Insectes Sociaux (2020) 67:411–418
https://doi.org/10.1007/s00040-020-00775-4

Insectes Sociaux

RESEARCH ARTICLE

Influence of RNA interference-mediated reduction of *Or*11 on the expression of transcription factor *Kr-h*1 in *Apis mellifera* drones

J. F. Liu[1,2] · X. J. Wan[3] · Z. L. Wang[1] · X. J. He[1] · Z. J. Zeng[1]

Received: 28 January 2020 / Revised: 17 June 2020 / Accepted: 2 July 2020 / Published online: 11 July 2020

Abstract

9-Oxo-2-decenoic acid (9-ODA, the predominant component of honeybee queen mandibular pheromones) acts as a sex pheromone attracting drones during mating flights in midair. *Odorant receptor* 11 (*Or*11), which is located on the membrane of antennal olfaction receptor neurons in bees, can specifically recognize 9-ODA. At present, the molecular pathway of honeybee drones responding to 9-ODA is still unclear. Studies have demonstrated that 9-ODA could downregulate the expression of *Krüppel-homolog*1 (*Kr-h*1, a transcription factor related to the regulation of reproduction and division of labor mediated by juvenile hormone) gene in the mushroom of honeybee brain. We speculate that *Kr-h1* may be the downstream gene of *Or*11, which is involved in the pathway of drones responding to 9-ODA. Therefore, we analyzed the influence of 9-ODA on the expression of *Or*11 and *Kr-h*1 in the antennae of sexually immature (4 days) and mature (14 days) male honeybees (*Apis mellifera*) by quantitative polymerase chain reaction (qPCR). The results demonstrated that 9-ODA significantly downregulated the expression of *Or*11 and *Kr-h*1 in the antennae of sexually immature and mature drones. Additionally, siRNA-*Or*11 was injected into the antennae and brain tissues of 8-day-old drone pupae, and the expression patterns of *Or*11, *Kr-h*1 and *Broad-Complex* (*Br–c*, downstream gene of *Kr-h*1) were determined by qPCR at 72 h. The RNAi-induced knockdown of *Or*11 significantly decreased the expression of *Or*11, *Kr-h*1 and *Br–c* in the antennae and brains of drones. This study suggests that the transcription factor *Kr-h*1 is downstream of *Or*11, *Kr-h*1, which may play an important role in the signal transduction process of drones responding to 9-ODA.

Keywords *Apis mellifera* · Drone · 9-ODA · *Odorant receptor* 11 · *Krüppel-homolog* 1 · RNA interference

Introduction

Pheromones, which are one of the major forms of communication for honeybees, can quickly trigger physiological and behavioral changes in honeybees (Grozinger et al. 2007). Queen mandibular pheromone (QMP) acts both as a social pheromone and a sex pheromone (Grozinger and Robinson 2007). As a social pheromone, QMP triggers long-term physiological and behavioral responses in worker honeybees through physiologically related systems (Rangel et al. 2016; Slessor et al. 2005). For example, the pheromone inhibits the development of worker bee ovaries and the brood of a new queen and delays the change of nurse worker to forage worker (Pankiw et al. 1998; Pankiw and Page 2003). QMP also acts as a sex pheromone by inducing rapidly behavioral responses in drones through the nervous system during mating flight (Grozinger et al. 2007). In mating flight, the virgin queen releases QMP to attract sexually mature drones for copulation (Butler 1971; Butler et al. 1962, 1959;

J. F. Liu and X. J. Wan contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00040-020-00775-4) contains supplementary material, which is available to authorized users.

✉ Z. J. Zeng
bees1965@sina.com

[1] Honeybee Research Institute, Jiangxi Agricultural University, Nanchang 330045, Jiangxi, People's Republic of China

[2] Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, Hainan, People's Republic of China

[3] Department of Laboratory Animal Science, Nanchang University, Nanchang 330006, Jiangxi, People's Republic of China

Gary 1962; Gary and Marston 1971). QMP is composed of five major functional secretions: (E)-9-oxodec-2-enoic acid (9-ODA) and two enantiomers of 9- hydroxydec-2-enoic acid (9-HDA), methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenyl ethanol (HVA) (Keeling et al. 2003). To date, 9-ODA is the most widely used and studied pheromone (Butler et al. 1962, 1959; Carlisle and Butler 1956; Gary 1962). Previous studies have shown that 9-ODA can produce multiple effects on the behaviors and physiology of drones (Brockmann et al. 2006; Loper et al. 1993). 9-ODA acts as a sex pheromone attracting drones during mating flights in midair (Gary 1962) and delays the development of sexually immature drones, such as the time for initial mating flight and decreases the number of flights (Villar and Grozinger 2017).

Drones have a highly specific and extremely sensitive olfactory system. Specifically, there are rich placoid sensilla on the surface of drone antennae, the number of which in drone is approximately seven times more than in worker (Sandoz et al. 2007). Brockmann et al. (1998) discovered that drone antennae were more sensitive to 9-ODA than worker antennae, and the electroantennography (EAG) response to 9-ODAin drone was higher than others components of QMP. Using a custom chemosensory-specific microarray and qPCR, Wanner et al. (2007) found high expression levels of candidate sex pheromone receptor genes *Or*10, *Or*11, *Or*18 and *Or*170 in the antennae of drones, and proved *Or*11 responds specifically to 9-ODA by injecting the cRNA of the four receptors into *Xenopus* oocytes and examining the sensitivity of each of the QMP components with two-electrode voltage-clamp electrophysiology in vitro. Claudianos et al. (2014) reported that the expression levels of *Or*11 in antennae declined after a proboscis extension reflex (PER) assay in which a worker encountered 9-ODA. The expression of *Ac*Or11 in brain of sexually mature drones was significantly higher than those of immature drones. Additionally, the expression of and *Ac*Or11 in brains of mature flying drones was higher than those of drones in hive, indicating that the expression levels of *Ac*Or11 in drone brains may be associated with sexual maturity and mating flight (Liu et al. 2019). In addition, the structure and functions of the brain tissues of drone are different from those of workers. The antennal lobe (AL) of drone includes both 103 ordinary glomeruli and 4 macroglomeruli (MG), but the AL of worker only consists of conventional glomeruli (Sandoz 2006). Moreover, by using calcium imaging technology, these authors identified that the MG2 of drones specifically responds to 9-ODA. Nonetheless, the mechanism of honeybee drones' response to 9-ODA remains unclear.

