Expression patterns of four candidate sex pheromone receptors in honeybee drones (*Apis mellifera*)

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In honeybee (*Apis mellifera*), odorant receptors (Ors) play a crucial role in special recognition of sex pheromones in honeybee mating activities. Four candidate sex pheromone Ors (*Am*Or10, *Am*Or11, *Am*Or18 and *Am*Or170) have been identified and found to be preferentially expressed in drone antennae. However, few studies have investigated the regulation of these four drone Ors on drone mating behaviour. This study characterised the expression patterns of these Ors across different sexual developmental stages (immature and sexually mature) and different physiological statuses (flying and crawling), using both the antennae and brains of drones. qRT-PCR results indicated that the expression of four Ors were not significantly different in drone antennae between flying and crawling statuses at immature stage. However, all four Ors expression levels in brains of flying drones were significantly higher than those of crawling drones at mature stage. Moreover, only the expression level of Or170 was significantly higher in antennae of mature flying drones than crawling ones. Therefore, this study demonstrated a link between four candidate sex pheromone Ors transcriptional expression in the brains of honeybee drones and behaviour associated with sexual maturity and mating flight. In addition, Or170 might be involved in the maturation of honeybee drones' olfactory system, and in the organisation of odour-mediated mating behaviours.

Key words: *Apis mellifera*, odorant receptors, drones, mating flight, sexual development.

INTRODUCTION

In insects, odorant receptors (Ors) play important roles in environmental odours recognition and social communication, being critical for feeding, oviposition, predator avoidance and mate recognition (Leary *et al*. 2012; Lebreton *et al*. 2017; Sakurai *et al*. 2011). Olfaction is mediated by the interaction of volatile ligands with a set of specialised membrane proteins in olfactory sensory neurons (OSNs) of their antennae (Dweck *et al*. 2015; Smart *et al*. 2008; van der Goes van Naters & Carlson 2007). Odorant molecule bound by these receptors results in OSNs depolarisation and produces a neuronal signal that is decoded by the insect brains, informing the decisions of behavioural responses (Carraher *et al*. 2015).

Honeybees (*Apis mellifer*a) originated in Africa and expanded into Eurasia and New World, it mainly includes African honeybees and European honeybees (Whitfield *et al*. 2006). Honeybees display a striking copulate behaviour in the air. During the mating seasons, sexually mature males gather in the air and form drone congregations area (DCA), searching for the virgin queens. Virgin queens fly to this DCA and release queen mating pheromones to attract males (Koeniger *et al*. 1989; Koeniger 1986; Koeniger *et al*. 2005; Koeniger *et al*. 1989). Therefore, the drone's mating behaviour is triggered by queen sex pheromones such as queen mandibular pheromone (QMP), highly relying on their Ors in the olfactory system (Brockmann & Brückner 2001; Gries & Koeniger 1996; Wanner *et al*. 2007). From the genome point of view, the honeybee *Apis mellifera* and *Apis cerana* encode 170 and 119 Ors respectively (Park *et al*. 2015; Robertson & Wanner 2006). Moreover, Wu *et al*. (2016) revealed that 16 Ors were up-regulated in the sexually matured drones of *A. mellifera* by using high-throughput RNA-Seq.

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Received 4 May 2019. Accepted 9 October 2019

ISSN 1021-3589 [Print]; 2224-8854 [Online]
DOI: https://doi.org/10.4001/003.028.0262

African Entomology **28**(2): 262–268 (2020)

Mounting evidence revealed that sex pheromone receptors play important roles in drones' physiological behaviours. Four candidate sex pheromone Ors (*Am*Or10, *Am*Or11, *Am*Or18 and *Am*Or170) have been identified from the honeybee genome based on their biased expression in male bee antennae. Particularly, *Am*Or11 can specifically bind 9-oxo-2-decenoic acid (9-ODA), which is one of the predominantly detected compounds of QMP (Wanner *et al*. 2007). A subsequent investigation reported that the expression level of *Am*Or11 was higher in the antennae of sexually mature drones than immature drones (Villar *et al*. 2015). Furthermore, our previous study showed that the *Ac*Or11 expression pattern in brains was dramatically higher in flying mature drones than crawling ones, suggesting that Or11 is associated with sexual maturity and mating behaviour (Liu *et al*. 2019). Despite the importance of candidate sex pheromone Ors in honeybees, their biological functions have not been deeply studied especially those Ors in drones at different behavioural statuses and mature stages.

