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Dissecting sex pheromone communication of *Mythimna separata* (Walker) in North China from receptor molecules and antennal lobes to behavior

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ABSTRACT

The Oriental armyworm, *Mythimna separata*, has been described to emit geographic population-specific sex pheromones, with either *Z*11-16:Ald or *Z*11-16:Ac as the major component. Using a comprehensive set of electrophysiological, behavioral, and genetic analyses, we study the sex pheromone communication of *M. separata* in North China from pheromone receptors and antennal lobe to behavior. GC-EAD results show that *Z*11-16:Ald is the only compound eliciting electrophysiological responses in pheromone gland extracts. Further *in vivo* optical imaging studies reveal that *Z*11-16:Ald activates the cumulus of the MGC and show dose-dependent responses. The wind tunnel tests demonstrate that *Z*11-16:Ald alone is sufficient to induce the entire sequence of male sexual behaviors. Transcriptome and q-PCR results show that *MsepOR3* is specifically and abundantly expressed in male antennae. By using the *Xenopus* oocytes and two-electrode voltage-clamp recording, we finally validate that the oocytes expressing *MsepOR3/ORco* gave dose dependent responses to *Z*11-16:Ald. We suggest single *Z*11-16:Ald could be used for monitoring the population of *M. separata* in North China.

1. Introduction

Moths have developed delicate and efficient sex pheromone communication systems, and female adults generally release a sex pheromone to attract males (Löfstedt, 1990). The sex pheromones of most moth species usually consist of multiple components in specific ratios, and plays substantial roles in intraspecific sexual communication and interspecific reproductive isolation (Roelofs et al., 1987; Leary et al., 2012). Sex pheromones have been used to monitor and control pest population dynamics by targeting the functional specificity and sensitivity of male responses (Witzgall et al., 2010).

Sex pheromone molecules released by females are detected by specialized olfactory receptor neurons (ORNs) housed in long trichoid sensilla on the antennae of male moths (Kaissling et al., 1986). The ability of ORNs is mainly given by pheromone receptors (PRs), which belong to a subclass of insect odorant receptors (ORs) (Krieger et al., 2004; Leal, 2013; Nakagawa et al., 2005). Unlike most broad tuning ORs, PRs are narrowly tuned to specific pheromone components (Fleischer et al., 2018; de Fouchier et al., 2017; Grosse-Wilde et al., 2007; Miura et al., 2010). Sex pheromone signals are transmitted through the ORN axons to the macroglomerular complex (MGC), which is an enlarged glomerular neuropil in antennal lobes (ALs) (Berg et al., 1998; Christensen et al., 1995; Hansson et al., 1991, 1992, 2011). The ORNs sharing the same PRs project to a subunit (glomerulus) of MGC, one for each PR type (Sakurai et al., 2011). After integration of olfactory information in the higher brain centers, including the lateral horns and mushroom bodies, male moths exhibit strong searching behaviors for the sex pheromone source (Sakurai et al., 2014).

The Oriental armyworm, *Mythimna separata* (Walker) (also called *Pseudaletia separata*) (Lepidoptera: Noctuidae) is a major agricultural pest of several cereal crops, such as maize, rice, millet, and wheat in China, Japan, South-east Asia, India, eastern Australia, New Zealand, and some Pacific islands, and annually causes substantial grain losses (Jiang et al., 2007; Sharma and Davies, 1983; Sharma and Youm, 1999; Zou, 1956). It seasonally migrates long distances in the eastern China (Li et al., 1964; Zhao et al., 2009; Jiang et al., 2011). The sex pheromone can be employed to monitor its activity and obtain detection, phenology, and relative density information. The *M. separata* sex pheromone was reported in several previous studies (Fónagy et al., 2011; Kou et al., 1992; Lebedeva et al., 2000; Takahashi et al., 1979; Zhu et al., 1987); however, the composition and proportion of the blend differ among populations. The sex pheromone blends identified from

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different geographic populations of *M. separata* show great differences and can be roughly grouped into two clusters, one uses (*Z*)-11-hexadecenyl acetate (*Z*11-16:Ac) as the major component (Fónagy et al., 2011; Kou et al., 1992; Takahashi et al., 1979), and another uses (*Z*)-11-hexadecenal (*Z*11-16:Ald) as the major component (Lebedeva et al., 2000; Zhu et al., 1987).

In this study, we introduced an integrative approach to study the sex pheromone communication of *M. separata* in North China from pheromone receptors and antennal lobes to behavior. Firstly, we examined the potential pheromone components in the pheromone gland extracts by using the gas chromatography-electroantennograhic detection (GC-EAD) and gas chromatography coupled with mass spectrometry (GC-MS). Next, we identified the glomeruli in male antennal lobes responding to these compounds by *in vivo* optical imaging and three-dimensional modelling. Thirdly, we investigated behavioral responses of males to a series of blends of these compounds in a wind tunnel, and uncovered that the single component *Z*11-16:Ald was substantial to attract males. Fourthly, we identified six putative PR transcripts and studied their expression levels in *M. separata* antennae by transcriptome sequencing and q-PCR. Finally, we validated that a most highly expressed PR in male antennae was tuned to *Z*11-16:Ald by using the *Xenopus* oocyte expression system and two-electrode voltage-clamp recording.

2. Materials and methods

2.1. Insects

Mythimna separata larvae were collected in the wheat cultivated field in Baoding, Hebei Province, China (N38.9°, E115.5°). Larvae were reared with an artificial diet in the laboratory at 25 ± 1 °C, 20–40% RH and a photoperiod of 16:8 h L:D. The artificial diet contains corn leave powder 100 g, wheat germ flour 40 g, yeast powder 34 g, sorbic acid 2 g, methyl-p-hydroxybenzoate 4 g, L-ascorbic acid 2.5 g, linoleic acid 0.2 g and agar 18 g. After pupating, pupae were distinguished by sex and kept in separated cages for emergence. Adults were fed with 10% honey water.

2.2. Chemicals

(*Z*)-11-hexadecenal (*Z*11-16:Ald), (*Z*)-11-hexadecenyl acetate (*Z*11-16:Ac), (*Z*)-11-hexadencenol (*Z*11-16:OH), hexadecenal (16:Ald), (*Z*)-9-hexadecenal (*Z*9-16:Ald), (*Z*)-9-hexadecenol (*Z*9-16:OH), (*Z*)-9-hexadecenyl acetate (*Z*9-16:Ac), and (*Z*)-9-tetradecenal (*Z*9-14:Ald) were purchased from Shin-Etsu Chemical (Tokyo, Japan) with purity level all ≥ 95%.