Krüppel-homolog 1 (*Kr-h*1), a nuclear receptor gene that was first identified in a study in *Drosophila melanogaster*, can regulate insect metamorphosis (Schuh et al. 1986). *Kr-h*1 is related to the regulation of the behavior by QMP-mediated ovary activation and labor division mediated by juvenile hormone (JH) in honeybee (Grozinger et al. 2007; Whitfield et al. 2003; Shpigleret al. 2010; Kilaso et al. 2017). Interestingly, 9-ODA, as effective as QMP, can inhibit the expression of the transcription factor *Kr-h*1 in the brain of worker bees (Grozinger et al. 2007). However, it is still unknown for the function of *Kr-h*1 in drone bees. Therefore, we speculate that *Kr-h*1 (nuclear receptor) may be the downstream gene of *Or*11 (membrane receptor), and involved in modulating the signal transduction process that the *Or*11 responds to 9-ODA in honeybee drones. To further explore the signal transduction pathway behind the responses of drones to 9-ODA, we further tested the expression level of *Broad-Complex* (*Br–c*, transcription factor) after knocking down *Or*11. *Br–c* is the downstream gene of *Kr-h*1 in many insects (Belles and Santos 2014; Huang et al. 2013; Minakuchi et al. 2009, 2008) and is a key gene in the JH and 20-hydroxyecdysone (20E) signaling pathway of fruit fly and honeybee (Abdou et al. 2011; Paul et al. 2006).

In this study, the influence of 9-ODA on the expression levels of *Or*11 and *Kr-h*1 in *A. mellifera* drones were detected by qPCR. Then siRNAs targeting the gene *Or*11 was injected into the antennae and head of honeybee drones. We determined the expression characteristics of *Or*11, *Kr-h*1 and *Br–c* by qPCR for exploring the relationship between *Kr-h*1 and *Or*11. The results help interpret the physiological functions of *Or*11 and *Kr-h*1 in drones and exploring the signaling pathway that drones respond to 9-ODA in honeybees.

Materials and methods

Insects

Western honeybee (*A. mellifera*) colonies were maintained at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46°N, 115.49°E), using standard beekeeping techniques.

General bee rearing, 9-ODA treatment and collection

Four *A. mellifera* colonies with the same genetic background and population were selected. Four-day-old (sexually immature) and 14-day-old (sexually mature) drones were produced by caging the queen on frames with drone-sized honeycomb; the queen was moved to another comb after 24 h. Twenty-three days later, the frames with capped cells were transferred to an incubator (34 °C and 70% humidity) to obtain emerging drone bees. Upon emergence, the drones were marked on their thorax with marking paint.

Rearing of 4-day-old drones in cages was performed as described (Villar et al. 2015). Groups of 10 newly emerged drones and 20 newly emerged workers were established in Plexiglass cages (10 × 7 × 6 cm; the insects were fed 50% sucrose, water and crushed pollen. The cages were maintained in a dark incubator at 34 °C and 50% relative humidity. The treatment group was stimulated by daily administration of a solution of synthetic 9-ODA (Contech International, Victoria, BC) ethanol equivalent to that of one virgin queen (approximately 70 µg, according to previously described (Plettner et al. 1997), which was dissolved in ethanol and placed on filter paper. The blank control group was treated only with the same dose of ethanol on filter paper. There were three replicate cages for both the treatment and control groups. Twenty-four 4-day-old drones were collected from the treatment and control groups, immediately frozen in liquid nitrogen in a centrifugal tube, and stored at − 80 °C.

The rearing of 14-day-old drones in cages was performed as previously described (Liu et al. 2019). Three hundred newly emerged drones with marked paint were placed back into their parent colony. When these drones reached 14 days of age, we collected those that returned to the entrance of the hive (hereafter referred to as flying drones); the drones crawling in the wall and comb of the hive (hereafter referred to as hive drones) were caught in hive with a 50-mL centrifugal tube. Moreover, to confirm the biological mating habits of adult drones, we collected drones during the peak flight period, between 13:00 and 16:00, during good weather that was clear and warm with light, variable breezes. A drone was then placed into the base of a Y-tube olfactometer (35 cm arm length and 5 cm internal diameter, customized by the Glass instrument factory of Nanchang University, China), and tests were performed in a dark room. For the treatment group, a solution of 9-ODA ethanol that was equivalent to that of one virgin queen was placed on filter paper in the left and right bottles of the Y-tube olfactometer. For the blank control group, only 5 µL of ethanol solution was added to the filter paper. After 5 min of testing, the drone was caught and put into a centrifuge tube, in which liquid nitrogen was added for quick freezing; the samples were stored at − 80 °C for subsequent isolation of RNA. There were six groups for each treatment, with eight drone repetitions for each group.

