle and Ras pathway.	(Cooley et al. 1992)
Flotillin-1 (**FLT**)	Marker for membrane microdomains (lipid "rafts"), participates in growth factor-induced regulation of the actin cytoskeleton formation.	(Haglund et al. 2004)
Transferrin (**TRF**)	Iron binding protein with multiple functions. Differentially expressed in young and foraging worker and in virgin and mature queens.	(Kucharski and Maleszka 2003)
Vitellogenin (**VIT**)	A female-specific glucolipoprotein yolk precursor produced by all oviparous animals, but other functions have also been implied (nurse bees use vitellogenin to produce royal jelly).	(Piulachs et al. 2003)
Nitric oxide synthase (**NOS**)	An enzyme responsive to oxygen related hypoxia. Synthesises nitric oxide, an important second messenger.	(Nilsson et al. 2004)
Arginine kinase (**ArgK**)	An important component of the energy releasing mechanism in tissues with high energy demands.	(Kucharski and Maleszka 1998)
Octopamine receptor (**OCTR**)	A binder of biogenic amines and neuromodulator of many physiological processes.	(Grohmann et al. 2003)
Take-out-like carrier protein (**JHBP-1**)	Take-out-like carrier protein with a juvenile hormone binding motif. Members of this family regulate embryogenesis, maintain the status quo of larva development and stimulate reproductive maturation in the adult forms.	(Kolodziejczyk et al. 2003)
Ribosomal protein (**RpS8**)	Component of ribosomes, but also multiple functions: chromosomal condensation, replication, transcription, RNA processing, DNA repair, and protein-protein interactions.	(Kucharski and Maleszka 2002)

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Table 2. Sequences of gene-specific primers used for qRT-PCR assays

Gene product (abbrev.)	GenBank Acc.	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
Profilin (Chickadee) (**PRF**)	AY545000	CAGGATTACGTTGACAAGCA	CGATCCGTACCTGACAGGTA	220
Flotillin-1 (**FLT**)	GroupUn10026	CCAAACGAAGCTCTCGTAGTA	TACTTGTCAAGGTGTACCA	168
Transferrin (**TRF**)	AY217097	AGCGGCATACTCCAGGGAC	CGTTGAGCCTGATCCATACGA	190
Vitellogenin (**VIT**)	CAD56944	CCGACGAGGAGCTGTTGATTA	CTAGGATACGTGGTCATGACA	148
Nitric oxide synthase (**NOS**)	GroupUn473	GACAAAGCTATGATAGAAGCTAA	CCATAGCCAATCAGCTGGACATCCA	156
Arginine kinase (**ArgK**)	AF023619	GTGCCTCGAGGAGTAACTCA	GCGTAAATGCCAACGCCAGA	267
Octopamine receptor (**OCTR**)	CAD67999	AGCTCACTCAAGTCACCGAGGA	GCGAATGCACAGTCGGTTCTGA	480
Take-out-like (**JHBP-1**)	AY736135	CGTCTTGACAACAATGTTGATGA	CCAATCGCTCGATCGGCAGAA	182
Ribosomal protein (**RpS8**)	AF080430	ACGAGGTCCGAAACTGACTGA	GCACTGTCCAGGTCTACTCGA	175

Quantitative PCR

Target sequences for PCR were selected from cDNAs and ESTs using the genomic scaffolds at the Baylor College of Medicine. To eliminate genomic DNA amplification we manually designed primers that either spaned intron/exon junctions or amplified exon sequences separated by long introns. Our primers were 40-60% GC-rich and contained 20-24 bases to maximize reaction efficacy. 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. These gene-specific primers are listed in Table 2.

The relative amount of specific cDNA template between treated and control samples was quantified using real time quantitative PCR (qRT-PCR). *Taq* polymerase (0.2 units) from Promega (www.promega.com) was used in 20 µl reactions (in triplicate) containing 2 mM $MgCl_2$, 200 µM of each dNTP, 0.25 µM each of forward and reverse primer, and cDNA template equivalent to approximately 25 ng of total RNA. For every reaction, an RNA sample without reverse transcriptase was included to control for genomic DNA contamination. Product formation was monitored by the inclusion of SYBR Green I (Fisher Biotech, www.fishersci.com) at a final dilution of 1:40,000. Thermocycling was conducted in a RotorGene 3000 Thermal Cycler (Corbett Research, www.corbettresearch.com) for 35 cycles consisting of denaturation for 30 s at 94° C, annealing at 60° C for 30 s, extension at 72° C for 30 s, and fluorescence acquisition at 84° C for 15 s. 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 $^{s-1}$ with fluorescence acquired after every 1° C increase.