2.3. Observations on the pheromone gland and calling behavior of females

To observe the pheromone gland under the microscope, we used a forcep to squeeze the abdominal tip of a female. It is also observable naturally when a female moth showing the calling behavior by fanning wings and stretching out the pheromone gland and ovipositor. Twenty newly emerged female moths were collected in a mesh cage (30 cm × 30 cm × 30 cm). During 0–8 h dark period at 25 ± 1 °C from day 1 to day 6 after emergence, the females showing the calling behavior were recorded under red light lit diffusely from the top at 10 lux. Four replicates were run and different batches of insects were used.

2.4. Extraction of sex pheromone

Pheromone gland extracts were obtained from 4 day old virgin females during 4–8 h dark period. When female calling behavior occurred, the pheromone gland-ovipositor complex was excised by micro-scissors. A collection of 25 pheromone gland-ovipositor complexes was immersed with 500 µl hexane (20 µl per female) solvent for 20 min in ambient temperature and then desiccated through $MgSO_4$ column, and samples were concentrated to 50 µl (2 µl per female) with nitrogen gas flow. Concentrated samples were stored in 2 ml glass vials (Agilent Technologies, Santa Clara CA, USA) in −20 °C refrigerator before used in chemical analysis and electric physiological experiments.

The single pheromone gland extracts were extracted from single pheromone glands without ovipositors this time because the samples were used to precisely determine pheromone component contents in the single gland. One pheromone gland excised from a calling female was immediately immersed in 6 µl hexane for 20 min under ambient temperature, and then the pheromone gland was removed before the sample was stored in −20 °C.

2.5. Gas chromatography-electroantennograhic detection (GC-EAD)

The pheromone gland extracts of female *M. separata* were used for GC-EAD analysis. The methods followed Arn et al. (1975), Byers (2004) and Lu et al. (2012). Agilent Technologies 6890N with a flame ionization detector (FID) equipped with a HP-5 capillary column (30 m × 0.25 mm ID, 0.25 µm film, J&W Scientific, Folsom, CA, USA) coupled with an electroantennographic detector (EAD; Syntech, Hilversum, The Netherlands) was used. Injector temperature was 220 °C, and the GC oven temperature was programmed from 50 °C for 1 min, then increased to 180 °C at 10 °C per min, and then increased to 220 °C at 5 °C per min, held at 220 °C for 5 min. The temperature of detector was at 250 °C. Nitrogen was used as carrier gas. Male antennae were excised with micro-scissors. After clipping off the tips, antennae were mounted on the antenna holder with two metal electrodes using conductive gel (Spectra 360, Parker Lab, NJ, USA), and then the electrode holder was inserted into the EAD probe. Testing began after a stable baseline was observed. The outlet of the GC column was split into a 1:1 ratio between the electroantennographic detector and the FID. One female equivalent (1 FE) of the pheromone gland extracts was used in the GC-EAD test. Autospike V3.9 (Syntech) was used for GC-EAD data analysis.

2.6. Gas chromatography coupled with mass spectrometry (GC-MS)

The pheromone gland extracts were also analyzed by Agilent Technologies 5973 MS (Agilent) coupled with an Agilent Technologies 6890 N GC (Agilent) equipped with HP-5 capillary column (30 m × 0.25 mm ID, 0.25 µm film, J&W Scientific, Folsom, CA, USA). The same temperature program was used as to the GC-EAD test. Injection was at splitless mode and helium was used as carrier gas. Windows NT/MASS Spectral Search Program (Version 1.7) software was used for data analysis. Voltage for electron impact (EI) ionization in mass spectra was 70 eV. The temperatures of the ion source and the interface were 230 °C and 280 °C respectively. To determine the *Z*11-16:Ald content in the single pheromone gland extract, we first used GC-MS to establish a standard curve of the synthetic *Z*11-16:Ald with gradient dosages under the same condition.

2.7. Dosage dependent electroantennography (EAG) responses

Synthetic *Z*11-16:Ald, *Z*11-16:OH, *Z*11-16:Ac and 16:Ald were used to test the dose-EAG responses of male antennae. They were diluted with paraffin oil into solutions of different gradient concentrations at 10 ng/µl, 100 ng/µl, 1 µg/µl and 10 µg/µl. Each was added onto a filter paper (0.2 cm × 1 cm) at 10 µl in a pasture tube, which made the final loading capacity doses of each chemical were 100 ng, 1 µg, 10 µg and 100 µg. Ten µl of paraffin oil was used as the control. An IDAC-2 amplifier (Syntech) was coupled to a computer installed with EAG data were analyzed with EAG2000 (Syntech) for data acquisition, recording and analysis.

2.8. In vivo optical imaging of male antennal lobes

The response patterns of the antennal lobe (AL) to potential sex pheromone components identified above (*Z*11-16:Ald, *Z*11-16:OH, *Z*11-16:Ac, and 16:Ald) were recorded by using *in vivo* optical imaging. Single compounds were dissolved in paraffin oil. A loading dosage of 100 μg was used for each compound at first, and paraffin oil was used as the control. Five replicates were run. In the dose-response tests, a series of dosages of *Z*11-16:Ald (1 μg, 10 μg, 100 μg, and 1000 μg) were used and eight replications were run.

The method was adopted from the previous studies (Bisch-Knaden et al., 2018; Deisig et al., 2006; Galizia et al., 2000; Hansson et al., 2003; Wu et al., 2015, 2018). Firstly, the moth was placed in a plastic tube and fixed with dental wax. Then, the tube was fixed in a custom-made chamber. After removing the head scales, mouthparts, and muscles, a window was made in the head between the two compound eyes. The brain was kept in Ringer solution during preparation (Christensen and Hildebrand, 1987). Before staining, the dye was dissolved in 20% Pluronic-127 in dimethyl sulfoxide and then diluted in Ringer solution to a final concentration of 30 mmol/L. The brain was stained with a calcium-sensitive dye, CaGR-2-AM (Molecular Probes, Eugene, OR, USA). Dye was dropped in the brain, and the moth was placed in the dark for 1 h at 12 °C. After staining, the brain was rinsed three times with Ringer solution and ready for imaging.

