mRNA expression and DNA methylation in three key genes involved in caste differentiation in female honeybees (*Apis mellifera*)

Xin-Liang SHAO[1,2,3], Shao-Yu HE[1,3,4], Xin-Ying ZHUANG[2], Ying FAN[1,3], Ya-Hui LI[1,3,4,*], Yong-Gang YAO[2]

1. *College of Animal Science and Technology*, *Yunnan Agricultural University*, *Kunming* 650201, *China*
2. *Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology*, *Kunming* 650223, *China*
3. *Eastern Bee Institute*, *Yunnan Agricultural University*, *Kunming* 650201, *China*
4. *College of Food Science and Technology*, *Yunnan Agricultural University*, *Kunming* 650201, *China*

Abstract: In honeybee (*Apis mellifera*) colonies, queens and workers are alternative forms of the adult female honeybee that develop from genetically identical zygotes but that depend on differential nourishment. Queens and workers display distinct morphologies, anatomies and behavior, better known as caste differentiation. Despite some basic insights, the exact mechanism responsible for this phenomenon, especially at the molecular level, remains unclear although some progress has been achieved. In this study, we examined mRNA levels of the *TOR* (target of rapamycin) and *Dnmt3* (DNA methyltransferase 3) genes, closely related to caste differentiation in honeybees. We also investigated mRNA expression of the *S6K* (similar to RPS6-p70-protein kinase) gene linked closely to organismal growth and development in queen and worker larvae (1-day and 3-day old). Last, we investigated the methylation status of these three genes in corresponding castes. We found no difference in mRNA expression for the three genes between 1st instar queen and worker larvae; however, 3rd instar queen larvae had a higher level of *TOR* mRNA than worker larvae. Methylation levels of all three genes were lower in queen larvae than worker larvae but the differences were not statistically significant. These findings provide basic data for broadening our understanding of caste differentiation in female honeybees.

Keywords: Caste differentiation; DNA methylation; DNA methyltransferase 3; Honeybee; Target of rapamycin

The honeybee (*Apis mellifera*) is one of the most socially advanced and hitherto best-studied species in *Hymenopterans* (Amdam et al, 2004; Omholt & Amdam, 2004). A honeybee colony is typically composed of a single queen, none to a few thousand drones (male) depending on reproductive requirements, and 20−40 thousand workers (Winston, 1987). Queens and workers develop from genetically identical fertilized eggs, however, queens are twice as big as workers, have a much longer lifespan, are equipped with specialized anatomy and develop faster from egg to adult. Queens are fertile but workers are sterile. Workers perform various tasks that change with their age such as nest construction and cleaning, larva nursing, food processing and foraging and guarding, while the queens' sole duty is laying eggs for the reproduction of their colonies (Camazine, 1991; Hoover et al, 2005; Kucharski et al, 2008; Page & Peng, 2001; Rueppell et al, 2005, 2007).

Caste differentiation in social insects has been investigated over several decades and the honeybee has been used as a model animal since the eighteenth century (Corona et al, 1999; Cristino et al, 2006; Page & Peng, 2001; Patel et al, 2007; Rembold et al, 1974; Wheeler et al, 2006). Consequently, the association between nouris-

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* Corresponding author, E-mail: liyh85@aliyun.com

hment and developmental trajectory has been recognized for more than 100 years (Corona et al, 1999; Patel et al, 2007). It is clear that queen-destined larvae are fed royal jelly, whereas other female larvae are fed worker jelly. Royal jelly contains approximately 12% sugar (wet mass) while worker jelly has only 4% sugar (Corona et al, 1999; Ishay et al, 1976). In addition, protein type and content are different between royal and worker jelly. In a recent study, Kamakura (2011) showed that royalactin in royal jelly induces the differentiation of honeybee larvae into queens through an epidermal growth factor receptor-mediated signaling pathway. However, whether other mechanisms regulate caste differentiation remains unknown.

