Insect Biochemistry and Molecular Biology 90 (2017) 34–42

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

The *microRNA ame-miR-279a* regulates sucrose responsiveness of forager honey bees (*Apis mellifera*)

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ARTICLE INFO

Article history:
Received 29 March 2016
Received in revised form
20 August 2017
Accepted 14 September 2017
Available online 20 September 2017

Keywords:
microRNA
Honey bee
Proboscis extension reflex
Sucrose responsiveness
Division of labour

ABSTRACT

Increasing evidence demonstrates that microRNAs (miRNA) play an important role in the regulation of animal behaviours. Honey bees (*Apis mellifera*) are eusocial insects, with honey bee workers displaying age-dependent behavioural maturation. Many different miRNAs have been implicated in the change of behaviours in honey bees and *ame-miR-279a* was previously shown to be more highly expressed in nurse bee heads than in those of foragers. However, it was not clear whether this difference in expression was associated with age or task performance. Here we show that *ame-miR-279a* shows significantly higher expression in the brains of nurse bees relative to forager bees regardless of their ages, and that *ame-miR-279a* is primarily localized in the Kenyon cells of the mushroom body in both foragers and nurses. Overexpression of *ame-miR-279a* attenuates the sucrose responsiveness of foragers, while its absence enhances their sucrose responsiveness. Lastly, we determined that *ame-miR-279a* directly target the mRNA of *Mblk-1*. These findings suggest that *ame-miR-279a* plays important roles in regulating honey bee division of labour.

1. Introduction

The honey bee (*Apis mellifera*. L) is a eusocial insect and a good model organism to study the mechanisms and evolution of social behaviours (Robinson et al., 2005). The workers in the colony exhibit age-related division of labour: young honey bees usually engage in within-nest tasks such as brood care ("nursing"), while the old honey bees forage outside for different resources (pollen, nectar, water and propolis) (Winston, 1987; Robinson, 1992). However, the division of labour is very flexible: bees can accelerate or reverse their behavioural development according to the colony needs (Robinson, 1992; Huang and Robinson, 1996).

Numerous studies have focused on the molecular mechanisms underpinning division of labour. Behavioural changes are associated with gene expression changes in the honey bee brain (Whitfield et al., 2003). A number of genes, such as *period* (Toma et al., 2000), *acetylcholinesterase* (Shapira et al., 2001), *foraging* (Ben-Shahar et al., 2002, Ben-Shahar, 2005) and *malvolio* (Ben-Shahar et al., 2004) are reported to be involved in the behavioural transition from nurse to forager. MicroRNAs (miRNAs) are endogenous small non-coding RNAs (18–24nt) which down-regulate gene expression by mRNA cleavage or translation repression (Bartel, 2004). One single miRNA may target many mRNAs, and a single mRNA may contain binding sites for many different miRNAs. This leads to a complex regulatory system for biological processes, such as cell proliferation, differentiation and apoptosis, embryonic development, neurogenesis, immunity response and disease resistance (Ambros, 2004; Pillai, 2005; Vasudevan et al., 2007; Legeai et al., 2010).

Several miRNAs were reported to be involved in the honey bee behavioural maturation process. Behura and Whitfield (2010) found that *miR-276* was upregulated in young nurses, and had obviously higher expression in young and old nurses than in young

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http://dx.doi.org/10.1016/j.ibmb.2017.09.008
0965-1748/

and old foragers, suggesting its involvement in the behavioural maturation from nurses to foragers. Hori et al. (2011) found that *ame-miR-276* and *ame-miR-1000* are enriched in the optic lobes and in small type Kenyon cells of honey bees and that their targets may encode neural function related genes. Greenberg et al. (2012) found that *miR-2796* is highly expressed in bee brain, and binds to the coding region of phospholipase C (PLC)-epsilon gene, which was implicated in neuronal development and differentiation in mammals (Wing et al., 2003), and reported to be transcriptionally regulated in association with division of labour in honey bees (Tsuchimoto et al., 2004). Nunes et al. (2013) identified more than 70 miRNAs that were regulated by the gene *vitellogenin*, and one of these was *ame-miR-279*, which may be associated with foraging behavior. Still, the precise mechanism of how miRNAs regulate the division of labour in honey bees is poorly understood.

Nine miRNAs were previously found to be significantly differentially expressed between nurses and foragers. One of these was *ame-miR-279a*, which was up-regulated in nurses, and *Mblk-1* was predicted as a candidate target of *ame-miR-279a* through bioinformatics (Liu et al., 2012). In the present study, we further investigate the role of *ame-miR-279a* in honey bee behavioural development. We show that *ame-miR-279a* is mainly localized in the Kenyon cells of the honey bee mushroom body, and overexpression of *ame-miR-279a* attenuates the sucrose responsiveness of foragers, while its inhibition enhances their sucrose responsiveness. Furthermore, we found that *ame-miR-279a* directly targets the mRNA of *Mblk-1*.

2. Materials and methods

2.1. Honey bees collections

European honey bees, *Apis mellifera*, were maintained according to standard beekeeping practices at Anhui Agriculture University, Hefei, China. Nurses were caught when they had their heads inside cells feeding the larvae. Foragers with pollens on their corbiculae were captured at the entrance of the hive. One-day-old honey bees were obtained by removing honeycombs with capped pupae from a typical colony to an incubator (33 °C) until adults emerged. Each one-day-old honey bee was marked with a paint dot on the thorax, and kept in the incubator for an hour before being put back into the original colony. A total of 200–300 one-day-old honey bees were marked from each typical colony, and three independent typical colonies were used in this study. Three single-cohort colonies were also made, each with about 1000 one-day-old honey bees obtained as described, an unrelated mated queen, an empty comb for queen to lay eggs, a comb containing some honey and pollen, all placed in small hive boxes (Whitfield et al., 2003).

