Differentially expressed microRNAs between queen and worker larvae of the honey bee (*Apis mellifera*)

Yuan-Yuan Shi, Hua-Jun Zheng, Qi-Zhong Pan, Zi-Long Wang, Zhi-Jiang Zeng

► To cite this version:

Yuan-Yuan Shi, Hua-Jun Zheng, Qi-Zhong Pan, Zi-Long Wang, Zhi-Jiang Zeng. Differentially expressed microRNAs between queen and worker larvae of the honey bee (*Apis mellifera*). *Apidologie*, 2015, 46 (1), pp.35-45. 10.1007/s13592-014-0299-9 . hal-01284426

HAL Id: hal-01284426

<https://hal.science/hal-01284426>

Submitted on 7 Mar 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Differentially expressed microRNAs between queen and worker larvae of the honey bee (*Apis mellifera*)

Yuan-Yuan SHI¹, Hua-Jun ZHENG², Qi-Zhong PAN¹, Zi-Long WANG¹, Zhi-Jiang ZENG¹

¹Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, China

²Shanghai-MOST Key Laboratory of Health and Disease Genomics, Chinese National Human Genome Center at Shanghai, Shanghai 201203, China

Received 4 March 2014 – Revised 17 May 2014 – Accepted 3 June 2014

Abstract – Although the honey bee queen and worker have identical genomes, they exhibit morphological, behavioral, and reproductive differences. Queen larvae (QL) and worker larvae (WL) have different gene and protein expression profiles and different DNA methylation profiles. MicroRNAs (miRNAs) play an important role in regulating gene expression and cell biological processes. To examine the roles of miRNAs in caste differentiation, we analyzed small RNA profiles in 4-day-old QL and WL (*Apis mellifera*). The results demonstrated that the small RNAs with a length of 22 nt showed significant difference between the two castes. By comparison with mirBase 19.0, we identified 61 known miRNAs in QL and WL; 17, 24 and 20 of these miRNAs were respectively up-regulated, equally expressed, and down-regulated in QL compared with WL. These differentially expressed miRNAs were involved in many signaling pathways related to caste differentiation.

honey bee / larvae / microRNA / caste differentiation

1. INTRODUCTION

The honey bee (*Apis mellifera*) is an important eusocial insect because of its high levels of social cohesion, precise division of labor, and dance communication for the effective use of natural resources (Seeley 1989). A normal honey bee colony has three castes: a single fertile queen, several thousands of almost sterile workers, and hundreds of haploid drones (Winston 1987). Although the queen and her workers have identical genomes, they exhibit significant differences in morphology, behavior,

physiology, longevity and reproduction (Weaver 1957).

Queen-worker differentiation is a fascinating phenomenon that has been extensively studied (Weaver 1966; Kucharski et al. 2008; Kamakura 2011; Shi et al. 2011). The main factor contributing to caste differentiation is royal jelly (RJ), the food provided to queen larvae (Winston 1987). The major RJ proteins play important roles in brain development and reproductive maturation (Drapeau et al. 2006). Royalactin is a critical RJ protein that increases body size, promotes ovarian development, shortens the developmental period and induces queen characters (Kamakura 2011). In addition, the differences in larval dietary sugar concentrations may influence caste dimorphism (Leimar et al. 2012). Worker larvae younger than 3 days of age will develop into normal queens if they are transferred to queen cups and

Corresponding author: Z.-J. Zeng,
bees1965@sina.com

Manuscript editor: Klaus Hartfelder

Yuan-Yuan Shi and Hua-Jun Zheng contributed equally to this paper.

raised inside the colony, but worker larvae older than 3.5 days are not totipotent (Weaver 1966).

MicroRNAs (miRNAs) are 20–24 nucleotides (nt) long, single-stranded, non-coding, endogenous RNA molecules, which regulate eukaryotic post-transcriptional gene expression and diverse cellular pathways (Bartel 2004; Moran et al. 2014). MiRNAs in the honey bee brain are correlated with age-related behavioral modifications as well as learning and memory (Greenberg et al. 2012; Qin et al. 2014). Nursing and foraging bees have 9 known differentially expressed miRNAs and 67 novel miRNAs (Liu et al. 2012). A total of 267 novel miRNAs have been observed in *A. mellifera* (Hori et al. 2011; Chen et al. 2010a). MiRNAs are present in the honey bee and in the RJ from *A. mellifera* (RJM) and *A. cerana* (RJC). There are 23 miRNAs specific to RJM, 2 miRNAs specific to RJC, and 46 miRNAs shared in both types of RJ, which may play a role in caste differentiation (Shi et al. 2012).

Queen larvae (QL) and worker larvae (WL) exhibit differentially expressed gene (Evans and Wheeler 2001; Hepperle and Hartfelder 2001; Barchuk et al. 2007; Chen et al. 2012; Cameron et al. 2013) and protein patterns (Wu and Li 2010) as well as DNA methylome profiles (Foret et al. 2012; Shi et al. 2013). The mechanism by which miRNAs regulate gene expression has been widely studied (Bartel 2004; Behura 2007; Laubinger et al. 2010), however, it is unclear whether there are differences in the small RNA profiles of QL and WL. In this study, we identified and characterized the differences in miRNAs between 4-day-old QL and WL.

2. MATERIALS AND METHODS

2.1. Experimental bee colonies

Honey bee colonies (*A. mellifera*) were maintained at the Honey Bee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46°N, 115.49°E), according to standard bee-keeping techniques.

2.2. Honey bee larvae

To decrease the effect of genetic background on miRNAs expression, 4-day-old QL and WL were sampled from three single-drone-inseminated colonies, with three biological replicates (ten larvae for each replicate) for both QL and WL. The six samples (QL1-3 and WL1-3) were flash frozen in liquid nitrogen and stored at -80 °C until use.