Many aspects of honeybee biology have benefited from new molecular biological technologies and the accomplishment of whole genome sequencing of *Apis mellifera* (Honeybee Genome Sequencing Consortium, 2006). The third to fourth day in larval development is a critical period of gene expression/regulation during diphenic caste development in female honeybees (Barchuk et al, 2007; Corona et al, 1999; Evans & Wheeler, 1999; Wheeler et al, 2006). TOR (target of rapamycin) is a nutrient- and energy-sensing kinase and a key player in the caste determination pathway in *Apis mellifera*; 3rd instar queen larvae have about two-fold higher *TOR* mRNA levels than 3rd instar worker larvae (Patel et al, 2007). Using rapamycin/FK506 pharmacology and RNA interference (RNAi) gene knockdown methods, Patel et al (2007) proved that in queen-destined larvae, rapamycin can specifically inhibit the activity of TOR and induce the development of worker characteristics, and that *TOR* gene knockdown shifts larval developmental fate from queen-destined to worker (Patel et al, 2007). Another important discovery is that fruit flies and mice deficient in S6K, another kinase governing cell growth, exhibit severe abnormalities such as developmental delay and smaller body sizes (Montagne & Steward, 1999; Shima et al, 1998).

DNA methyltransferase Dnmt3, which is in charge of *de novo* methylation of cytosines in CpG dinucleotides of genomic DNA, is a possible key driver of epigenetic global reprogramming in honeybees (Kucharski et al, 2008). When treated with Dnmt3 siRNA in newly hatched honeybee larvae, most worker-destined individuals emerge as queens with fully developed ovaries (Kucharski et al, 2008). Further, Kucharski et al (2008) have shown that silencing Dnmt3 can cause widespread gene expression changes and shifts the larvae developmental trajectory.

Based on these findings, we speculated that the *TOR*, *S6K* and *Dnmt3* genes are differentially expressed in 3rd instar queen and worker larvae and were eager to know if the activities of *TOR* and *S6K* are regulated through DNA methylation by Dnmt3. Lower methylation levels of DNA can promote mRNA expression (Jones & Gonzalgo, 1997; Razin, 1998; Weiss & Cedar, 1997; Wolffe et al, 1999; Yamada et al, 2008) in vertebrates. Here, we measured mRNA expression levels of *TOR, S6K* and *Dnmt3* and examined CpG methylation levels for these genes in queen and worker 3-day old larvae (using gene expression patterns of 1st instar larvae as the control). Our results indicate the relative quantity of *TOR* mRNA level in 3rd instar queen larvae is higher than in worker larvae, but there is no difference in *S6K* or *Dnmt3* mRNA levels between queens and workers. The CpG methylation quantities of the three genes in queen larvae are lower (~20%) than in worker larvae, but the differences are not statistically significant.

MATERIALS AND METHODS

Honeybee larvae

Honeybee larvae were raised in a strong double 8 frame hive using a queen excluder. The colony was fed 1:1 w/v sugar water at the rate of 500 mL every second day. The queen was confined to lay eggs in a square (20 cm×20 cm) of empty comb using wire gauze. Newly emerged larvae were grafted into queen cups using a transferring tool and remaining larvae in the comb were raised as worker larvae. Queen cups were carefully placed in the centre of the top box. After 24 and 72 h larvae were collected for experimentation (Kucharski et al, 2008).

DNA extraction

Nine larvae (queen or worker) from the hive were pooled together for DNA extraction using the TIANamp Genomic DNA kit (DP304). The procedure was performed as described by the manufacturer.

RNA isolation and reverse transcription

Live larvae were grafted into 1.5 mL Eppendorf centrifuge tubes and frozen in liquid nitrogen immediately. RNA extraction was performed using Trizol according to the manufacturer's instructions (Invitrogen, Cat.No.15596026). We collected larvae from two bee

colonies (A and B). In colony A, a total of 48 1st instar larvae (queen, *n*=24; worker, *n*=24) and 52 3rd instar larvae (queen, *n*=26; worker, *n*=26) were used for total RNA isolation; for colony B, 46 1st instar larvae (queen, *n*=23; worker, *n*=23) and 47 3rd instar larvae (queen, *n*=24; worker, *n*=23) were used. Reverse transcription was performed using reverse transcriptase (Promega, Catalog# M1701) following the manufacturer's instructions.