Twenty 12-day-old nurses (12N) and 30-day-old foragers (30F) were captured respectively from each of the three typical colonies, while another twenty of 12-day-old nurses (12N) and 12-day-old ("precocious") foragers (12PF), and 30-day-old ("overaged") nurses (30ON) and 30-day-old foragers (30F) were captured from the three single-cohort colonies. The collected honey bees were kept in an incubator (33 °C) before their heads were removed for brain dissection to extract RNA for real-time quantitative polymerase chain reaction (RT-qPCR) and northern blot analysis. The honey bees for behavioural experiments were collected from typical colonies. More details are provided later in Section 2.6.

2.2. Oversupply/inhibition of ame-miR-279a in honey bees

A mimic of *ame-miR-279a* with the sense strand (5′ugacuaga uccacacucauuaa3′) and the antisense strand 5′aaugaguguggau cuagucauu3′) including a 2 nt-3′overhang (UU) and 2 nt-5′trim was synthesized by GenPharma (Shanghai, China). An inhibitor (5′uuaaugaguguggaucuaguca3′), a single stranded RNA exactly complementary to *ame-miR-279a* sequence was also synthesized. A mimic control by using nonsense sequence (sense: 5′uucuccg aacgugucacgutt3′, antisense: 5′acgugacacguucggagaatt3′) and an inhibitor control using nonsense sequence (5′caguacuuuugug uaguacaa3′) were also synthesized.

Twenty foragers from a typical colony were used in each treatment and feeding treatments were carried out in three independent experiments. The bees were cold-anaesthetized, secured in 0.5-ml Eppendorf tubes with a strip of insulating tape (Supplementary Fig. S1), and kept in an incubator (28 °C, 70% relative humidity) for at least an hour to recover. There were four groups of foragers in the experiment, namely groups fed with the mimic of *ame-miR-279a* (M), the mimic control of nonsense sequences (NS), the inhibitor of *ame-miR-279a* (I) and the inhibitor control of nonsense sequences (INS) respectively. Each forager was fed with 10 μl 50% sucrose solution containing 6.6 μg of each synthetic reagent. All the foragers were fed to satiety with 50% sucrose solution after treatments (Fig. S2), and kept in the incubator in darkness (28 °C, 70% relative humidity). The *ame-miR-279a* expression in the brains of the foragers was measured 24 h after feeding.

2.3. RT-PCR and qRT-PCR analysis

Bee brains were dissected according to Whitfield et al. (2003), then processed for total RNA extraction using a miRNeasy Mini Kit (Qiagen, Germany). The sample quality and quantity were confirmed using a NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA), and the samples were stored at −80 °C.

Total RNA (0.5μg per sample) was reverse transcribed with a universal adaptor primer and primeScript RTase. PCR was performed at the same time with specific forward primer (Table 1) and Uni-miR qPCR primer according to the instructions of the SYBR PrimeScript miRNA RT-PCR Kit (TakaRa). The reactions were performed in a TC PCR Thermocycle Instrument (BIOER) under the following conditions: 50 °C for 60 min, 85 °C for 5 s. The qRT-PCR assays were performed in the ABI StepOnePlus™ Real-Time PCR system. Amplification was carried out in 25-μl reaction volume, containing 10 μl SYBR premix Ex *Taq*II, 2 μl first strand cDNA, 6 μl RNase free water, 0.8 μl of 10 μM of each of F and R of the specific primer (Table 1). PCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, followed by the melting curve (60 °C–95 °C). *β-actin* was used as the reference gene. For each gene, test reactions were amplified in quadruplicate along with a no-template and a no-enzyme control. Relative gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001).

2.4. Northern blot

Total RNA (15 μg per sample) from 20 honey bees brains was separated through a 15% denaturing polyacrylamide gel, then transferred to Hybond-N nylon membranes by Mini Tans-Blot

Table 1
Primer sequences used for qRT-PCR validation of *ame-miR-279a* and *Mblk-1*.

Primer	5′ to 3′
Mblk-1 -F	AACACCAAATACGACCCAAAAC
Mblk-1 -R	CAACAGAGCCTTCTCCACTTCT
ame-miR-279a-F	CTTTCTAAGTATCAATAATG
ame-miR-279a–R	TCTTAAAATTCATATTCATA
β-actin-F	TGCCAACACTGTCCTTTCTG
β-actin-R	AGAATTGACCCACCAATCCA

(Liuyi, Beijing, China) and cross-linked by exposing to ultraviolet light. DNA oligonucleotides with reverse complementarity to specific sequences were incorporated with a single digoxigenin-labeled dideoxyuridine-triphosphate (DIG-ddUTP) (Schmitz et al., 1991) by terminal transferase. The sequence of *ame-miR-279a* probe was *5′uuaaugaguguggaucuaguca3′*. The probe hybridizations and washes were performed at 65 °C according to the instructions of DIG Northern Starter Kit (Roche, Shanghai, China). Finally, the blots were exposed to Kodak film according to the method established by Ramkissoon et al. (2006).

2.5. In situ hybridization

The honey bee brains were prepared according to Olivier et al. (2008), with the modification that each brain was fixed in 4% paraformaldehyde (PFA, Sigma) at 4 °C for 30 min, and dehydrated in ascending concentrations of ethanol, embedded in paraffin, then sectioned 10 μm from the frontal side. In situ hybridization was performed according to the kit instructions of BOSTER (# MK10197). The main steps were as follows: the endogenous enzymes in the brain sections were firstly inactivated with 3% H_2O_2; then the sections were treated with pepsin diluted with 3% citric acid for 20 min at room temperature, and washed using PBS; each section was incubated with 20 μl hybrid liquid of ame-*miR-279a* probe (*5′ttaatgagtgtggatctagtca3′*) overnight in 40 °C; the reactions were blocked and sample incubated with biotinylated anti-mouse digoxin. Colour development was carried out according to the instructions of DAB kit. Finally, sections were dehydrated through a graded series of methanol, soaked with xylene, mounted with neutral gum and examined with a TissueFAXS plus microscope (TissueGnostics, Austria).

