CHAPTER III

RESULTS

3.1 Total RNA Extraction

The selected honeybee workers, *A. cerana*, from Chumporn province, Thailand, including newly emerged (n = 23), 5-10 –day–old nurse (n = 150), 11-15 –day–old nurse (n = 100) and forager bees (n = 70) were immediately shock-frozen in liquid nitrogen. From the head of each bee, the hypopharyngeal glands were dissected under a binocular microscope on ice cold tray and placed in a tube containing pre-chille TriZol reagent and stored at -70 °C until used. One sample tube of pooled hypopharyngeal glands from each stage, total RNA was then extracted using TriZol reagent (Invitrogen life Technologies, UK). The quantity of extracted total RNA was determined by spectrophotometer at the wavelength of 260 nm. Following the formula: [Total RNA] = A_{260} x 40 µg/ml x dilution factor, approximate concentration of extracted total RNA of each stage was shown in Table 3.1. In addition, the amount of extracted total RNA per individual bees were calculated in every stages as newly emerged, 5-10 –day–old nurse, 11-15 –day–old nurse and forager bees (Table 3.1). The extracted total RNA per individual bees of forager bees was about 4-5 times lower than those in newly emerged, 5-10 –day–old nurse, 11-15 –day–old nurse bees.

The integrity and purity of RNA was analyzed by formaldehyde-agarose gel electrophoresis and the ratio of absorbancy at the wavelength of 260 nm and 280 nm was measured. The result from agarose gel showed that extracted RNA did not contain high molecular weight DNA (Figure 3.1) and the two predominant bands of small and large ribosomal RNA were found. The OD_{260}/OD_{280} ratio of 1.64 -1.90

indicated that acceptable quality of extracted RNA was obtained. The RNA solution was kept at -70°C until used.

3.2 First Stranded cDNA Synthesis

To prepare the template for quantification, 2 μg of total RNA at various stages of worker bees was reverse transcribed to 1st stranded cDNA template. The concentration of 1st stranded cDNA was estimated by comparing its florescence intensity under a long wavelength UV light with 12 -28 ng λ DNA (Figure 3.2).

Table 3.1 Concentration of extracted total RNA

Extracted total RNA of	Calculated concentration (μg/μl)	Total RNA (μg)	Total RNA per individual bee (μg)
Emerged bees (n = 23)	1.44	57.79	2.51
5-10 –day–old nurse bees (n = 150)	9.06	362.34	2.42
11-15 –day–old nurse bees (n = 100)	5.28	211.20	2.11
Forager bees (n = 70)	0.86	34.56	0.49

Figure 3.1 The total RNA extracted from the hypopharyngeal glands of 11-15 –day–old nurse and forager bees of *A. cerana* analyzed by 1% denaturing agarose gel electrophoresis

Lane N = total RNA from 11-15 –day–old nurse bees

Lane F = total RNA from forager bees

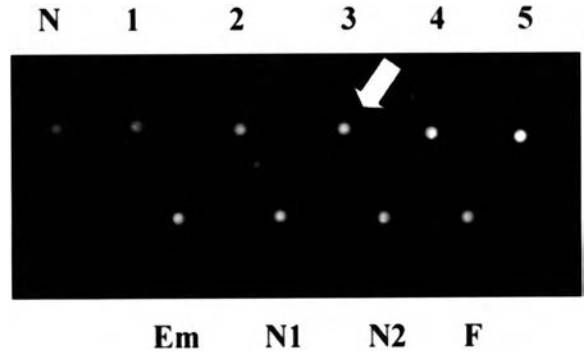


Figure 3.2 Determination of synthesized 1st stranded cDNA concentration using ethidium bromide intensity comparison

Lane N = A negative control

Lane 1-5 = 12, 16, 20, 24, and 28 ng of λ DNA, respectively.

Lane Em-F = synthesized 1st stranded cDNA from total RNA of emerged, 5-10 –day–old nurse, 11-15 –day–old nurse, and forager bees

3.3 Quantification of mRNA Level of AcMRJPs and AcApisimin by Using Internal Standard as Endogenous Sequence

3.3.1 Designation of Specific Primer and Optimization of PCR Condition

To determine the expression level of AcMRJPs and AcApisimin mRNA, specific primers for target (AcMRJP4, AcMRJP5, AcMRJP6 DNA sequence) and internal standard (28S rRNA gene sequence) were designed from *A. cerana* cDNA sequences. The specific primers for amplification of AcMRJP 1, 2 and 3 DNA sequences were from the previous study (Srisuparbh, 2002). Each pair of designed primers was optimized for the most appropriate primer and $MgCl_2$ concentrations.

Specific primer of AcMRJP4 was designed with forward primer as 5′-ACAG AGTTTTTGGAATGGCAC-3′ and reverse primer as 5′-CAGATTGTGGAAGGTTT TGCT-3′, the PCR product size was 342 bp. Optimal $MgCl_2$ concentration was chosen at 1.5 mM when using $MgCl_2$ range of 0-4.0 mM and a primer concentration was constantly used at 0.15 μM of each primer (Figure 3.3). The amount of PCR product gradually increased with the increasing of $MgCl_2$ from 0.5 to 1.5 mM and slightly decreased when more than 2 mM of $MgCl_2$ concentration was used. Non-specific PCR product was not found in any used range of $MgCl_2$ concentration. Subsequently, primer concentration range 0-0.3 μM was optimized at the constant concentration of 1.5 mM $MgCl_2$ (Figure 3.4). PCR product was firstly visualized at the primer concentration of 0.05 μM and increased with higher primer concentration of 0.10 μM. Primer dimer was initially observed at 0.15 μM. Thus, an optimal primer concentration for amplification of an AcMRJP4 gene was selected at 0.10 μM.

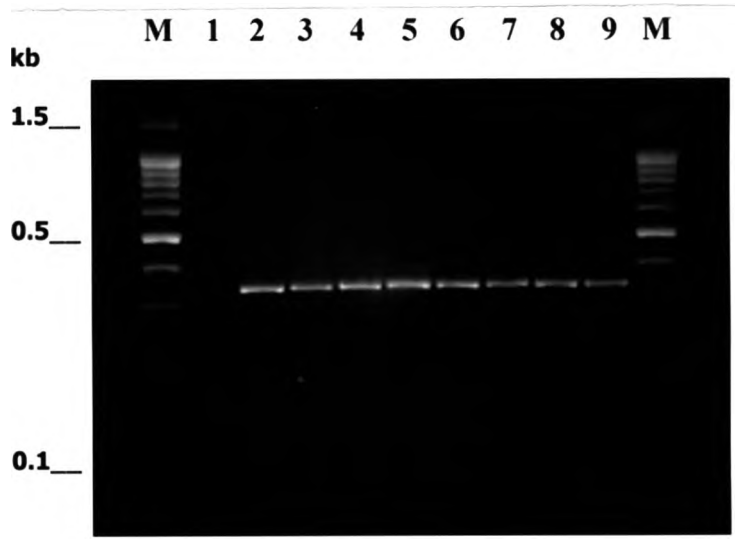


Figure 3.3 Agarose gel electrophoresis showing an optimization of $MgCl_2$ concentration at a constant primer concentration of 0.15 μM used for AcMRJP4 amplification.

Lanes M = A 100 bp DNA ladder

Lane 1-9 = Amplification product using of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM of $MgCl_2$, respectively.

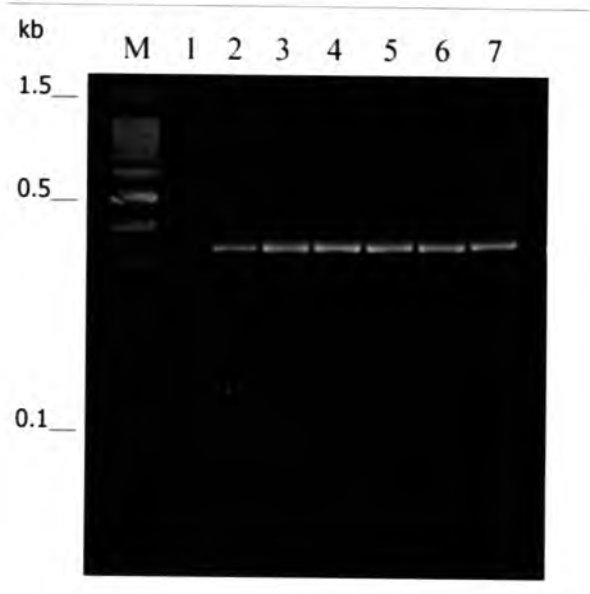


Figure 3.4 Agarose gel electrophoresis showing an optimization of primer concentration at 1.5 mM $MgCl_2$ concentration used for AcMRJP4 amplification.

Lanes M = A 100 bp DNA ladder

Lane 1-7 = Amplification products using 0, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 μM of each primer, respectively.

. As mention above, the optimum condition for amplification of AcMRJP5, AcMRJP6, and AcApisimin DNA and internal standard, 28S ribosomal DNA sequences were performed like those for AcMRJP4 for suitable condition. The results were summarized and shown in the Table 3.2 and the agarose gel electrophoresis was revealed in appendix A (II and III). The optimal primer and $MgCl_2$ concentrations were chosen under condition with highest product yield and formation of primer-dimer and non-specific product was not found.

For quantification method as described previously, the data from this type of experiment must be obtained before the amplification reactions reach the plateau phase. Therefore, cycle number of amplification of each gene was determined in 11-15 –day–old nurse bees. PCR reaction for AcMRJP1-6, AcApisimin and 28S ribosomal RNA was carried out for 4 – 24 cycles. The yield of the PCR product was gradually increased until the 14^{th} cycles for AcMRJP1, 16^{th} cycles for AcMRJP2, 20^{th} cycles for AcMRJP3, AcMRJP4 and 28S ribosomal RNA, 24^{th} cycles for AcMRJP5, 22^{nd} cycles for AcMRJP6 and 18^{th} for AcApisimin genes (Figure 3.5 and 3.6).

In addition, the log of the intensity of PCR product in each cycle was plotted versus a number of consecutive amplification cycles (n) (appendix B, I). To determine the relative difference in mRNA expression level, a value of n was chosen at a point where each curve was parallel. Therefore, at 16^{th} cycles was selected only for AcMRJP3, AcMRJP4, AcMRJP5, and AcMRJP6. In AcMRJP1, AcMRJP2 and AcApisimin quantification, the exponential phase of these target genes and internal standard could not be compared because of PCR product of internal standard could not be detected at the cycle in exponential phase of these target genes and when cycle of internal standard was in exponential phase, the target was already in plateau phase.