Estimation of Changes in Transcript Abundance

The amplification efficiencies of primer pairs used in qRT-PCR assays varied by less than 10% compared with that of the normalizer (ribosomal protein S8; Table 1), which was ~1.85. These values were determined by the slope of the curve generated by amplification of serially diluted cDNA over at least three orders of magnitude (data not shown). Accordingly, all primer pairs were nominated as having approximately equal efficiencies and, under this simplifying assumption, fold changes in relative transcript abundance were calculated using the method of Livak and Schmittgen (2001).

Results

CO_2 narcosis and ovary activation - Experiment 1

Very young workers, bees narcotised on days 2 and 3 and examined just 4 hrs after treatment, had relatively low mean ovary scores, with no individual scored as having well-defined ova (i.e. all scores ≤ 1). Neither the mean score (Figure 1A) nor the categorical distribution of ovary activation scores (X^2_1= 0.069, P = 0.8) differed between treated and control cages. At 24 hrs post-treatment a general increase to mean score was observed in both treated and control groups. However, this increase did not differ between treatments X^2_1= 0.271, P = 0.6, (Fig.1).

By 48 hrs post-treatment, however, an effect was apparent. Control workers showed significantly greater levels of ovary activation than did treated workers, X^2_2= 7.735, P = 0.021 (Fig. 1A). Finally, bees examined 96 hrs post-treatment showed the greatest difference ovary activation between treated and control cages X^2_3= 10.764, P = 0.013 (Fig. 1A), again with the control group showing significantly higher levels of ovary activation.

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CO_2 narcosis and ovary activation - Experiment 2

For bees narcotised 4 and 5 days post emergence, the pattern was similar. At 4 hrs after treatment bees had low and similar ovary scores, $X^2_2 = 1.077$, $P = 0.6$, while at 48 hrs following treatment controls showed significantly greater ovary activation than did treated workers $X^2_3 = 16.831$, $P = 0.001$ (Fig. 1B).

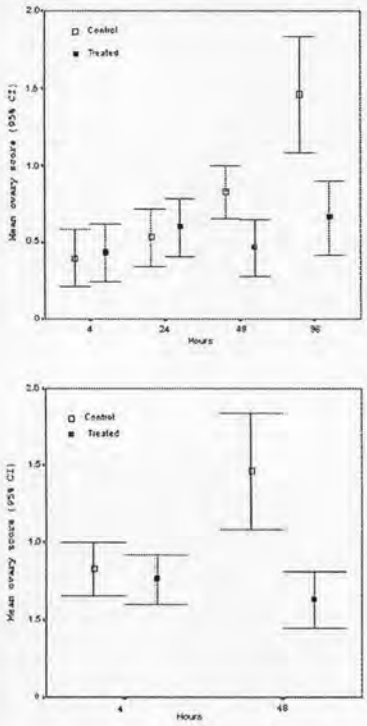

Figure 1. Effects of CO_2 on ovary activation of worker honeybees

A) In *Experiment 1 Apis mellifera* workers were narcotized on days 2 and 3 of adult life, and their ovaries examined after 4 hrs, 24 hrs, 48 hrs, and 96 hrs. The mean ovary score of narcotized bees was significantly lower than controls at 48 hrs (t-test, $P = 0.005$) and 96 hrs (t-test, $P = 0.003$).

B) In *Experiment 2* workers were narcotized on days 4 and 5 of adult life, and their ovaries examined after 4 hrs and 48 hrs. The mean ovary score of narcotized bees was significantly lower than controls at 48 hrs (t-test, $P < 0.001$).