Quantification of *TOR*, *S6K* and *Dnmt3* mRNA levels in queen and worker larvae

TOR, *S6K* and *Dnmt3* mRNA levels were determined by using real-time PCR following previously described methods (Amdam et al, 2004; Patel et al, 2007) (Table 1). In brief, real-time PCR was performed in a total volume of 20 μL reaction solution, containing SYBR mix 10 μL, cDNA 1 μL, Primer 1 (1 μmol/L) 1 μL, Primer 2 (1 μmol/L) 1 μL and ddH_2O 7 μL. Target fragments were amplified under the following thermal cycle conditions: a denaturation cycle for 3 min at 95 °C, followed by 40 cycles of 10 s at 94 °C, 10 s at 58 °C and 10 s at 72 °C. The signal was collected at 72 °C per cycle.

Table 1 Primers for quantifying mRNA levels and DNA methylation levels of the *TOR*, *S6K* and *Dnmt3* genes

Gene	Primer sequence (5'−3')	Length of fragment (bp)
For real-time PCR		
TOR	F: CTGCCACATTACCAAAGAAAGG R: AACTTGACGTTGAACACTCAATG	170
S6K	F: TAAATGCTAGAAGTCCACGTAGAG R: TTAGCCTATTTCGATCATTTCTG	150
Dnmt3	F: ACTCGAATGTGGAACACCTGG R: GTCTTGGTCTATCTCGCTCGC	155
Actin	F: GTATGCCAACACTGTCCTTTCTG R: AAGAATTGACCCACCAATCCA	160
For examination of DNA methylation		
TOR	Distal primer pair F: GATGGTTTTAGGTTATGATTA R: ACACTACATAATACTCCTTTC	615
	Proximal primer pair F: TTATGATTATTTTATGTTTATG R: ATAACAATAAAAAAATCTAATAC	573
S6K	Distal primer pair F: AAGTGAATTTGTTAATAGAG R: CGATCATTTCTATATCTTCTAC	481
	Proximal primer pair F: GTTAATAGAGTATTTTAAGTGTG R: ATCCAACTCCATTATATCTAC	441
Dnmt3	Distal primer pair F: TTTTTGGGATGGTGATTATTTG R: ACAAACCTAAAAACATCTATCCTCT	1205
	Proximal primer pair F: GATAAAGAAGATTGTTTAAGGT R: AATTTCTAAAAACATCTATCTTC	476

All primers were designed in this study.

Examination of DNA methylation

Total DNA Bisulfite conversion was carried out using the QIAGEN Epitect^R Bisulfite Kit (Code No. 59104) as recommended by the manufacturer. Converted DNA was amplified using gene-specific primers (Table 1) in a volume of 20 μL containing 10×PCR Buffer 2 μL, dNTP 1.6 μL, LA Taq DNA polymerase 0.2 μL, Primer 1 (10 μmol/L) 0.4 μL, Primer 2 (10 μmol/L) 0.4 μL, DNA template 1 μL, ddH_2O 14.4 μL. PCR amplification was performed with an initial denaturation cycle for 5 min at 95 °C, followed by 40 cycles of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 °C. We used 0.5 μL of the first PCR product as template for the second round of PCR amplification under the same thermal cycle conditions. Purified PCR products were cloned into PGM-T vector (TIANGEN, VT202-02). Recombinant clones were selected by plating the transformed DH5α cells (TIANGEN, CB101) onto Amp^+ LB plates. Fourteen to 20 clones were chosen for each fragment for sequencing.

Data analysis

Analyses were performed with Prism 4.0. *t*-test was performed on differential expression in queen and worker larvae. A P value < 0.05 was considered statistically significant.