Table 3.2 Primer sequences and PCR conditions for quantification of AcMRJPs and Apisimin mRNA level using 28S rRNA as internal standard

Gene	Primer sequence (5′ to 3′)	PCR product size (bp)	$MgCl_2$ concentration (mM)	Primer concentration (µM)	Annealing temperature (°C)
AcMRJP1	**For**: TCA AGG TAC GAC AAG CAG CAT TC **Rev**: TTG TCG ATC GCA AGT TTT GTG G	351	1.5	0.15	54.0
AcMRJP2	**For**: CTT GGA AAA TTC GTT GAA CGT A **Rev**: GTA TTT CGA TTT GCT TAA GGT GC	379	1.5	0.15	54.0
AcMRJP3	**For**: GCG CAG CTG TGA ACC ATC AAA G **Rev**: AGA AGA GGT CCA CCT TTG CCT T	271	1.5	0.15	54.0
AcMRJP4	**For**: ACA GAG TTT TTG GAA TGG CAC **Rev**: CAG ATT GTG GAA GGT TTT GCT	342	1.5	0.10	54.0
AcMRJP5	**For**: TAC AGG AAA AGG AGG ACT AGA AAA CC **Rev**: CAT TCT GTT GGA TTG TGG GAG ATG	559	2.0	0.05	54.0
AcMRJP6	**For**: GCA GAT GAC AGA GGT GAC **Rev**: ACA AGT CCG AAG AAA AGG	350	1.5	0.15	54.0
AcApisimin	**For**: TTG GTC AGC GAT GTG TCC **Rev**: GCG TCG ATG AGA ATT TGG	170	1.5	0.10	54.0
28S rRNA	**For**: GTG CTG ACT CCT GTT GAT GC **Rev**: GAA TTG GTT GTT GCC CGA TA	255	1.5	0.15	54.0

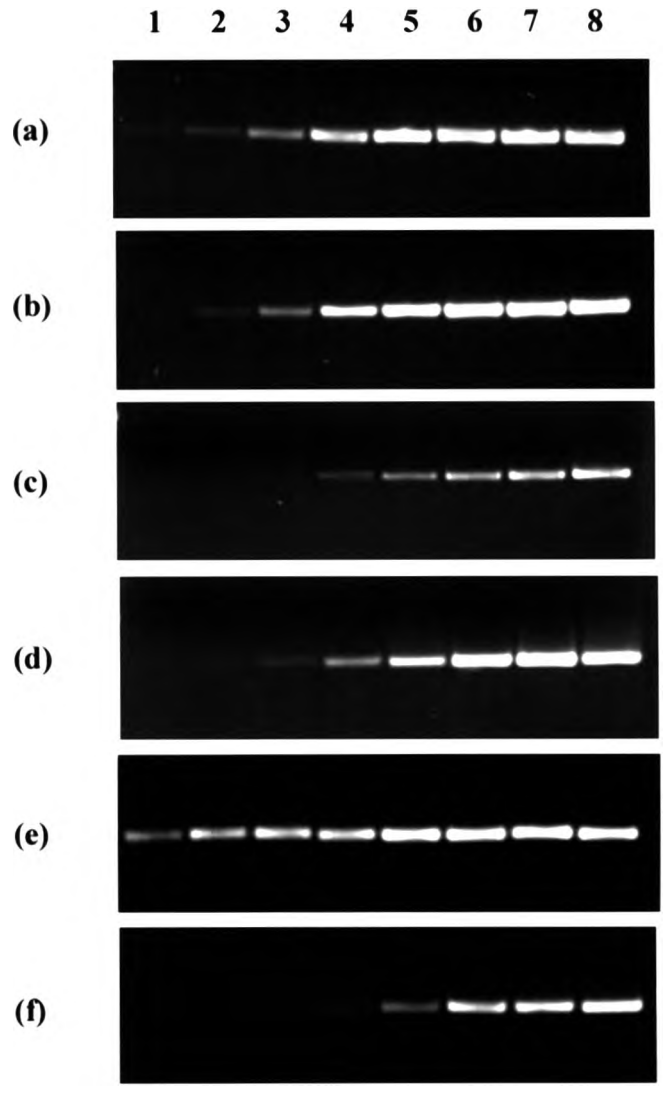


Figure 3.5 Agarose gel electrophoresis showing PCR products determined from various cycle numbers for amplification of AcMRJP3 (a), AcMRJP4 (b), AcMRJP5 (c), AcMRJP6 (d), AcApisimin (e), and 28S ribosomal RNA (f).

Lanes M = A 100 bp DNA ladder

Lane 1-8 = PCR product amplified at 10, 12, 14, 16, 18, 20, 22 and 24 cycles, respectively.

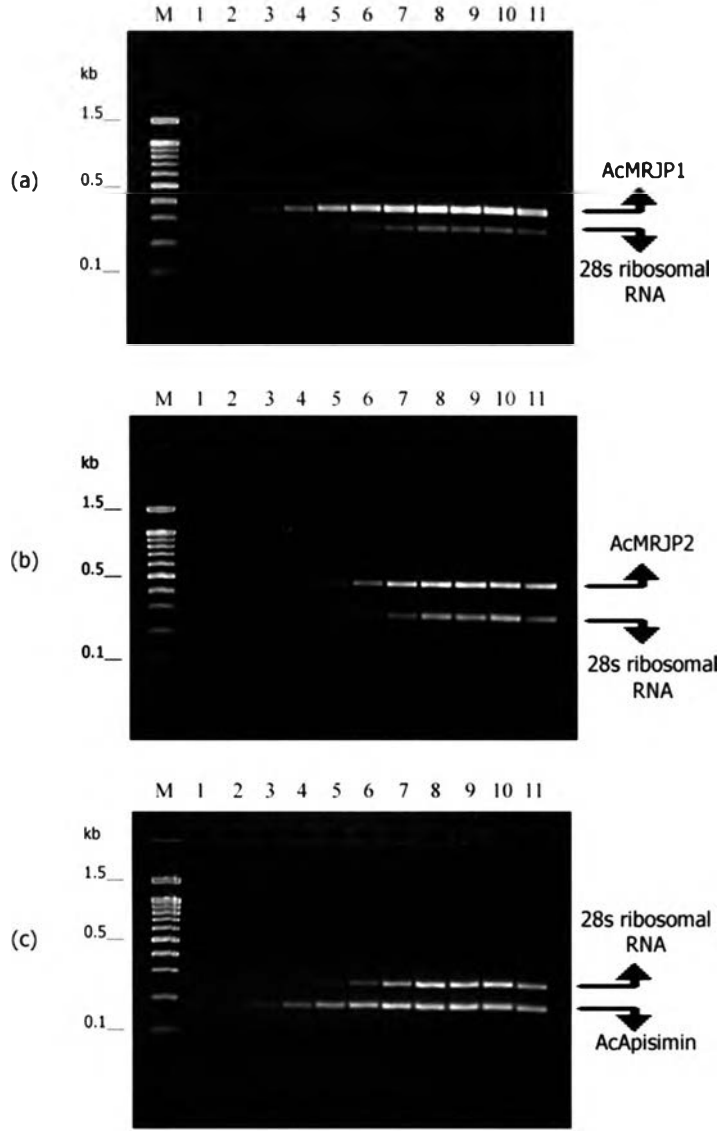


Figure 3.6 Agarose gel electrophoresis showing PCR products determined from various cycle numbers for amplification of AcMRJP1 (a), AcMRJP2 (b), and AcApisimin (c).

Lanes M = A 100 bp DNA ladder

Lane 1-11 = PCR product amplified at 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 cycles, respectively.

At 16^{th} cycles in exponential phase of AcMRJP3, AcMRJP4, AcMRJP5, AcMRJP6, and 28S ribosomal RNA amplification, the fluorescence intensity of PCR product band was determined and compared between AcMRJPs and 28S ribosomal RNA for each individual reaction. The relative initial amounts of that was 6.8 for AcMRJP3, 3.2 for AcMRJP4, 0.5 for AcMRJP5 and 0.85 for AcMRJP6. Therefore, the ratio of AcMRJP3 : AcMRJP4 : AcMRJP5 : AcMRJP6 in 11-15 –day–old nurse bees was 13.6 : 6.4 : 1 : 1.7, respectively. For expression level of AcMRJP1, AcMRJP2, and AcApisimin, the ratio was calculated relative to AcMRJP2 as 4 : 1 : 13.

3.4 Quantification of mRNA Level of AcMRJPa and AcApisimin Using Internal Standard as Added Exogenous DNA

As a result of quantification using endogenous sequence as internal standard could not be performed for AcMRJP1, AcMRJP2 and AcApisimin gene, quantification using added exogenous DNA (genomic DNA competitor) was subsequently used.

3.4.1 Designation of Specific Primers for Target and Genomic DNA Competitor and Optimization of PCR Condition

Specific primers for genomic DNA competitor in this approach must be put over the small introns in the genomic DNA sequences. From gene sequence of *A. cerana* (Imjongjairak, 2005), introns position was marked before primer designation of AcMRJP4, AcMRJP5 and AcMRJP6 genes. By the same way, optimization of PCR condition was adjusted for the best appropriate primer and $MgCl_2$ concentrations. The results were summarized in Table 3.3 and appendix A, (IV). The

optimal primer and $MgCl_2$ concentrations were choose at 0.15 μM and 1.5 mM for all AcMRJPs amplification. In addition, annealing temperature was also optimized, amplification at 52°C, 54°C and 56°C were carried out for AcMRJP1-3 (Figure 3.7, panel a) and at 40°C, 50°C and 55°C were used for AcMRJP4-6 amplification (Figure 3.7, panel b). As the result, product yield was not significantly differed in AcMRJP1, and AcMRJP3, whereas AcMRJP2 was slightly increased when comparing between 52°C and 54°C. For AcMRJP4-6, the product yield at 40°C was the lowest and higher at 50°C which equal to the yield at 55°C (Figure 3.7, panel b). For simplification of the experiment, the annealing temperature for PCR was selected at 54°C for all primer sets.

3.4.2 Competitor Preparation and Quantification

To produce genomic DNA competitor as internal standard, total DNA was extracted from the thorax of each *A. cerana* bee. Using a pair of each family-specific primer, extracted DNA was amplified in separately PCR tube. Subsequently, the PCR product of each AcMRJP gene family was purified and estimated its quantity by comparing fluorescent intensity to λDNA which known concentrations. Due to the existence of intervening sequences in the genomic DNA, the sizes of amplification product are larger than that from cDNA target.

To perform a competitive PCR, a dilution series of the genomic DNA competitor of target gene was prepared, and a constant amount of the target cDNA was added to each of the optimized PCR reactions and simultaneously amplified. As the result of these competitive PCR, non-specific PCR products were found in all of reaction when co-amplified target and genomic DNA competitor. In contrast to

separate amplification of target or genomic DNA competitor, the non-specific PCR products were not found (Figure 3.8).

3.5 Quantification of mRNA Level of AcMRJP and AcApisimin Using Internal Standard as Added Exogenous cDNA

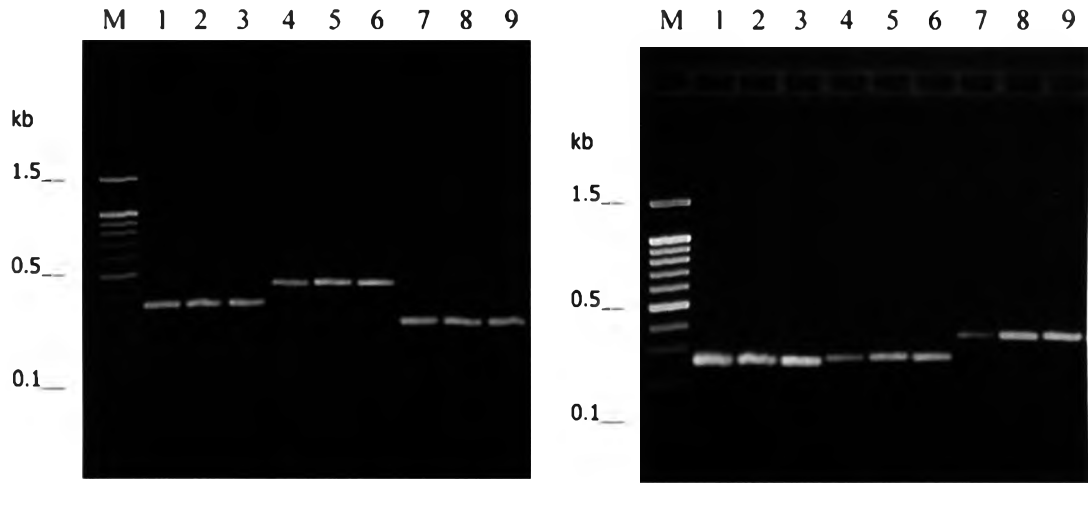
The non-specific PCR product was found in competitive RT-PCR using added genomic DNA competitor as an exogenous internal standard, therefore, an alternative competitor was used. The competitor size is smaller than the cDNA target.

3.5.1 Designation of Specific Primer and Optimization PCR

By this methods, an extraordinary specific reverse primer was designed and used for preparation of internal standard or cDNA competitor (Table 3.4) by PCR using the first stranded cDNA (2.8) as a template. The expected PCR product sizes of both cDNA target and cDNA competitor were also summarized in Table 3.4.