2.6. Behavioural experiments

Foragers (N = 60–70) were captured from three independent typical colonies, with over 20 foragers per colony. The bees were restrained as mentioned above. The foragers were divided into two groups, one group was fed with *ame-miR-279a* mimic (279a-M), and another one was fed with the mimic control nonsense sequences (279aM-NS). Similarly, another group of foragers (N = 60–70) was collected from the same colonies. One half of these foragers were fed with *miR-279a* inhibitor (279aI), another half were fed with the inhibitor control nonsense sequence (279aI-NS). Each forager was fed with 4.5 μl 50% sucrose solution containing 1 μg of each synthetic reagent. The foragers were fed to satiety with 50% sucrose solution after being fed the reagents, then put back into the incubator. The bees were tested for sucrose responsiveness using the proboscis extension reflex (PER) assay 24 h and 48 h after treatment. Both antenna of foragers was touched with a droplet of ascending concentrations of sucrose: 0.1, 0.3, 1, 3, 10 and 30% (w: w) to test their sucrose responsiveness according to previous studies (Pankiw et al., 2001; Page et al., 1998). Analysis of variance (ANOVA) was used to analyze the data with PER response as a dependent variable. PER response (%) was analyzed after arcsine-square root transformation. Sugar concentration was treated as a repeated measures variable.

Bee brains in the 279aM and 279aM-NS groups were dissected immediately after PER for total RNA extraction according to Section 2.3. The expression of *ame-miR-279a* and *Mblk-1* were quantified using qRT-PCR with *β-actin* as a control gene (Table 1).

2.7. Western blot

Proteins (90 μg per samples) were extracted from 15 honey bee heads using the Tissue or Cell Total Protein Extraction Kit (Sangon Biotech, Shanghai, China). The protein samples were separated through a 5% denaturing polyacrylamide gel, and transferred to nitrocellulose membranes (Pall Life Sciences, Shanghai, China). Non-specific binding-sites on the membranes were blocked with 5% nonfat milk in TBST for 2 h at room temperature. The membrane was incubated with TBST containing 5% nonfat milk and diluted rabbit anti-*Mblk-1* polyclonal antibody (1: 200) (SBS, Beijing, China) overnight at 4 °C. It was then washed, incubated with horseradish peroxidase-labeled anti-rabbit IgG (1: 500) (BeyotimeBiotech, Shanghai, China) for an hour at room temperature, and washed again. The immunological detection was carried out according to instructions of the Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen Biotech, Beijing, China).

2.8. S2 cell culture and luciferase reporter assay

A 421-bp fragment from *Mblk-1* 3′UTR and its mutant sequence and a 249-bp coding region of *ame-miR-279a* were synthesized and amplified using 2 × PCR Mix (TaKaRa) (Fig. S3). The *Mblk-1* 3′ UTR and its mutant were cloned into a pAc5.1-firefly luciferase-V5-His vector respectively (Fig. S4A), and the *ame-miR-279a* coding region was cloned into a pAc5.1-V5-His vector (Fig. S4B), *Xho*I and *Not*I restriction sites were added to the 5′ end of the forward and reverse primers, respectively (Table 2). *Drosophila* S2 cells were cultured with 10% fetal bovine serum (HyClone) in Schneider's Insect Medium (Invitrogen, Carlsbad, USA). Cells were seeded at 1×10^6 cells per well in a 12-well plate. One day later, *ame-miR-279a* expression vector (pAc-*ame-miR-279a*) was co-transfected with either pAc-fluc-*Mblk-13′*UTR, pAc-fluc-*Mblk-13′*UTRm, or an empty vector (pAc) in the cells using the calcium phosphate transfection method as described by Tiscornia et al. (2006). In all cases, 12 μl $CaCl_2$ (2 M) and 6 μg transfer vector were mixed, and 1.5 μg of pCopia-Renilla luciferase was added as internal control. Forty eight hours after transfection, luciferase assays were performed using a dual-specific luciferase assay kit (#RG027, Biyuntian, Shanghai, China). Renilla luciferase activity provided normalization for firefly luciferase activity.

2.9. Statistical analysis

Statistical analysis was conducted as indicated in the text and in figure legends. All t-tests used were two tailed. All tests were done by SPSS 16.0.

3. Results

3.1. The expression of ame-miR-279 paralogs in the brains of nurse and forager bees

We had previously detected a significantly higher expression level of *ame-miR-279a* in the heads of nurses compared to foragers in normal colonies (Liu et al., 2012), and *ame-miR-279b*, *ame-miR-279c*, *ame-miR-279d* were also detected in honey bees (Chen et al., 2010; Qin et al., 2014). What might be the differences in expression among these *miR-279* paralogs between nurses and foragers? As shown in Fig. 1, there was a significantly higher level of *ame-miR-*

Table 2
Primer sequences used for RT-PCR amplification of 3′UTR and *pri-miR-279a*.

Primer	5′ to 3′
Mblk-1 3′UTR-F	CGCCCGAAACCGCGAAAGAA
Mblk-1 3′UTR-R	GACGTCGAATCACGCCTTGT
pri-miR-279a-F	CTTTCTAAGTATCAATAATG
pri-miR-279a–R	TCTTAAAATTCATATTCATA

279a in the brain of nurses and foragers than *ame-miR-279b*, *ame-miR-279c*, *ame-miR-279d*. It reveals the important role of *ame-miR-279a* in the brain function of the honey bee.