2.3. Measurement of miRNAs between QL and WL

The total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. The small RNA single-end sequencing was performed by the Chinese National Human Genome Center at Shanghai using the Illumina HiSeq™ 2500 (Illumina, CA, USA). Briefly, RNA fragments 10–45 nt in length were separated from total RNA using a Novex 15 % TBE-urea gel and followed by a 10 % TBE-urea gel. The resulting small RNAs were combined with 5' adaptors and then ligated to 3' adaptors. These integrative products were amplified by PCR amplification and excised from a 6 % TBE-urea gel. The sequencer-produced image files were then converted into digital-quality data. To further analyze the RNA secondary structure associated with the matching Solexa reads, digital-quality sequences with perfect match or one mismatch were maintained (Shi et al. 2012). Genomic sequences 100 nt in length were randomly selected from these sequences, and the secondary structure was analyzed and predicted using the Mireap and RNAfold software (Chen et al. 2010b) with default parameters. Finally, the candidate miRNA reads were analyzed using miRBase database 19.0. Clean reads were obtained by removing adaptor reads, low-quality reads, reads that were shorter than 10 nt and single-copy reads. These clean reads were deposited in the National Center for Biotechnology Information (NCBI) sequence read archive (SRR1105795 for QL1, SRR1105799 for QL2, SRR1105800 for QL3, SRR1105796 for WL1, SRR1105797 for WL2, and SRR1105798 for WL3).

The known miRNA expression between QL and WL samples were further analyzed to find out the differentially expressed miRNAs according to R statistics

software and the DEG-Sequencing package (Wang et al. 2010). The following formulae were used to calculate

fold-changes and P values from the normalized miRNA expression of QL and WL samples:

$$\text{QL normalized miRNA expression} = \text{QL miRNA clean reads} / \text{QL total clean reads} * 1000000$$

(Shi et al. 2012; Qin et al. 2013),

$$\text{WL normalized miRNA expression} = \text{WL miRNA clean reads} / \text{WL total clean reads} * 1000000$$

(Shi et al. 2012; Qin et al. 2013),

$$\text{Fold-change} = \log_2(\text{QL normalized miRNA expression} / \text{WL normalized miRNA expression})$$

(Shi et al. 2012; Qin et al. 2013),

$$p(x|y) = \binom{N_2}{N_1}^y \frac{(x+y)!}{x!y! \left(1 + \frac{N_2}{N_1}\right)^{(x+y+1)}}$$

$$C(y \leq y_{\min} | x) = \sum_{y=0}^{y \leq y_{\min}} p(y|x)$$

$$D(y \geq y_{\max} | x) = \sum_{y \geq y_{\max}}^{\infty} p(y|x)$$

where x and y indicate the mapped clean reads number for the same miRNA in QL and WL library, N_1 and N_2 represent the total reads number for the two libraries respectively (Shi et al. 2012; Qin et al. 2014). In this study, absolute $\log_2$ (fold change) > 1 and $P < 0.001$ were considered to represent significant differences in miRNA expression (Shi et al. 2012; Qin et al. 2014). A miRNA was considered “altered” only when Solexa sequencing analysis identified ten copies in all samples (Shi et al. 2012).

Using the RNA hybrid software (Rehmsmeier et al. 2004), we identified potential target sequences of the differentially expressed miRNAs in QL and WL, which

enabled identification of unique putative interaction sites with minimum free energy (Shi et al. 2012; Qin et al. 2014). To examine the functions of the differentially expressed miRNAs, all target genes were analyzed using the KEGG database (Ogata et al. 1999).

2.4. Public data used and data analyses

The *A. mellifera* reference genome (*Amel* 4.5) and gene information were obtained from the NCBI database (ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera/). The length distributions of small RNAs were examined by analysis of variance (ANOVA) using StatView (v 5.01;

Table 1. Summary of clean data produced by small RNA sequencing.

Sample	Raw reads	Total clean reads	Unique reads	Mapping rate	miRNA	siRNA	piRNA
4d-QL1	10,786,848	3,376,950	413,305	23.67 %	21.30 %	6.06 %	1.56 %
4d-QL2	8,738,265	4,763,431	360,434	25.41 %	24.34 %	3.91 %	0.88 %
4d-QL3	9,889,510	7,837,410	364,191	13.79 %	21.77 %	8.81 %	1.61 %
4d-WL1	4,218,342	3,065,428	162,074	8.35 %	40.04 %	4.40 %	1.31 %
4d-WL2	7,645,726	3,415,229	313,871	10.53 %	41.38 %	3.22 %	0.98 %
4d-WL3	5,327,012	4,647,577	208,101	9.28 %	48.06 %	2.83 %	1.02 %

Table II. Length distribution of clean reads in 4-day-old queen and worker larvae (%).

	22 nt	23 nt	24 nt	30 nt
4d-QL	22.47±0.94 a	17.66±0.21 a	6.26±1.42 a	1.14±0.24 a
4d-WL	43.16±2.48 b	12.72±2.23 a	3.48±0.47 a	1.10±0.11 a
P-value	0.0015	0.0917	0.1362	0.3921

Values are means±SE
The *different letters* in the same column indicate significant difference ($P<0.05$), *same letter* indicates no significant difference ($P>0.05$)

SAS Institute, Gary, NC, USA). Multiple comparisons of the mean values were performed using Fisher’s Protected Least Significant Difference only after ANOVA showed a significant effect ($P<0.05$).