Table 3.3 Primer sequences and PCR conditions for quantification of AcMRJP4-5 using genomic DNA competitor as internal standard.

Gene	Primer sequence (5′ to 3′)	PCR product size (bp)	$MgCl_2$ concentration (mM)	Primer concentration (µM)	Annealing temperature (°C)
AcMRJP4	**For**: CCG ATT GGT CAT TTG CTA AGT **Rev**: AGC TTG AAC AGC TAA AGA TGC	254	1.5	0.15	54.0
AcMRJP5	**For**: GTC ACC GTA CTA AGA TAT AAA G **Rev**: TAA TCT GCT GTG AGG TAT TG	265	1.5	0.15	54.0
AcMRJP6	**For**: TAA GAC TTT TGT CGC TGT AA **Rev**: CTT GAA CAA CTA ATG AGA CG	353	1.5	0.15	54.0



(a) (b)

Figure 3.7 Agarose gel electrophoresis showing an optimization of annealing temperature.

Lanes M = A 100 bp DNA ladder

Panel a, Lane 1-3 = Amplification products of AcMRJP1 using annealing temperature as 52 °C, 54°C and 56°C, respectively.

Lane 4-6 = Amplification products of AcMRJP2 using annealing temperature as 52 °C, 54°C and 56°C, respectively.

Lane 7-9 = Amplification products of AcMRJP3 using annealing temperature as 52 °C, 54°C and 56°C, respectively.

Panel b, Lane 1-3 = Amplification products of AcMRJP4 using annealing temperature as 40 °C, 50°C and 55°C, respectively.

Lane 4-6 = Amplification products of AcMRJP5 using annealing temperature as 40 °C, 50°C and 55°C, respectively.

Lane 7-9 = Amplification products of AcMRJP6 using annealing temperature as 40 °C, 50°C and 55°C, respectively.

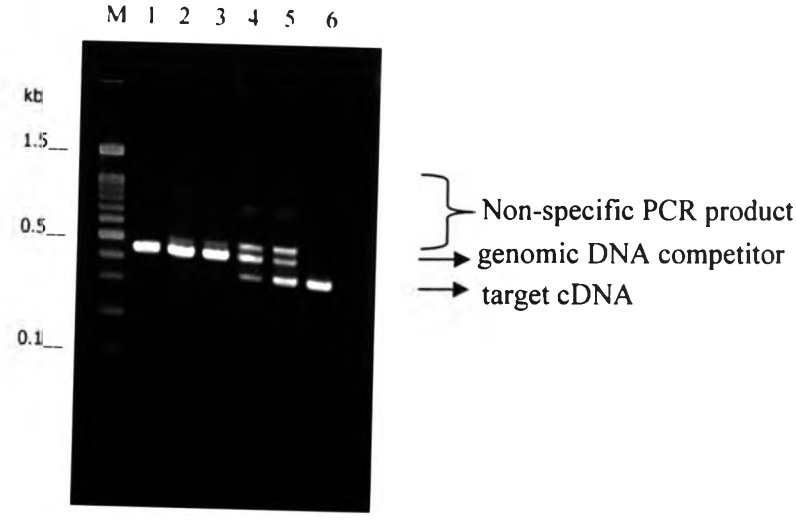


Figure 3.8 Agarose gel electrophoresis showing the result of competitive PCR using genomic DNA competitor.

Lanes M = A 100 bp DNA ladder.

Lane 1 = Amplification products of AcMRJP6 genomic DNA competitor.

Lane 2-5 = Amplification products of AcMRJP6 cDNA target co-amplified in the presence of 1000, 500, 250, 125 pg of genomic DNA competitor, respectively.

Lane 6 = Amplification products of AcMRJP6 cDNA target.

For PCR optimization, $MgCl_2$ concentrations were optimized between 0.5-2.0 mM and 1.0-2.0 mM at constant primer concentration of 0.15 and 0.1 μM for AcMRJP2 (Figure 3.9) and AcMRJP5 cDNA (Figure 3.10), respectively. The PCR products were observed in all concentrations and the highest yield was obtained when using 1.5 mM $MgCl_2$. Together with this, an optimal primer concentration was also examined (0.05-0.20 μM) at 1.5 mM $MgCl_2$. The highest PCR product of AcMRJP2 was at the 0.15 μM primers concentration, while AcMRJP5 was at 0.05 μM and gradually decreased with primer dimer when higher concentration was used. Thus, the optimal primers concentration for amplification of AcMRJP2 and AcMRJP5 cDNA were chosen at 0.15 and 0.05 μM, respectively.

As mention above, amplification of AcApisimin cDNA (211 bp) was also optimized with $MgCl_2$ concentration ranging from 1.0 to 2.0 mM at constant 0.15 μM primers concentration (Figure 3.11). Then, the primers concentrations ranging from 0.05-0.20 μM at constant $MgCl_2$ concentration (1.5 mM) were performed. The result was summarized in Table 3.5.

Additional, the amplification products were also mapped by digestion with restriction endonucleases to verify the target amplification fragment. The amplified products of AcMRJPs and AcApisimin genes were individually digested with restriction endonucleases as shown in Table 3.6. For example, the expected digestion patterns of AcMJRP4 cDNA with *Rsa* I, and *Hinf* I were obtained (Figure 3.12). This suggested specificity of family-specific primers was developed in this study.

3.5.1 Competitive PCR Template Preparation

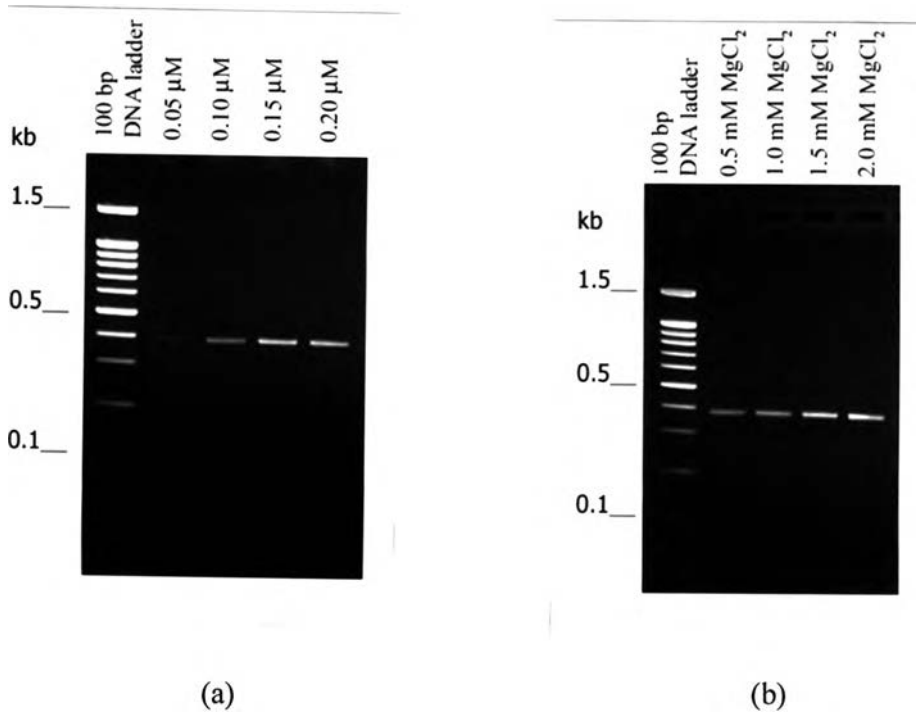
There are two types of the templates for competitive PCR; target and competitor. The target was synthesized using each pair of family-specific primers as described previously in 2.10.3.

Preparation of the internal standard for competitive-PCR, cDNA competitor was separately generated by a pair of the specific primers; a forward PCR primer and an internal standard reverse PCR primer (Table 3.4). From an agarose gel electrophoresis, the expected product sizes of competitor were found (Figure 3.13) which were smaller than that from the target. The PCR product of each AcMRJPs gene was then purified from gel and checked by agarose electrophoresis (Figure 3.14). Subsequently, its quantity was estimated by comparing the fluorescent intensity to the known concentrations of λDNA (Figure 3.15). Then, the cDNA competitor concentration was finally adjusted to a final concentration of 20 ng/μl and preliminary used in the PCR reactions.

Table 3.4 Primer sequences for competitive PCR of AcMRJPs and Apisimin mRNA using exogenous cDNA as internal standard.

	Forward PCR primer sequence (5' to 3')	Reverse PCR primer sequence (5' to 3')	Internal standard* reverse PCR primer sequence (5' to 3')	Target size (bp)	Competitor size (bp)	Δn
AcMRJP1	TCA AGG TAC GAC AAG CAG CAT TC	TTG TCG ATC GCA AGT TTT GTG G	**TTG TCG ATC GCA AGT TTT GTG** GTC GGG ATA AGG TTG AAG AAG AG	351	307	44
AcMRJP2	CTT GGA AAA TTC GTT GAA CGT A	TGG AGC ACA TAT AGG TTC AGT TC	**TGG AGC ACA TAT AGG TTC AGT TCC** AAA ACC CAC AAT CTG TCG AA	379	360	19
AcMRJP3	GCG CAG CTG TGA ACC ATC AAA G	AGA AGA GGT CCA CCT TTG CCT T	**AGA AGA GGT CCA CCT TTG CCT TGA** AGA AGG TAC ACC ATC GAA CC	271	248	23
AcMRJP4	ACA GAG TTT TTG GAA TGG CAC	CAG ATT GTG GAA GGT TTT GCT	**CAG ATT GTG GAA GGT TTT GCT** AGC GTC TCA TTT CGA GCT ACA	342	316	26
AcMRJP5	CAC TCA GTC CCA TGA CAA A	CAT TCT GTT GGA TTG TGG G	**CAT TCT GTT GGA TTG TGG GCT CTT** CAT TCT GAG CGA CC	335	297	38
AcMRJP6	GCA GAT GAC AGA GGT GAC	ACA AGT CCG AAG AAA AGG	**ACA AGT CCG AAG AAA AGG** GCG GAT GAT TGA GTG TTG	350	326	24
Apisimin	TCA TTG CTG TCG TCG TCC TA	GCG TCG ATG AGA ATT TGG	**GCG TCG ATG AGA ATT TGG** GAC ACG TTG GCA CCA GAT AC	211	175	36

* cDNA Competitor



(a) (b)

Figure 3.9 Agarose gel electrophoresis showing an optimization of primer concentration at constant 1.5 mM $MgCl_2$ concentration (a) and optimization of $MgCl_2$ concentration at a constant primer concentration of 0.15 µM (b) used for AcMRJP2 gene amplification.

Panel a = Amplification product using of 0.05, 0.10, 0.15, and 0.20 µM of each primer concentration.

Panel b = Amplification product using of 1.0, 1.5, and 2.0 mM of $MgCl_2$ concentration.

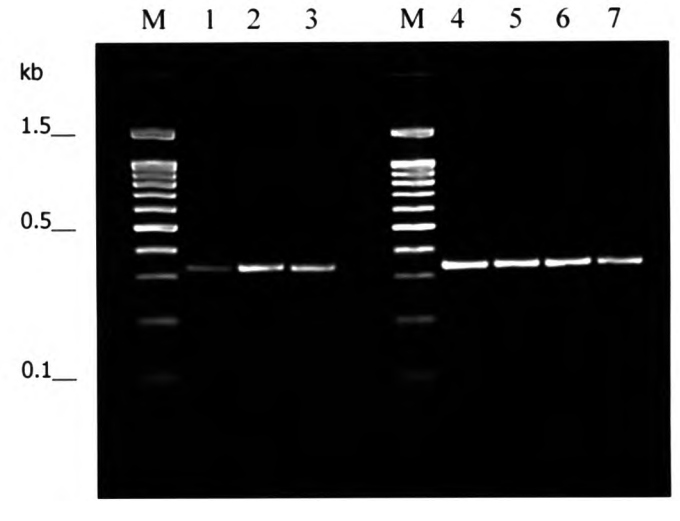


Figure 3.10 Agarose gel electrophoresis showing an optimization of $MgCl_2$ concentration at a constant primer concentration of 0.15 μM and optimization of primer concentration at constant 1.5 mM $MgCl_2$ concentration used for AcMRJP5 gene amplification.