An upright microscope (Olympus BX51WI, Tokyo, Japan) with a 206 water-immersion objective (NA 0.95, Olympus) was used to observe and record male ALs. All the imaging data were collected by the Till Photonics imaging system (Till Photonics, Gräfelfing, Germany). Till-vision (Till photonics), ImageJ (NIH, USA) and custom-made programs in MATLAB (The Math Works, Inc) was used to acquire and analyze *in vivo* optical imaging data. The stimulus delivery setup was similar to the EAG above. For each stimuli, 40 continuous frames were acquired at a sampling rate of 4 Hz and the stimulation occur at the frame 12. The mean fluorescence (F) of frames 2–11 was defined as the background. For each frame, the ratio of the frame against the background (F_n/F, n = 1–40) was calculated. Then, to reduce the noise, the value of F_n/F was treated with smooth function of Matlab and define as F_n/F (smooth, 30 frames). The fluorescence change value ($\Delta F/F$) was define as $\Delta F/F = F_n/F - F_n/F$ (smooth, 30 frames).

2.9. Male antennal lobe reconstruction

To locate the part of the MGC that reacted, we reconstructed a model of the male AL. Moth brains were dissected and processed as previously described (Kvello et al., 2009). After fixation in 4% paraformaldehyde and rinsing in a phosphate-buffered saline (684 mM NaCl, 13 mM KCl, 50.7 mM Na_2HPO_4, 5 mM KH_2PO_4, pH 7.2), an anti-synapsin SYNORF1 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was used to label glomeruli in ALs. Labeled ALs were visualized with Alexa 488 goat anti-mouse secondary antibody (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA), and photo stacks were obtained with a Zeiss LSM710 Meta laser scanning microscope (Zeiss, Oberkochen, Germany). The male brain atlas was constructed using segmentation and volume statistics tools in AMIRA 6.0 (Zuse Institute Berlin, Berlin, Germany), volumes of the selected glomeruli were calculated in AMIRA and data were later processed using SPSS. Means of the volumes were compared with the generalized linear model (GLM) to see whether there was difference among the glomeruli and, successive multiple comparisons were done using Tukey HSD.

2.10. Wind tunnel tests

The wind tunnel tests were carried out to measure male attractiveness of a series of pheromone blends. The lures tested include: (1) the mixture of *Z*11-16:Ald, 16:Ald, and *Z*11-16:OH at a ratio of 100:10:0.1 reported by Zhu et al. (1987), n = 58; (2) the mixture of *Z*11-16:Ac and *Z*11-16:OH at a ratio of 100:12.5 reported by Takahashi et al. (1979), n = 44; (3) the pheromone gland extract (1 FE), n = 48; (4) the mixture of synthetic *Z*11-16:Ald, *Z*11-16:Ac, *Z*11-16:OH, and 16:Ald at a ratio of 100:21:21:11, based on our GC-MS results, n = 57; (5) the mixture of *Z*11-16:Ald, *Z*11-16:Ac, and *Z*11-16:OH (100:21:21), n = 30; (6) the mixture of *Z*11-16:Ald, *Z*11-16:Ac, and 16:Ald (100:21:11), n = 30; (7) the mixture of *Z*11-16:Ald, *Z*11-16:OH, and 16:Ald (100:21:11), n = 30; (8) the mixture of *Z*11-16:Ald and *Z*11-16OH (100:21), n = 30; (9) the mixture of *Z*11-16:Ald and *Z*11-16:Ac (100:21), n = 30; (10) the mixture of *Z*11-16:Ald and 16:Ald (100:11), n = 30: (11) *Z*11-16:Ald alone based on our GC-EAD results, n = 63. Hexane was used as the control, n = 55.

The whole experiment were conducted in a wind tunnel with a dimension of 2.5 m × 1 m × 1 m (L × W × H). The method was adopted from Miller and Roelofs (1978) and Vickers and Baker (1997). Naive male moths (4-day-old) were moved into the wind tunnel chamber to acclimate to the conditions for at least 40 min prior to the experiment. All synthetic compounds were dissolved in hexane, and then loaded on a piece of filter paper in a fixed volume of 10 μl. For each mixture, the dosage of the first component (either *Z*11-16:Ald or *Z*11-16:Ac) loaded was always 10 ng, and the other compounds in the blends were loaded in a corresponding ratio with comparing to the first component. Pheromone gland extracts were made as described above, and 1 FE was used to test each male. 10 μl hexane was used as the control.

A piece of filter paper loaded with chemicals were used as a lure. The behavioral responses of male moths to lures were classified and recorded according to the following typical categories: (1) Flight: male moths took off from the release cage; (2) Upwind: male moths flew at the height of the lure and showed a characteristic zigzag pursuing flight pattern toward the pheromone source and reached ≤ 70 cm from the lure; (3) Closing: male moths continued upwind behavior and reached 10 cm from the pheromone source; (4) Landing: male moths landed on the lure; (5) Mating: male moths climbed the lure and extruded the hair-pencils from the abdominal cavity. Each male moth was observed for successive 5 min. The lure changed after each male was tested. The experimental conditions were as follows: 22–25 °C, 20–40% humidity, and 0.5 lux of diffusive red light from the top. Wind speed was 0.5 m/s.

2.11. Tissues collection and transcriptome sequencing

Antennae were dissected from about 60 virgin female or male moths during the scotophase of the 4th day after emergence, and pooled and stored in the −80 °C freezer until RNA extraction. Total RNA was extracted following the Trizol manufacturer's instructions and dissolved in H_2O. All the sequencing were accomplished in the Beijing Genome Institute (Shenzhen, China) on the Illumina sequencing platform (Hiseq2000).

Raw reads were removed the adapter sequences and low-quality bases. The reads were *de novo* assembled using Trinity and the contigs were annotated using Trinotate (https://github.com/trinotate) (Grabherr et al., 2011; Haas et al., 2013). Through the BLASTX analysis in GenBank, candidate odorant receptors were found out by their homologies. The putative protein sequences were then compared to orthologous genes from other insect species using BLASTP.