3.2. The expression pattern of ame-miR-279a in the brains of nurses and foragers

There was a significantly higher expression of *ame-miR-279a* in the heads of nurses than in those of foragers in typical colonies (Liu et al., 2012), and it showed a high degree of temporal specificity during the development of adult workers, with the highest expression in the 12-day-old nurses and remaining stable in over 30-day-old foragers (Shi et al., 2014). These suggest a possible important function of *ame-miR-279a* in honey bee behavior plasticity. To confirm this hypothesis, the expression and localization of *ame-miR-279a* in the brains of nurses and foragers were investigated. We first measured the *ame-miR-279a* expression in the brains of nurses and foragers exhibiting normal behavior in typical colonies. A *t*-test showed that *ame-miR-279a* was significantly highly expression in 12-day-old nurses compared to the 30-day-old foragers ($t = 3.79$, $P < 0.05$) (Fig. 2A). However, the differential expression of *ame-miR-279a* between nurses and foragers may be associated with their ages but not their different behavior. To resolve this question, we created the single-cohort colonies, and tested *ame-miR-279a* expression in foragers and nurses of the same age. As expected, this pattern stayed the same regardless whether nurses and foragers were both young (12 days old) or both old (30 days old) in single cohort colonies (Fig. 2B). The *ame-miR-279a* expression between nurses of different ages (12 vs. 30 days old) was not significantly different, nor was it between foragers of different ages (Fig. S5). Northern blot further confirmed that *ame-miR-279a* had a higher expression in nurses than in foragers, regardless of whether both groups were 12 days old or 30 days old (Fig. 2C).

To determine the localization of *ame-miR-279a* in adult honey bee brains (nurses and foragers), *in situ* hybridization was performed using LNA (locked nucleic acid) miRNA. The results showed that *ame-miR-279a* (brown staining) was predominantly expressed in the Kenyon cells of the mushroom bodies (Fig. 3A and B) and in the lamina of the optic lobes in nurse and forager (Fig. 3A, C). The blank control produced no brown staining (Fig. 3D). Moreover, *ame-miR-279a* expression in the brain showed no obvious spatial difference between the nurse bees and forager bees even when they were of the same age (Fig. S6). Taken all together, these results confirmed the important role of *ame-miR-279a* in the bee behavioural maturation.

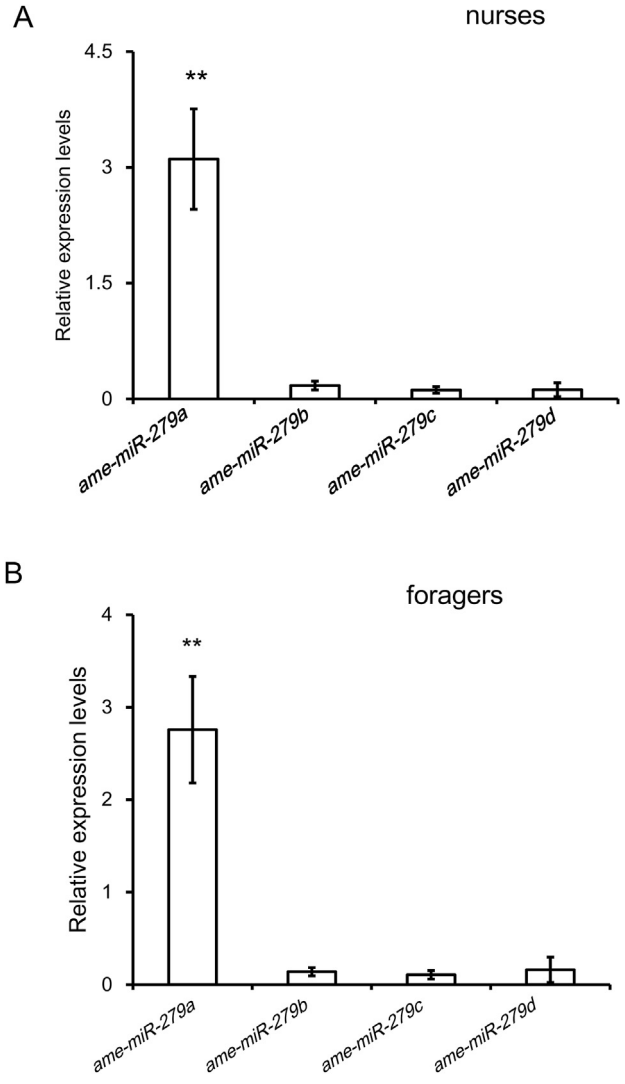

Fig. 1. Expression levels of four *miR-279* paralogs (*miR-279a*, *miR-279b*, *miR-279c*, *miR-279d*) in the brains of nurses and foragers.

3.3. Inhibition and overexpression of ame-miR-279a in the honey bee

Considering the importance of *ame-miR-279a* in behavioural maturation, we decided to overexpress and inhibit the miRNA in honey bees to examine possible effects on behavior. The synthetic inhibitor (anti-miRNA) and mimic of *ame-miR-279a* were fed to foragers together with 50% sucrose solution. The qRT-PCR confirmed the overexpression and inhibition of *ame-miR-279a* in the brains of honey bee in the presence of the mimic and inhibitor respectively. As shown in Fig. 4, the *ame-miR-279a* expression in foragers from the M group was significantly higher than in the NS group, while *ame-miR-279a* expression in foragers from the I group was significantly lower than that of the INS group.

3.4. ame-miR-279a affects the sucrose responsiveness of foragers

To further investigate the possible function of *ame-miR-279a* in the honey bees' behavioural maturation, we tested the effect of *ame-miR-279a* on PER first by using a mimic. As was no significant difference in PER between 24 and 48 h ($F = 3.22$, $df = 1, 48$; $P = 0.08$), we analyzed the two sets of data together. PER response varied significantly with sugar concentrations ($F = 15.78$, $df = 5, 48$; $P < 0.001$). PER response was significantly lower in bees fed with a mimic (279aM) compared to a control group fed with nonsense control (279aM-NS) ($F = 13.12$, $df = 1, 5$; $P < 0.001$, Fig. 5A).

