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Full Length Article

# Effect of royal jelly on longevity and memory-related traits of *Apis mellifera* workers

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ABSTRACT

Royal jelly (RJ) is a key factor for honey bee caste determination. The queen bee is fed with RJ by worker bees throughout her life, while the worker bees eat bee bread themselves. This study was designed to explore the effect of nutrient-rich RJ on longevity and learning and memory abilities of workers of the western honey bee *Apis mellifera*. The newly emerged worker bees were randomly divided into three groups and were fed 50% sucrose solution containing 0%, 10%, and 20% RJ. We found that worker bees fed with 10% and 20% RJ showed significantly improved longevity and higher proboscis extension response success rate compared to bees fed with 50% sucrose containing 0% RJ. Additionally, bees fed with 20% RJ showed significantly higher level of expression of memory related genes (*GluRA* and *Nmdar1*) compared to the control group. Furthermore, expression of the *Nmdar1* gene of worker bees fed with 10% RJ was also significantly higher than in the control group. These results indicate that RJ has potential effects on the longevity and learning and memory abilities of *A. mellifera*.

## Introduction

Royal jelly (RJ) is an acerbic, spicy, and sweet substance with a special aroma, which is secreted by the subpharyngeal glands as well as mandibular glands of young worker honey bees as is used as food for larvae and adult queens (Cai and Lin, 2007; Ma et al., 2012). It is rich in bioactive substances such as proteins, steroids, enzymes, vitamins, trace elements, and polyphenols (Liu et al., 2008; Gao et al., 2011; Li et al., 2016). If larvae were fed with RJ all the time, they will develop into queens (Kamakura, 2011) and will have a long longevity of up to several years (Song et al., 2006). If the larvae stopped receiving RJ after they are three days old, they will develop into a worker bee with a longevity of about 35 days during the foraging period (Zeng, 2007). Researches have shown that the amount of food, the amount of sugars fed to individuals, and the amount of fatty acids fed to larvae are factors that determine whether the adult will be a queen or a worker (Leimar et al., 2012; Buttstedt et al., 2016; Buttstedt et al., 2018). Moreover, earlier studies have reported that RJ is the key driver for caste differentiation in honey bees (Bloch et al., 2002). It can also prolong the lifespan, increase anti-aging ability, and improve memory in flies, nematodes, mice, and other organisms (Chen et al., 2009; Peng et al., 2011; Wang et al., 2014a, 2014b; Ji et al., 2016).

Although honey bees have a relatively small brain and simple central nervous system, they have complex and rich behaviors, and an excellent learning ability (Liu et al., 2014). The learning ability in bees is directly related to their foraging efficiency, and the proboscis extension response (PER) is a classical behavioral method to measure the bee's olfactory learning and memory abilities (Letzkus et al., 2006). However, little is known about the effect of RJ on the longevity, learning, and memory of worker bees. Therefore, in the current study, the effects of RJ on the longevity, learning, and memory abilities of worker bees were tested through caged rearing and PER tests. At the same time, the effect of RJ on the expression of the learning and memory related genes glutamate receptor (*GluRA*) and *N*-methyl-D-aspartic acid receptor (*Nmdar1*) were quantified through real-time quantitative PCR. The aim of this study was to investigate the impact of RJ on the longevity and memory-related traits of *Apis mellifera* worker bees.

## Materials and methods

### *Experimental design*

The queen was restricted to lay eggs for 24 h in one comb devoid of any eggs and larvae in the apiary of Jiangxi Agricultural University. After 19 days, the frame with capped brood was selected from the

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**Table 1**
Description of primer sequences and genes used for RT-qPCR.

| Gene name | GenBank accession number | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---|---|---|---|
| *Nmdar1* | NM_001011573.1 | ACTGACGGTACCGAAGAGGA | CCCATACCATGCCCAACACT |
| *GluRA* | XM_395227.4 | GGATGAAAGAAGGAAAAGGATA | ACAGTAACAATAACAACAGCGAT |
| *GAPDH* | AF023666.1 | GCTGGTTTCATCGATGGTTT | ACGATTTCGACCACCGTAAC |

colonie and was transferred into an incubator (T 34 °C; RH 75%). Newly emerged worker bees were obtained and were randomly divided into three groups. Each group contained approximately 200 bees and was kept in a wooden cage with mesh on one side. Following the method reported in an earlier study on *Drosophila melanogaster* by Shorter et al. (2015), newly emerged worker bees of each group were fed 50% sucrose solution containing 10% and 20% RJ while worker bees were fed 50% sucrose solution without RJ (0%) were used as control group. The sucrose solution with different concentrations of RJ was provided every 24 h to ensure fresh and adequate food. All cages were kept in an incubator (T 34 °C; RH 75%) during the experimental period. The RJ used in this experiment was freshly collected from the apiary of the Honeybee Research Institute, Jiangxi Agricultural University (28.46°N, 115.49°E).