2.12. Quantitative real-time PCR

The quantitative real-time PCR was performed on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA US) with SYBR Premix Ex Taq (TaKaRa, Shiga, Japan). The gene-specific primers to amplify an 80–150 bp product were designed by Primer3plus (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi), and primers are listed in Table 1. The q-PCR reaction program was: 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s, followed by the measurement of fluorescence during a 55 °C–95 °C melting curve

Table 1
The q-PCR primer sequences of pheromone receptor genes in *Mythimna separata* in North China

Gene	F/R	Primer sequences (5'-3')
MsepOR1	F	ACTTTCTTCGAGCTTGGCCA
	R	GGTGGGCAATTCGATGGACT
MsepOR2	F	GCCGATGCTGTTCGTGTATTAC
	R	AGCTTGCGTTGTCATCTGTG
MsepOR3	F	AGGGGTGGAGAAAACAGAAGAC
	R	TTTGGGTTCTTTGGCCAAGC
MsepOR4	F	TAGCCGCTTGCATGTTCAAC
	R	ACTGGTCTCAAAGTTCCAAGGG
MsepOR5	F	ACACGTACAAGAGCTCATCGG
	R	ATTCCCTGCGAAATGATCCC
MsepOR6	F	AGATGCGGTATACGGTTTGC
	R	AACATTGACAACGCCCAAGG
MsepORco	F	AAAATGCCGGCATGTCGAAC
	R	TACTGTGCCATGTTGATGCC
β-Actin	F	ATGAATGCGGACCTTCCATC
	R	AATTTGAGCCGCCCATTCAG

F: forward strand; R: reverse strand.

to detect a single gene-specific peak, and to check the absence of primer dimer peaks. The *β-Actin* was used as the reference gene. Each reaction was run in triplicate (technical replicates) and the means and standard errors were obtained from three independent biological replicates. The relative copy numbers of PR genes were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

2.13. Expression of MsepOR3 in Xenopus laevis oocytes and electrophysiological recordings

Total RNA of the antennae collected from 80 male adult moths was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. M-MLV Reverse Transcriptase (Promoga, Wisconsin, WI, USA) was used to synthesis cDNA. PCR was carried out using gene specific primers with Kozak consensus sequence and Restriction Enzyme cutting site based on the mRNA sequences of *MsepOR3* and *MsepORco*. The primer sequences are listed in Table 2. The PCR program included initial denaturation 98 °C 30s; 98 °C 10s, 52 °C 30s, 72 °C 30s for 35 cycles; 72 °C 2min. The coding sequences of *MsepOR3* and *MsepORco* were cloned into pGEM-T easy vector (Promega, Madison, WI, USA), then cut by related enzymes and cloned into pCS2+ vector. The pCS2+ vectors were linearized by using NotI (Takara shuzo, Shiga, Japan), cRNAs were synthesized from the linearized pCS2+ vectors with mMESSAGE mMACHINE SP6 (Ambion, Austin, TX, USA). cRNA were dissolved in RNAase-free water and stored at −80 °C.

Xenopus laevis was anesthetized 30 min by bathed in the ice, the oocytes were surgically collected and the wounds were carefully treated to avoid infection. The surgery was performed following the reported protocols (Nakagawa and Touhara, 2013). All procedures on *Xenopus laevis* were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences for the care and use of laboratory animals. Mature healthy oocytes were treated with 2 mg/mL of collagenase typeI (Sigma-Aldrich) in Ca^{2+} free saline solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5) for 1–2 h at room temperature. Each oocyte was microinjected with 23.6 nl (50 ng) of *MsepOR3* and *MsepORco* cRNA mixtures with the ratio of 1:1. Oocytes injected with RNase-free water were used as a negative control. Injected oocytes were incubated for 3–4 days at 18 °C in a bath solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) supplemented with 100 mg/mL gentamycin and 550 mg/mL sodium pyruvate.

Table 2
The full length primers of *MsepOR3* and *MsepORco*.

Gene	Enzyme cutting site	Primer sequences (5′-3′)
MsepORco	EcoRI/F	GAATTC**GCCACC**ATGATGACCAAAGTGAAGGC
	XhoI/R	CTCGAGTTACTTGAGTTGCACCAAC
MsepOR3	EcoRI/F	GAATTC**GCCACC**ATGAAATTAGTATCCGATGC
	XhoI/R	CTCGAGTTATTCTTCTTCCTCTGCT

F: forward strand; R: reverse strand; the underlined indicate restriction recognition sites, the bold indicate Kozak sequence.

Two-electrode voltage-clamp recording was used to detect the whole cell current of the oocytes responding to the stimulations of a range of chemicals (Jiang et al., 2014). The chemicals include *Z*11-16:Ald, *Z*11-16:Ac, *Z*11-16:OH, *Z*9-16:Ald, *Z*9-16:Ac, *Z*9-14:Ald, 16Ald. The concentration of 0.1 mM for each chemical was used at first, and concentration gradients were used later when a clear current response was detected. Intracellular glass electrodes were filled with 3 M KCl and presented resistances of 0.2–2.0 MΩ. Signals were amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT, USA) at a holding potential of −80 mV, low-pass filtered at 50 Hz and digitized at 1 kHz. Data acquisition and analysis were carried out with Digidata 1322A and pCLAMP (Axon Instruments Inc.).

2.14. Statistics and data analysis

Statistical analysis was mainly carried out by using SPSS 16.0 (IBM Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Linear regression was used for modelling the relationship between the Z11-16:Ald content and the integrated area of *Z*11-16:Ald in GC-MS. The data of EAG responses, calcium activities in optical imaging, and two-electrode voltage-clamp recording were analyzed by one-way ANOVA for analysis of variance, and the Tukey HSD test was used for means multiple comparisons ($P < 0.05$). The percentages of males performing sequential behaviors in the wind tunnel experiment were subjected to Chi-square 2 × 2 test of independence with Yates' continuity correction ($P < 0.05$).

3. Results

3.1. The pheromone gland and calling behavior of M. separata females

The pheromone gland in females of *M. separata* in North China is located at the dorsal inter-segmental membrane between the abdominal segment 8 and 9. The pheromone gland displays the 'I' shape (Fig. 1A). At the first day after emergence, just a few virgin females (6.46%) exhibited the calling behavior at night. At the 4th day, the percentage of the calling females reached to a peak at night, and there were more than 60% individuals showing calling behavior from the 2–8 h into scotophase (Fig. 1B). Therefore, in the following experiment we collected pheromone glands from the calling females at 4 day old to make the pheromone gland extracts.