We then tested the effect of *ame-miR-279a* on PER by using its inhibitor. There was no significant difference in PER between 24 and 48 h ($F = 1.07$, $df = 1, 48$; $P > 0.1$), and we analyzed the two sets of data together. PER response varied significantly with sugar concentrations ($F = 14.71$, $df = 5, 48$; $P < 0.001$). PER response was significantly higher in bees fed with an inhibitor (279aI) compared to a control group fed with nonsense control (279aI-NS) ($F = 4.96$, $df = 1, 5$; $P < 0.04$, Fig. 5B).

3.5. Quantification of the expression of ame-miR-279a and Mblk-1

Mblk-1 was predicted as the target of *ame-miR-279a* (Liu et al., 2012). In order to confirm their interaction, we detected the expression of *ame-miR-279a* and *Mblk-1* in the brains of honey bees

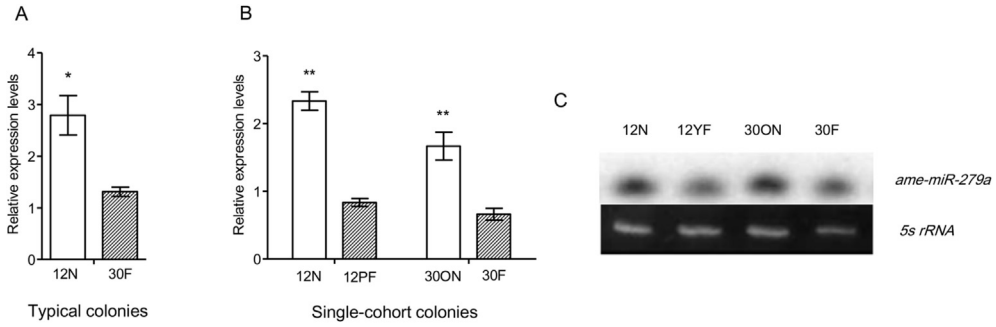

Fig. 2. Expression levels (±SE) of *ame-miR-279a* in the brain of 12 and 30 days old age-matched nurses and foragers from typical colonies (A) and single-cohort colonies (B). Student t-test results were shown, with * denoting $P < 0.05$ and ** denoting $P < 0.01$. Data based on three replicates (colonies). (C) Northern blot analysis of *ame-miR-279a* in brains of age-matched 12-day-old young nurses (12N) and young ("precocious") foragers (12PF), and age-matched 30 days old foragers (30F) and old ("overage") nurses (30ON) from single-cohort colonies. 5s rRNA was used as a reference.

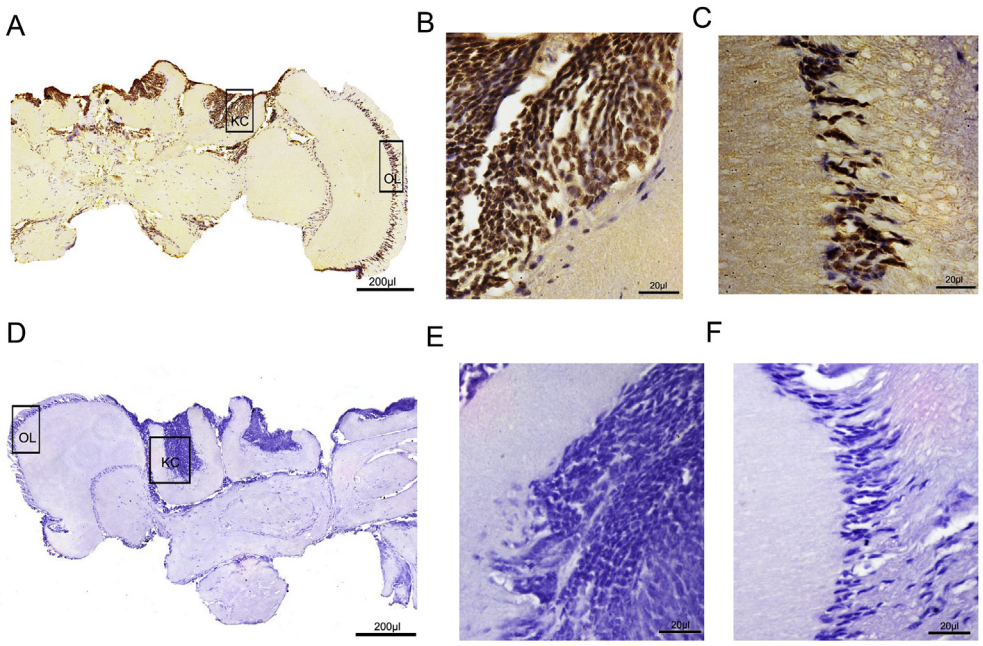

Fig. 3. Expression of *ame-miR-279a* in the honey bee brain. OL, optic lobe; KC, Kenyon cells. *ame-miR-279a* is highly expressed in the Kenyon cells of the mushroom bodies and in the lamina of the optic lobes (brown colour) with the positive probe (A). No brown labeling was seen in sections probed with a blank control (D). Squares delineate regions in shown magnified in BC and EF. There were no obvious spatial differences between nurses and foragers; these images are from a nurse brain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