In this study, we detected and compared the expression patterns of four sex pheromone receptors (*Am*Or10, *Am*Or11, *Am*Or18 and *Am*Or170) during sexual developmental stages (immature and sexually mature) and different physiological statuses (flying and crawling) in antennae and brains of drones. The results allow further exploration of the biological functions of these Ors in drone copulation behaviour and eventually to provide the basis for elucidating the regulation mechanism of honeybee mating flights.

MATERIAL AND METHODS

Honeybee

Three honeybee (*A. mellifera*) colonies from the Honeybee Research Institute of Jiangxi Agricultural University (28.46°N 115.49°E) were used for all experiments. These colonies were maintained using standard keeping practices. Three healthy mated queens were caged to lay in the empty drone frames for 24 h, and then these frames with drone eggs were removed into queenless area (the top box of the hive) in colonies. The sealed frames were moved into a dark incubator at 34 °C and 50 % relative humidity until the day prior to adult emergence. The sampling methods were modified from Villar *et al*. (2015). After drones emerged, they were paint-marked on their thorax and placed back into their natal colonies. We collected 10 crawling drones (inside the hive) and 10 flying drones (return back to the hive) from each colony at two age points of day 4 (sexually immature) and day 14 (sexually mature), respectively. All bees were snap-frozen in liquid N_2 until processed. Ten pairs of drone antennae or brains were collected from each group (total four groups: 4-day flying and crawling groups, and 14-day flying and crawling groups) for RNA extraction. Each group had three biological replicates from three honeybee colonies.

Gene expression quantification and statistical analysis

Total RNA was isolated from the antennae and brains of drones using TransZol reagent (Transgen Biotech, www.transgen.com.cn) according to the manufacturer's instructions, and stored in a freezer at –80 °C until use. The cDNA was synthesised from the total RNA isolated from antennae using the Primer-Script RT reagent Kit (TaKaRa, www.takara-bio.com) according to the manufacturer's instructions. Then, the expression levels of these *Am*Ors (*Am*Or10, *Am*Or11, *Am*Or18 and *Am*Or170) were determined using quantitative Real-Time PCR and normalised to the β-actin gene. The primers for these *Am*Ors and *Am*-β-actin were designed by the primer premier 5.0 software (Table 1). Finally, the relative expression levels of these *Am*Ors mRNAs were calculated using the $2^{-\Delta\Delta t}$ comparative CT method.

Statistical analysis

Differences in the relative expression of the *Am*Ors genes were determined using a *t*-tests in SPSS 17.0 (IBM, Armonk, NY, U.S.A.). Values of $P < 0.05$ were considered significant in all treatments.

RESULTS

In antennae, the expression levels of four *Am*Ors were not significantly different between 4-day-old flying and crawling drones (Or10: $t = –1.832$, d.f. = 4, $P = 0.208$; Or11: $t = –1.052$, d.f. = 4, $P = 0.352$; Or18: $t = –2.044$, d.f. = 4, $P = 0.110$; Or170: $t = –0.202$, d.f. = 4, $P = 0.850$; Fig. 1a). The expression levels of three genes (*Am*Or10, *Am*Or11 and *Am*Or18) also were not different in mature drones from flying/crawling comparisons (Or10: $t = 2.689$, d.f. = 4, $P = 0.055$; Or11: $t = 1.155$, d.f. = 4, $P = 0.313$; Or18: $t = 0.251$, d.f. = 4, $P = 0.814$; Fig. 1b).

Table 1. Primers used to *Am*Ors and β-actin gene for qRT-PCR.