Quantitative real-time PCR

Figure 2 shows the responsiveness of eight candidate genes to CO_2 treatment. Bees narcotised on days 4 and 5 (Experiment 2) and collected 48 hrs later were used as the basis for examining gene expression differences. For all eight genes, the level of expression was calculated for treated bees relative to non-treated controls. Thus, genes whose relative expression was estimated at '1' were deemed to show no expression difference between treated and control groups, whereas genes significantly above or below this value, as evidenced by their 95% confidence intervals, were considered to be over- or under-expressed in treated workers, respectively. Genes encoding profilin (aka chickadee; PRF), flotillin (FLT), nitric oxide synthase (NOS), arginine kinase (ArgK), octopamine receptor (OCTR), and take-out-like carrier protein (JHBP-1) showed no consistent difference in expression (Figure 2), even though differences in ovary activation were now apparent among the workers. Therefore these genes do not appear to be associated with experimentally induced differences in ovary activation, at least not by this assay. In contrast, the genes encoding vitellogenin (VIT) and transferrin (TRF) were down-regulated (~ 4-5 fold; Fig. 2) in treated relative to control groups. The expression of these two genes is therefore associated with differences in functional ovary activation among workers.

Discussion

CO_2-induced changes in ovary activation

Our study confirms that double CO_2 narcosis inhibits ovary activation in workers (Biedermann, 1964; Harris and Harbo, 1990; Harris et al., 1996; Kropàcovà et al., 1968). This was true regardless of whether workers were narcotised when young (on days 2 and 3; Fig. 1A) or slightly older (on days 4 and 5; Fig. 1B), and all narcotised groups had mean ovary scores of less than '1'. In contrast, Mackensen (1947) showed that double CO_2 narcosis of virgin queens actually accelerated ovary activation and egg laying. It is well known that in the absence of mating or narcosis, queens will not activate their ovaries or initiate oviposition for many weeks, if ever. Thus the contrasting effects of narcosis on ovary activation in queens versus workers provides a potentially powerful model system for studying the molecular mechanisms that regulate worker sterility in honeybees, and potentially other social insects.

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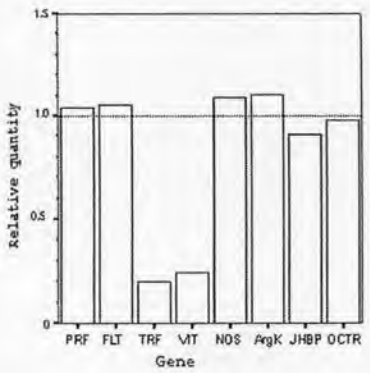

Figure 2. Relative quantification of gene expression
The expression levels of eight candidate genes in *Apis mellifera* as estimated using real-time quantitative PCR. Relative quantities are normalized to an endogenous reference gene (S8, see text). Note that vitellogenin (VIT) and transferrin (TRF) are down-regulated following CO_2-induced changes in reproductive state. Relative quantities represent an average from three assays of a single group of 8 individuals.

Molecular correlates to ovary activation in worker honeybees

This study shows that abdominal expression of vitellogenin and transferrin is reduced in narcotised workers compared to controls. This suggests that these two genes may be part of the network that regulates functional sterility in worker honeybees.

The process of ovary activation probably involves a hierarchy of events and hundreds of genes (Bownes, 1986), some of which may not be directly associated with the synthesis of ovarian tissue *per se*. For example, oocyte development depends on the synthetic activities of other, non-oocytic cells. For most insects, yolk is synthesised outside of the ovary and secondarily imported into the oocyte (Raikhel and Dadhialla, 1992). Oocytes then take up the yolk proteins, such as vitellogenin, from the extracellular fluid by receptor-mediated endocytosis (Raikhel and Dadhialla, 1992). Transferrin too can be incorporated via this mechanism into developing eggs, and likely functions to mediate iron-uptake (Kucharski and Maleszka, 2003; Kurama et al., 1995). By contrast, genes encoding proteins that are essential for the regulation of the actin cytoskeleton (i.e. flotillin) or linking the cytoskeleton with major signalling pathways (i.e. profilin) are expressed directly within the ovarian tissue and are tightly regulated during development (Cooley et al., 1992). By sampling whole abdomens in our assay genes involved in different functional categories related to egg production are potentially detected.