3. RESULTS

3.1. Global mapping of small RNAs from QL and WL in the honey bee genome

A total of 8,738,265–10,786,848 reads from the QL libraries and 4,218,342–7,645,726 reads from the WL libraries were obtained after discarding adapter sequences. After filtering low-quality and single-read sequences as well as sequences shorter than 10 nt, 3,376,950–7,837,410 and 3,065,428–4,647,577 total reads were obtained for the QL and WL, respectively, and 360,434–413,305 and 162,074–313,871 unique reads were obtained, respectively (Table I). The RNAs sequenced by HiSeqTM 2500 were 10–45 nt long, and small

RNAs with a length of 22 nt showed significant difference between the two castes. In general, miRNAs are 20–24 nt long (Moran et al. 2014), and WL expressed more 22 nt RNAs compared with QL (Table II). Both QL and WL contained several small RNA species, which included miRNAs, degraded rRNA fragments and mRNA fragments (Table III). As shown in Table IV, the unique and total sRNAs shared in QL and WL were 86,814 and 12,241,528 reads, respectively.

3.2. Differentially expressed microRNAs in QL and WL

By referencing to the mirBase 19.0 database (<http://microrna.sanger.ac.uk/>), we identified 61 known miRNAs in QL and WL (Table V); 17, 24 and 20 of these miRNAs were respectively up-regulated, equally expressed, and down-regulated in QL compared with WL (Table VI). The index number of the linear relationships (Lu et al. 2010) between miRNAs expression levels in

Table III. Distribution of unique and total sRNAs categories in 4-day-old queen and worker larvae.

Category	4d-QL1	4d-QL2	4d-QL3	4d-WL1	4d-WL2	4d-WL3
Unique sRNAs						
miRNAs	3,668	3,591	3,462	1,549	3,887	2,275
rRNA, etc.	12,117	9,289	9,926	6,656	8,453	10,279
Other	65,078	50,401	53,394	30,554	44,577	51,308
Total sRNAs						
miRNAs	134,950	74,128	147,051	58,149	77,824	86,746
rRNA, etc.	36,139	19,767	40,320	15,572	18,953	21,686
Other	77,118	55,672	88,998	47,082	61,562	71,259

Table IV. Common and specific sequences in 4-day-old queen and worker larvae.

	Unique sRNAs (%)	Total sRNAs (%)	372
Total sRNAs	964,523 (100.00)	19,811,504 (100.00)	373
4d-QL1 specific	215,667 (22.36)	1,428,409 (7.21)	374
4d-QL2 specific	198,402 (20.57)	1,339,257 (6.76)	375
4d-QL3 specific	201,618 (20.91)	1,872,895 (9.45)	376
4d-WL1 specific	87,868 (9.11)	1,208,501 (6.10)	377
4d-WL2 specific	176,441 (18.29)	1,305,578 (6.59)	378
4d-WL3 specific	150,176 (15.57)	1,672,090 (8.44)	379
Shared all samples	86,814 (8.98)	12,241,528 (61.79)	380
			381

QL and WL replicates were $0.88 < R^2 < 0.94$ and $0.84 < R^2 < 0.94$, respectively. Using KEGG annotations, we observed that several differentially expressed larval miRNAs were related to development, growth, metabolic processes and signaling pathways (Table VII).

4. DISCUSSION

Worker larvae younger than 3 days of age are believed to develop into normal queens if they are transferred to queen cups and raised inside the colony (Weaver 1966). Using cDNA microarrays and digital gene expression (DGE) tag profiling, several DEGs were observed in 4-day-old QL and WL (Barchuk et al. 2007; Chen et al. 2012). Our previous study showed that DNA methylation

levels were higher in 4-day-old QL compared with 2-day-old QL, and the levels then decreased in 6-day-old larvae, whereas methylation levels increased with age in WL (Shi et al. 2013). These results indicate that the differences in gene expression and methylation level mostly occur in 4-day-old larvae. In *A. mellifera*, miRNAs are correlated with age-dependent behavioral changes (Behura and Whitfield 2010; Greenberg et al. 2012; Liu et al. 2012). In this study, we focused on the role of known miRNAs in caste differentiation in 4-day-old QL and WL. All three types of small RNAs (miRNA, 20–24 nt; siRNA, 24–26 nt; piRNA, 30–32 nt) were expressed in both WL and QL (Shi et al. 2012; Moran et al. 2014). However, WL has a higher percentage of 22 nt miRNA (Table II).

Table V. Differentially expressed miRNAs between 4-day-old queen and worker larvae.

Up-regulated in QL (17)
<i>ame-bantam</i> , <i>ame-let-7</i> , <i>ame-mir-10</i> , <i>ame-mir-100</i> , <i>ame-mir-12</i> , <i>ame-mir-125</i> , <i>ame-mir-133</i> , <i>ame-mir-14</i> , <i>ame-mir-184</i> , <i>ame-mir-2</i> , <i>ame-mir-252b</i> , <i>ame-mir-275</i> , <i>ame-mir-276</i> , <i>ame-mir-281</i> , <i>ame-mir-375</i> , <i>ame-mir-3785</i> , <i>ame-mir-6001-3p</i>
Equally expressed (24)
<i>ame-mir-11</i> , <i>ame-mir-1175</i> , <i>ame-mir-190</i> , <i>ame-mir-277</i> , <i>ame-mir-278</i> , <i>ame-mir-279a</i> , <i>ame-mir-279c</i> , <i>ame-mir-31a</i> , <i>ame-mir-3477</i> , <i>ame-mir-3719</i> , <i>ame-mir-3720</i> , <i>ame-mir-3747b</i> , <i>ame-mir-3786</i> , <i>ame-mir-3791</i> , <i>ame-mir-6000b</i> , <i>ame-mir-6001-5p</i> , <i>ame-mir-6040</i> , <i>ame-mir-6041</i> , <i>ame-mir-6047a</i> , <i>ame-mir-6051</i> , <i>ame-mir-6053</i> , <i>ame-mir-6060</i> , <i>ame-mir-6065</i> , <i>ame-mir-989</i>
Down-regulated in QL (20)
<i>ame-mir-13b</i> , <i>ame-mir-252a</i> , <i>ame-mir-2765-5p</i> , <i>ame-mir-2788</i> , <i>ame-mir-2796</i> , <i>ame-mir-3049-3p</i> , <i>ame-mir-3049-5p</i> , <i>ame-mir-305</i> , <i>ame-mir-306</i> , <i>ame-mir-316</i> , <i>ame-mir-317</i> , <i>ame-mir-3715</i> , <i>ame-mir-34</i> , <i>ame-mir-3718a</i> , <i>ame-mir-750</i> , <i>ame-mir-8</i> , <i>ame-mir-92b</i> , <i>ame-mir-993</i> , <i>ame-mir-996</i> , <i>ame-mir-9a</i>