Primer names	Primer sequences	GenBank accession
*Am*Or10- *Am*Or10-R	5′-CCGCATCTGAACAGTATCGTG-3′ 5′-ATTCTCCTCCGTGGCTATCG-3′	NM_001242961.2
*Am*Or11- *Am*Or11-R	5′-ATGTGCGGTTTGCTGAAGA-3′ 5′-CGAGAAGGTGCCAATGACG-3′	NM_001242962.1
*Am*Or18 *Am*Or18-R	5′-TTTTATTACATCGCTTTGCC-3′ 5′-TCTTCCTTCCATCCACCA-3′	XM_003250678.4
*Am*Or170-F *Am*Or170-R	5′-CCAGTGTTGCCTCGCTC-3′ 5′-TTTCGTTATCTCACGCTCC-3′	NM_001242993.1
Am-β-actin-F *Am*-β-actin-R	5′-GGTATTGTATTGGATTCGGGTG-3′ 5′-TGCCATTTCCTGTTCAAAGTCA-3′	NM_001185146.1

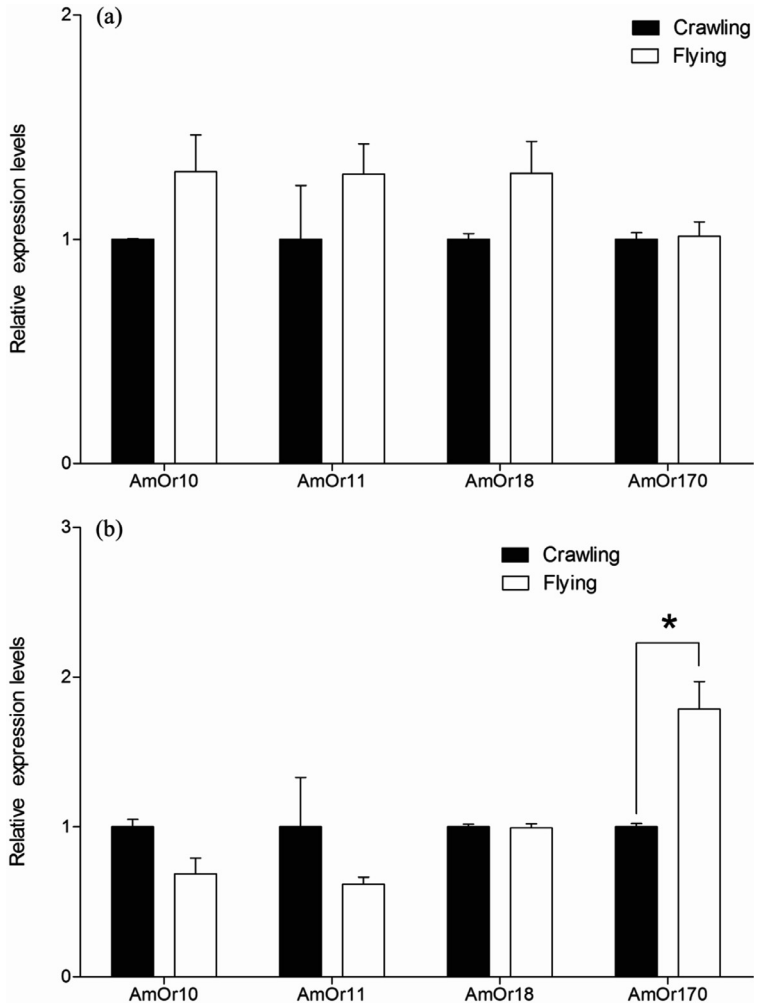

Fig. 1. Four candidate sex pheromone receptor gene expression patterns in antennae of immature (**a**) and mature (**b**) drones ($n = 3$ pools). The black bars represent the normalised expression level of crawling drones. The open bars represent the relative expression of flying drones. The data are expressed as mean ± S.E., and * indicates a significant difference ($P < 0.05$).