Lanes M = A 100 bp DNA ladder

Lane 1-3 = Amplification product using of 1.0, 1.5, and 2.0 mM of $MgCl_2$, respectively.

Panel 4-7 = Amplification product using of 0.05, 0.10, 0.15, and 0.20 μM of each primer concentration.

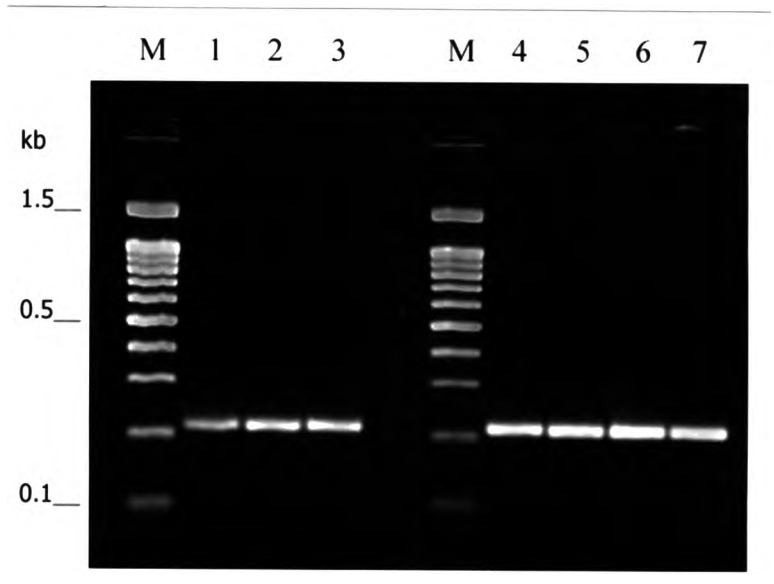


Figure 3.11 Agarose gel electrophoresis showing an optimization of $MgCl_2$ concentration at a constant primer concentration of 0.15 μM and optimization of primer concentration at constant 1.5 mM $MgCl_2$ concentration used for AcApisimin gene amplification.

Lanes M = A 100 bp DNA ladder

Lane 1-3 = Amplification product using of 1.0, 1.5, and 2.0 mM of $MgCl_2$, respectively.

Panel 4-7 = Amplification product using of 0.05, 0.10, 0.15, and 0.20 μM of each primer concentration.

Table 3.5 PCR conditions for quantification of AcMRJPs expression level in 40 cycles.

	$MgCl_2$ concentration (mM)	Primer concentration (μM)	Annealing temperature (°C)
AcMRJP1	1.5	0.15	54.0
AcMRJP2	1.5	0.15	54.0
AcMRJP3	1.5	0.15	54.0
AcMRJP4	1.5	0.10	54.0
AcMRJP5	1.5	0.05	54.0
AcMRJP6	1.5	0.15	54.0
AcApisimin	1.5	0.15	54.0

Table 3.6 A summary of digestion pattern of amplification products on various restriction enzymes.

PCR products	Restriction enzymes	Digested size (bp)			Undigested size (bp)
AcMRJP1	*Rsa* I	209	135	7	351
	Hinf I	286	33	32	
AcMRJP2	*Rsa* I	209	170		379
	Alu I	302	77		
AcMRJP3	*Rsa* I	217	54		271
	Alu I	265	6		
AcMRJP4	*Rsa* I	196	146		342
	Hinf I	208	134		
AcMRJP5	*Rsa* I	226	109		335
	Alu I	207	128		
AcMRJP6	*Hinf* I	304	46		350
	*Eco*R I	275	75		
AcApisimin	*Hinf* I	124	87		211
	Alu I	150	40	21	

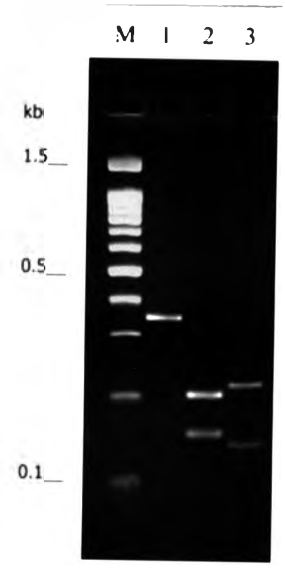


Figure 3.12 The restriction analysis of the amplification products from AcMRJP4 cDNA.

Lane M = A 100 bp DNA ladder

Lane 1 = Undigested AcMRJP4 cDNA

Lane 2 = AcMRJP4 cDNA digested with *Rsa* I

Lane 3 = AcMRJP4 cDNA digested with *Hinf* I

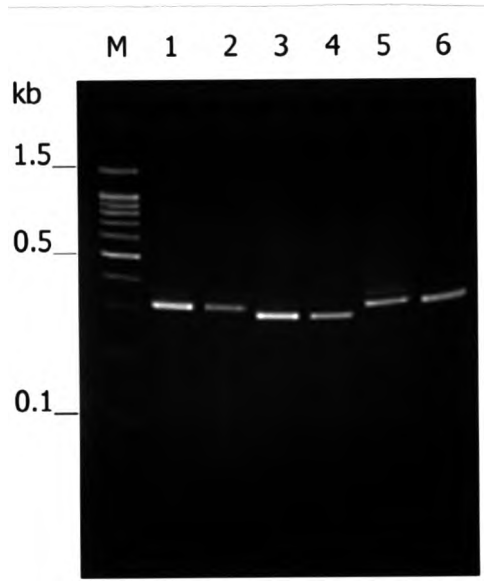


Figure 3.13 Agarose gel electrophoresis of AcMRJP4-6 cDNA competitor prepared from PCR using in competitive PCR.

Lane 1-2 = PCR product for cDNA competitor preparation of AcMRJP4.

Lane 3-4 = PCR product for cDNA competitor preparation of AcMRJP5.

Lane 5-6 = PCR product for cDNA competitor preparation of AcMRJP6.

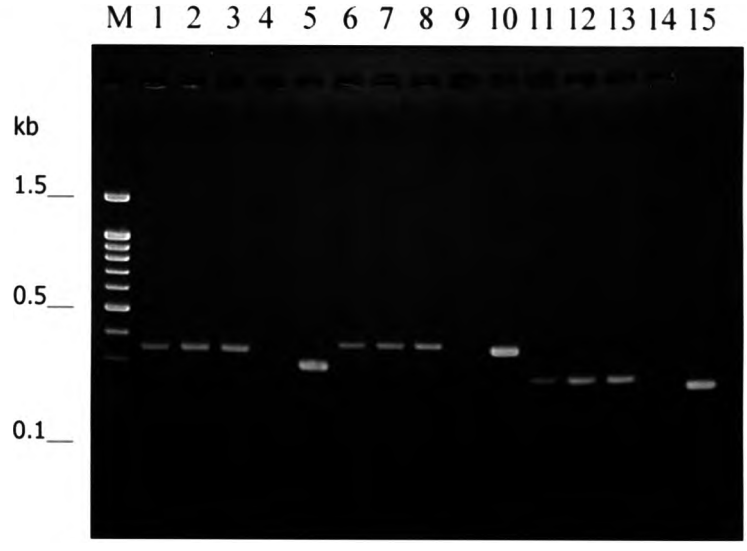


Figure 3.14 Agarose gel electrophoresis of the cDNA target amplified using 1st cDNA as template and their corresponding competitor.

Lane 1-5 = Amplification product of AcMRJP1 using cDNA of forager, 5-10 -day-old nurse, 11-15 -day-old nurse, emerged honeybees and competitor as template, respectively.

Lane 6-10 = Amplification product of AcMRJP2 using cDNA of forager, 5-10 -day-old nurse, 11-15 -day-old nurse, emerged honeybees and competitor as template, respectively.

Lane 11-15 = Amplification product of AcMRJP3 using cDNA of forager, 5-10 -day-old nurse, 11-15 -day-old nurse, emerged honeybees and competitor as template, respectively.

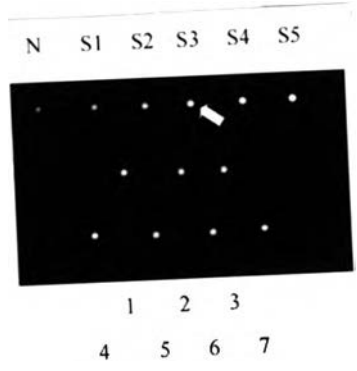


Figure 3.15 Determination of competitor concentration using ethidium bromide intensity comparison.

Lane N = A negative control

Lane S1-S5 = 16, 18, 20, 24, and 26 ng of λDNA, respectively.

Lane 1-7 = competitors of AcMRJP1, 2, 3, 4, 5, 6, and AcApisimin

3.6 Quantitative PCR assay

The expression level of AcMRJPs and AcApisimin genes in hypopharyngeal glands were determined by semi-quantitative PCR. The total RNA was used as the template to construct first and second stranded AcMRJPs and AcApisimin cDNA (sections 2.8, 2.10.3). This second stranded cDNA was used as cDNA target and co-amplified with the known concentrations of internal standard or cDNA competitor.

To quantify mRNA level, competitive PCR was performed, in the PCR reactions which consisted of two types of the template as a dilution series of the competitor of each gene, and a constant amount of the target cDNA. The PCR product was electrophoretically analyzed through an agarose gel and stained with 2.5 µg/ml of ethidium bromide. PCR fragments product in each reaction were visualized under a UV transilluminator and photographed with Gel Documentation System (GeneCam FLEX1, SynGene). The fluorescence intensity was quantified using GeneTools analysis software (Synoptics) as described previously. The ratio of amount of PCR products which generated by the competitor standard DNA ([S]) and the target cDNA ([T]) were determined for each individual reaction and converted in logarithm form for plotting versus the known amounts of the competitors. The amount of target cDNA was equal to the amount of the added competitors where the log of [T]/[S] = log of 1/1=0.

Due to the suitable range of competitor amount providing competitive results; the dilution series of competitor were prepared and co-amplified with constant amount of target gene. In suitable competitor range, the result was showed competitive relationships between the intensity of target gene and its competitor. However, the competitor range as adjusted when using inappropriate competitor amount (Figure

3.16). Expressions level of AcMRJPs and AcApisimin mRNA in hypopharyngeal glands were quantified at 4 different developmental stages of the above-mentioned honeybee samples. The relative ratio was calculated and compared with the same AcMRJPs genes in each stage.

In 11-15 -day-old nurse bees when 100 ng of target cDNA was used for PCR amplification, the expression amount of AcMRJP1 mRNA was 24.6 pg/ 1 ng total RNA and ratio relative to AcMRJP3 mRNA was 3.9 : 1 which was similar to previous report of Srisuphab (2000) (3.3 : 1). Subsequently, a two-fold dilution of cDNA target was performed which expected result that the amount was in a half. In contrary, the result showed higher amount of AcMRJP1 mRNA approximately at 66.5 pg/1 ng total RNA. Then, all the quantification of AcMRJPs and AcApisimin mRNA were repeated by a two-fold dilution of target cDNA, base on the value must be decreased in half when the target template was two-fold diluted.

At suitable competitor range, the transcription level of AcMRJP1 mRNA in 5-10 -day-old nurse bees was 66.2 pg/ 1 ng total RNA (Figure 3.17 and 3.18) which resembled to 11-15 -day-old nurse bees at 66.5 pg/ 1 ng total RNA (Figure 3.19 and 3.20) whereas lowest value of 16.6 pg/ 1 ng total RNA was found in forager bees (Figure 3.21 and 3.22).

Likewise, other AcMRJPs and AcApisimin expression level was quantitated as above. The agarose gel electrophoresis at the suitable competitive fashion was showed in appendix C.