Table 2 Primers used for qRT-PCR

Primer name	Primer sequence	TM (°C)
*AmOr*11-F	5′- CTTTTACCGAACAACATGACAG -3’	54
*AmOr*11-R	5′- TTATCTCGTAATTAGGTGTGG -3’	
*AmKr-h*1-F	5′- GCACTGGCAGTGACAAGGAA -3’	60
*AmKr-h*1-R	5′- CGTGGAGTGTTATCGTAAGTAGCA A-3’	
AmBrc-F	5′- GACAGGTGGCAACAGCGGTAAC-3’	60
AmBrc-R	5′- TGGACGTGTGCTCGGACTCG -3’	
Amβ-actin-F	5′- GGTATTGTATTGGATTCGGGTG -3’	60
Amβ-actin-R	5′- TGCCATTTCCTGTTCAAAGTCA -3’	

Design and synthesis of siRNA sequences and qPCR primers

Three siRNA sequences (siRNA-*AmOr*11) were designed and synthesized by Shanghai GenePharma Co., Ltd (*AmOr*11 GenBank accession number: NM_001242962.1), as negative control siRNA (siRNA-NC). The siRNA sequences are listed in Table 1. qPCR primers were designed by Primer 5.0 software and synthesized by Shanghai Sangon Biotechnology Co. Ltd.; these primers are shown in Table 2.

Injecting siRNA into antennae and head tissues of drone pupae

Gene knockdown using siRNA is a powerful experimental approach to identify gene function in honeybee. Knockdown of target genes have been successfully achieved by injecting siRNA into eggs, larvae or adults of honeybee (Beye et al. 2002; Erezyilmaz et al. 2006; Guo et al. 2018; Minakuchi et al. 2009). Our previous test found that drones easily die when they were injected with siRNA or water at the adult stage,the reason is that cutting the ommateum for injecting injured their brains, and these injured adult drones were easily attacked by worker bees. Thus, we used 8-day-old drone pupa to perform the RNAi experiment and found that this method of RNAi is feasible.

Drone pupae from four *A. mellifera* colonies were produced by caging the queen on frames with drone-sized honeycomb for 24 h. Twenty days later, the drone pupae on the

Table 1 siRNA sequence designed for RNA interference

siRNA	Sense	Antisense
siRNA-*Or*11-329	GCAACGGGCUAAGGAAUUUTT	AAAUUCCUUAGCCCGUUGCTT
siRNA-*Or*11-528	CCGAACAACAUGACAGUAATT	UUACUGUCAUGUUGUUCGGTT
siRNA-*Or*11-1143	GCAGGAAGAAUUAUGGAUUTT	AAUCCAUAAUUCUUCCUGCTT
siRNA-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

frames with capped cells were transferred to 12-well sterile cell culture plates.

Approximately 100 ng/μL siRNA-*Or*11, siRNA-NC and diethyl pyrocarbonate water (DEPC water) solutions were injected into two antennae (0.25 μL each) and the head (0.5 μL, at the base of antennas) by using a microinjector under 3 × bench magnifiers, and the culture plate containing the pupae was placed in a constant-temperature (34 °C) and humidity (50%) incubator for approximately 72 h until emergence. Statistical analysis of the survival rates of the drone pupae in each group was performed by one-way ANOVA in SPSS 17.0. For each colony, eight emerged drones were added to a centrifuge tube and quickly frozen by liquid nitrogen; the centrifuge tube was stored at − 80 °C.

Preparation of antennae and brain tissue

Preparation of antennae samples occurred as follows. Drones were removed from the liquid nitrogen. One pair of complete antennae was removed from the head using clean tweezers and blades and placed into a 1.5-mL RNase-free EP tube. Eight pairs of antennae from eight drones of the same colony were pooled as one sample, quickly frozen in liquid nitrogen, and stored at − 80 °C for extraction of RNA.

The brain samples were prepared as follows. The remaining heads after the antennae were removed were placed in phosphate-buffered saline (PBS). Head shell and ommateum tissues were eliminated by using clean tweezers and blades, and the tissue was quickly placed in a 1.5-mL RNase-free EP tube. Liquid nitrogen was added to freeze the samples quickly. Eight pairs of brain tissues from eight drones in the same colony were used as one sample, which was stored at − 80 °C for subsequent extraction of RNA.

Total RNA isolation, cDNA synthesis and qPCR experiments

Antennae and brain tissues were collected from each group of samples, and RNA was extracted according to the operating instructions of a TransZol Up kit (Transgen Biotech). The RNA concentration and mass of each sample were measured and tested by spectrophotometry and agarose gel electrophoresis (AGE), respectively. cDNA was synthesized from total antenna RNA using a reverse transcription kit (TaKaRa) according to the manufacturer's instructions.

Relative expression of *AmOr*11 and *AmKr-h*1 in the antennae of *A. mellifera* drones between 4 and 14 days, as well as relative expression of *AmOr*11, *AmKr-h*1 and *AmBr-c* in antennae and brains of drones after injection of siRNA, was tested by qPCR. qPCR was performed with an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of 94 °C for 15 s, 60 °C for 40 s, and 72 °C for 35 s; a melting curve analysis was conducted to verify the specificity of the amplification. Experiments for test samples, an endogenous control, and a negative control were performed in triplicate to ensure reproducibility.

To quantify the expression levels of the target genes, the *actin* gene was often used as a single reliable reference gene in honeybees, *Apis mellifera*, in many studies (Harwood et al. 2019; Lourenço et al. 2008; Martins et al. 2010; Nunes and Simões 2009; Scharlaken et al. 2008; Wang et al. 2013). Especially, in the study performed by (Wang et al. 2013), they observed a high consistency between behavioral changes of adult bees and expression changes of target genes after RNAi of *vitellogenin* (*vg*) and *ultraspiracle* (*usp*), in which the *actin* was used as a single reference gene for qRT-PCR. These results suggest that it is reliable using *actin* as a single reference gene for adequate normalization. Therefore, we used *actin* as an endogenous control in this study.