3.2. Potential pheromone components and their EAG activities in male antennae

In the GC-EAD experiment, we found only one component elicited an electrophysiological activity in the male antenna (Fig. 2A). The mass spectra of this component showed the same characteristic fragments to *Z*11-16:Ald with a molecular ion at m/z 238 (M^+), at m/z 220 ($[M-18]^+$), the base peak at m/z 55 and others m/z 41, m/z 69, m/z 81, and m/z 95 (Fig. 3). Besides, its retention time (Rt. 16.646 min) in GC-MS is about same with the synthetic *Z*11-16:Ald (Rt. 16.647 min) (Table 3). The previously reported pheromone components, *Z*11-16:Ac, *Z*11-16:OH and 16:Ald were also found through GC-MS, with a retention time of 18.559 min, 17.336 min, 16.753 min, respectively (Fig. 3; Table 3). The ratio of *Z*11-16:Ald, *Z*11-16:OH, *Z*11-16:Ac, and 16:Ald was 100:21:21:11 (Table 3). In order to know the precise content of *Z*11-16:Ald in the single sex pheromone gland extract, we established a

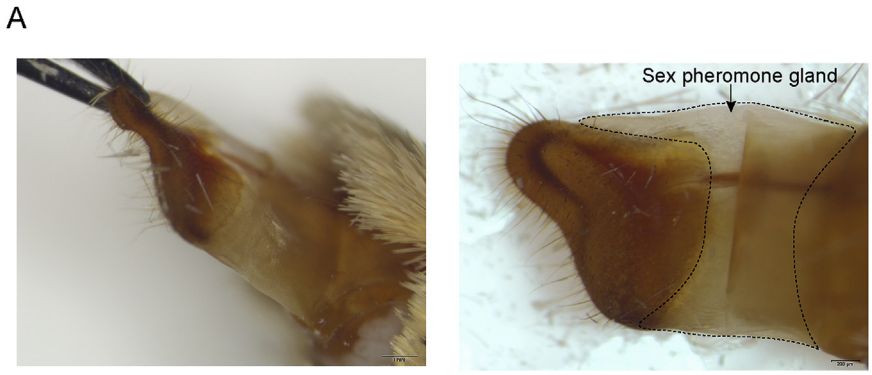
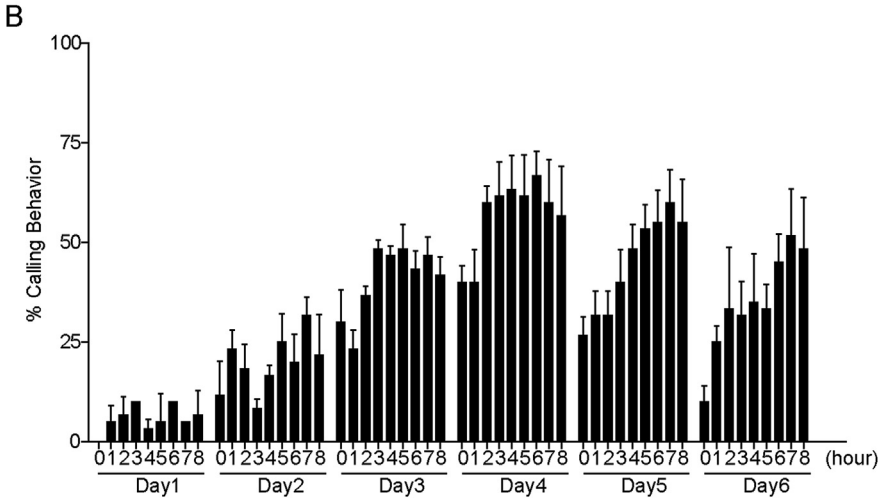


Fig. 1. Calling behavior of *M. separata* females during dark periods and sex pheromone gland observation. (A) The left panel shows the pheromone gland and ovipositor in a live moth. The right panel shows the isolated pheromone gland and ovipositor. The dashed area indicated the pheromone gland. (B) Twenty females per cage were observed from 1 to 6 d. In total, four cages were observed.