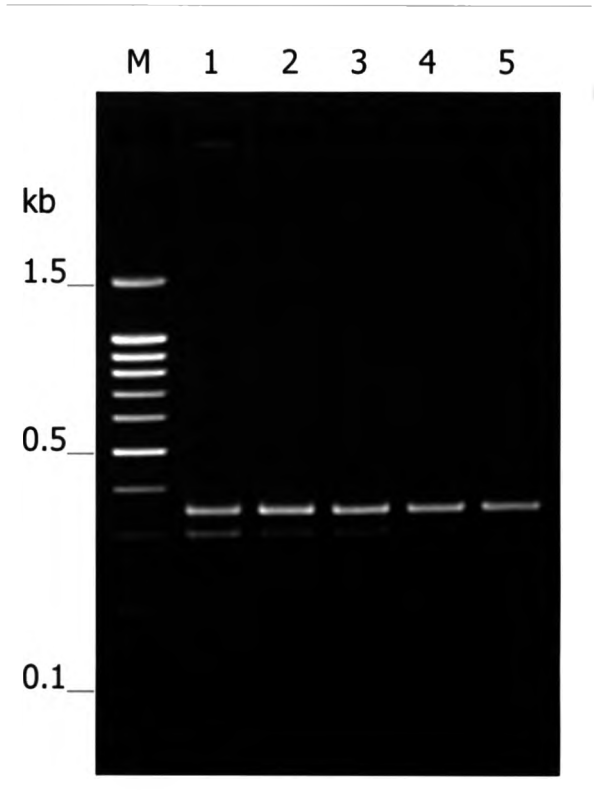


Figure 3.16 Agarose gel electrophoresis for quantification of MRJP1 mRNA level in hypopharyngeal gland of *A. cerana* 5-10 -day-old nurse bees using competitive PCR at the constant 2 ng of the cDNA target with unsuitable competitor range.

Lane M = A 100 bp DNA ladder

Panel a; lane 1-5 = AcMRJP1 cDNA target co-amplified in the presence of 20, 10, 5, 2.5, and 1.25 pg of AcMRJP1 cDNA competitor, respectively

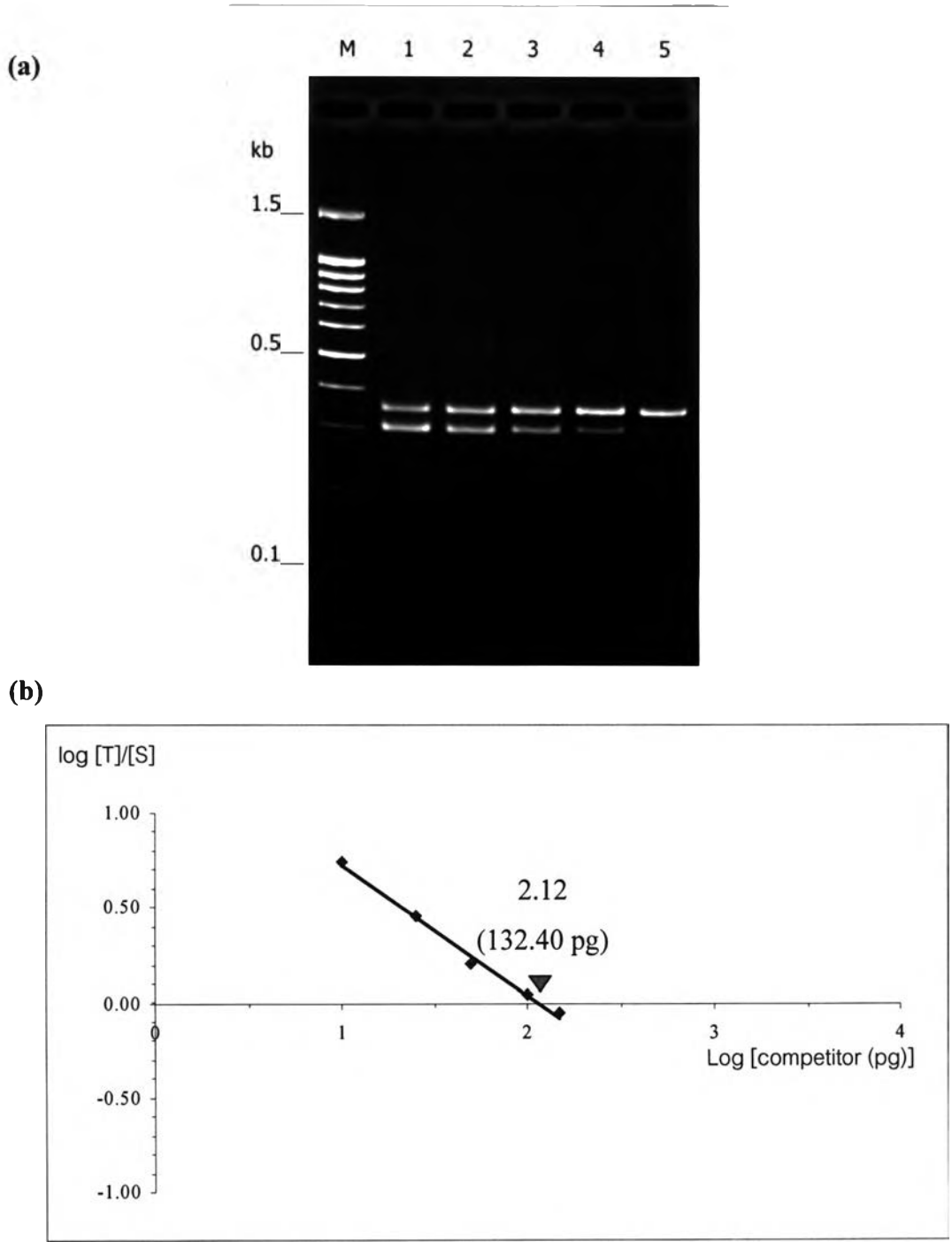
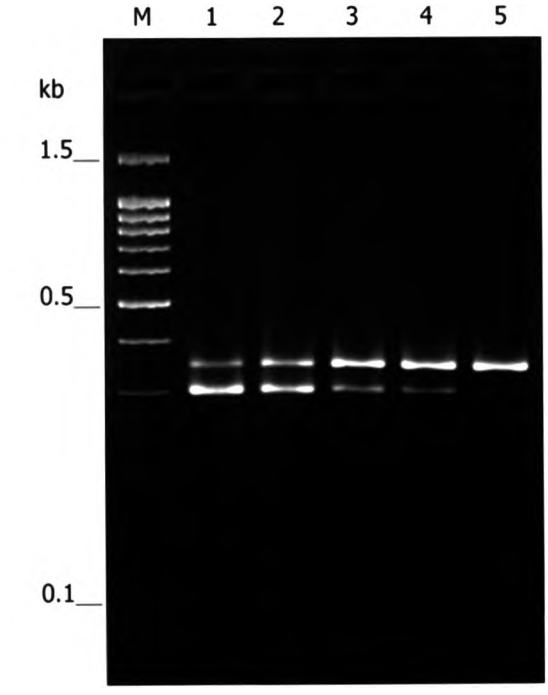


Figure 3.17 Agarose gel electrophoresis (a) and log graph (b) for quantification analysis of MRJP1 mRNA in hypopharyngeal gland of *A. cerana* 5-10 -day-old nurse bees using competitive PCR at the constant 2 ng of the cDNA target.

Lane M = A 100 bp DNA ladder
Panel a; lane 1-5 = AcMRJP1 cDNA target co-amplified in the presence of 150, 100, 50, 25 and 10 pg of AcMRJP1 cDNA competitor standard DNA, respectively.

(a)



(b)

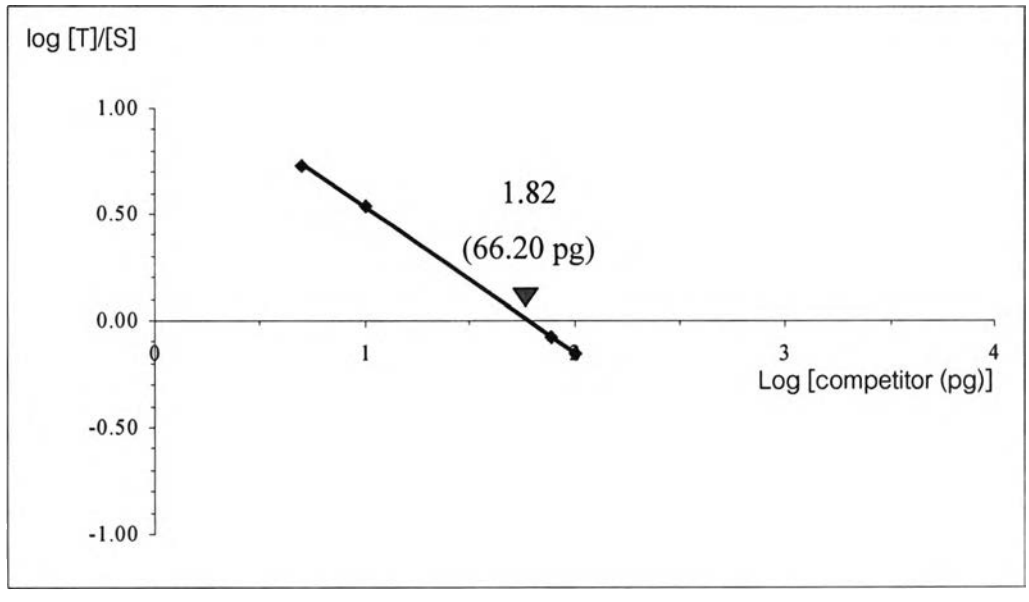


Figure 3.18 Agarose gel electrophoresis (a) and log graph (b) for quantification analysis of MRJP1 mRNA in hypopharyngeal gland of *A. cerana* 5-10 -day-old nurse bees using competitive PCR at the constant 1 ng of the cDNA target.

Lane M = A 100 bp DNA ladder
Panel a; lane 1-5 = AcMRJP1 cDNA target co-amplified in the presence of 100, 75, 10, 5 and 1 pg of AcMRJP1 cDNA competitor, respectively.