Statistical analysis

Statistical analysis of the survival rates of drone pupae was carried out using one-way ANOVA in SPSS17.0. The CT values of the target genes and *β-actin*, which was used as an internal control, were collected. The relative expression levels of target genes in honeybees were calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). The statistical analyses of qPCR were performed by *t* tests and one-way ANOVA. $P < 0.05$ represents a significant difference.

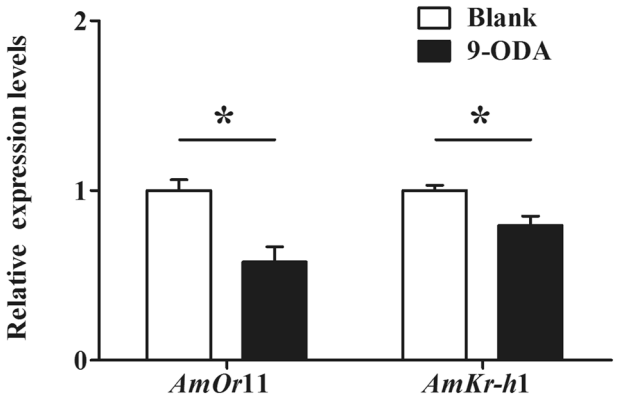

Fig. 1 Effects of 9-ODA on *Or*11 and *Kr-h*1 expression in antennae of 4-day-old *A. mellifera* drones. The open bars represent normalized expression levels. The black bars represent relative expression levels of the 9-ODA tested group. Data are expressed as the mean ± SE, as normalized to a blank sample. Statistical analysis was performed with a *t* test, and "*" indicates a significant difference ($P < 0.05$)

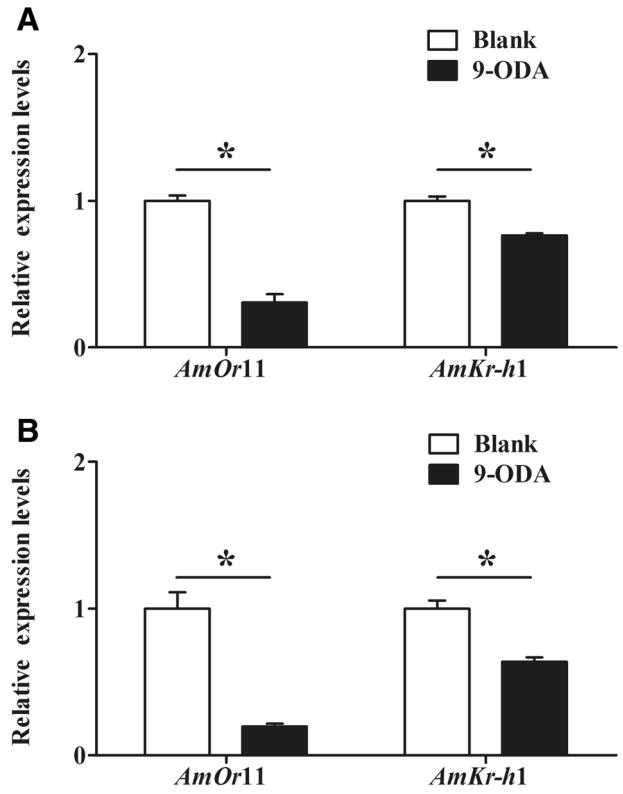

Fig. 2 Effects of 9-ODA on *Or*11 and *Kr-h*1 expression levels in antennae of flying (**a**) and hive (**b**) *A. mellifera* drones at 14 days. The open bars represent normalized expression levels. The black bars represent relative expression levels of the 9-ODA tested group. Data are expressed as the mean ± SE, as normalized to the blank sample. Statistical analysis was performed with a *t* test, and the "*" indicates a significant difference ($P < 0.05$)

Results

Effects of 9-ODA on expression of *AmOr11* and *AmKr-h1* in antennae of 4- and 14-day-old drones

According to qPCR results, sustained exposure to 9-ODA significantly inhibited expression of *AmOr*11 and *AmKr-h*1 in the antennae of 4-day-old drones ($P < 0.05$, Fig. 1). Similarly, acute exposure to 9-ODA significantly repressed expression of *AmOr*11 and *AmKr-h*1 in the antennae of flying and hive drones at 14 days ($P < 0.05$, Fig. 2).

siRNA screen and the effects of injection on the survival of drone pupae

The effectiveness of RNA interference by three siRNAs targeting *Or*11 was compared through a pilot experiment. We found that siRNA-*Or*11-528 downregulated relative expression of *Or*11 in drone antennae at 72 h after injection; in contrast, the expression levels of *Or*11 after the other two siRNA-*Or*11s were injected were similar to those of the blank control group (SI Fig 1). The three siRNA-*Or*11s all repressed *Or*11 expression in drone brains at different stages after injection (SI Fig 2). Therefore, we carried out subsequent RNA interference experiments using siRNA-*Or*11-528.

siRNA-*Or*11, siRNA-NC and DEPC water solutions were injected into the antennae and brain tissues of *A. mellifera* drone pupae, and the survival rate of the drone pupae at 72 h was observed. The survival rates after the injection of siRNA-*Or*11, siRNA-NC and DEPC water solutions into antennae were 72.92 ± 0.02%, 77.08 ± 0.02% and 79.17 ± 0.02%, respectively, with no significant differences ($P > 0.05$, SI Fig 3 A). The survival rates after injection of siRNA-*Or*11, siRNA-NC and DEPC water solutions into brain tissues were 75.00 ± 0.05%, 70.83 ± 0.04% and 70.83 ± 0.02%, respectively, also with no significant differences ($P > 0.05$, SI Fig 3 B).