*Effect of RJ on the longevity of worker bees*

The syrup with different concentrations of RJ was replaced every day and the number of dead bees was counted and cleared away every day until all bees had died. This experiment had three independent biological replicates using newly emerged bees from three original colonies.

*Effect of RJ on learning and memory abilities of worker bees*

The proboscis extension response (PER) conditioning experiment was initiated on the seventh day. Thirty bees were captured from each group and immobilized on ice for 5 min. Each bee was fixed in a U-shaped metal tube with thin strips. The bees were fed with 50% sugar solution and put into an incubator (T: 35 °C; RH: 75%) to recover. The worker bees were trained and tested for olfactory learning following the method reported in Letzkus et al. (2006). We used two scents (limonene and vanilla) as a rewarding and punishing unconditioned stimulus respectively. The reward stimulus was a scent mixed with sugar solution while the punishment was the different scent mixed with saturated salt solution. The bees were trained with the reward and punishment for three times in different order. And then, the bees were fed enough sugar and returned to the incubator for overnight. The bees were tested for three times with the punishing and reward on the morning of second day. If a bee's correct responses to the punishing and reward stimuli were more than incorrect responses, this bee was considered PER success (Liao et al., 2018). This experiment was repeated three times using newly emerged bees from three original colonies.

*Effect of RJ on mRNA expression of genes related to learning and memory*

*Sample collection*

After PER training, the worker bees that passed the memory retrieval test were collected. A total of nine bees were collected from each group, heads of three bees were pooled to form a sample, and three of these pools were used per group, consequently, each group included three samples. And we run this operation three times using three honeybee colonies for each group at the same time. All the samples were stored in liquid nitrogen.

*Extraction of total RNA and cDNA synthesis*

The total RNA was extracted using an RNA extraction kit (*TransZol* Up Plus RNA Kit). RNA purity was determined based on the OD260/280 ratio and the integrity of RNA was assessed by visualizing the 18S, 28S, and 5S rRNA bands by agarose gel electrophoresis (1.5%). Reverse transcription of total RNA was performed using a reverse transcription kit (PrimeScript™ RT reagent Kit with gDNA Eraser) and the cDNAs were preserved in refrigerator at −80 °C.

*Design of quantitative PCR primer and real-time quantitative PCR methods*

RT-qPCR primers of *A. mellifera Nmdar1* and *GluRA* genes were designed using Primer 5.0 software using mRNA sequences obtained from GenBank. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as internal control (Table 1).

The RT-qPCRs were performed on an Applied Biosystems ABI 7500 machine. The PCR reaction mixture (10 μL) contained 1 μL cDNA, 5 μL SYBR® Premix ExTaq™ II, 0.2 μL Rox Reference Dye, 0.4 μL each of the upstream and downstream primers, and 3 μL ultra-pure sterile water. The reaction thermal profile included an initial denaturation (95 °C 30 s), quantification for 40 cycles (95 °C 10 s, 60 °C 1 min), and a dissociation from 50 °C to 90 °C (Elevated by 1 °C every 6 s). When the fluorescent quantitative PCR machine ran out, the error of three technical replicates' values of each reaction was validated within 0.5. The dissolution curve was established to collect the Ct values of the target gene and the internal reference gene (Wang et al., 2014a, 2014b), and the relative expression of each gene was calculated following the method by Huang et al. (2012).

*Statistical analysis*

We tested for differences in survival among three groups using the log-rank tests and the Cox proportional hazards model using the statistics software SPSS17.0. PER and gene expressions were further analyzed via ANOVA and multivariate ANOVA, using $P < .05$. LSD tests were used to determine if there were any differences among different groups. In all figures, data are shown as means ± SE.

## Results

*Effect of RJ on the longevity of worker bees*

The worker bees fed with 10% RJ (hazard ratio, HR = 0.408) and 20% RJ (HR = 0.315) both showed significantly improved longevity than the control group ($X^2 = 365.68$, $P < .001$). The longevity of bees fed with 20% RJ was significantly longer than the bees fed with 10% RJ (Fig. 1). The highest mortality of bees in the control group occurred on day 17, while the highest mortality of bees fed with RJ occurred on day 23.

*Effects of RJ on the learning and memory abilities of worker bees*

As shown in Fig. 2, the PER success rate was significantly higher in worker bees fed with RJ compared to the control group ($F_{2,6} = 21.757$, $P = .002$). The worker bees fed with 20% RJ showed higher PER success rate than worker bees fed with 10% RJ (LSD: $P < .05$).