RESULTS

mRNA levels for *TOR* are different between queen and worker 3rd instar but not 1st instar larvae

Using the 1st and 3rd instar queen and worker larvae reared in colony, we measured the relative mRNA levels of the *TOR*, *S6K* and *Dnmt3* genes. We found no difference in expression between 1st instar queen and worker larvae (Figure 1). However, *TOR* mRNA expression levels in 3rd instar queen larvae were higher than in worker larvae, whereas there were no differences for *S6K* and *Dnmt3* mRNA levels between 3rd instar queen and worker larvae (Figure 2). To confirm our results, we examined another colony (Colony B) and obtained similar results (Figure 1, Figure 2).

In 3rd instar larvae, DNA methylation level is lower in queen larvae than in worker larvae but the differences are not significant

To ascertain whether the mRNA expression of *TOR*, *S6K* and *Dnmt3* are regulated by DNA methylation we examined methylation levels for these genes in 3rd instar

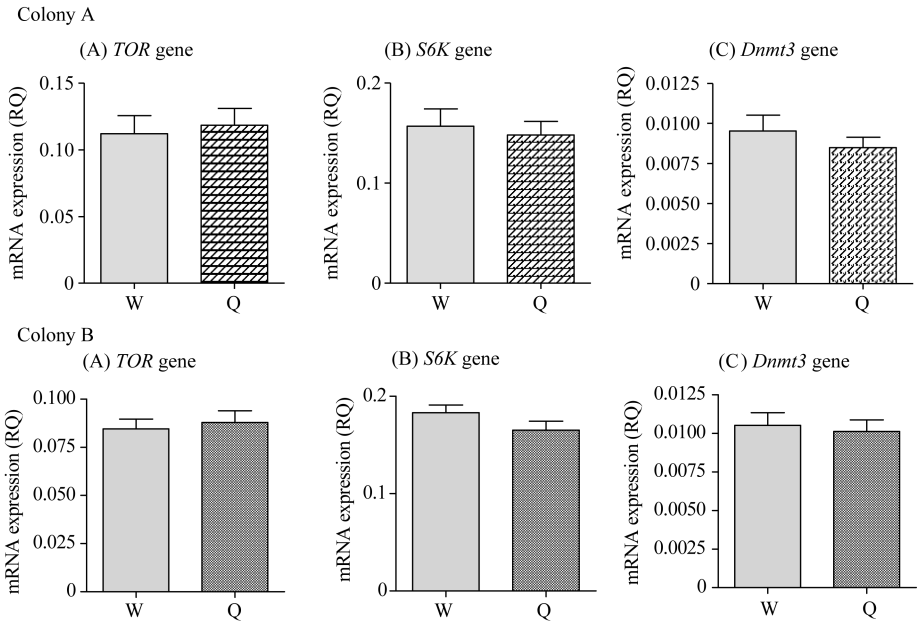

Figure 1 Comparison of relative quantity (RQ) of *TOR* gene (A), *S6K* gene (B) and *Dnmt3* gene (C) mRNA levels in 1st instar larvae

In a natural environment, there was no difference between queen and worker mRNA expression levels for the three genes in 1st instar animals (Colony A: *TOR* gene, t=0.3534, df=46, P= 0.3395; *S6K* gene, t=0.4003, df=46, P=0.1220; *Dnmt3* gene, t=0.8774, df=46, P=0.0288; Colony B: *TOR* gene, t=0.4189, df=44, P=0.6773; *S6K* gene, t=1.475, df=44, P=0.1472; *Dnmt3* gene, t=0.3535, df=44, P=0.7254). W: Worker; Q: Queen; Bars are mean + s. e.; 1st : 1-day old larvae.