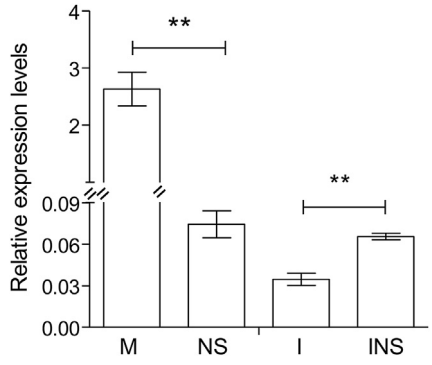

Fig. 4. *Ame-miR-279a* expression in the brains of foragers after oral feeding with *mimic-mir-279a* (M) or nonsense sequence (NS), or *inhibitor-mir-279a* (I) or inhibitor nonsense sequence (INS). An independent *t*-test result is shown, data represent the mean from three independent experiments ± s. e.m * means $P < 0.05$, ** means $P < 0.01$.

from the experimental foragers above. As expected, *ame-miR-279a* had much higher expression in the brains of foragers in group 279aM than in group 279aM-NS ($t = 14.924$, $P < 0.05$) (Fig. 6), while *Mblk-1* had significantly lower expression in the brains of foragers from the 279aM group than from the 279aM-NS group ($t = 3.884$, $P < 0.05$) 24 h after treatment (Fig. 6). The *Mblk-1* protein level in forager heads from the corresponding honey bees was further examined by western blot, as shown in Fig. 6. Honey bees in 279aM group showed a lower Mblk-1 protein level than the 279aM-NS group 24 h after treatment (Fig. 6). Similar results were obtained 48 h after treatment (Fig. S7).

3.6. Confirmation of the interaction of ame-miR-279a with Mblk-1 using a luciferase reporter assay

To test whether *ame-miR-279a* actually targets the *Mblk-1* 3′ UTR, we subcloned a 421-bp fragment of the 3′UTR region of *Mblk-1* mRNA that included the predicted *ame-miR-279a* recognition site

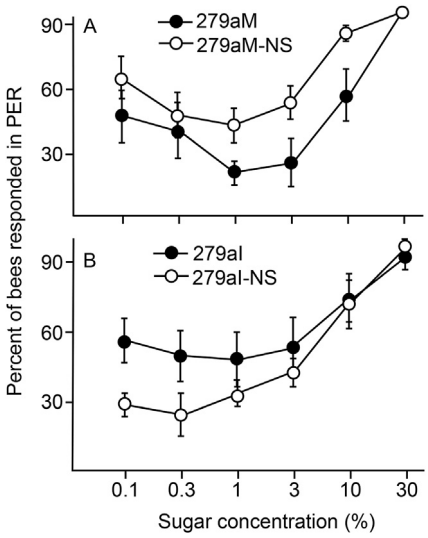

Fig. 5. Mean score (%±SE) of bees responding with proboscis extension response to various sugar concentrations after bees treated with a mimic (A) or inhibitor (B) of *ame-miR-279a*. The effect of *ame-miR-279a* on foragers' responsiveness to sucrose. Responsiveness to sucrose was significantly lower ($P < 0.01$) in foragers fed on a *miR-279a mimic* (279aM) compared to those fed with a nonsense sequence (279aM-NS). Conversely, response to sucrose was significantly ($P < 0.01$) enhanced in foragers fed on a *miR-279a inhibitor* (279al) compared to those fed with a nonsense sequence (279al-NS). Data from three colonies were analyzed after arsine-square root transformation but presented here without transformation.

(Fig. 7) into a luciferase reporter plasmid designated as pAc-fluc-*Mblk-1*′UTR (Fig. 8A). A sequence with mutations (m) was also constructed as the negative control for the same reporter assay, named as pAc-fluc-*Mblk-1*′UTR-m. The coding region of *ame-miR-279a* was cloned into a pAc5.1-V5-His vector designated as pAc-*ame-miR-279a*. When pAc-*ame-miR-279a* was co-transfected with pAc-fluc-*Mblk-1*′UTR in S_2 cells, the luciferase activity significantly decreased compared to the assay involving co-transfection with pAc-fluc-*Mblk-1*′UTR m and pAc ($t = 10.07$, $P < 0.0001$, Fig. 8B). Moreover, *ame-miR-279a* expression directly reduced the *Mblk-1* mRNA and protein levels (Fig. 4). All these results support the conclusion that *Mblk-1* is a direct target of *ame-miR-279a*.

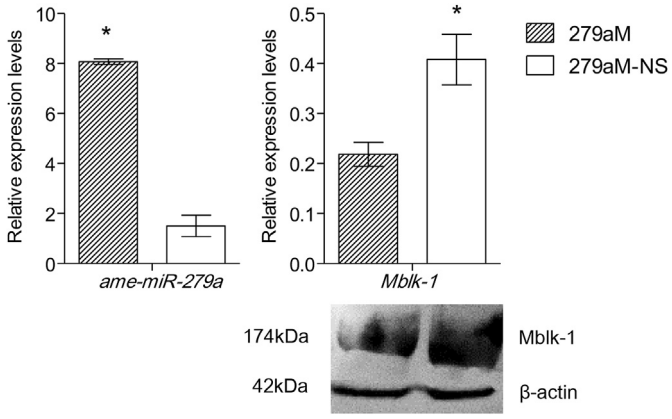

Fig. 6. Relative expression levels (±SE) of *ame-miR-279a* and *Mblk-1* from group 279aM and 279aM-NS at 24 h after treatment. Student t-test results are shown with * denoting $P < 0.05$, ** denoting $P < 0.01$. Data are from three replicates (colonies). Western blot analysis of Mblk-1 protein in foragers' heads from 279aM to 279aM-NS at 24 h after treatment, *β-actin* was used as the reference protein.