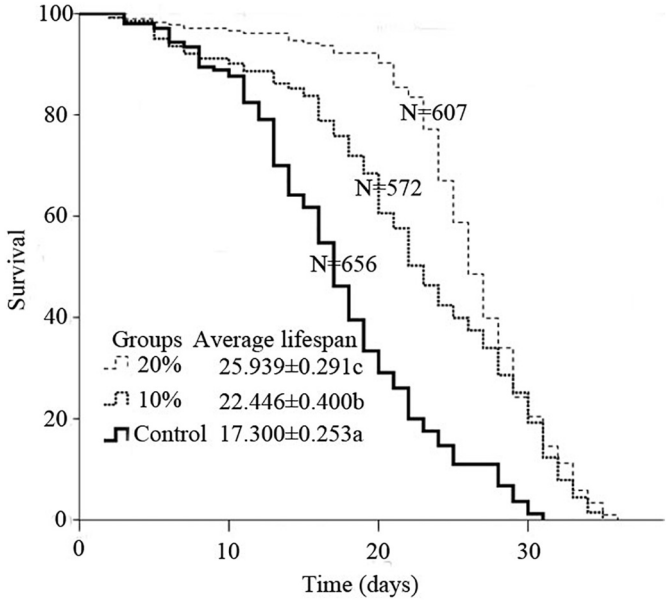


**Fig. 1.** Effect of RJ on the survival rate of *Apis mellifera* workers. Bees were fed with different concentrations of RJ (20%, 10%, or 0%) in three groups corresponding to three colors, respectively. Bees fed with 0% RJ were used as control group. Different letters in the column of average lifespan indicate significant difference ($P < .05$). N represents the sample size marked on the trend line.

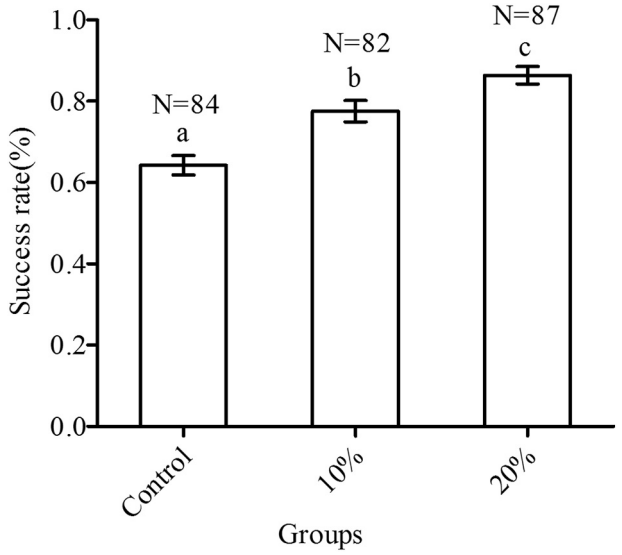


**Fig. 2.** Effect of RJ on learning and memory abilities of worker bees. Effects of different concentrations of RJ on the success rate of PER conditioning. The histogram shows the percentage of 7-day-old worker bees that achieved correct score in the PER memory test. Each group has a single error bar, which indicates the mean ± *SE* of three biological replicates. Different letters above the bars indicate significant difference ($P < .05$). N represents the sample size.

### *Effect of RJ on the relative expression of learning and memory related genes in worker bees*

The relative expression level of *GluRA* of worker bees in the 20% RJ group was significantly higher than that in the control group ($F_{2,24} = 26.162$, $P < .001$), while there was no difference between the 10% RJ group and the control group (LSD: $P < .05$). There was also no significant difference in the expression level between the 20% group and the 10% group. The expression level of *Nmdar1* of worker bees in both the 10% group and the 20% group were significantly higher than that in control group ($F_{2,24} = 9.853$, $P = .001$). However, there was no significant difference between the 10% group and the 20% group (LSD, $P > .05$; Fig. 3).

## Discussion and conclusion

The findings of the current study showed that the longevity of worker bees fed with syrup mixed with RJ was significantly higher than that of the control group, which suggests a potential role for RJ to prolong the lifespan or to improve the longevity of worker bees. RJ contains valuable nutrients, such as acetylcholine and enzymes, which helps to prolong the lifespan (Peng et al., 2011). Furthermore, the average longevity of workers in the 20% group was higher than that in the 10% group, which indicates that increased RJ intake by worker bees also increases their longevity.

Our study also found that with increasing proportion of RJ in the food, the PER success rate increased. These results are similar to the reports by Wang et al. (2015) who reported that bees fed with pure RJ demonstrated much better learning and memory abilities than those fed with syrup only. Both studies suggest that RJ not only affects the longevity of honey bees, but also improves their abilities for learning and memory. The study by Pyrzanowska et al. (2014) reported that RJ improves the spatial memory ability of senile mice. All these animal studies show that RJ can improve learning and memory abilities.