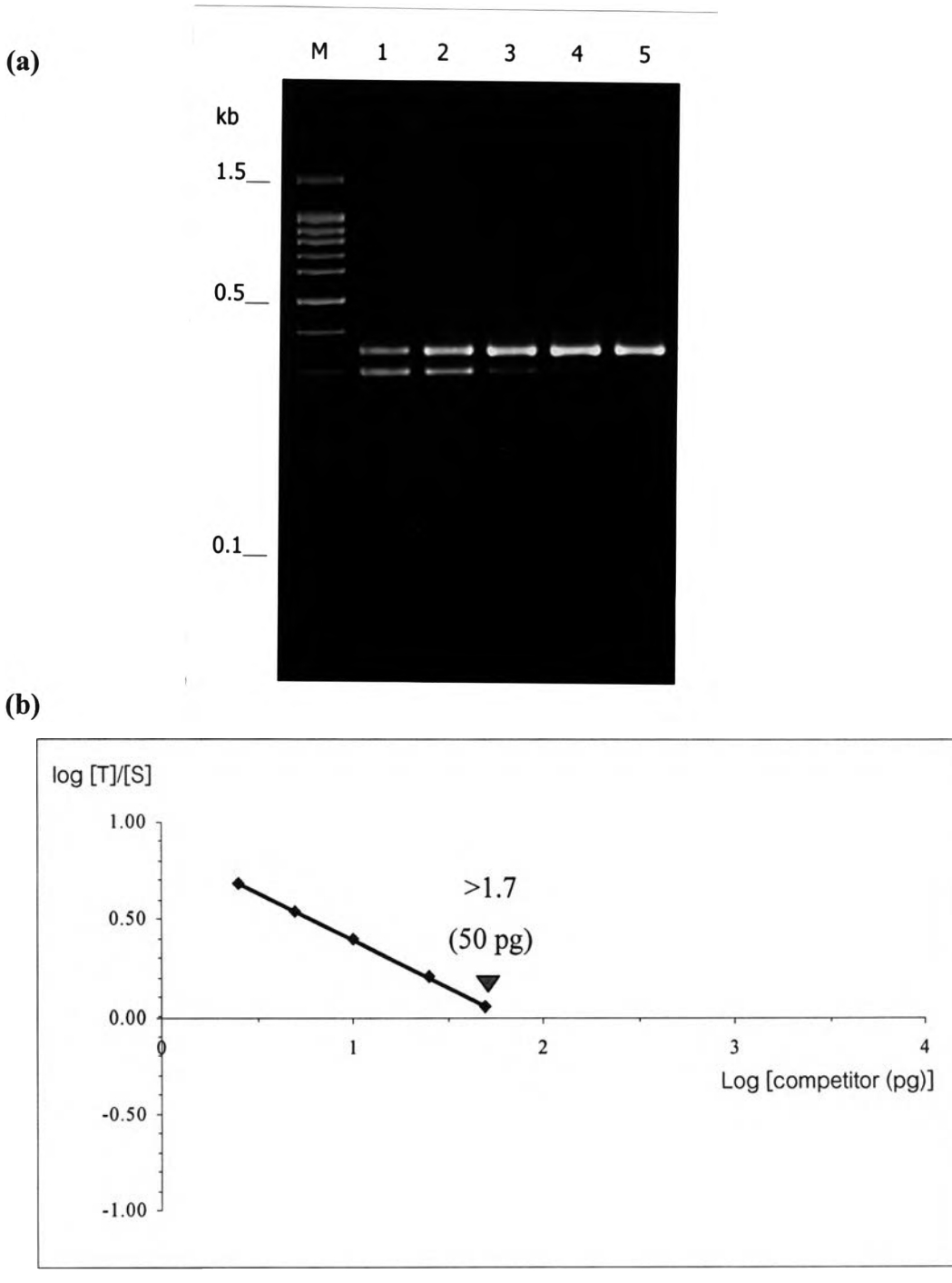


Figure 3.19 Agarose gel electrophoresis (a) and log graph (b) for quantification analysis of MRJP1 mRNA in hypopharyngeal gland of *A. cerana* 11-15 -day-old nurse bees using competitive PCR at the constant 1 ng of the cDNA target.

Lane M = A 100 bp DNA ladder
Panel a; lane 1-5 = AcMRJP1 cDNA target co-amplified in the presence of 50, 25, 10, 5 and 2.5 pg of AcMRJP1 cDNA competitor, respectively.

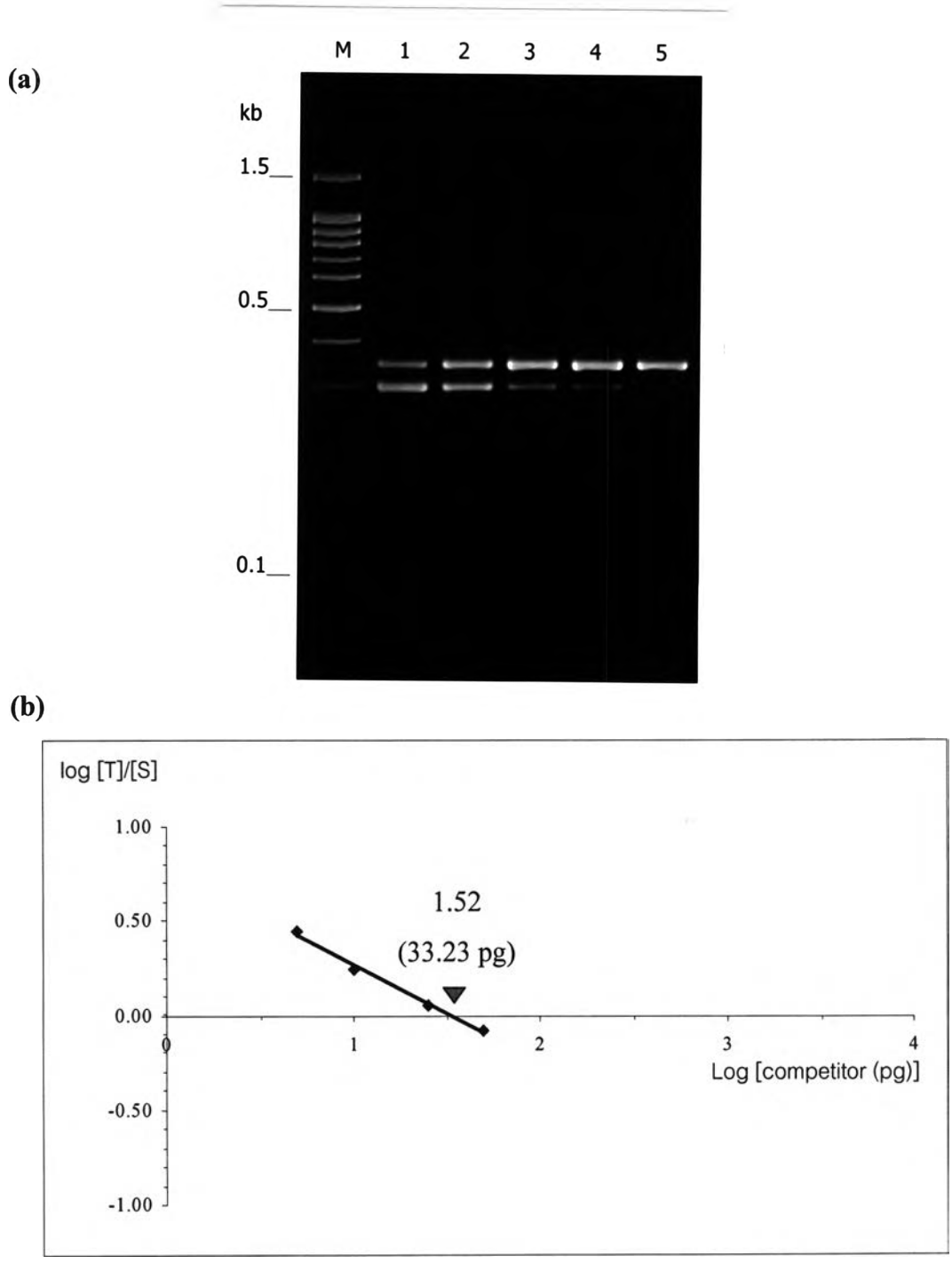
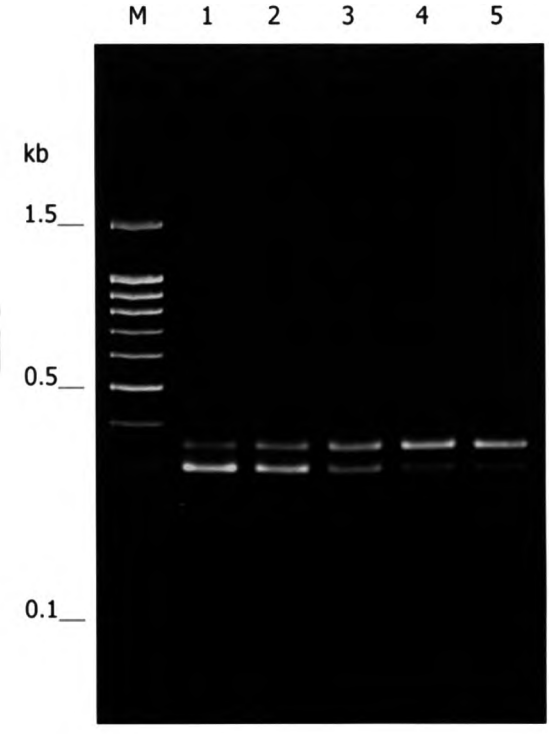


Figure 3.20 Agarose gel electrophoresis (a) and log graph (b) for quantification analysis of MRJP1 mRNA in hypopharyngeal gland of *A. cerana* 11-15 -day-old nurse bees using competitive PCR at the constant 0.5 ng of the cDNA target.

Lane M = A 100 bp DNA ladder
Panel a; lane 1-5 = AcMRJP1 cDNA target co-amplified in the presence of 50, 25, 10, 5 and 2.5 pg of AcMRJP1 cDNA competitor, respectively.

(a)



(b)

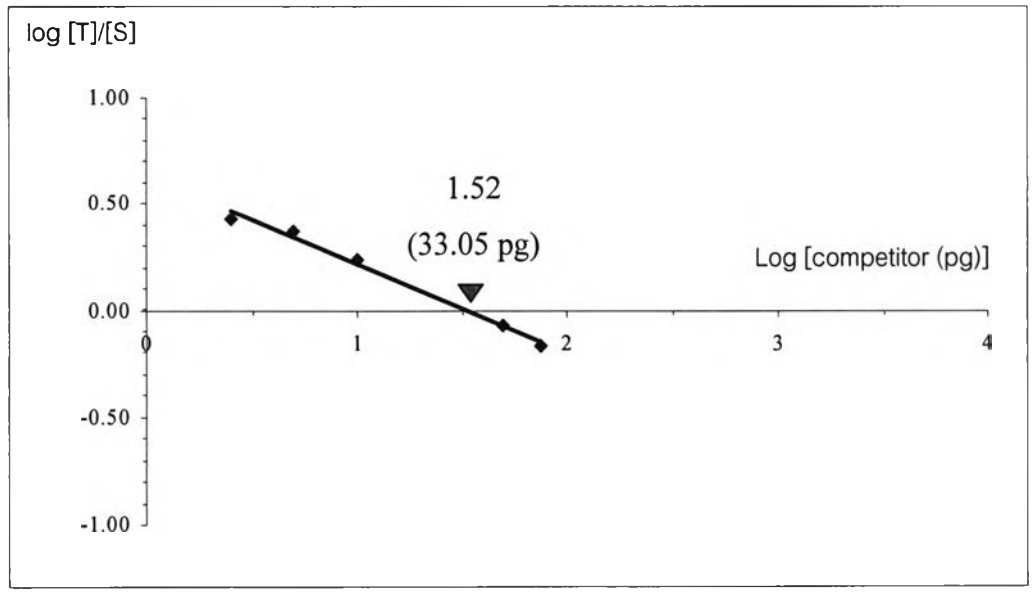


Figure 3.21 Agarose gel electrophoresis (a) and log graph (b) for quantification analysis of MRJP1 mRNA in hypopharyngeal gland of *A. cerana* forager bees using competitive PCR at the constant 2 ng of the cDNA target.

Lane M = A 100 bp DNA ladder
Panel a; lane 1-5 = AcMRJP1 cDNA target co-amplified in the presence of 75, 50, 10, 5, and 2.5 pg of AcMRJP1 cDNA competitor, respectively.