Table VI. The read number, fold change and *P*value of differentially expressed miRNAs in 4-day-old queen larvae and worker larvae.

mir names	QL1	WL1	Fold change 1	<i>P</i> value 1	QL2	WL2	Fold change 2	<i>P</i> value 2	QL3	WL3	Fold change 3	<i>P</i> value 3
bantam	1,987	254	-2.97	2.15E-101	1,935	428	-2.18	4.07E-107	2613	738	-1.82	9.51E-09
ane-let-7	405	42	-3.27	7.45E-28	394	96	-2.04	4.16E-20	388	98	-1.99	0.0008827
ane-miR-10	1,938	145	-3.74	4.15E-169	2,109	190	-3.47	8.04E-262	479	256	-1.90	7.33E-10
ane-miR-100	13,787	2,061	-2.74	0	13,787	2,206	-2.64	0	14938	2,639	-2.50	0
ane-miR-11	236	234	1.18	0.0045642	224	224	0	0.5781247	165	164	-0.14	0.0188690
ane-miR-1175	2	6	1.58	0.0035641	7	7	0	0.3471446	11	10	-0.14	0.0179690
ane-miR-12	715	282	-3.34	0.0005626	611	117	-2.38	4.10E-42	631	172	-2.88	0.0001510
ane-miR-125	28,547	3,082	-3.21	0	26,631	3,242	-3.04	0	22,668	2,259	-3.33	0
ane-miR-133	616	136	-2.18	2.82E-11	766	113	-2.76	2.08E-68	688	135	-2.35	6.66E-14
ane-miR-13b	22	111	2.33	5.12E-43	30	156	2.38	4.04E-37	19	122	2.68	4.76E-53
ane-miR-14	7,741	1,296	-2.58	2.90E-252	7,197	1,996	-1.85	2.28E-268	7974	1,288	-2.63	1.03E-236
ane-miR-184	131,703	59,535	-1.15	0	199,431	55,577	-1.84	0	146,271	51,911	-1.49	4.26E-13
ane-miR-190	60	57	-1.77	0.0300068	88	78	-0.17	0.0144299	122	124	-1.10	0.0658514
ane-miR-2	279	82	-1.07	7.44E-08	313	99	-1.66	4.08E-10	235	99	-1.25	3.28E-16
ane-miR-252a	875	143	-2.61	3.90E-31	893	262	-1.77	7.02E-31	876	147	-2.58	3.18E-25
ane-miR-252b	139	666	2.26	1.79E-245	168	615	1.87	6.76E-118	148	619	2.06	3.45E-236
ane-miR-275	61,671	9,290	-2.73	0	60,323	8,451	-2.84	0	58,100	8,464	-2.78	0
ane-miR-276	15,036	7,218	-1.06	2.83E-89	15,634	6,628	-1.24	4.60E-157	14,346	6,894	-1.06	1.01E-141
ane-miR-2765-5p	199	1,295	2.70	0	172	1,209	2.81	6.81E-308	128	1,115	3.12	0
ane-miR-277	18	16	-0.17	0.0070894	31	30	-0.05	0.0668970	27	26	-0.05	0.0617724
ane-miR-278	30	26	-0.11	0.0017967	61	53	-0.20	0.0550404	31	30	-0.05	0.0583324
ane-miR-2788	14	83	2.57	7.73E-34	11	83	2.92	3.12E-23	11	84	2.93	4.27E-38
ane-miR-2796	23	144	2.64	4.39E-58	36	197	2.45	2.16E-47	25	146	2.55	5.31E-62
ane-miR-279a	99	91	-0.12	0.0037388	165	165	-0.38	0.2411758	93	94	0.02	0.0048723
ane-miR-279c	18	17	-0.08	0.0034567	39	32	-0.29	0.2048180	11	10	0.74	0.0019347
ane-miR-281	467	69	-2.76	1.74E-20	539	91	-2.57	7.58E-43	514	83	-2.63	1.16E-16
ane-miR-3049-3p	54	222	2.04	2.36E-79	34	289	3.09	3.76E-79	40	211	2.40	7.43E-87
ane-miR-3049-5p	37	231	2.64	5.18E-92	34	213	2.65	9.97E-54	33	241	2.87	3.12E-105
ane-miR-305	63	418	2.73	5.83E-167	72	496	2.78	1.25E-126	79	433	2.45	9.05E-178
ane-miR-306	94	846	3.17	0	156	856	2.46	5.56E-200	114	893	2.97	0
ane-miR-316	158	1,184	2.91	0	122	1,229	3.33	0	163	1,212	2.89	0
ane-miR-317	51	540	3.40	7.87E-230	100	832	3.06	5.00E-223	109	942	3.11	0