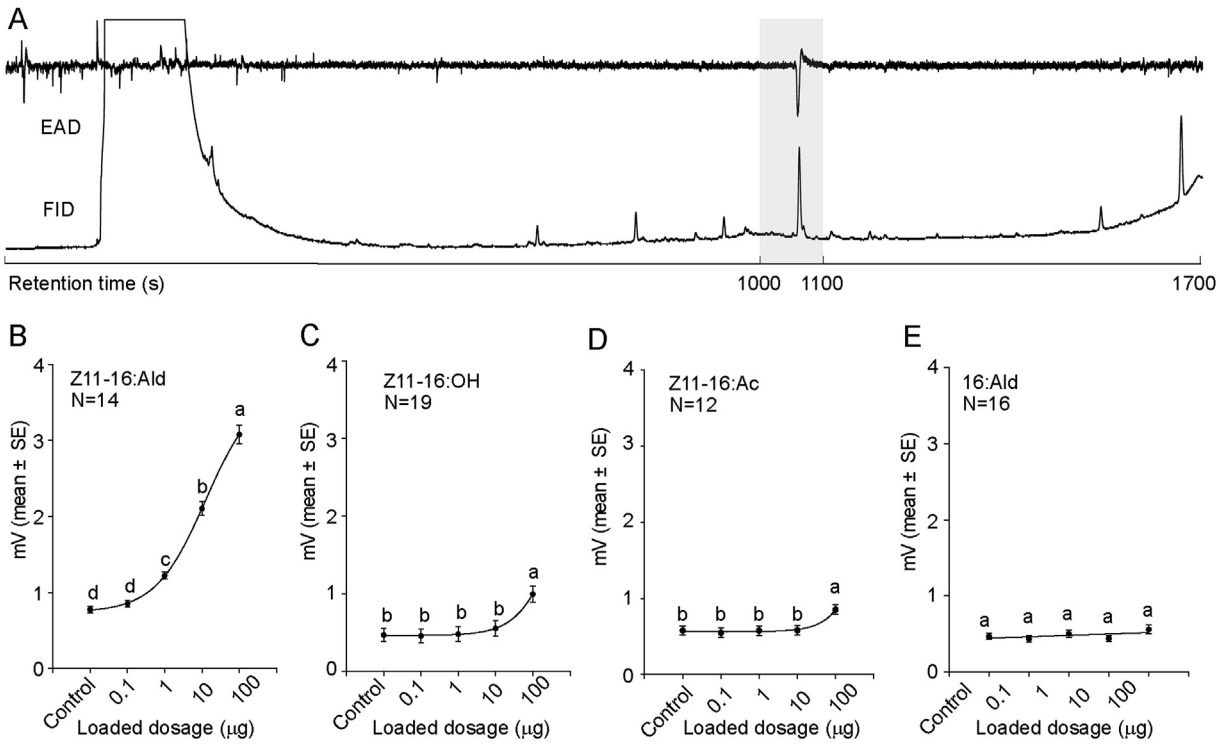


Fig. 2. Electrophysiological responses of *M. separata* males to female pheromone gland extracts and related compounds. (A) Sample GC-EAD responses of a male antenna to female pheromone gland extracts. (B, C, D, E) The EAG dose response curve of male antennae to synthetic compounds *Z*11-16:Ald, *Z*11-16:OH, *Z*11-16:Ac, 16:Ald. Different letters indicate significant differences between different dosages (ANOVA & Tukey HSD multiple comparison, $P < 0.05$).

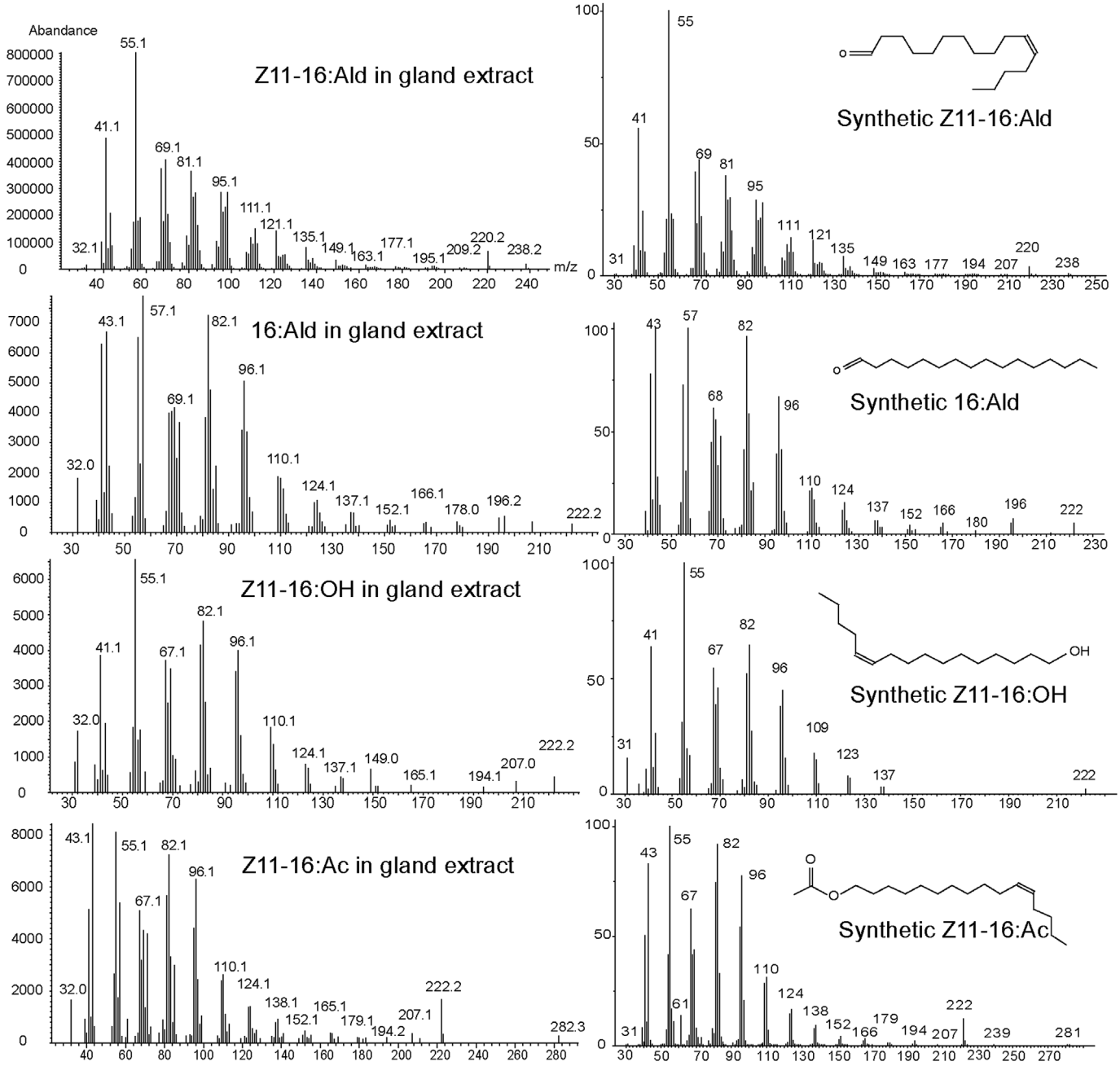


Fig. 3. Mass spectrometry of potential sex pheromone components in the pheromone gland extracts of *M. separata* female.

Table 3
The retention time of the compounds in female pheromone gland extracts of *Mythimna separata* in North China.

Sex pheromone component	Gland extract R.t (min)	Synthetic sample R.t (min)	Integrated area (Mean ± SE)
*Z*11-16:Ald	16.646	16.647	358972.57 ± 50616.00
16:Ald	16.753	16.758	40616.07 ± 10874.51
*Z*11-16:OH	17.336	17.320	74927.79 ± 14295.19
*Z*11-16:Ac	18.559	18.567	78274.21 ± 20254.22

R.t: retention time.

linear regression of the *Z*11-16:Ald content (x) (from 1 ng to 50 ng) and the integrated area (y) of *Z*11-16:Ald in GC-MS (Fig. 4). Accordingly, the content of *Z*11-16:Ald in the single pheromone gland extract was determined as 16.2 ± 6.1 ng on average.

We further tested the dose-EAG responses of *Z*11-16:Ald, *Z*11-16:Ac, *Z*11-16:OH and 16:Ald. *Z*11-16:Ald elicited a significant EAG response in male antennae from a dose of 1 μg. Comparing to the strong response induced by *Z*11-16:Ald, *Z*11-16:Ac and *Z*11-16:OH induced a much weaker response at a dose of 100 μg (Fig. 2C and D). No EAG response was detected for 16:Ald in the tested dose range (Fig. 2E, $P = 0.294$).