However, the expression level of *Am*Or170 in antennae was significantly higher in 4-day-old flying drones than those of crawling ones ($t = –4.211$, d.f. = 4, $P = 0.014$, Fig. 1b).

In brains, the expression levels of three genes (*Am*Or11, *Am*Or18 and *Am*Or170) were not different in mature drones by compared flying with crawling drones (Or11: $t = –2.004$, d.f. = 4, $P = 0.116$; Or18: $t = 0.860$, d.f. = 4, $P = 0.479$; Or170: $t = 0.813$, d.f. = 4, $P = 0.462$, Fig. 2a). But, the expression level of *Am*Or10 in brains of 4-day-old flying drones was significantly higher than those of crawling drones ($t = –4.397$, d.f. = 4, $P = 0.048$, Fig. 2a). Moreover, the expression levels of four *Am*Ors in mature drone brains in flying status were significantly higher than those in crawling status (Or10: $t = –4.520$, d.f. = 4, $P = 0.045$; Or11: $t = –3.433$, d.f. = 4, $P = 0.026$; Or18: $t = –6.026$, d.f. = 4, $P = 0.026$; Or170: $t = –4.833$, d.f. = 4, $P = 0.008$, Fig. 2b).

DISCUSSION

In honeybees, odorant receptors which are expressed in male neurons are critical for detecting the queen sex pheromones released during mating flights (Park *et al*. 2015; Robertson & Wanner 2006). In this study, we documented the

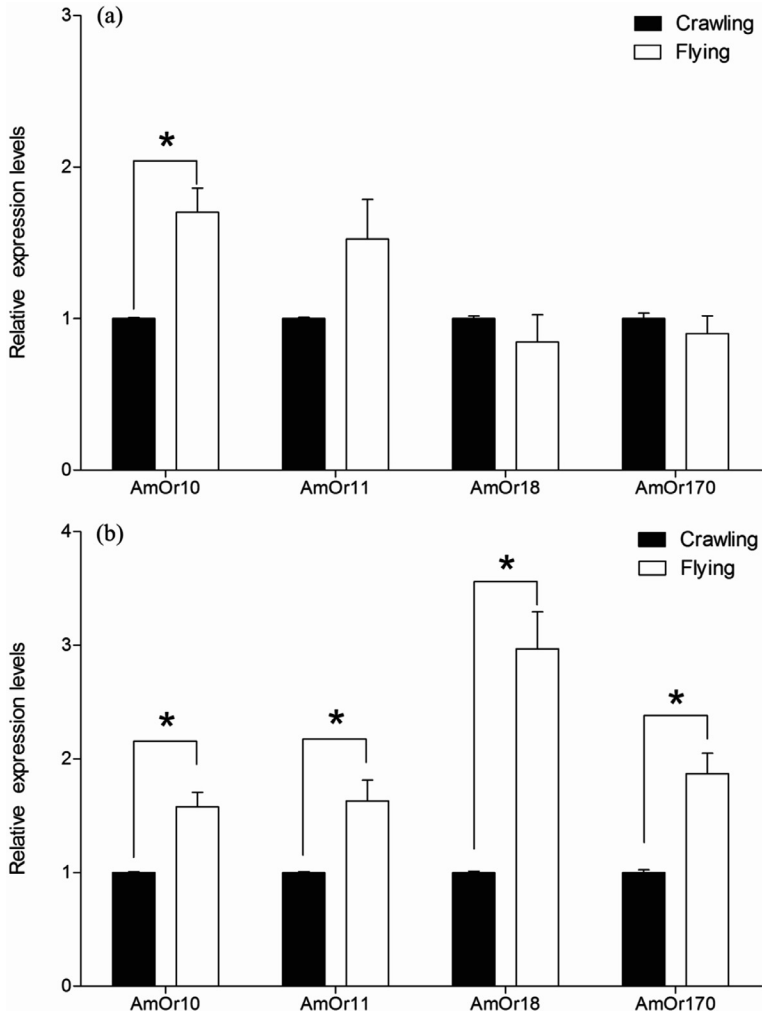

Fig. 2. Four candidate sex pheromone receptor gene expression patterns in brains of immature (**a**) and mature (**b**) drones ($n = 3$ pools). The black bars represent the normalised expression level of crawling drones. The open bars represent the relative expression of flying drones. The data are expressed as mean ± S.E., and * indicates a significant difference ($P < 0.05$).

expression patterns of four candidate sex pheromone receptor genes (*Am*Or10, *Am*Or11, *Am*Or18 and *Am*Or170) in drone brains and antennae under different sexual developmental stages and physiological statuses.