ame-miR-31a	19	12	-0.66	0.1226139	54	56	0.05	0.0046713	30	29	-0.05	0.0017389
ame-miR-34	12	226	4.24	3.74E-100	27	220	3.03	4.90E-60	19	227	3.58	1.56E-104
ame-miR-3477	5	4	-0.32	0.2243271	27	26	-0.05	0.0910955	30	29	-0.05	0.0017389
ame-miR-3715	24	186	2.95	2.22E-77	24	180	2.91	2.45E-48	24	197	3.04	8.59E-88
ame-miR-3718a	251	958	1.93	0	290	999	1.78	3.09E-183	219	972	2.15	0
ame-miR-3719	3	2	-0.58	0.4931715	3	3	0	0.5382483	3	2	-0.58	0.4375301
ame-miR-3720	20	19	-0.07	0.0018962	29	28	-0.05	0.0779973	14	15	0.19	0.0014717
ame-miR-3747b	16	15	-0.09	0.0063372	14	16	0.19	0.0808835	14	11	-0.35	0.0288668
ame-miR-375	1,210	121	-3.32	7.53E-83	1,532	163	-3.23	6.19E-173	1,526	102	-3.90	6.26E-135
ame-miR-3785	1,592	272	-2.55	9.51E-51	1,453	222	-2.71	4.18E-124	1,555	299	-2.38	3.25E-31
ame-miR-3786	49	48	-0.14	0.0336812	65	65	0	0.0041690	53	53	0	0.0077583
ame-miR-3791	21	19	-0.14	0.0028768	32	30	-0.09	0.0854158	27	27	0	0.0077583
ame-miR-6000b	19	16	-0.25	0.0104690	34	30	-0.18	0.1335135	85	84	-0.13	0.0124935
ame-miR-6001-3p	132	39	-1.18	3.606E-05	174	44	-1.98	3.43E-09	154	49	-1.65	0.5545430
ame-miR-6001-5p	92	91	-0.02	0.0731856	73	70	-0.06	0.0059616	223	229	0.04	0.7785935
ame-miR-6040	12	12	0	0.0101645	11	13	0.24	0.1002728	11	11	0	0.0077583
ame-miR-6041	11	11	0	0.0138645	11	12	0.13	0.1567193	13	11	-0.24	0.0193344
ame-miR-6047a	11	12	0.13	0.0062180	12	11	-0.13	0.3208465	12	11	-0.13	0.0124935
ame-miR-6051	10	12	0.26	0.0036350	14	14	0	0.1836582	14	12	-0.22	0.0134999
ame-miR-6053	11	13	0.24	0.0027091	17	14	-0.28	0.3963150	12	10	-0.26	0.0277734
ame-miR-6060	17	15	-0.18	0.0096031	14	12	-0.22	0.3783815	11	11	0	0.0077583
ame-miR-6065	32	33	0.14	0.0137759	83	84	0.02	0.0008812	11	11	0	0.0077583
ame-miR-750	3,979	12,362	1.64	0	3,723	14,728	1.98	0	3597	12,773	1.83	0
ame-miR-8	32,950	9,716	-1.76	3.61E-130	44,686	14,970	-1.58	0	42904	19,591	-1.13	0
ame-miR-92b	51,369	17,171	-1.58	1.39E-49	58,241	148,304	1.35	0	57812	12,118	-2.25	0
ame-miR-989	13	13	0	0.0074699	29	27	-0.10	0.1077567	75	74	-0.13	0.0124935
ame-miR-993	159	1,156	2.86	0	124	1,437	3.53	0	226	1,122	2.31	0
ame-miR-996	46	320	2.79	2.13E-129	58	392	2.76	7.28E-100	32	330	3.37	2.05E-149
ame-miR-9a	968	1,208	1.32	7.19E-197	1,234	2,350	1.93	7.17E-251	690	1,891	1.45	0

Table VII. The signaling pathway of different expressed miRNAs in 4-day-old queen and worker larvae.