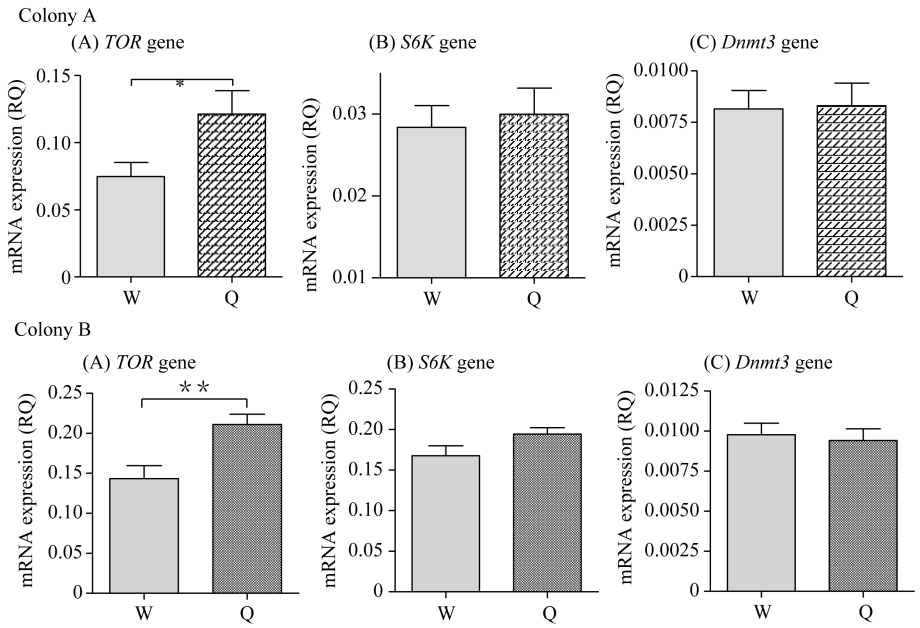

Figure 2 Comparison of relative quantity (RQ) of *TOR* gene (A), *S6K* gene (B) and *Dnmt3* gene (C) mRNA levels in 3rd instar larvae

In a natural environment, *TOR* mRNA expression in the 3rd instar queen larvae is higher than in worker larvae (Colony A: t=2.294, df=50, P=0.0260; Colony B: t=3.289, df=45, P=0.0020), whereas mRNA expression levels of the *S6K* gene (Colony A: t=0.3951, df=50, P=0.6944; Colony B: t=1.874, df=45, P=0.0674) and *Dnmt3* gene (Colony A: t=0.1135, df=50, P=0.9101; Colony B: t=0.3640, df=45, P=0.7176) did not differ between queen and worker larvae. W: Worker; Q: Queen; Bars are means + *s.e.*; 3rd: 3-day old larvae; asterisk: significant difference.

larvae (1st instar larvae were not analyzed as we did not see any change in mRNA expression for these three genes). Previous studies found that CpG methylation in *Apis* targets most coding exons (Kucharski et al, 2008; Wang et al, 2006). Therefore, for the *TOR* gene, we chose exon 4 which contains 18 CpG dinucleotides as the target region for examination. The overall amount of *TOR* methylation is 14.3% in queen larvae versus 17.5% in worker larvae (Figure 3A). For the *S6K* gene, we screened exon 10, which contains 7 CpG dinucleotides,

and we found that the overall amount of *S6K* methylation in the queen larvae (17.5%) was lower than that of worker larvae (19.8%) (Figure 3B). For the *Dnmt3* gene, we also observed a lower level of methylation in queen larvae (5%) than in worker larvae (6.2%) when we focused on exon 9 of this gene, which harbors 13 CpG dinucleotides (Figure 3C). Overall, the CpG methylation quantities of all three genes in queen larvae were around 20% lower when compared with worker larvae (Figure 3). However, these data were not statistically different, probably due to the limited number of individuals analyzed in this study.