Effects of siRNA-*Or*11 injection on the expression of *AmOr*11, *AmKr-h*1 and *AmBr-c* in the antennae and brains of drones

We knocked down the *AmOr*11 gene by RNAi to further study its function in vivo. Seventy-two hours after siRNA injection in both antennae and brains, the expression levels of *AmOr*11, *AmKr-h*1 and *AmBr-c* were significantly reduced compared with those of the siRNA-NC-injected and water-injected controls ($P < 0.05$, Fig. 3; $P < 0.05$, Fig. 4). The transcript levels of *AmOr*11, *AmKr-h*1 and *AmBr-c* in the siRNA-NC-injected or water-injected drones remained unchanged.

Discussion

According to the results of this study, 9-ODA inhibits gene expression of *Or*11 and *Kr-h*1 in antennae of 4- (Fig. 1) and 14-day-old (Fig. 2) *A. mellifera* drones. This result is consistent with the responses of the honeybee workers (Claudianos et al. 2014; Grozinger et al. 2007). Wanner et al. (2007) found that *Or*11, a sex pheromone receptor, is a specific receptor for 9-ODA. *AmOr*11 expression was significantly downregulated after honeybee worker was conditioned with 9-ODA in an olfactory learning paradigm, and EAG recordings showed that the neural response of the antenna was similarly reduced after 9-ODA learning (Claudianos et al. 2014). Moreover, 9-ODA also inhibits the expression of transcription factor *AmKr-h*1 in the brains of *A. mellifera*

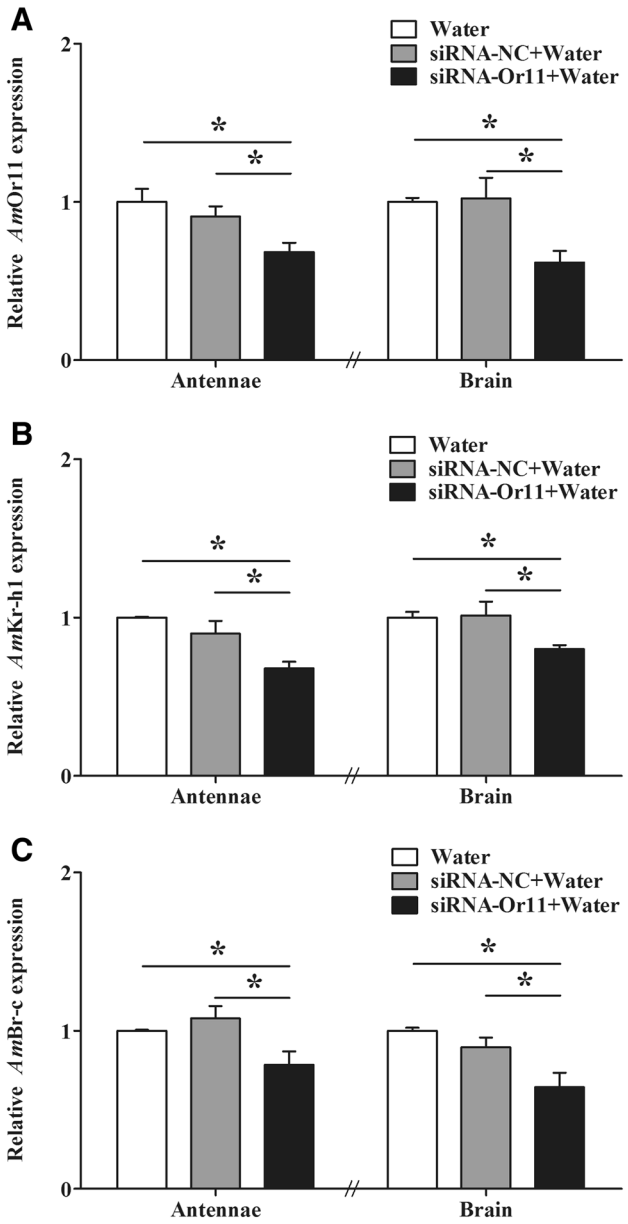

Fig. 3 Effects of injecting antennae with siRNA-*Or*11 on the expression of *AmOr*11 (**a**), *AmKr-h*1 (**b**) and *AmBr-c* (**c**) in the antennae and brains of drones. The open bars represent the group injected with water. The gray bars represent the group injected with siRNA-NC. The black bars represent the group injected with siRNA-*Or*11. Data are expressed as the mean ± SE, as normalized to the water control. Statistical analysis was performed by one-way ANOVA, and the "*" indicates a significant difference ($P < 0.05$)