miRNA	Signaling pathway
ame-bantam	Axon guidance; Basal transcription factors; Calcium; Endocytosis; GnRH; Insulin; Metabolic; Olfactory transduction; Wnt
ame-let-7	Focal adhesion; Lysosome; Metabolic; Neuroactive ligand-receptor interaction; Phagosome; Regulation of actin cytoskeleton; Wnt pathway
ame-mir-10	Cell adhesion molecules (CAMs); Lysosome; Metabolic pathways
ame-mir-12	Dorso-ventral axis formation; Endocytosis; GnRH; Insulin; MAPK; Metabolic; Olfactory transduction;p53; Wnt pathway
ame-mir-13b	Endocytosis; GnRH; Insulin; mTOR; MAPK; Metabolic; Ubiquitin mediated proteolysis; Wnt pathway
ame-mir-14	Adherens junction; Antigen; CAMs; Endocytosis; GnRH; Insulin; mTOR; MAPK; Metabolic; Ubiquitin mediated proteolysis; Wnt pathway
ame-mir-100	CAMs; ECM-receptor interaction; Lysosome; Metabolic pathways; Regulation of actin cytoskeleton; Tight junction
ame-mir-125	Adipocytokine; Calcium; CAMs; Gap junction; GnRH; MAPK; Insulin; mTOR; Vitamin digestion and absorption
ame-mir-133	Endocytosis; MAPK; Metabolic pathways; Metabolism of cytochrome P450
ame-mir-184	Gap junction; Insulin; Lysosome; Metabolic; Neuroactive ligand-receptor; Phagosome; Vitamin digestion and absorption
ame-mir-2	Insulin; Metabolic; Phagosome; Vitamin digestion and absorption
ame-mir-252a	Metabolic pathways; Metabolism of cytochrome P450; Regulation of actin cytoskeleton
ame-mir-252b	Metabolic pathways; Metabolism of cytochrome P450
ame-mir-275	Cell cycle; Insulin; MAPK; Metabolic pathways; Metabolism of cytochrome P450; Olfactory transduction; Regulation of actin cytoskeleton
ame-mir-276	Metabolic pathways; Olfactory transduction; Phototransduction – fly; Regulation of actin cytoskeleton; Vitamin digestion and absorption
ame-mir-2765-5p	Lysosome; Metabolic pathways; TCA cycle
ame-mir-2788	Focal adhesion; Regulation of actin cytoskeleton; Wnt signaling pathway
ame-mir-2796	p53 signaling pathway; Phagosome
ame-mir-281	B and T cell receptor pathway; Lysosome; MAPK; Metabolic; TCA cycle; Wnt pathway
ame-mir-34	Endocytosis; GnRH; Insulin; Lysosome; MAPK; Metabolic pathways; Protein digestion and absorption
ame-mir-305	Antigen; CAMs; Endocytosis; GnRH; MAPK; Metabolic; Olfactory; Regulation of actin cytoskeleton; Taste transduction; TCA; Wnt
ame-mir-306	Metabolic pathways; Oxidative phosphorylation
ame-mir-316	Actin cytoskeleton; Endocytosis; Focal adhesion; Lysosome; MAPK; Metabolic; Neuroactive ligand-receptor interaction; Wnt
ame-mir-317	Insulin signaling pathway; Metabolic pathways
ame-mir-375	Calcium; CAMs; Focal adhesion; MAPK; Neuroactive ligand-receptor interaction
ame-mir-3049-3p	Actin cytoskeleton; Focal adhesion; Lysosome; Metabolic pathways; Neuroactive ligand-receptor interaction; Wnt signaling pathway
ame-mir-3049-5p	Focal adhesion; Metabolism of cytochrome P450; Purine metabolism; Vitamin digestion and absorption; Wnt signaling pathway
ame-mir-3715	Focal adhesion; Regulation of actin cytoskeleton
ame-mir-3718a	Lysosome; Metabolic pathways
ame-mir-3785	Lysosome; MAPK signaling pathway; Metabolic pathways

Table VII. (continued)

miRNA	Signaling pathway
ame-mir-6001-3p	ECM-receptor interaction; Metabolic pathways; Regulation of actin cytoskeleton
ame-mir-750	Antigen processing; CAMs; Dorso-ventral axis; GnRH; MAPK; Metabolic pathways; Olfactory transduction; Regulation of actin cytoskeleton
ame-mir-8	Metabolic pathways; Ubiquitin mediated proteolysis; Wnt signaling pathway
ame-mir-9a	B and T cell receptor; Calcium; Endocytosis; Insulin; mTOR; MAPK; Metabolic; Protein processing in endoplasmic reticulum
ame-mir-92b	B and T cell receptor; Insulin; mTOR; MAPK; Metabolic; Protein processing in endoplasmic reticulum
ame-mir-993	Insulin; Metabolic;p53 signaling
ame-mir-996	Basal transcription factors; Insulin; Metabolic;p53 signaling; Phagosome; Salivary secretion; Vitamin digestion and absorption

In this study, we used three biological replicates for small RNA sequencing analysis. As shown in Table VI, the three replicates were analyzed independently. The index number of the linear relationships (R^2) indicates variation in miRNAs expression. Higher R^2 values indicate better repeatability (Lu et al. 2010). When R^2 are high, it is not necessary to validate differences in miRNA expression by using qRT-PCR (Lu et al. 2010).

We observed 17 up-regulated and 20 down-regulated miRNAs in QL compared with WL. Among the differentially expressed miRNAs, *ame-let-7*, *ame-mir-100*, *ame-mir-34* and *ame-mir-375* were widely found in humans (Ragan et al. 2009), mouse (Ragan et al. 2009), *Caenorhabditis elegans* (Hunter et al. 2013), *Drosophila melanogaster* (Luhur et al. 2013), hemimetabolon insect (Rubio and Belles 2013) and RJ (Shi et al. 2012), which were also presented in QL and WL. Consistent with a previous study (Chen 2012), we observed three up-regulated (*ame-let-7*, *ame-mir-184*, *ame-mir-375*) and four down-regulated miRNAs (*ame-mir-2788*, *ame-mir-2796*, *ame-mir-305*, *ame-mir-316*) in QL. By identifying the potential target genes of these miRNAs, we found that *ame-let-7* was most likely related to the expression of *Dnmt1* (GB48403), *HDAC1* (GB53438), *p38b* (GB43914), *Sirt6* (GB51490), which might regulate DNA methyltransferase activity, Na-dependent histone deacetylase activity,

reactive oxygen species and the insulin signaling pathway, respectively (Shi et al. 2013). The expression of *HDAC6* (GB42847), which is related to Na-dependent histone deacetylase activity, was regulated by *ame-mir-184* and *ame-mir-375* (Shi et al. 2013).