Of the eight candidate genes examined, six did not change their expression with changes in reproductive state. These genes represent different, but overlapping functional categories: receptor/sensing molecules (octopamine receptor, take-out-like carrier protein), signalling cascades (nitric oxide synthase, profilin, take-out-like), structural dynamics (profilin, flotillin), and energy transfer (arginine kinase). Broadly, these genes can be regarded as developmental regulators (Table 1), but either they are not differentially expressed between CO_2-treated and control worker groups, or their differential expression was not detected by the assay.

Two genes, vitellogenin and transferrin, which are both involved in oocyte packaging, did show differences in expression (> 4-fold; Fig. 2) following the double CO_2 treatment. For vitellogenin, this response was temporally consistent with previous observations on like-treated virgin queens (Engels et al., 1976). Unlike queens, however, the change in worker vitellogenin expression was not conditional on bees being re-introduced into host colonies (Engels et al., 1976). Instead, workers responded to CO_2 as they matured within cages, albeit as a group member rather than as lone individuals. More notably, the direction of the transcriptional response is opposite to that known for queens: workers decrease, rather than increase, vitellogenin synthesis in the abdomen following CO_2 narcosis. This contrasting pattern of transcription in queens versus workers in response to CO_2 treatment parallels the physiological pattern concerning ovary activation itself, and again accentuates the kin-selected difference in reproductive potential between these two castes.

Vitellogenin is a prerequisite for ovary activation in honeybees (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

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heads of workers. Nurse-age workers feed this jelly to dependent but related larvae, a behaviour that constitutes a form of kin-selected alloparental care. Amdam et al. (2003) speculate 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 reproductive division of labour (Amdam et al., 2004). Our finding that vitellogenin synthesis is down-regulated in abdomens of ovary-deactivated workers implies that this molecule is actively linked to the reproductive status of individual workers. In these experiments caged bees were not exposed to larvae and thus it is not certain whether they would show a concomitant up-regulation of vitellogenin in their heads associated with nursing and the production of royal jelly. Nontheless, these results, together with available knowledge from queens, suggests that there is a general link between CO_2 exposure, vitellogenin synthesis, and caste-specific ovary activation in honeybees. Vitellogenin is a promising new candidate component in the regulatory pathway that controls functional sterility in workers, a pathway of great theoretical significance that has never been empirically deconstructed.

The relationship between transferrin and ovary activation is less clear because transferrin is truly multifunctional in the honey bee (Kucharski and Maleszka, 2003). However, its co-regulation with vitellogenin in the current study, and it's selective incorporation into eggs during oogenesis of *Sarcophaga* (Kurama et al., 1995) and *Riptortus* (Hirai et al., 2000), suggests that transferrin 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). Our finding that transferrin is down-regulated in ovary deactivated workers implies that, like vitellogenin, it is intimately associated with ovary activation in honey bee workers. It seems less likely that transferrin would have played an active role in the evolution of honey bee alloparental care or the reproductive division of labour, but nonetheless its known function and observed kinetics suggest it too could be a component in the regulatory pathway that controls functional sterility in workers.

Speculation on regulation of ovary activation

The mechanism by which CO_2 affects ovary activation via molecular intermediates in workers and queens is unknown, but there is some evidence to suggest that honeybees are sensitive to CO_2 and that the gas is an important exogenous factor that modulates several aspects of social life. Firstly, honeybees are equipped with sensitive CO_2 receptors on their antennae (Strange and Diesendorf, 1973). Workers use these antennal receptors to tightly regulate CO_2 concentrations within their colonies (Seeley, 1974). Second, CO_2 narcosis is known to affect honey bee foraging (Ebadi et al., 1980), hoarding (Mardan and Rinderer 1980), fanning behaviour (Seeley, 1974), sound production (Schneider and Gary, 1984), and some age-related polyethisms (Heran, 1952; Ribbands, 1950). Combined, these studies suggest that CO_2 may be an important factor in the modulation of honeybee task specialization. Third, note that honeybee queens are kin-selected for extremely high fecundity and react to narcosis by accelerating reproductive development (Engels et al., 1976; Engels and Ramamurty, 1976; Mackensen, 1947), whereas workers are selected for low fecundity or sterility and react to narcosis by retarding reproductive development (Biedermann, 1964: Harris and Harbo, 1990; Harris et al., 1996; Kropàcovà et al., 1968; the present study). This contrasting effect of CO_2 on queen versus worker reproduction suggests a caste-specific response to CO_2 and, more generally, it suggests that CO_2 differentially affects the regulatory mechanism underpinning honeybee reproductive division of labor.