The gene *GluRA* is considered one of the major excitatory neurotransmitters in the vertebrate brain and plays a key role in cell differentiation and synapse formation during the development of the nervous system, which affects the learning and memory abilities of honey bees. It is a key neurotransmitter in the physiological process underlying learning and memory in bees (Danbolt, 2001; Kucharski et al., 2007). The *Nmdar1* receptor is one of the important excitatory amino acid receptors in the central nervous system and it is an ionotropic receptor, which is vital for learning and memory (Chen et al., 2003; Zachepilo et al., 2008; Zannat et al., 2006). So far, no study reported a role for RJ in regulating learning and memory related genes. The results of the PER test from the three groups in the current study indicate that worker bees showed different gene expression levels, which are similar to the results of PER. Expression of *Nmdar1* in worker bees fed with 10% RJ and 20% RJ were both significantly higher than the control group and the expression of *GluRA* in worker bees fed with 20% RJ was also higher than the control group. This suggests that RJ can increase the expression of learning and memory related genes and improve the learning and memory ability of honey bees, which proposes a putative role for RJ in the development of the bees' nervous system. Zhang and Yu (1996) reported that RJ contains rich neurotransmitters such as acetylcholine and norepinephrine, which play a key role in brain strengthening and intelligence. RJ also contains rich enzymes such as lipase and superoxide dismutase, which can enhance the body's ability to remove free radicals and which are conducive in improving intelligence and memory (Peng et al., 2011). RJ contains the unique natural unsaturated fatty acid 10-hydroxy-2-decenoic acid (10-HDA). Researchers found that 10-HDA has a significant promoting effect on the development and proliferation of hippocampal neurons (Tian and Zhong, 2010). The expression of *GluRA* in worker bees fed with 10% RJ was not different from the control group, while the expression of *Nmdar1* in worker bees fed with 10% RJ was significantly higher than in the control group. The main reason may be that the mechanism or threshold of the two genes responding to the RJ stimulation signal are different; however, this requires further study and analysis.

## Conflict of interest

No conflict of interest exits in the submission of this manuscript.

## Acknowledgments

Xiaobo WU designed the experiment, Chunhua Liao and Jing-liang

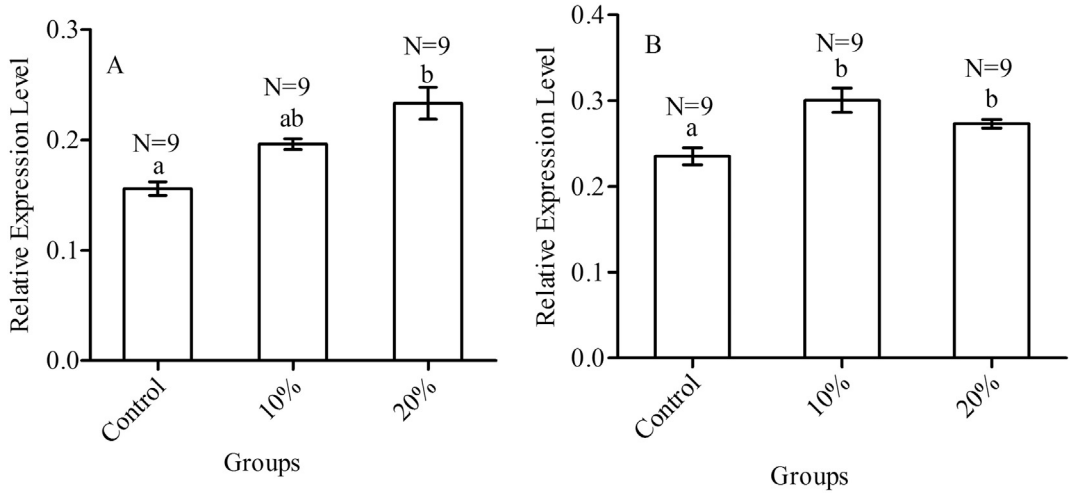


**Fig. 3.** Effect of RJ on the relative expression of learning and memory related genes GluRA (A) and Nmdar1 (B) of *Apis mellifera* workers. Each error bar below the letter indicates the mean ± *SE* of three biological replicates.

Shi conducted the experiment. Xiaobo WU, Jing-liang Shi, and Chunhua Liao wrote the paper. Zilong Wang revised the paper. All authors read and approved the final manuscript. This work was supported by the National Natural Science Foundation of China (No. 31760714), the outstanding young talent program of Jiangxi Province (No. 20162BCB23029), and the Earmarked Fund for China Agriculture Research System (No. CARS-44-KXJ15).

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