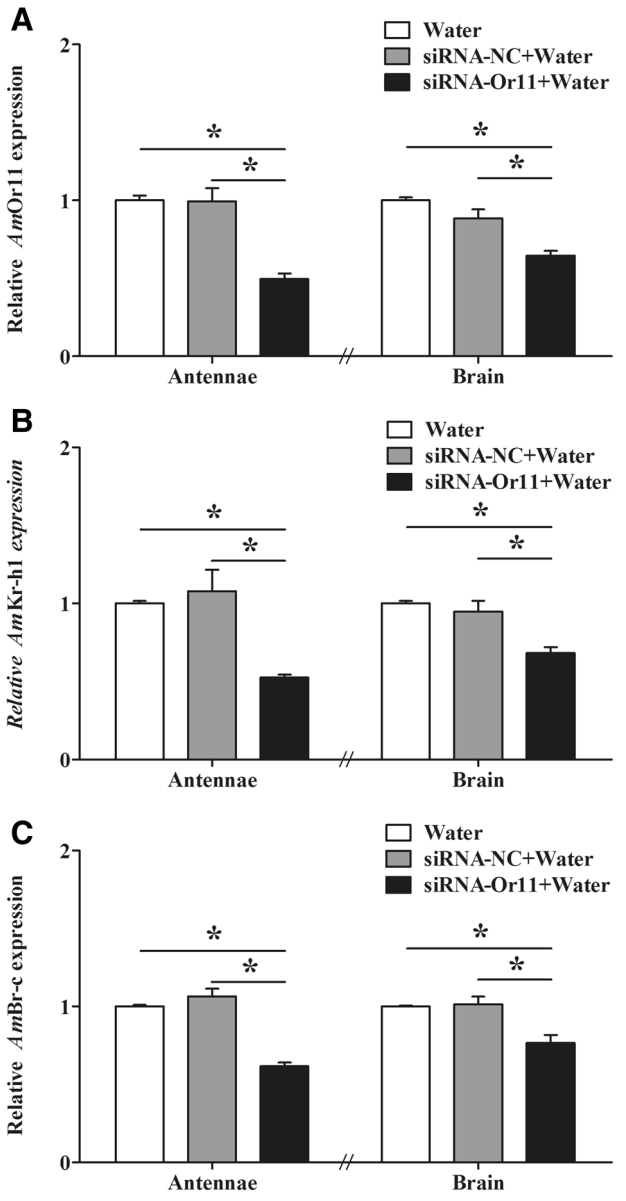

Fig. 4 Effects of injecting the head with siRNA-*Or*11 on the expression of *AmOr*11 (**a**), *AmKr-h*1 (**b**) and *AmBr-c* (**c**) in the antenna and brains of drones. The open bars represent the group injected with water. The gray bars represent the group injected with siRNA-NC. The black bars represent the group injected with siRNA-*Or*11. Data are expressed as the mean ± SE, as normalized to the water control. Statistical analysis was performed by one-way ANOVA, and the "*" indicates a significant difference ($P < 0.05$)

worker bees (Grozinger et al. 2007). These results indicated that *Or*11 (membrane receptor) and *Kr-h*1 (nuclear receptor) were downregulated by 9-ODA both in immature and mature drone, and *Kr-h*1 may be involved in the signaling pathway of responding to 9-ODA in drones.

Relative expression levels of *AmOr*11 and *AmKr-h*1 in the antennae and brains of drones at 72 h after injection with siRNA-*Or*11 into the antennae and head of drone pupae were tested by qPCR. Our results showed that siRNA-*Or*11 significantly downregulated the expression of *AmOr*11 and *AmKr-h*1 in both the antennae (Fig. 3) and brains (Fig. 4) of drones. We preliminarily speculated that *AmOr*11 and *AmKr-h*1 were components of the same signaling pathway responding to 9-ODA. To validate this speculation, we further determined the expression level of *Br–c,* which is the

downstream gene of *Kr-h*1 in *D. melanogaster* and *T. castaneum* (Belles and Santos 2014; Minakuchi et al. 2009, 2008). Our results of RNAi experiments substantiated this hypothesis: siRNA-*Or*11 treatment also inhibited expression of *AmBr-c* in the antennae (Fig. 3) and brains (Fig. 4) of drones. *Br–c* plays roles in the regulation of metamorphosis in insects by JH and 20E (Abdou et al. 2011; Paul et al. 2006). *Br–c* expression was downregulated by knocking down of *Kr-h*1 in *Blattella germanica* at the nymph stage (Huang et al. 2013). During the pupal stage of *T. castaneum*, exogenous JH analogs mediated upregulation of *Kr-h*1 and induced transcription of *Br–c* (Minakuchi et al. 2009). Therefore, this result further confirmed our speculation that *Kr-h*1 is the downstream gene of *Or*11. Studies have showed that the expression level of *Kr-h*1 in the brains of foraging workers is significantly higher than that in the brains of juvenile workers and brood workers in hives, suggesting that *Kr-h*1 participates in regulating the foraging behavior of workers (Grozinger and Robinson 2007; Whitfield et al. 2003). DNA methylation regulation of *Kr-h*1 plays an important role in the regulatory gene network of ovary activation in honeybee workers (Kilaso et al. 2017). It is worth noting that whether the signal pathway responding to 9-ODA is the same between male and female honeybees needs further exploration.

In conclusion, our results indicate that *Kr-h*1 is a downstream gene of *Or*11, and is negatively regulated by 9-ODA. It suggests that *Kr-h*1 is a key player in the response of honeybee drones to sex pheromone 9-ODA. This study provides a new insight into the molecular mechanisms of the mating flight of drones.

Acknowledgements We thank Frederick Partridge and anonymous reviewers for their comments that improved this manuscript. This work was supported by the National Natural Science Foundation of China (31572469) and the Earmarked Fund for the China Agricultural Research System (CARS-44-KXJ15).

Author contributions Conceived and designed the experiments: ZJZ. Performed the experiments: JFL. Analyzed the data: JFL XJW. Contributed reagents/materials/analysis tools: ZLW XJH. Wrote the paper: JFL XJW.

Funding This work was supported by the National Natural Science Foundation of China (31572469) and the Earmarked Fund for the China Agricultural Research System (CARS-44-KXJ15).