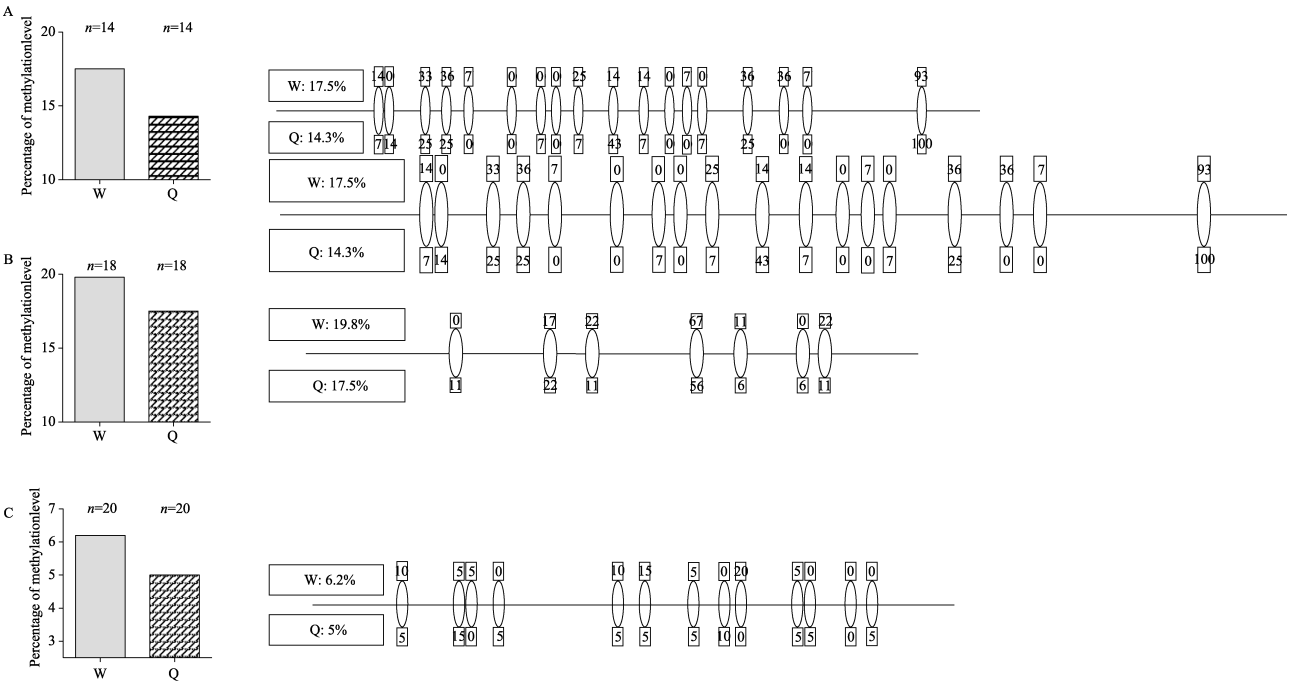

Figure 3 Comparison of methylation level of cytosines in CpG dinucleotides in exon 4 of the *TOR* gene (A), exon 10 of the *S6K* gene (B) and exon 9 of the *Dnmt3* gene (C)

Overall methylation for each gene is shown in the left graph and the percentage of methylation for individual CpGs is listed in boxes on the right. DNA was isolated using 9 pooled 3rd instar worker larvae (W) or 3rd instar queen larvae (Q) collected from the hive. The number of clones sequenced for each fragment for worker larvae or queen larvae is listed on the top of the bar in each figure. Ellipses indicate CpGs. W: worker, Q: queen. 3rd : 3-day old larvae.