MiRNAs might inhibit mRNA transcription in specific combinations (Valadi et al. 2007; Laubinger et al. 2010). Larval development involves several signaling pathways such as the insulin (Wolschin et al. 2011), MAPK (Kamakura 2011), mTOR (Patel et al. 2007) and Wnt signaling pathways (Shi et al. 2013). With using KEGG annotations, we found that 37 miRNAs that were differentially expressed in QL and WL were related to insulin pathway (*ame-bantam*, *ame-mir-12*, *ame-mir-13b*, *ame-mir-14*, *ame-mir-125*, *ame-mir-184*, *ame-mir-2*, *ame-mir-275*, *ame-mir-34*, *ame-mir-317*, *ame-mir-9a*, *ame-mir-92b*, *ame-mir-993*, *ame-mir-996*), MAPK pathway (*ame-mir-12*, *ame-mir-13b*, *ame-mir-14*, *ame-mir-125*, *ame-mir-133*, *ame-mir-275*, *ame-mir-281*, *ame-mir-34*, *ame-mir-305*, *ame-mir-316*, *ame-mir-375*, *ame-mir-3785*, *ame-mir-750*, *ame-mir-9a*, *ame-mir-92b*), mTOR pathway (*ame-mir-13b*, *ame-mir-14*, *ame-mir-125*, *ame-mir-9a*, *ame-mir-92b*) and Wnt pathway (*ame-bantam*, *ame-let-7*, *ame-mir-13b*, *ame-mir-14*, *ame-mir-2788*, *ame-mir-281*, *ame-mir-3049-3p*, *ame-mir-3049-5p*, *ame-mir-305*, *ame-mir-316*, *ame-mir-8*), all these pathways were involved in caste differentiation.

Caste differentiation is a fascinating natural phenomenon, in which completely distinct biological characteristics are derived from identical genomes. A significant factor in this process is the regulation of gene expression by nutrient intake (Shi et al. 2011; Leimar et al. 2012). The effects of the methylation on caste differentiation have been well studied (Foret et al. 2012; Shi et al. 2013). This study suggests that differentially expressed miRNAs in the larvae might also function in caste differentiation.

ACKNOWLEDGMENTS

We thank Dr. Qiang Huang for reviewing this manuscript. This work was supported by the Earmarked Fund for China Agriculture Research System (No. CARS-45-KXJ12), the National Natural Science Foundation of China (No. 31260524).

MicroARN exprimés différemment entre larves de reine et larves d'ouvrière de l'abeille (*Apis mellifera*)

Apidae / expression génique / différenciation des castes / développement

Differentielle Expression von microRNAs bei Königinnen- und Arbeiterinnenlarven der Honigbiene (*Apis mellifera*)

Honigbiene / Larven / microRNA / Kastendifferenzierung

REFERENCES

- Barchuk, A.R., Cristino, A.S., Kucharski, R., Costa, L.F., Simões, Z.L., Maleszka, R. (2007) Molecular determinants of caste differentiation in the highly eusocial honey bee *Apis mellifera*. *BMC Dev. Biol.* **7**, 70
- Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**(2), 281–297
- Behura, S.K. (2007) Insect microRNAs: structure, function and evolution. *Insect Biochem. Mol. Biol.* **37**(1), 3–9
- Behura, S.K., Whitfield, C.W. (2010) Correlated expression patterns of microRNA genes with age-dependent behavioral changes in honeybee. *Insect Molecular Biology* **19**(4), 431–439
- Cameron, R.C., Duncan, E.J., Dearden, P.K. (2013) Biased gene expression in early honeybee larval development. *BMC Genomics* **14**, 903
- Chen, X., Yu, X., Cai, Y., Zheng, H., Yu, D., Liu, G., Zhou, Q., Hu, S., Hu, F. (2010a) Next-generation small RNA sequencing for microRNAs profiling in the honey bee *Apis mellifera*. *Insect Mol. Biol.* **19**(6), 799–805
- Chen, X., Gao, C., Li, H., Huang, L., Sun, Q., et al. (2010b) Identification and characterisation of microRNAs in raw milk during different periods of lactation, commercial fluid, and powdered milk products. *Cell Res.* **20**(10), 1–10
- Chen, X., Hu, Y., Zheng, H., Cao, L., Niu, D., Yu, D., Sun, Y., Hu, S., Hu, F. (2012) Transcriptome comparison between honey bee queen- and worker-destined larvae. *Insect Biochem. Mol. Biol.* **42**(9), 665–673
- Chen, X. (2012) Genome-wide identification of microRNAs and their regulation of transcriptome on female caste determination of honey bee (*Apis mellifera*). Zhejiang University, National Science Library. 210p
- Drapeau, M.D., Albert, S., Kucharski, R., Prusko, C., Maleszka, R. (2006) Evolution of the Yellow/Major Royal Jelly Protein family and the emergence of social behavior in honeybees. *Genome Res.* **16**(11), 1385–1394
- Evans, J.D., Wheeler, D.E. (2001) Expression profiles during honeybee caste determination. *Genome Biol.* **2** (1), Research0001.1-0001.6
- Foret, S., Kucharski, R., Pellegrini, M., Feng, S., Jacobsen, S.E., Robinson, G.E., Maleszka, R. (2012) DNA methylation dynamics, metabolic fluxes, gene splicing, and alternative phenotypes in honeybees. *Proc. Nat. Acad. Sci. USA* **109**(13), 4968–4973
- Greenberg, J.K., Xia, J., Zhou, X., Thatcher, S.R., Gu, X., et al. (2012) Behavioral plasticity in honey bees is associated with differences in brain microRNA transcriptome. *Genes Brain Behav.* **11**(6), 660–670
- Hepperle, C., Hartfelder, K. (2001) Differentially expressed regulatory genes in honeybee caste development. *Naturwissenschaften* **88**(3), 113–116
- Hori, S., Kaneko, K., Saito, T.H., Takeuchi, H. (2011) Expression of two microRNAs, ame-mir-276 and -1000, in the adult honeybee (*Apis mellifera*) brain. *Apidologie* **42**, 89–102
- Hunter, S.E., Finnegan, E.F., Zisoulis, D.G., Lovci, M.T., Melnik-Martinez, K.V., Yeo, G.W., Pasquinelli, A.E. (2013) Functional genomic analysis of the let-7 regulatory network in *Caenorhabditis elegans*. *PLoS Genetics* **9**(3), e1003353