Data availability All data and material are fully available without restriction.

Compliance with ethical standards

Conflicts of interest No conflict of interest exits in the submission of this manuscript. The authors have declared that no competing interests exist.

Ethics approval All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Jiangxi Agricultural University (JXAULL-2017002).

Consent to participate All authors have consented to participate.

Consent for publication All authors have consented for publication.

References

Abdou MA, He Q, Wen D et al (2011) *Drosophila* Met and Gce are partially redundant in transducing juvenile hormone action. Insect Biochem Mol Biol 41:938–945. https://doi.org/10.1016/j.ibmb.2011.09.003

Belles X, Santos CG (2014) The MEKRE93 (Methoprene tolerant-Krüppel homolog 1–E93) pathway in the regulation of insect metamorphosis, and the homology of the pupal stage. Insect Biochem Mol Biol 52:60–68. https://doi.org/10.1016/j.ibmb.2014.06.009

Beye M, Hartel S, Hagen A et al (2002) Specific developmental gene silencing in the honey bee using a homeobox motif. Insect Mol Biol 11:527–532. https://doi.org/10.1046/j.1365-2583.2002.00361.x

Brockmann A, Brückner D, Crewe RM (1998) The EAG response spectra of workers and drones to queen honeybee mandibular gland components: the evolution of a social signal. Naturwissenschaften 85:283–285. https://doi.org/10.1007/s001140050500

Brockmann A, Dietz D, Spaethe J, Tautz J (2006) Beyond 9-ODA: SEX pheromone communication in the European honey bee *Apis mellifera* L. J Chem Ecol 32:657–667. https://doi.org/10.1007/s10886-005-9027-2

Butler CG (1971) The mating behaviour of the honeybee (*Apis mellifera* L.). J Entomol Ser A, Gen Entomol 46:1–11. https://doi.org/10.1111/j.1365-3032.1971.tb00103.x

Butler CG, Callow RK, Johnston NC (1962) The isolation and synthesis of queen substance, 9-oxodec-trans-2-enoic acid, a honeybee pheromone. Proceed Royal Soc B: Biol Sci 155:417–432. https://doi.org/10.1098/rspb.1962.0009

Butler CG, Callow RK, Johnston NC (1959) Extraction and purification of "queen substance" from queen bees. Nature 184:1871–1871. https://doi.org/10.1038/1841871a0

Carlisle DB, Butler CG (1956) The "queen-substance" of honeybees and the ovary-inhibiting hormone of crustaceans. Nature 178:323

Claudianos C, Lim J, Young M et al (2014) Odor memories regulate olfactory receptor expression in the sensory periphery. Eur J Neurosci 39:1642–1654. https://doi.org/10.1111/ejn.12539

Erezyilmaz DF, Riddiford LM, Truman JW (2006) The pupal specifier broad directs progressive morphogenesis in a direct-developing insect. Proc Natl Acad Sci USA 103:6925–6930. https://doi.org/10.1073/pnas.0509983103

Gary NE (1962) Chemical mating attractants in the queen honey bee. Science 136:773–774. https://doi.org/10.1126/science.136.3518.773

Gary NE, Marston J (1971) Mating behaviour of drone honey bees with queen models (*Apis mellifera* L.). Anim Behav 19:299–304. https://doi.org/10.1016/S0003-3472(71)80010-6

Grozinger CM, Fischer P, Hampton JE (2007) Uncoupling primer and releaser responses to pheromone in honey bees. Naturwissenschaften 94:375–379. https://doi.org/10.1007/s00114-006-0197-8

Grozinger CM, Robinson GE (2007) Endocrine modulation of a pheromone-responsive gene in the honey bee brain. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 193:461–470. https://doi.org/10.1007/s00359-006-0202-x

Grozinger CM, Sharabash NM, Whitfield CW, Robinson GE (2003) Pheromone-mediated gene expression in the honey bee brain. Proc Natl Acad Sci 100:14519–14525. https://doi.org/10.1073/pnas.2335884100

Guo X, Wang Y, Sinakevitch I et al (2018) Comparison of RNAi knockdown effect of tyramine receptor 1 induced by dsRNA and siRNA in brains of the honey bee, *Apis mellifera*. J Insect Physiol 111:47–52. https://doi.org/10.1016/j.jinsphys.2018.10.005

Harwood G, Amdam G, Freitak D (2019) The role of Vitellogenin in the transfer of immune elicitors from gut to hypopharyngeal glands in honey bees (*Apis mellifera*). J Insect Physiol 112:90–100. https://doi.org/10.1016/j.jinsphys.2018.12.006

Huang JH, Lozano J, Belles X (2013) Broad-complex functions in postembryonic development of the cockroach *Blattella germanica* shed new light on the evolution of insect metamorphosis. Biochim Biophys Acta Gen Subj 1830:2178–2187. https://doi.org/10.1016/j.bbagen.2012.09.025

Kaatz HH, Hildebrandt H, Engels W (1992) Primer effect of queen pheromone on juvenile hormone biosynthesis in adult worker honey bees. J Comp Physiol B 162:588–592. https://doi.org/10.1007/BF00296638

Keeling CI, Slessor KN, HigoWinston HaML (2003) New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone. Proc Natl Acad Sci USA 100:4486–4491. https://doi.org/10.1073/pnas.0836984100

Kilaso M, Remnant EJ, Chapman NC et al (2017) DNA methylation of Kr-h1 is involved in regulating ovary activation in worker honeybees (*Apis mellifera*). Insectes Soc 64:87–94. https://doi.org/10.1007/s00040-016-0518-7

