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**Original article**

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

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**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

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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 (28.46ºN, 115.49ºE), according to standard beekeeping 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 HiSeqTM 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:

QL normalized miRNA expression = QL miRNA clean reads/QL total clean reads*1000000 (Shi et al. 2012; Qin et al. 2013),

WL normalized miRNA expression = WL miRNA clean reads/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),

$$p(x|y) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}} \qquad C(y \le y_{\min}|x) = \sum_{y=0}^{y \le y\min} p(y|x)$$

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

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 I.** 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)

*ame-bantam, ame-let-7, ame-mir-10, ame-mir-100, ame-mir-12, ame-mir-125, ame-mir-133, ame-mir-14, ame-mir-184, ame-mir-2, ame-mir-252b, ame-mir-275, ame-mir-276, ame-mir-281, ame-mir-375, ame-mir-3785, ame-mir-6001-3p*

Equally expressed (24)

*ame-mir-11, ame-mir-1175, ame-mir-190, ame-mir-277, ame-mir-278, ame-mir-279a, ame-mir-279c, ame-mir-31a, ame-mir-3477, ame-mir-3719, ame-mir-3720, ame-mir-3747b, ame-mir-3786, ame-mir-3791, ame-mir-6000b, ame-mir-6001-5p, ame-mir-6040, ame-mir-6041, ame-mir-6047a, ame-mir-6051, ame-mir-6053, ame-mir-6060, ame-mir-6065, ame-mir-989*

Down-regulated in QL (20)

*ame-mir-13b, ame-mir-252a, ame-mir-2765-5p, ame-mir-2788, ame-mir-2796, ame-mir-3049-3p, ame-mir-3049-5p, ame-mir-305, ame-mir-306, ame-mir-316, ame-mir-317, ame-mir-3715, ame-mir-34, ame-mir-3718a, ame-mir-750, ame-mir-8, ame-mir-92b, ame-mir-993, ame-mir-996, ame-mir-9a*

**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 | *P*value 1 | QL2 | WL2 | Fold change 2 | *P*value 2 | QL3 | WL3 | Fold change 3 | *P*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 |
| ame-let-7 | 405 | 42 | −3.27 | 7.45E-28 | 394 | 96 | −2.04 | 4.16E-20 | 388 | 98 | −1.99 | 0.0008827 |
| ame-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 |
| ame-miR-100 | 13,787 | 2,061 | −2.74 | 0 | 13,787 | 2,206 | −2.64 | 0 | 14938 | 2,639 | −2.50 | 0 |
| ame-miR-11 | 236 | 234 | 1.18 | 0.0045642 | 224 | 224 | 0 | 0.5781247 | 165 | 164 | −0.14 | 0.0188690 |
| ame-miR-1175 | 2 | 6 | 1.58 | 0.0035641 | 7 | 7 | 0 | 0.3471446 | 11 | 10 | −0.14 | 0.0179690 |
| ame-miR-12 | 715 | 282 | −3.34 | 0.0005626 | 611 | 117 | −2.38 | 4.10E-42 | 631 | 172 | −2.88 | 0.0001510 |
| ame-miR-125 | 28,547 | 3,082 | −3.21 | 0 | 26,631 | 3,242 | −3.04 | 0 | 22,668 | 2,259 | −3.33 | 0 |
| ame-miR-133 | 616 | 136 | −2.18 | 2.82E-11 | 766 | 113 | −2.76 | 2.08E-68 | 688 | 135 | −2.35 | 6.66E-14 |
| ame-miR-13b | 22 | 111 | 2.33 | 5.12E-43 | 30 | 156 | 2.38 | 4.04E-37 | 19 | 122 | 2.68 | 4.76E-53 |
| ame-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 |
| ame-miR-184 | 131,703 | 59,535 | −1.15 | 0 | 199,431 | 55,577 | −1.84 | 0 | 146271 | 51,911 | −1.49 | 4.26E-13 |
| ame-miR-190 | 60 | 57 | −1.77 | 0.0300068 | 88 | 78 | −0.17 | 0.0144299 | 122 | 124 | −1.10 | 0.0658514 |
| ame-miR-2 | 279 | 82 | −1.07 | 7.44E-08 | 313 | 99 | −1.66 | 4.08E-10 | 235 | 99 | −1.25 | 3.28E-16 |
| ame-miR-252a | 875 | 143 | −2.61 | 3.90E-31 | 893 | 262 | −1.77 | 7.02E-31 | 876 | 147 | −2.58 | 3.18E-25 |
| ame-miR-252b | 139 | 666 | 2.26 | 1.79E-245 | 168 | 615 | 1.87 | 6.76E-118 | 148 | 619 | 2.06 | 3.45E-236 |
| ame-miR-275 | 61,671 | 9,290 | −2.73 | 0 | 60,323 | 8,451 | −2.84 | 0 | 58,100 | 8,464 | −2.78 | 0 |
| ame-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 |
| ame-miR-2765-5p | 199 | 1,295 | 2.70 | 0 | 172 | 1,209 | 2.81 | 6.81E-308 | 128 | 1,115 | 3.12 | 0 |
| ame-miR-277 | 18 | 16 | −0.17 | 0.0070894 | 31 | 30 | −0.05 | 0.0668970 | 27 | 26 | −0.05 | 0.0617724 |
| ame-miR-278 | 30 | 26 | −0.11 | 0.0017967 | 61 | 53 | −0.20 | 0.0550404 | 31 | 30 | −0.05 | 0.0583324 |
| ame-miR-2788 | 14 | 83 | 2.57 | 7.73E-34 | 11 | 83 | 2.92 | 3.12E-23 | 11 | 84 | 2.93 | 4.27E-38 |
| ame-miR-2796 | 23 | 144 | 2.64 | 4.39E-58 | 36 | 197 | 2.45 | 2.16E-47 | 25 | 146 | 2.55 | 5.31E-62 |
| ame-miR-279a | 99 | 91 | −0.12 | 0.0037388 | 165 | 165 | −0.38 | 0.2411758 | 93 | 94 | 0.02 | 0.0048723 |
| ame-miR-279c | 18 | 17 | −0.08 | 0.0034567 | 39 | 32 | −0.29 | 0.2048180 | 11 | 10 | 0.74 | 0.0019347 |
| ame-miR-281 | 467 | 69 | −2.76 | 1.74E-20 | 539 | 91 | −2.57 | 7.58E-43 | 514 | 83 | −2.63 | 1.16E-16 |
| ame-miR-3049-3p | 54 | 222 | 2.04 | 2.36E-79 | 34 | 289 | 3.09 | 3.76E-79 | 40 | 211 | 2.40 | 7.43E-87 |
| ame-miR-3049-5p | 37 | 231 | 2.64 | 5.18E-92 | 34 | 213 | 2.65 | 9.97E-54 | 33 | 241 | 2.87 | 3.12E-105 |
| ame-miR-305 | 63 | 418 | 2.73 | 5.83E-167 | 72 | 496 | 2.78 | 1.25E-126 | 79 | 433 | 2.45 | 9.05E-178 |
| ame-miR-306 | 94 | 846 | 3.17 | 0 | 156 | 856 | 2.46 | 5.56E-200 | 114 | 893 | 2.97 | 0 |
| ame-miR-316 | 158 | 1,184 | 2.91 | 0 | 122 | 1,229 | 3.33 | 0 | 163 | 1,212 | 2.89 | 0 |
| ame-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), hemimetabolan 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.

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**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**

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