It was observed that the expression patterns of four *Am*Ors in antennae have no difference between flying and crawling immature drones (Fig. 1a). Many insects utilise floral scents and other chemical signals for orientation at immature stage (Phelan & Baker 1987; Hern & Dorn 1999; Anton *et al*. 2007). Therefore, the flights of sexually immature drones (less than 12 days) are generally orientation flights rather than mating flights, which is also consistent with a previous study (Graham 2015). Therefore, these results suggested that the biological foundation of these odorant receptors related to copulation may be underdeveloped in antennae of drones at the juvenile stage. The expression levels of three genes (*Am*Or10, *Am*Or11 and *Am*Or18) from all flying/crawling comparisons were not significantly different in antennae of mature drones (Fig. 1b). Interestingly, only the expression of *Am*Or170 was dramatically higher in antennae of mature flying drones than the mature crawling ones, though there was no significant difference in the immature drone comparison. Presumably because *Am*Or170 gene is sensitive to physiological variation and employed by mature drones to detect environmental scents for mating flights. A similar result is reported in male *Drosophila melanogaster* that the expression level of sex pheromone receptor Or67d also is highly correlated to the physiological variation (Kurtovic *et al*. 2007; Zhou *et al*. 2009).

In addition, the physiological status of honeybee drones strongly affected their Ors expression in brains at the mature stage (Fig. 2b). In many insects, a number of olfactory neurons expressed sex pheromone receptors in order to increase sensitivity to respond to pheromone in the antennae lobe (the first level of olfactory processing), which can effectively transfer odour molecules to the sensory receptors distributed on the dendritic of olfaction receptor neuron (Graham 2015). Particularly, *Am*Or11 was the most highly expressed of the four candidate sex pheromone receptors and specially responded to 9-ODA, the major QMP component. When the antennae of drones were specifically stimulated with 9-ODA, the enlarged macroglomerulus 2 (MG2, the largest of the four macroglomeruli) in the drone antennae lobe was activated, suggesting that olfactory neurons projecting to MG2 express *Am*Or11 in drone brain (Sandoz 2006; Sandoz *et al*. 2007). Moreover, these results are similar to those of previous studies on Asian honeybees that *Ac*Or10 and *Ac*Or11were significantly higher expressed in the brains of mature flying drones than those of crawling drones (Liu *et al*. 2019; Yang *et al*. 2018). This study indicated that there were not only Or10 and Or11 taking part in the daily mating flights of mature drones, but also Or18 and Or170 possibly perform as important roles in improving the mating behaviour of honeybee drones. Although we preliminary observed the characteristic of four Or genes, the molecular mechanism of how Ors play a role in the sophisticated mating behaviour of honeybees is still obscure. This requires further investigations in future work.

In summary, the expression patterns of candidate sex pheromone receptor genes (*Am*Or10, *Am*Or11, *Am*Or18 and *Am*Or170) in the antennae and brains of *A. mellifera* drones were analysed at different stages of sexual maturity and physiological statuses. These Ors expression patterns in drone brains were closely correlated with both sexual development and physiological status, suggesting that they were involved in drone sexual maturity and mating behaviour. Our results provide an insight into the molecular basis underlying mating flights of honeybee drones.

ACKNOWLEDGEMENTS

All authors read and approved the final manuscript. This work is supported by the National Natural Science Foundation of China (31572469), and the Earmarked Fund for China Agriculture Research System (CARS-44-KXJ15).

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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