3.3. Neural representation of pheromone compounds in male antennal lobes

Using *in vivo* optical imaging, we tested the activities of male glomeruli to *Z*11-16:Ald, *Z*11-16:OH, *Z*11-16:Ac, and 16:Ald at the dosage of 100 μg. *Z*11-16:Ald activated a large area and elicited a strong response at the entrance ventral part of the AL (Fig. 5A and B). The dose response curve shows that the ΔF/F value was increased when the dosage of *Z*11-16:Ald becomes higher, and the lowest responding

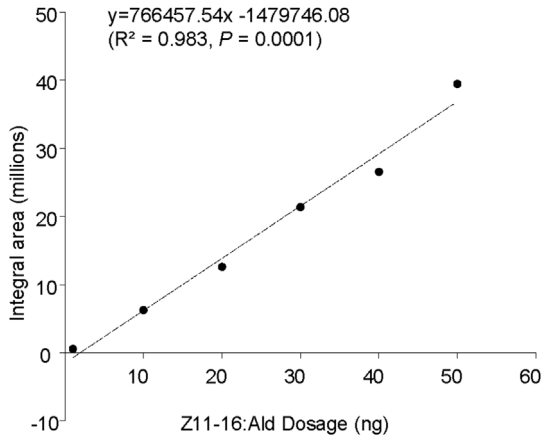


Fig. 4. Linear regression analysis between the contentsand the integrated areas of *Z*11–16:Ald in GC-MS.

dosage is 1 μg (Fig. 5C). *Z*11-16:OH and *Z*11-16:Ac at 100 μg each also activated a small area and elicited a weak response at the entrance of the AL, but the locations of three areas were different (Fig. 5A and B). We failed to find any response in AL to 16:Ald at the dose of 100 μg (Fig. 5A and B).

In order to know which glomeruli activated by Z11-16:Ald, *Z*11-16:OH, *Z*11-16:Ac, we built the morphological atlas of antennal lobes (ALs) of male *M. separata*. The macroglomerular complex (MGC) at the entry site of the antennal nerve contains three large subunits (Fig. 5D and F). Based on their enlarged volumes and positions, we adopted the terminology used in previous studies on other moth species (Berg et al., 1998, 2005; Hansson et al., 1991, 1992). In the MGC, the subunit with the largest volume located on the most ventral part of the AL is called the "cumulus (CU)"; the second subunit adjacent to the CU was referred to as the "dorso-anterior part (DA)"; and the third subunit was distorted and called the "dorso-posterior part (DP)". The volumes of CU, DA, and DP were 273329.60 ± 26009.79 μm^3, 85271.48 ± 10930.55 μm^3, and 72428.55 ± 14569.31 μm^3, respectively (Fig. 5E).

When we overlapped the gray photographs with the fake color photographs based on our results of *in vivo* optical imaging and morphological atlas, we found that the area (the green part) activated by *Z*11-16:Ald overlapped considerably with the CU, the area (the red part) activated by *Z*11-16:OH best fitted to DA which is close to the CU, and *Z*11-16:Ac activated the distal area (the purple part) in MGC which best fitted to DP (Fig. 5G).

3.4. Behavioral valence of pheromone blends in wind tunnel tests

The pheromone gland extract, the mixture of *Z*11-16:Ald, 16:Ald and *Z*11-16:OH (100: 10: 0.1) reported by Zhu et al. (1987), and the mixture of *Z*11-16:Ald, *Z*11-16:Ac, *Z*11-16:OH and 16:Ald (100:21:21:11) all induced the entire sequence of male sexual behaviors with no significant difference in upwind, closing, landing, and mating, while the mixture of *Z*11-16:Ac and *Z*11-16:OH (100:12.5) reported by Takahashi et al. (1979) failed to attract any male moths (Fig. 6).

Based on the mixture of *Z*11-16:Ald, *Z*11-16:Ac, *Z*11-16:OH, and 16:Ald with a ratio of 100:21:21:11), a series of trinary and binary blends of *Z*11-16:Ald with the other components and *Z*11-16:Ald alone were also tested for their male attractiveness. Surprisingly, all these blends and single *Z*11-16:Ald attracted males as well as the sex pheromone gland extract (Fig. 6), indicating that *Z*11-16:Ald can sufficiently induce the entire sequence of male sexual behaviors, and *Z*11-16:Ac, *Z*11-16:OH, 16:Ald had no synergetic or antagonistic effects on the attractiveness of *Z*11-16:Ald in wind tunnel at the testing dosage. We conclude that *Z*11-16:Ald is the essential sex pheromone component of *M. separata* in North China.

3.5. Pheromone receptors and their expression patterns

Based on the antennal transcriptome data, the putative PRs and odorant receptor co-receptor (ORco) of *M. separata* were identified and called MsepOR1, MsepOR2, MsepOR3, MsepOR4, MsepOR5, MsepOR6, and MsepORco. Neighbor-joining analysis with data of other noctuid species shows the amino acids sequences of these PRs were aligned with those of previously reported PRs, including MsOR1, MsOR2, and MsOR3 of a Japanese *M. separata* population (Mitsuno et al., 2008) (Fig. 7A). MsepORco and MsOR2 shared 99% identity, and were identified as the ORco of the two population; MsepOR1 shared 92% identity with MsOR1, which was identified as the PR tuned to *Z*11-16:Ac (Mitsuno et al., 2008); MsepOR3 shared 98% identity with MsOR3, and 63% and 65% identity with HarmOR13 and HassOR13 (Jiang et al., 2014; Liu et al., 2013; Zhang et al., 2010), respectively.

By analyzing the fragments per kilobase of exon per million fragments mapped (FPKM) (Mortazavi et al., 2008) of the antenna transcriptome, we found that *MsepOR1*, *MsepOR2*, and *MsepOR3* were specifically expressed in the male antennae, and the expression of *MsepOR3* was the highest among them. *MsepORco, MsepOR4*, *MsepOR5*, and *MsepOR6* were expressed in both male and female antennae, and the expression of *MsepORco* was the highest (Fig. 7B). In further q-PCR experiments, we tested 6 putative PRs in both male and female antennae. The results proved that *MsepOR1*, *MsepOR2*, and *MsepOR3* are male-specifically expressed, and the expression level of *MsepOR3* was the highest among that of 6 putative pheromone receptors in male antennae (Fig. 7C).