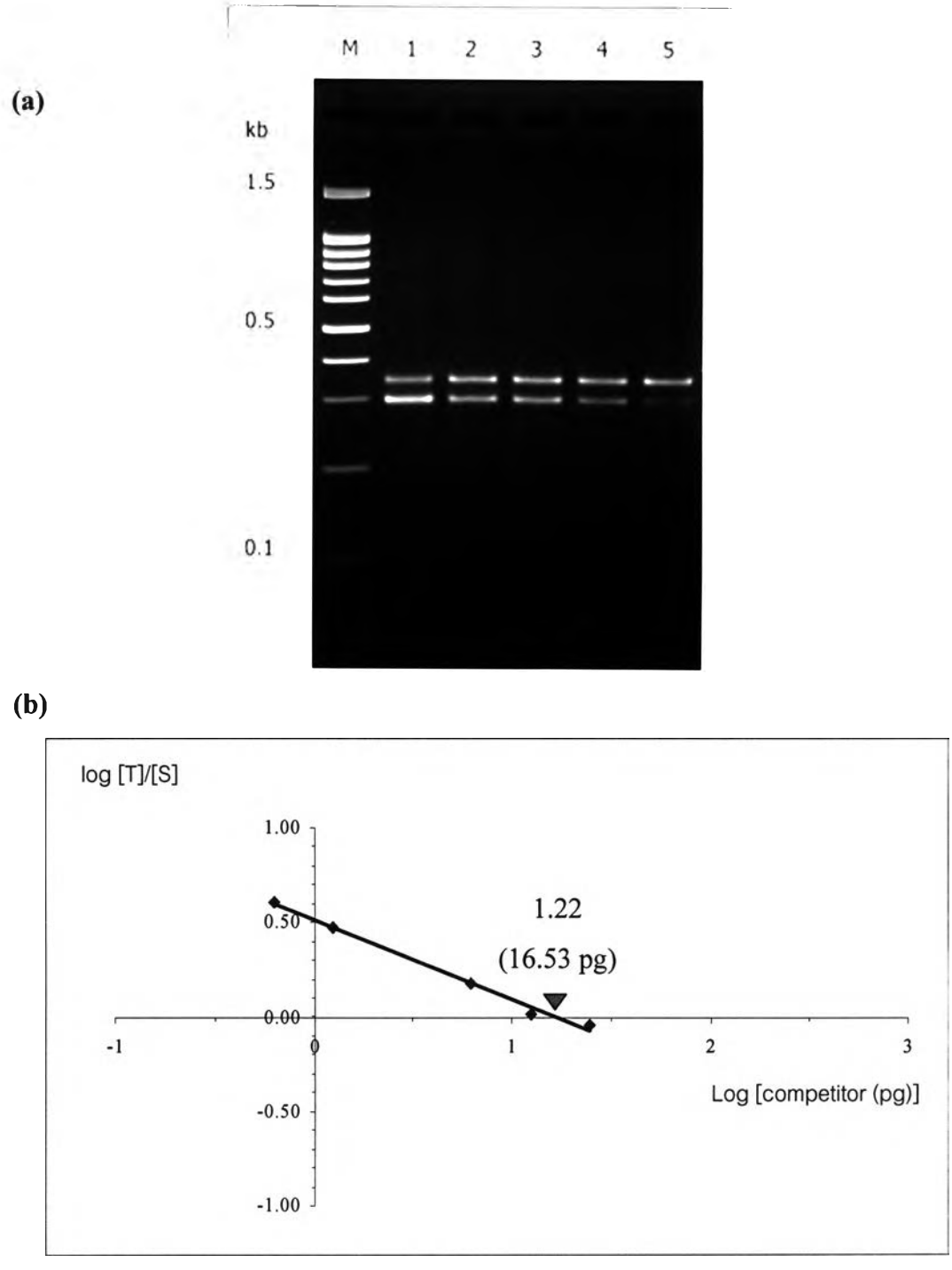


Figure 3.22 Agarose gel electrophoresis (a) and log graph (b) for quantification analysis of MRJP1 mRNA in hypopharyngeal gland of *A. cerana* forager bees using competitive PCR at the constant 1 ng of the cDNA target.

Lane M = A 100 bp DNA ladder
Panel a; lane 1-5 = AcMRJP1 cDNA target co-amplified in the presence of 25, 12.5, 6.25, 1.25, and 0.625 pg of AcMRJP1 cDNA competitor, respectively.

At suitable competitor range, the transcription level of AcMRJPs and AcApisimin mRNA was evaluated in newly emerged bees, 5-10- day-old nurse bees, the 11-15- day-old nurse bees and the forager bees. In the newly emerged bees, the PCR products of all AcMRJPs mRNA were not found, whereas AcApisimin mRNA was observed in newly emerged bees and also in all study stages. The summary of estimated transcription levels of AcMRJPs and AcApisimin mRNA per 1 ng total RNA were shown in Table 3.7.

In 5-10 -day-old nurse bees, the highest relative ratio of mRNA level (Table 3.8) was AcMRJP1 mRNA (approximately 400 times higher than the lowest), followed by AcMRJP2 mRNA (approximately 250 times), AcMRJP3 mRNA similar to AcMRJP4 mRNA (approximately 22 times), AcMRJP5 mRNA (approximately 5 times) and the lowest was AcMJRP6 mRNA. In 11-15 -day-old nurse (Table 3.9), AcMRJP1 mRNA had highest level (128 times higher than the lowest) followed by AcMRJP2 mRNA (64.7 times), AcMRJP4 mRNA (5.7 times), AcMRJP3 mRNA (5.4 times), AcMRJP5 mRNA (2.2 times), and the lowest was AcMJRP6 mRNA. For forager bees (Table 3.10), the highest mRNA level was AcMRJP1 mRNA (107 times higher than the lowest) followed by AcMRJP2 mRNA (92.6 times), AcMRJP6 mRNA (86.3 times), AcMRJP4 mRNA (9.6 times), AcMRJP3 mRNA (3.0 times) and the lowest AcMRJP5 mRNA.

Transcription level of AcApisimin mRNA was found to be lowest in newly emerged bees (Table3.11). The level was highest in 11-15 -day-old nurse bees then gradually decreased in 5-10 -day-old nurse bees and forager bees, respectively. The transcription level of AcApisimin mRNA was higher than AcMRJP1 mRNA about 10 times in nurse bees and 70 times in forager bees.

Since, the total RNA amount per individual forager bees was approximately 4-5 times less than the newly emerged and nurse bees. Therefore, the transcription profiles of AcMRJPs and AcApisimin mRNA were adjusted and shown in Table 3.11 and Figure 3.23, 3.24, 3.25 and 3.26. The relative ratio between different stages as newly emerged bees : 5-10 -day-old nurse bees : 11-15 -day-old nurse bees : forager bees was 0 : 20.0: 20.1 : 1 for AcMRJP1, 0 : 13.9 : 11.7: 1 for AcMRJP2, 0 : 37.5 : 29.7 : 1 for AcMRJP3, 0 : 12.0 : 10.1 : 1 for AcMRJP4, 0 : 28.5 : 36.8 : 1 for AcMRJP5, 0 : 1 : 3.3 : 16.7 for AcMRJP6 and 1 : 15.3 : 15.9 : 5.3 for AcApisimin, respectively.

Table 3.7 The transcription levels of AcMRJPs and AcApisimin mRNA.

Gene	Estimated transcription levels (pg per 1 ng total RNA)			
	newly emerged bees	5-10 -day-old nurse bees	11-15 -day-old nurse bees	forager bees
AcMRJP1	NF*	66.2	66.5	16.5
AcMRJP2	NF*	39.7	33.6	14.3
AcMRJP3	NF*	3.5	2.8	0.5
AcMRJP4	NF*	3.5	3.0	1.5
AcMRJP5	NF*	0.9	1.1	0.2
AcMRJP6	NF*	0.2	0.5	13.3
AcApisimin	45.1	691.2	716.2	1,201.3

* NF = Not Found

Table 3.8 The relative ratio of AcMRJPs mRNA in 5-10 -day-old nurse bees.

Gene	AcMRJP1	AcMRJP2	AcMRJP3	AcMRJP4	AcMRJP5	AcMRJP6
Relative ratio	415.6 :	249.3 :	22.0 :	22.2 :	5.5 :	1.0

Table 3.9 The relative ratio of AcMRJPs mRNA in 11-15 -day-old nurse bees.

Gene	AcMRJP1	AcMRJP2	AcMRJP3	AcMRJP4	AcMRJP5	AcMRJP6
Relative ratio	128.1 :	64.7 :	5.4 :	5.7 :	2.2 :	1.0

Table 3.10 The relative ratio of AcMRJPs mRNA in forager bees.

Gene	AcMRJP1	AcMRJP2	AcMRJP3	AcMRJP4	AcMRJP5	AcMRJP6
Relative ratio	107.1 :	92.6 :	3.0 :	9.6 :	1.0 :	86.3

Table 3.11 The transcription levels of AcMRJPs and AcApisimin mRNA per individual bees.

Gene	Estimated transcription levels (pg per individual bees)			
	newly emerged bees	5-10 -day-old nurse bees	11-15 -day-old nurse bees	forager bees
AcMRJP1	NF*	63.6	55.9	3.2
AcMRJP2	NF*	38.2	28.2	2.8
AcMRJP3	NF*	3.4	2.3	0.1
AcMRJP4	NF*	3.4	2.5	0.3
AcMRJP5	NF*	0.8	1.0	0.03
AcMRJP6	NF*	0.2	0.4	2.6
AcApisimin	45.1	664.6	602.0	236.0

* NF = Not Found

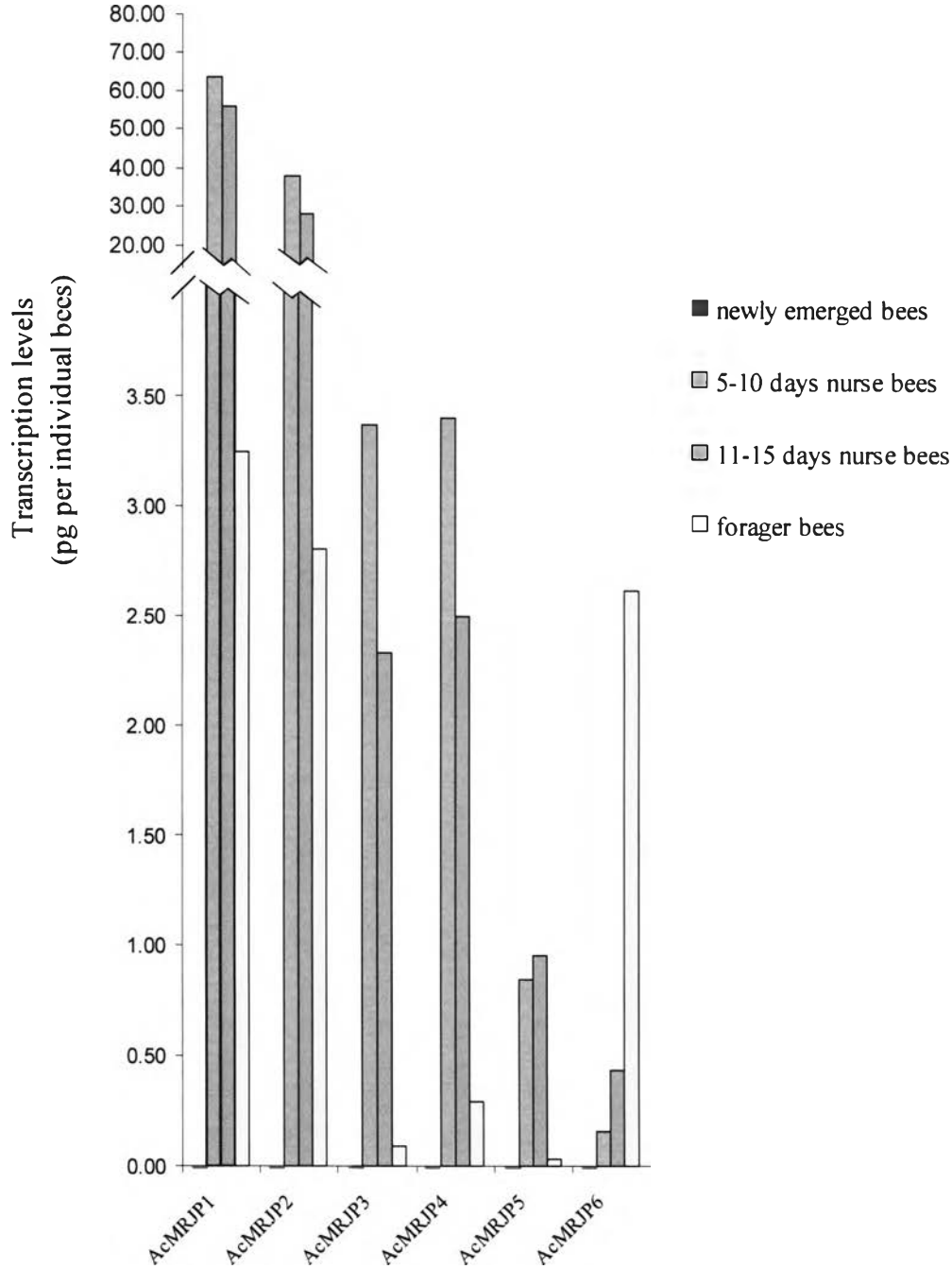


Figure 3.23 The AcMRJPs mRNA levels at various stages of worker bees.