One possibility is that exposure to CO_2 affects the level of neurosecretions in worker brains (Harris et al., 1996). Levels of dopamine and serotonin, for example, co-vary with changes in worker ovarian development (Harris and Woodring, 1995) and even with CO_2-induced changes in ovarian development (Harris et al., 1996). It is possible therefore that CO_2 triggers the regulatory mechanism that controls worker sterility by affecting, for example, the level of dopamine or serotonin, which are putative up-stream components in the regulatory pathway that controls ovary activation in workers. A second or dual

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possibility is that CO_2 affects the titre of juvenile hormone (JH) in the hemolymph (Bühler et al., 1983), which in turn affects age-related behavior in workers, possibly including reproduction (Robinson et al., 1991). Note that, unlike for insects generally, JH has an inverse relationship with vitellogenin in honey bee workers: high JH titre turns off vitellogenin synthesis (Pinto et al., 2000). Thus, JH and vitellogenin are causally linked, and CO_2 may stimulate the regulatory mechanism that normally controls worker sterility by causing an increase in JH, which causes a decrease in vitellogenin, which presumably retards ovary activation, as has observed been here.

Whatever the initial changes are that narcosis causes within the nervous system, and that ultimately gives rise to observed differences in ovary activation, we can begin to infer down-stream regulatory components via their differential expression on ovary activation or deactivation, in particular as a consequence of CO_2 treatment. This approach will give a first indication of molecules important to the maintenance of worker sterility.

Concluding remarks

Gene expression involves a cascade of events, from transcription factor transactivation to RNA processing and maturation. The candidate genes approach (Fitzpatrick et al., 2005) 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 treatment. 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.

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NOTE

Expression of Vitellogenin and Transferrin in Activated Ovaries of Worker Honey Bees, *Apis mellifera*

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Introduction

The European honey bee (*Apis mellifera* L.; Hymenoptera: Apoidea) is a eusocial species within the insect order Hymenoptera. 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, males (drones) and additional queens are produced but rapidly leave the colony. A new virgin queen will typically make several mating flights, during which she will mate with 10–40 (average 12) drones (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 on the developing larval feeding regime and duration. However, if the sperm utilized was from a matched male with respect to the complementary 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). In contrast, in a queenless colony, the portion of unmated workers with fully active ovaries is markedly increased, with up

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to 10% of the workers being capable of laying male eggs (haploid) after seven or more days of queenlessness (Robinson et al. 1990).

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 nondeveloped ovaries due to CO_2 narcosis, revealed an increased expression level of vitellogenin (*vtg*) and transferrin (*trf*) transcripts (but not of profilin, flotillin, nitric oxide synthase, arginine kinase, octopamine receptor, and take-out-like) in workers with activated ovary development (Koywiwattrakul et al. 2005). This study extends and tests that observation by analyzing the same set of eight genes (see Koywiwattrakul et al. 2005 for the rationale behind their selection) in another group of sterile worker bees without activated ovaries, avoiding CO_2 narcosis by analyzing workers from queenright colonies (workers without activated ovaries), and comparing them to those from a queenless colony with different stages of ovarian development.

Materials and Methods

Experimental Treatment

An emerging sealed brood from wild-type colonies of the European honey bee (*Apis mellifera* L.) was incubated overnight at 35°C and 40% rh. In the morning, newly emerged workers were marked and transferred into queenright and queenless colonies (the queen was removed at least 10 days before). At 2, 4, 6, 10, 15, and 21 days, groups of 20 workers were collected at random and snap-frozen in liquid N_2 until processed.

Honey bee abdomens were dissected according to Dade (1977) to remove the ovaries, which were examined and assigned a numerical score reflecting the state of ovary activation: 0 for ovaries thin and lacking defined ova, 1 for ovaries slightly thickened but still lacking defined ova, 2 for ovaries thick with clearly defined ova, and 3 for ovaries thick with at least one fully mature ovum.