Liu JF, Yang L, Li M et al (2019) Cloning and expression pattern of odorant receptor 11 in Asian honeybee drones, *Apis cerana* (Hymenoptera, Apidae). J Asia-Pac Entomol 22:110–116. https://doi.org/10.1016/j.aspen.2018.12.014

Loper GM, Wolf WW, Taylor OR (1993) Radar detection of drones responding to honeybee queen pheromone. J Chem Ecol 19:1929–1938. https://doi.org/10.1007/BF00983797

Lourenço AP, Mackert A, Cristino ADS, Simões ZLP (2008) Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. Apidologie 39:372–385. https://doi.org/10.1051/apido:2008015

Martins JR, Nunes FMF, Cristino AS et al (2010) The four hexamerin genes in the honey bee: structure, molecular evolution and function deduced from expression patterns in queens, workers and drones. BMC Mol Biol 11:23. https://doi.org/10.1186/1471-2199-11-23

Minakuchi C, Namiki T, Shinoda T (2009) Krüppel homolog 1, an early juvenile hormone-response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle *Tribolium castaneum*. Dev Biol 325:341–350. https://doi.org/10.1016/j.ydbio.2008.10.016

Minakuchi C, Zhou X, Riddiford LM (2008) Krüppel homolog 1 (Kr-h1) mediates juvenile hormone action during metamorphosis of *Drosophila melanogaster*. Mech Dev 125:91–105. https://doi.org/10.1016/j.mod.2007.10.002

Nunes FMF, Simões ZLP (2009) A non-invasive method for silencing gene transcription in honeybees maintained under natural conditions. Insect Biochem Mol Biol 39:157–160. https://doi.org/10.1016/j.ibmb.2008.10.011

Pankiw T, Huang Z, Winston M, Robinson G (1998) Queen mandibular gland pheromone influences worker honey bee (*Apis mellifera* L.) foraging ontogeny and juvenile hormone titers. J Insect Physiol 44:685–692. https://doi.org/10.1016/S0022-1910(98)00040-7

Pankiw T, Page RE (2003) Effect of pheromones, hormones, and handling on sucrose response thresholds of honey bees (*Apis mellifera* L.). J Comp Physiol A 189:675–684. https://doi.org/10.1007/s00359-003-0442-y

Paul RK, Takeuchi H, Kubo T (2006) Expression of two ecdysteroid-regulated genes, broad-complex and E75, in the brain and ovary of the honeybee (A*pis mellifera* L.). Zoolog Sci 23:1085–1092. https://doi.org/10.2108/zsj.23.1085

Plettner E, Otis GW, Wimalaratne PDC et al (1997) Species- and caste-determined mandibular gland signals in honeybees (*Apis*). J Chem Ecol 23:363–377. https://doi.org/10.1023/B:JOEC.0000006365.20996.a2

Rangel J, Böröczky K, Schal C, Tarpy DR (2016) Honey bee (*Apis mellifera*) queen reproductive potential affects queen mandibular gland pheromone composition and worker retinue response. PLoS ONE 11:1–16. https://doi.org/10.1371/journal.pone.0156027

Sandoz J-C (2006) Odour-evoked responses to queen pheromone components and to plant odours using optical imaging in the antennal lobe of the honey bee drone *Apis mellifera* L. J Exp Biol 209:3587–3598. https://doi.org/10.1242/jeb.02423

Sandoz J-C, Deisig N, de Brito Sanchez MG, Giurfa M (2007) Understanding the logics of pheromone processing in the honeybee brain: from labeled-lines to across-fiber patterns. Front Behav Neurosci 1:5. https://doi.org/10.3389/neuro.08.005.2007

Scharlaken B, de Graaf DC, Goossens K et al (2008) Reference gene selection for insect expression studies using quantitative real-time PCR: the head of the honeybee, *Apis mellifera*, after a bacterial challenge. J Insect Sci 8:1–10. https://doi.org/10.1673/031.008.3301

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108. https://doi.org/10.1038/nprot.2008.73

Schuh R, Aicher W, Gaul U et al (1986) A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Krüppel, a *Drosophila* segmentation gene. Cell 47:1025–1032. https://doi.org/10.1016/0092-8674(86)90817-2

Slessor KN, Winston ML, Le Conte Y (2005) Pheromone communication in the honeybee (*Apis mellifera* L.). J Chem Ecol 31:2731–2745. https://doi.org/10.1007/s10886-005-7623-9

Villar G, Baker TC, Patch HM, Grozinger CM (2015) Neurophysiological mechanisms underlying sex- and maturation-related variation in pheromone responses in honey bees (*Apis mellifera*). J Comp Physiol A 201:731–739. https://doi.org/10.1007/s00359-015-1006-7

Villar G, Grozinger CM (2017) Primer effects of the honeybee, *Apis mellifera*, queen pheromone 9-ODA on drones. Anim Behav 127:271–279. https://doi.org/10.1016/j.anbehav.2017.03.023

Wang Y, Baker N, Amdam GV (2013) RNAi-mediated double gene knockdown and gustatory perception measurement in honey bees (*Apis mellifera*). J Vis Exp 77:1–9. https://doi.org/10.3791/50446

Wanner KW, Nichols AS, Walden KKO et al (2007) A honey bee odorant receptor for the queen substance 9-oxo-2-decenoic acid. Proc Natl Acad Sci USA 104:14383–14388. https://doi.org/10.1073/pnas.0705459104

Whitfield CW, Cziko A-M, Robinson GE (2003) Gene expression profiles in the brain predict behavior in individual honey bees. Science 302:296–299. https://doi.org/10.1126/science.1086807