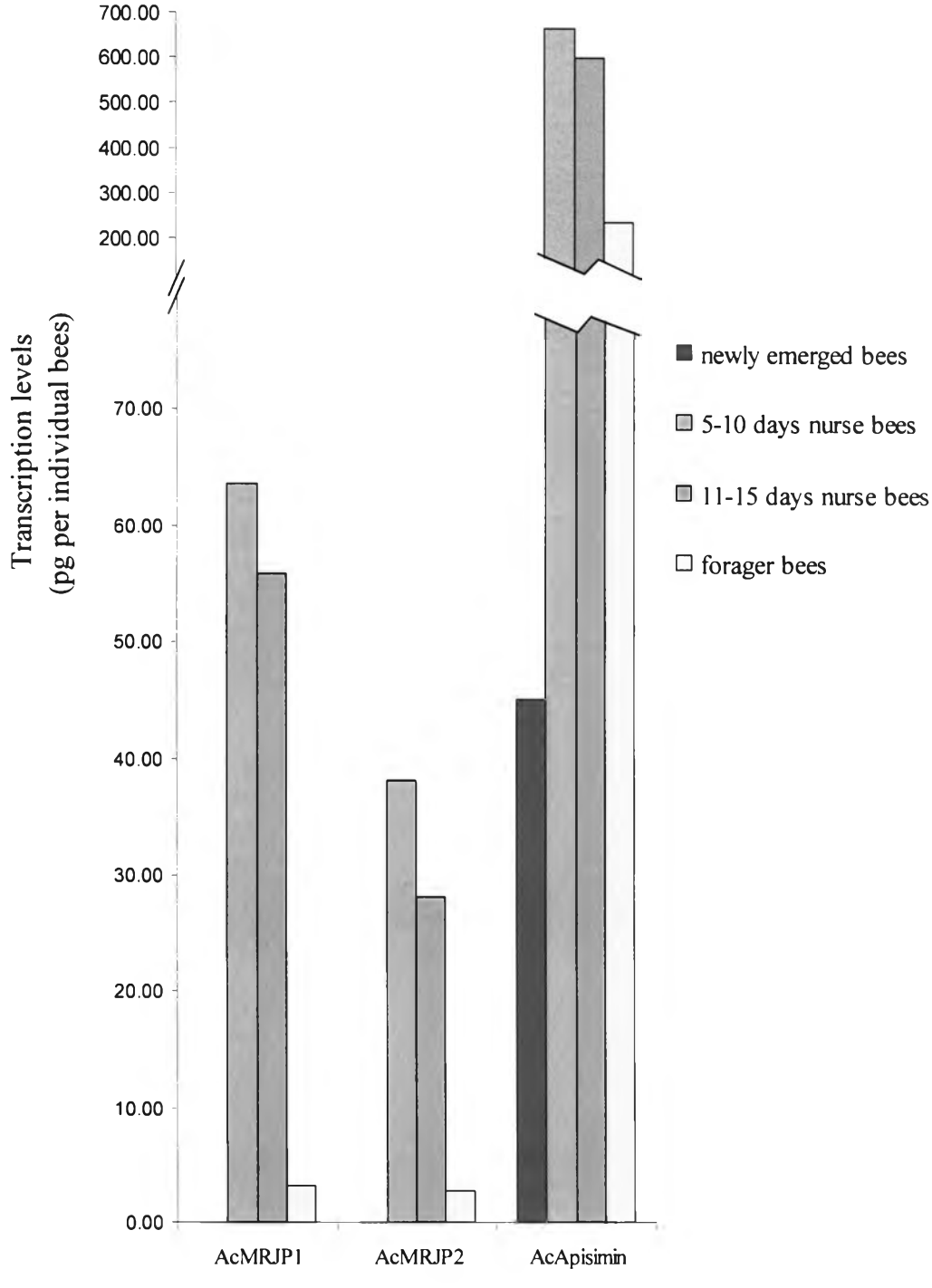


Figure 3.24 The AcMRJP1, AcMRJP2 and AcApisimin mRNA levels at various stages of worker bees.

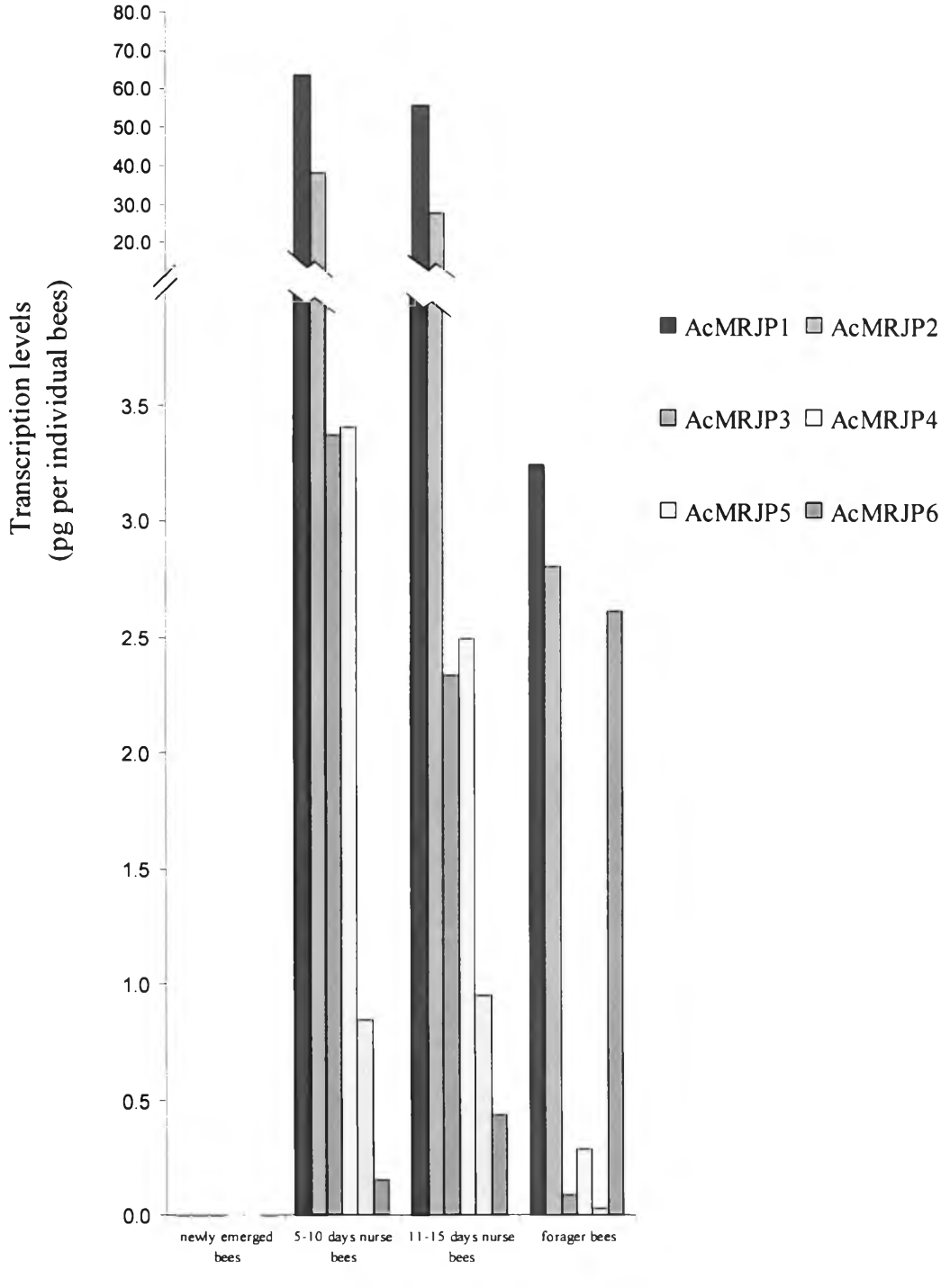


Figure 3.25 The transcription profiles of AcMRJPs mRNA at various stages of worker bees.

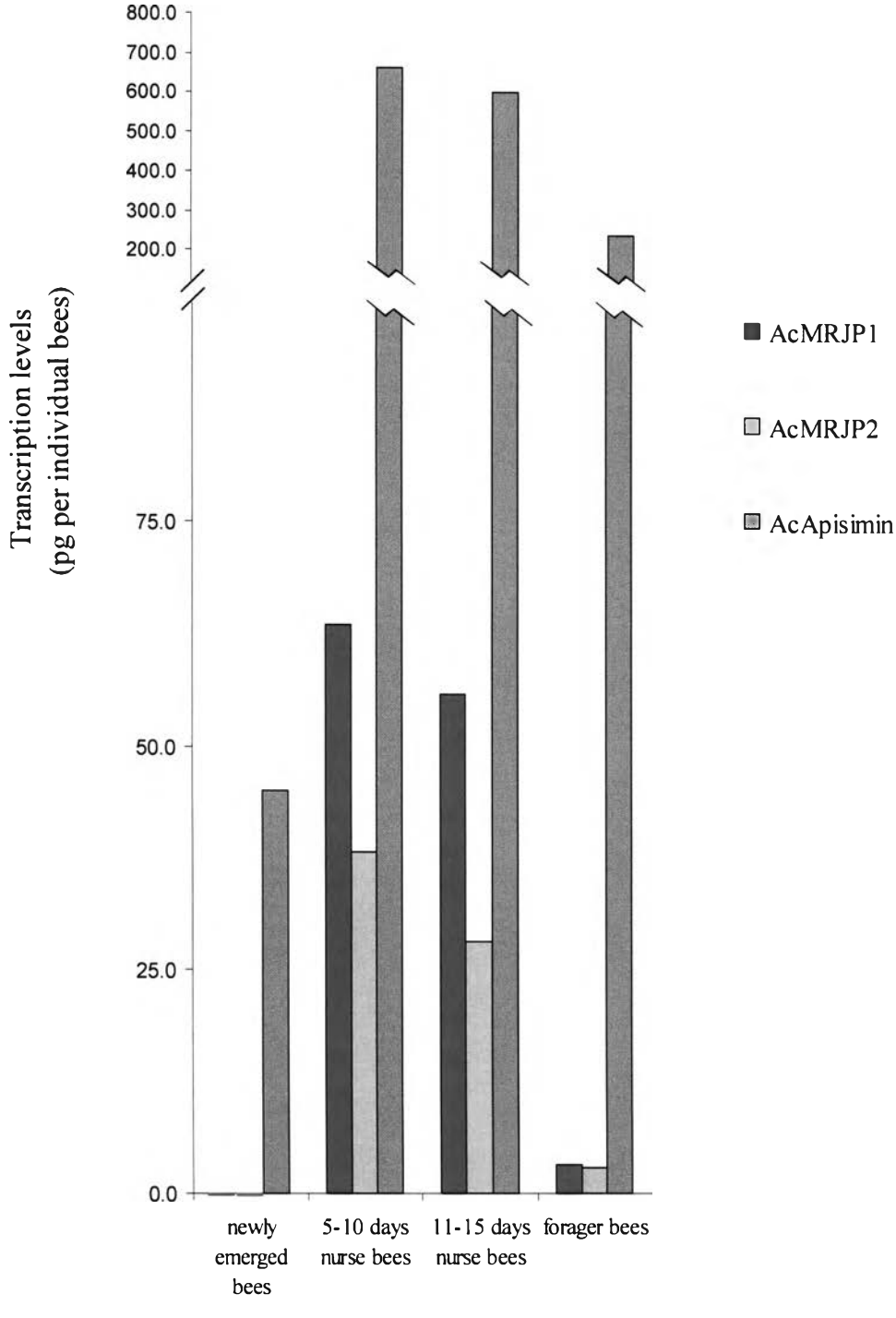


Figure 3.26 The transcription profiles of AcMRJP1, AcMRJP2 and AcApisimin mRNA at various stages of worker bees.