4. Discussion

The role of miRNA in insect behavior has been well established in recent years (Lucas and Raikhel, 2013). The miR-iab4/iab8 locus controls self-righting behavior in larvae of *Drosophila* by repressing the Hox gene *Ultrabithorax* (Picao-Osorio et al., 2015). Ecdysone controls *let-7 -Complex* to repress the circadian gene clockwork orange to regulate the circadian rhythms of *Drosophila* (Chen et al., 2014). *MicroRNA-133* inhibits the behavioural aggregation of locusts by controlling dopamine (Yang et al., 2014). *MicroRNA-932* regulates the memory of honey bee by targeting *actin* (Cristino et al., 2014>). *Dme-miR-279* regulates the JAK/STAT pathway to drive the rest: activity rhythms in *Drosophila* (Luo and Sehgal, 2012). In this study, we concentrated on *ame-miR-279a* since its expression was significantly higher in nurses than that of foragers, and showed a high degree of temporal specificity in typical colonies (Liu et al., 2012; Shi et al., 2014). However, it was not clear whether the expression of *ame-miR-279a* was associated with task performance (nursing) or age (young bees). We decoupled the task performance and age in honey bees by using single cohort colonies, a method regularly used to accomplish this (e.g. Robinson and Page, 1989; Ben-Shahar et al., 2002). We determined that the *ame-miR-279a* expression was always higher in nurses than in foragers regardless of whether they were young (typical nurses vs. precocious foragers), or were both old (overaged nurses vs. typical foragers). These results are consistent with another study in honey bees, in which the *foraging* gene was shown to regulate the behavioural transition between nurses and foragers (Ben-Shahar et al., 2002). Thus, we deduced that there is a good correlation between *ame-miR-279a* and honey bee behavioural changes.

Mushroom bodies (MBs) are higher-order brain centres thought to be important for sensory integration, learning and memory formation in the honey bee (Giurfa, 2007; Menzel, 1999, 2012). MBs have a high degree of structural plasticity depending on caste and task performance, suggesting that they are associated with honey bee social behaviours (Robinson et al., 1997; Withers et al., 1993). The MBs are famous as important brain regions of olfactory learning in the vinegar fly, *Drosophila melanogaster* (Hayashi et al., 2009). It has been reported that *dme-miR-279* was detected with strongest expression in the head epidermis in regions adjacent to where the sensory organ progenitors form in *Drosophila* (Stark et al., 2005). A putative orphan receptor (HR38) homologue that mediates ecdysteroid-signaling, showed higher expression in the MBs of forager brains compared to nurse bees, suggesting its involvement in regulation of the division of labour of the workers (Yamazaki et al., 2006). In this study, we demonstrated that *ame-miR-279a* is expressed more in the Kenyon cells of the mushroom bodies, suggesting that *ame-miR-279a* may play a role in social behavior. However, there were no obvious spatial differences between nurses and foragers when we used *in situ* hybridization. This suggests that the differences in *ame-miR-279a* levels between nurses and foragers detected with RT-qPCR may represent increased expression in the same cells. This is consistent with the expression pattern of the *foraging* gene in nurse and forager bees, which was proved to regulate the division of labour of honey bees (Ben-Shahar et al., 2002).

It was reported that *dme-miR-279* can regulate the formation of carbon dioxide (CO_2) neurons by targeting the transcription factor Nerfin-1 in *Drosophila* (Cayirlioglu et al., 2008), and that *Prospero* restricts CO_2 neuron formation indirectly via *miR-279* and directly by repressing the common targets, Nerfin-1 and Esg, suggesting the importance of *dme-miR-279* in the neuron and olfactory system development in *Drosophila* (Hartl et al., 2011). In this study, we found that overexpression of *ame-miR-279a* attenuated the sucrose responsiveness of foragers (Fig. 5A), while its reduction enhanced

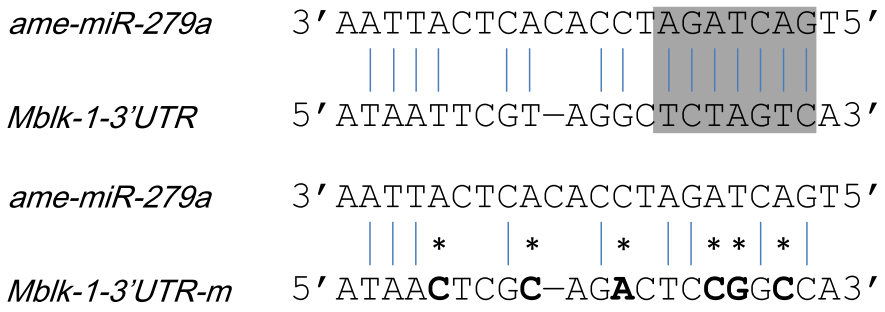

Fig. 7. Sequences of the interaction sites between *ame-miR-279a* and *Mblk-1-3′UTR*. Asterisks indicate mutated site, mutated nucleotide bases are shown in bold. Grey shaded areas indicate canonical 7mer "seed" region that aligns with the target site, the vertical lines indicate contiguous Watson-Crick pairing.

their sucrose responsiveness (Fig. 5B). Responsiveness to sucrose is associated with foraging choices, as bees with high sucrose responsiveness preferentially collect pollen or water while bees with low sucrose responsiveness mainly collect nectar (Pankiw and Page, 1999; Scheiner et al., 2001a), suggesting the importance of *ame-miR-279a* in regulating honey bee olfactory behavior. Moreover, we found that nurses always had higher expression of *ame-miR-279a* than foragers regardless of their age (Fig. 2). It has been demonstrated that nurse bees are less responsive than foragers to gustatory stimuli (Scheiner et al., 2001a,b), and water foragers have higher responsiveness to sucrose than both of pollen and nectar foragers (Pankiw, 2005). In our study, overexpression of *ame-miR-279a* in foragers may make them physiologically similar to nurses, resulting in lower sucrose responsiveness (Fig. 5A and B), and suggesting that *ame-miR-279a* may modulate the honey bee behavioural transition from nurses to foragers, or stimulate foragers to change their behavior from nectar collection to water or pollen foraging when colony conditions demand so.