- Kamakura, M. (2011) Royalactin induces queen differentiation in honeybees. *Nature* **473**(7348), 478–483
- Kucharski, J.R., Maleszka, J., Fore, S., Maleszka, R. (2008) Nutritional control of reproductive status in honeybees via DNA methylation. *Science* **319**(5871), 1827–1829
- Laubinger, S., Zeller, G., Henz, S.R., Buechel, S., Sachsenberg, T., Wang, J.W., Rätsch, G., Weigel, D. (2010) Global effects of the small RNA biogenesis machinery on the *Arabidopsis thaliana* transcriptome. *Proc. Natl. Acad. Sci. USA* **107**(41), 17466–17473
- Leimar, O., Hartfelder, K., Laubichler, M.D., Page Jr., R.E. (2012) Development and evolution of caste dimorphism in honeybees—a modeling approach. *Ecol. Evol.* **2**(12), 3098–3109
- Liu, F., Peng, W., Li, Z., Li, W., Li, L., Pan, J., Zhang, S., Miao, Y., Chen, S., Su, S. (2012) Next-generation small RNA sequencing for microRNAs profiling in *Apis mellifera*: comparison between nurses and foragers. *Insect Mol. Biol.* **21**(3), 297–303
- Lu, T., Lu, G., Fan, D., Zhu, C., Li, W., et al. (2010) Function annotation of the rice transcriptome at single-nucleotide resolution by RNA-seq. *Genome Res.* **20**(9), 1238–1249
- Luhur, A., Chawla, G., Sokol, N.S. (2013) MicroRNAs as components of systemic signaling pathways in *Drosophila melanogaster*. *Curr. Top. Dev. Biol.* **105**, 97–123
- Moran, Y., Fredman, D., Praher, D., Li, X.Z., Wee, L.M., Rentzsch, F., Zamore, P.D., Technau, U., Seitz, H. (2014) Cnidarian microRNAs frequently regulate targets by cleavage. *Genome Res.* **24**(4), 651–663
- Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., Kanehisa, M. (1999) KEGG: Kyoto encyclopedia of genes and genomes. *Nucl. Acids Res.* **27**(1), 29–34
- Patel, A., Fondrk, M.K., Kaftanoglu, O., Emore, C., Hunt, G., Frederick, K., Amdam, G.V. (2007) The making of a queen: TOR pathway is a key player in diphenic caste development. *PLoS One* **2**(6), e509
- Qin, Q.H., Wang, Z.L., Tian, L.Q., Gan, H.Y., Zhang, S.W., Zeng, Z.J. (2014) The integrative analysis of microRNA and mRNA expression in *Apis mellifera* following maze-based visual pattern learning. *Insect Sci.* doi:10.1111/1744-7917
- Ragan, C., Cloonan, N., Grimmond, S.M., Zuker, M., Ragan, M.A. (2009) Transcriptome- wide prediction of miRNA targets in human and mouse using Fasth. *PLoS One* **4**(5), e5745
- Rehmsmeier, M., Steffen, P., Höchsmann, M., Giegerich, R. (2004) Fast and effective prediction of microRNA/target duplexes. *RNA* **10**(10), 1507–1517
- Rubio, M., Belles, X. (2013) Subtle roles of microRNAs let-7, miR-100 and miR-125 on wing morphogenesis in hemimetabolous metamorphosis. *J. Insect Physiol.* **59**(11), 1089–1094
- Seeley, T.D. (1989) The honey bee colony as a superorganism. *Am. Sci.* **77**, 546–553
- Shi, Y.Y., Huang, Z.Y., Zeng, Z.J., Wang, Z.L., Wu, X.B., Yan, W.Y. (2011) Diet and cell size both affect queen-worker differentiation through DNA methylation in honeybees (*Apis mellifera*, Apidae). *PLoS One* **6**(4), e18808
- Shi, Y.Y., Wu, X.B., Huang, Z.Y., Wang, Z.L., Yan, W.Y., Zeng, Z.J. (2012) Epigenetic modification of gene expression in honey bees by heterospecific gland secretions. *PLoS One* **7**(8), e43727
- Shi, Y.Y., Yan, W.Y., Huang, Z.Y., Wang, Z.L., Wu, X.B., Zeng, Z.J. (2013) Genome-wide analysis indicates that queen larvae have lower methylation levels in the honey bee (*Apis mellifera*). *Naturwissenschaften* **100**(2), 193–197
- Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J., Lötvall, J.O. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell. Biol.* **9**(6), 654–659
- Wang, L., Feng, Z., Wang, X., Wang, X., Zhang, X. (2010) DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics* **26**(1), 136–138
- Weaver, N. (1957) Effects of larval honey bee age on dimorphic differentiation of the female honeybee. *Ann. Entomol. Soc. Am.* **50**, 283–294
- Weaver, N. (1966) Physiology of caste determination. *Annu. Rev. Entomol.* **11**, 79–102
- Winston, M.L. (1987) Biology of the honey bee, p. 296. Harvard University Press, Cambridge
- Wolschin, F., Mutti, N.S., Amdam, G.V. (2011) Insulin receptor substrate influences female caste development in honeybees. *Biol. Lett.* **7**(1), 112–115
- Wu, J., Li, J.K. (2010) Proteomic analysis of the honeybee (*Apis mellifera* L.) caste differentiation between worker and queen larvae. *Sci. Agric. Sin* **43**(5), 176–181