DISCUSSION

The involvement of nutrition in diphenic development in female honeybees has long been recognized (Maeterlinck 1901), but insufficient knowledge has been accumulated regarding the molecular mechanisms regulating diverse developmental fates. Recently, studies have demonstrated that two proteins, TOR and DNA methyltransferase Dnmt3, play pivotal roles in caste differentiation in *Apis mellifera* (Kucharski et al, 2008; Patel et al, 2007). TOR is known to be a serine/threonine kinase that controls growth in response to nutrition. Patel et al (2007) found that high expression of the *TOR* gene is essential for a larva to become a queen and Kucharski et al (2008) indicated that knockdown of the *Dnmt3* gene in worker-destined larvae results in queen development. Some reports demonstrate that DNA methyltransferase can inactivate or suppress a gene by DNA methylation (Jones & Gonzalgo,1997; Razin,1998; Weiss & Cedar, 1997; Wolffe et al, 1999; Yamada et al, 2008). Consistent with the report by Patel et al (2007), the mRNA expression levels of *TOR* measured here were higher in 3rd instar queen larvae than worker larvae (Figure 2). However, there was no difference for *TOR* mRNA levels in 1st instar queen and worker larvae (Figure 1). This result suggests that TOR plays its regulatory role in a critical period of development, providing new molecular evidence to confirm the previous finding that the 3rd instar is a critical point for honeybee caste division (de Wilde & Beetsma, 1982; Handre & Lees, 1985). No significant difference in DNA methylation levels of the *TOR* gene was found between queen and worker larvae, although worker larvae had a relatively lower level of DNA methylation (Figure 3). In addition, there was no difference in mRNA expression of *Dnmt3* in both larvae

(Figure 2) and this observation implies that *TOR* mRNA expression may not be mediated by *Dnmt3* via DNA methylation.

S6K is another kinase that controls cell growth. The fact that knockout of the *S6K* gene in fruit flies and mice results in extreme developmental delay and smaller bodies (Montagne & Steward, 1999; Shima et al, 1998) led us to hypothesize that S6K may be involved in caste differentiation in honeybees. We showed that mRNA expression of the *S6K* gene in queen larvae was slightly higher than worker larvae at the 3rd instar (a critical developmental stage for caste determination), although this did not reach statistical significance (Figure 2). Consistent with mRNA expression patterns, we found a slightly lower level of DNA methylation of the *S6K* gene in queen larvae than in worker larvae. Combined with the report by Kamakura (2011) that suppression of *S6K* with RNAi inhibited the increase to adult size induced by royal jelly, we speculate that S6K may play a role in honeybee caste differentiation. Further study regarding the role of S6K in honeybee caste diphenism is clearly essential.

In our DNA methylation experiment we chose one exon as the target to estimate the overall methylation quantity of each gene. Although CpG methylations may be heterogeneous throughout the gene, at least 7 CpGs in our target fragment are capable of representing the overall methylation level by and large, as demonstrated in previous studies (Kucharski et al, 2008; Wang et al, 2006). In spite of the fact that some special genes have alternative exons, DNA methylation or other epigenetic modifications are correlated with alternative splicing patterns (Chittka & Chittka, 2010; Luco et al, 2011; Lyko et al, 2010) and methylation promotes expression of some neurogenic genes by functionally antagonizing Polycomb repression (Wu et al, 2010). Reducing the DNA methylation quantity of a gene can facilitate its expression for the overwhelming majority of genes. Nevertheless, it is difficult to determine the direct relationship between DNA methylation and mRNA expression of certain genes because widespread gene expression differences can be caused by changes to a small number of upstream regulators (Carone et al, 2010). Differences in protein expression between 3-day old worker and queen larvae are known, indicating that the fate of queen and worker larvae have already been decided before the 3rd instar (Li et al, 2010).

In conclusion, we investigated mRNA expression and DNA methylation of *Dnmt3*, *TOR* and *S6K* in queen and worker-destined larvae in order to determine if mRNA expression of *TOR* and *S6K* is controlled by Dnmt3 through DNA methylation. Our results showed no difference in gene expression (except for *TOR*) between 3rd instar queen and worker larvae. In addition, DNA methylation levels of these three genes in both larvae did not differ, although queen larvae had a relatively lower level of methylation. These results suggest that mRNA expression of *TOR* might not be regulated by Dnmt3; whether S6K is regulated by Dnmt3 remains obscure. Thus, the significance of CpG methylation in exons in honeybees needs to be reevaluated.

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