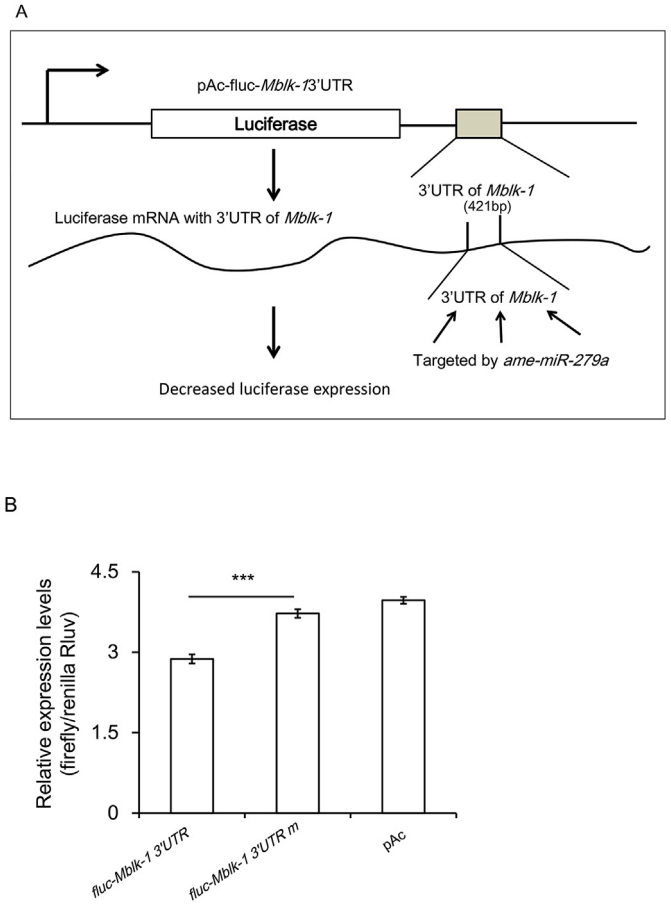

Fig. 8. (A) A schematic representation of the principle behind the luciferase assay. (B) co-transfection of *pAc-fluc-Mblk-1*3′UTR resulted in dramatic suppression of the luciferase activity. A normalized firefly/renilla luciferase value was plotted with ±s.e.m.

We have previously predicted *Mblk-1* to be a possible target for *ame-miR-279a* (Liu et al., 2012). The expression of *ame-miR-279a* is largely confined to the mushroom body of the honey bee brain (Fig. 3), and overexpression of *ame-miR-279a* significantly inhibited the mRNA and protein expression of *Mblk-1* in forager brains (Fig. 6). Moreover, our luciferase assay confirmed that *ame-miR-279a* targets the 3′UTR of *Mblk-1* because transfection of pAc-fluc-*Mblk-1*3′UTR reduced the luciferase activity and pAc-fluc-*Mblk-1*3′UTRm rescued this suppression to the same level as that of the blank control (Fig. 8). These results strongly indicate that *ame-miR-279a* directly targets *Mblk-1*. The *Mblk-1* gene, encoding a putative transcription factor is also expressed preferentially in the large-type Kenyon cells of honey bee MBs. It contains several motifs characteristic of transcription factors, including RHF1 and RHF2, a nuclear localization signal and glutamine-run motifs (Takeuchi et al., 2001). Thus, Mblk-1 is thought to be involved in brain function by regulating transcription of its target genes. It has been reported that Mblk-1 may function in MB neural circuits directly modulated by the Ras/MAPK pathway (Park et al., 2003). E93, a homologue of Mblk-1 in *Drosophila*, expressed highly in the brain of the fly, has been shown to affect olfactory sensory neurons (Jafari et al., 2012). MBR-1, another homologue of Mblk-1 in the nematode *Caenorhabditis elegans*, was also reported to have neuronal functions, in which it is required for the pruning of specific neurites that occur during larval development (Kage et al., 2005). Moreover, it was also shown that MBR-1 is required for olfactory plasticity in adult animals (Hayashi et al., 2009; Takayanagi-Kiya et al., 2017). Taken together, we deduce that Mblk-1 may be involved in the regulation of behavioural plasticity of honey bee through its target gene *ame-miR-279a* in the MBs.

In summary, we found that *ame-miR-279a* showed significantly higher expression in nurses than in foragers regardless of their ages, and *ame-miR-279a* was primarily localized in the Kenyon cells of the mushroom body of foragers and nurses; overexpression of *ame-miR-279a* attenuated the sucrose responsiveness of foragers, while its inhibition enhanced their sucrose responsiveness. Moreover, we determined that *ame-miR-279a* directly targets the mRNA of *Mblk-1*. These findings suggest that *ame-miR-279a* plays important roles in regulating honey bee division of labour.

Author's contributions

F.L. planned the experiments, performed *In Situ Hybridization*, the reporter assay, data analysis and wrote the manuscript. T.F.S. performed RNA extraction, RT-PCR and qRT-PCR *analysis*, western

blot. W.Y., X.S. and L.Q. performed behavioural experiments. Z.Y.H. was involved in experimental design, data analysis and manuscript revision. S.W.Z. and L.S.Y. performed manuscript revision. All authors have read the final draft of the manuscript.

Acknowledgements

This work was supported by grants of National Natural Science Foundation of China (31302039), Education Department Research Project of Anhui Province (2013SQRL018ZD), and the Open Fund of Anhui Province Key Laboratory of Local Livestock and Poultry, Genetical Resource Conservation and Breeding (AKLGRCB2017007). We thank Wenfeng Chen for his kindly provide with luciferase reporter plasmid, and thank Tiande Liang for technical assistance in collecting honey bees and preparing samples, Aung Si, Zhiguo Li and Shoujun Huang for critically reading the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2017.09.008.

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