RNA Extraction and cDNA Synthesis

Worker bees were selected for total RNA extraction using the same strategy as Koywiwattrakul et al. (2005). Eight workers from a queenright colony (four bees from stage 0 and four from stage 1) and eight from a queenless colony (two each from stages 0, 1, 2, and 3), all at 15 days old, were individually extracted for total RNA from the abdomen using a combined Trizol/Qiagen protocol (Koywiwattrakul et al. 2005). The total RNA extractions from individual bees from the same group were pooled to minimize interindividual variations and subjected to cDNA synthesis according to Omniscript Reverse Transcription from Qiagen's protocol (www.qiagen.com) using $T_{(20)}MN$ (M = A, G, or C; N = A, G, C, or T) as the primer.

Estimation of Changes in Transcription Abundance

The resulting cDNA preparations (see above) were used for quantitative measurement by real-time quantitative PCR (qRT-PCR) of the level of the eight specific gene transcripts and the internal control ribosomal protein RpS8, as their cDNA templates, between queenright and queenless workers using the specific primers and conditions described by Koywiwattrakul et al. (2005). The changes in relative transcript abundance were calculated using the method of Livak and Schmittgen (2001).

Results and 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 important, the prevention of development of the few (2–12) primordial ovarioles (anlagen) that survive metamorphosis to reach maturity, thus preventing all offspring production. The genes for sterility in the latter 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 (Fig. 1), including workers with fully active ovaries (stage 3), which first appeared in workers from 10 days posteclosure. Indeed, 5, 15, 55, and 75% of queenless workers had developed ovaries (stage 2 and 3) by 6, 10, 15, and 21 days of age, respectively. In contrast, no well-developed (stage 2) and functionally active (stage 3) ovaries 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 posteclosure), 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 stages 0 and 1, consistent with earlier findings (Fig. 1).

Figure 2 shows the standardized relative transcript levels of the eight 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 one, whereas genes significantly above or below this value, as evidenced by their 95% confidence intervals, are considered to be over or under expressed in queenright workers, respectively. The abdominal expression levels for six of the

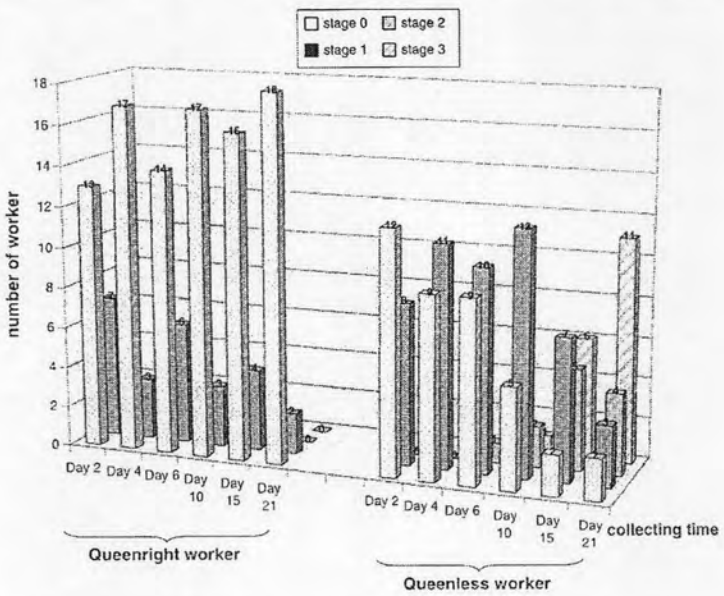

Fig. 1 Stage of ovary development in *Apis mellifera* queenright and queenless workers at various times. All workers from queenright and queenless colonies were snap-frozen in liquid N_2 at the times indicated below the bars, then dissected and scored for ovary activation

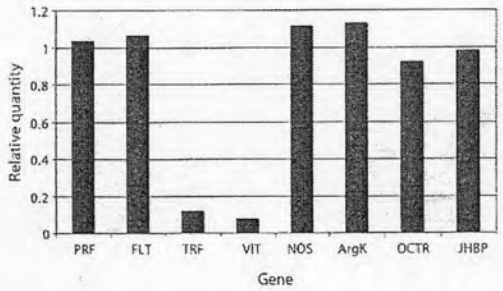

Fig. 2 Relative quantification of gene transcript expression levels of queenright worker abdomens relative to those of queenless bees. The transcript expression levels of eight candidate genes in the abdomens of 15-day-old (posteclosure) queenright and queenless *Apis mellifera* workers were estimated using qRT-PCR: profilin (PRF), flotillin (FLT), transferrin (TRF), vitellogenin (VIT), nitric oxide synthase (NOS), arginine kinase (ArgK), octopamine receptor (OCTR), and take-out-like carrier protein (JHBP). Relative transcript quantities were first normalized in each bee to the endogenous reference gene (Ribosomal protein S8) and then that of the queenright bees relative to transcript levels seen in queenless bees

eight candidate gene transcripts (profilin, flotillin, take-out-like carrier protein, nitric oxide synthase, arginine kinase, and octopamine receptor) did not significantly differ between queenright and queenless workers. The remaining two gene transcripts, however, were downregulated, transferrin about 8-fold and vitellogenin 12-fold, in queenright workers with nondeveloped ovaries compared with queenless workers with developed ovaries.

This result is consistent with a previous study, which suggested that *vit* and *trf* gene transcript levels were downregulated in the abdomen of queenless worker bees without developed ovaries following CO_2 narcosis (Koywiwattrakul et al. 2005). In contrast, these studies are incongruent with the report of Thompson et al. (2007), where no significant change in vitellogenin transcript levels was detected in queenless worker bees after inhibition of ovary development by CO_2 narcosis. Moreover, the same study revealed a significant increase of some 5-fold in the transcriptional expression level of the *trf* gene in 12-day-old queenless workers following inhibition of ovary activation by CO_2 narcosis, in direct contrast to the 5- to 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 stage 3 ovaries. Within the age range of 8 to 12 days posteclosure, they indeed showed a temporal dependence for transcriptional levels. In addition, we worked with natural in-hive conditions that are ideal for ovary development, compared with out-of-hive rearing conditions. The former scenario may imply a potentially biphasic response, with *trf* transcript levels upregulated during initial ovary development (stages 0–2; Thompson et al. 2007) and downregulated 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 ovary 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 pleiotropic in the honey bee, including antimicrobial and antiparasite 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, b). It is becoming clear, however, that at least in the honey bee, this protein has evolved pleiotropic 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,

including reduced longevity and immunity of foragers, thermotolerance, and coordination of worker behavior between nurse and forager bees and within foragers between pollen and nectar foraging (Amdam et al. 2003a, b, 2004a, b, 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), while vitellogenin gene activity influences the 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 that a key adaptation to eusociality by honey bees was the diversion of vitellogenin from its primary role as a yolk protein to a secondary role in the production of brood food (Amdam et al. 2003a, b). 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), and so favored the uncoupling of juvenile hormone control of expression levels.

This study supports the previous result found in queenless worker bees with suppressed ovary development following CO_2 treatment (Koywiwattrakul et al. 2005) and thus likely removes the possibility that the altered *trf* and *vit* levels are the result of an artifactual altered alternative redundant pathway by CO_2 narcosis. It further supports the correlation between *trf* and *vit* transcript downregulation in the activation of worker ovaries. In contrast to most of the effects on worker bee biology outlined above, all the current data linking *vit* transcript or vitellogenin protein levels to ovary maturation or the division of reproduction are at best correlative. For example, all reports of knockdown using *vit* RNAi to date have not examined the effects on the developmental activation and maturity of worker (or queen) ovaries, but rather focused on worker behavior traits, immunity, antioxidative stress, and longevity (e.g., Nelson et al. 2007; Amdam et al. 2003a, b, 2006a, b; 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; they may be part of the network that regulates functional sterility in worker honey bees.

Regardless, it may be suggested that workers in the queenless colonies with active ovaries that have an upregulation 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. 2004a; Seehuus et al. 2006) and suggested role in the division between colony-level traits and reproduction (Amdam 2003a, b, 2004a, b).

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