3.6. The pheromone receptor tuned to Z11-16:Ald

Because MsepOR3 was the most abundantly expressed in male antennae (Fig. 7B and C) and shared over 63% identity with HvirOR13, HarmOR13, and HassOR13, which are all tuned to *Z*11-16:Ald in *Heliothis/Helicoverpa* species (Grosse-Wilde et al., 2007; Jiang et al., 2014; Liu et al., 2013; Wang et al., 2011; Zhang et al., 2010), we predicted that MsepOR3 is the receptor tuned to *Z*11-16:Ald in *M. separata*. To test this, we first obtained the full length cDNA sequences of *MsepOR3* and *MsepORco* (GenBank accession numbers: MH717241 and MH717243), and then co-expressed cDNA of *MsepOR3* and *MsepORco* in *Xenopus* oocytes and used two-electrode voltage-clamp recording to test the oocyte response to *Z*11-16:Ald and related compounds. The results showed that *Z*11-16:Ald elicited a strongest electrophysiological response among four candidate sex pheromone components and related compounds (Fig. 8A). *Z*11-16:Ald induced currents in a dose-dependent manner from 10^{-6} M, with an EC_{50} value of 4.66×10^{-5} M (Fig. 8B). Besides, *Z*9-14:Ald which is not in the pheromone gland extracts also elicited a weak response (Fig. 8A).

4. Discussion

4.1. Geographic populations of M. separata use different sex pheromones

The sex pheromone of *M. separata* has been reported in several previous studies, and there are some significant differences in the composition and proportion of the pheromone blend among geographic populations. We summarize the results in the literature together with ours in Table 4. Takahashi et al. (1979), Kou et al. (1992) and Fónagy et al. (2011) found that *Z*11-16:Ac is the major pheromone component of *M. separata*. However, Zhu et al. (1987), Lebedeva et al. (2000) and the present study showed that *Z*11-16:Ald is the major pheromone component of *M. separata*. Furthermore, we find that *Z*11-16:Ald alone is sufficient to attract males, and *Z*11-16:Ac as the major component failed to attract any male moths in the wind tunnel. On the other hand, the PRs tuned to the major pheromone components show disparate in both the amino acid sequence and ligand selectivity between the Tokyo population and the Baoding population of *M. separata*. Mitsuno et al.

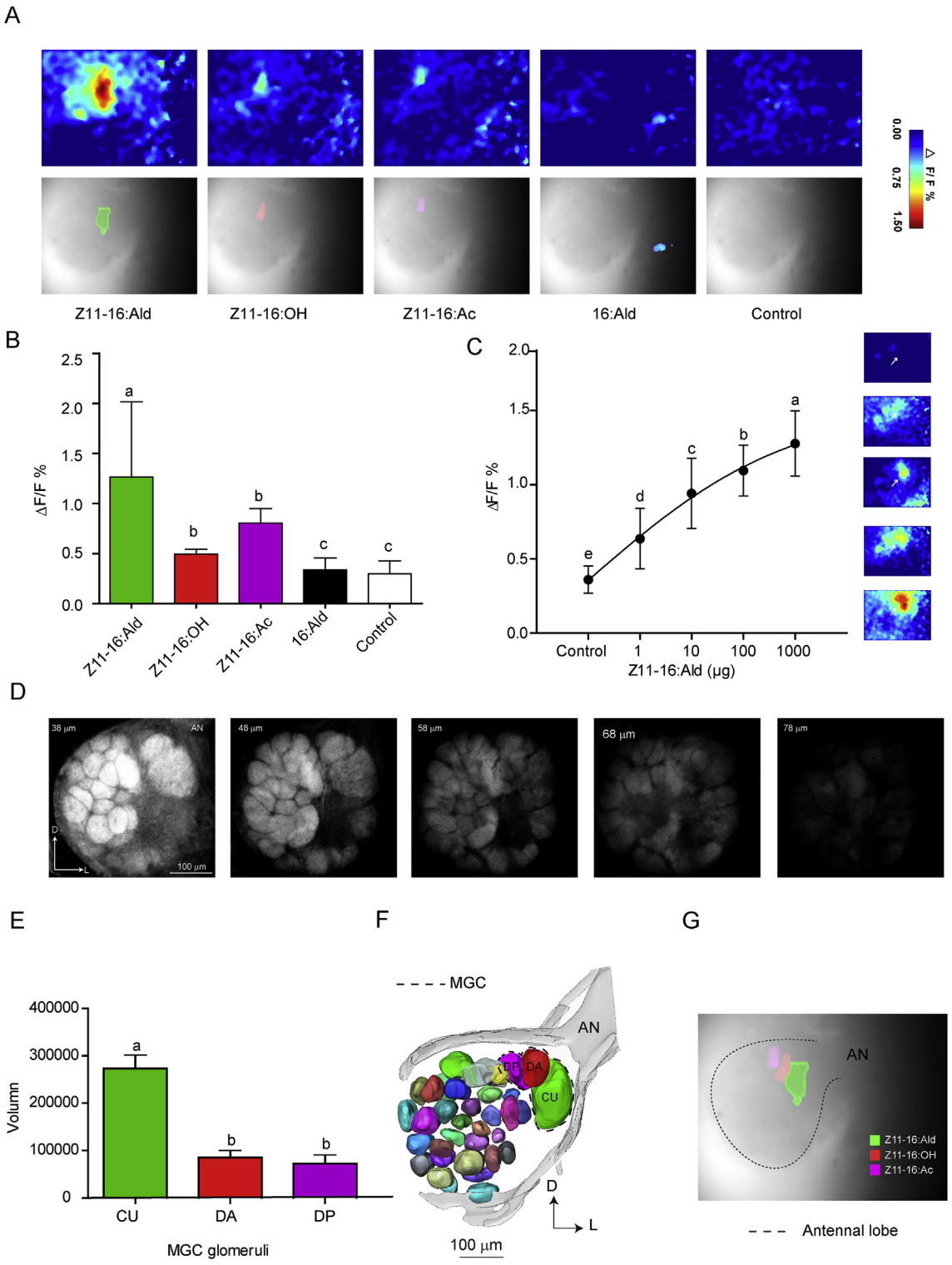


Fig. 5. Response patterns of the antennal lobes (ALs) to potential sex pheromone components in *M. separata* males. (A) Activated patterns in the ALs. Up panels show false-color-coded spatial response activities. Down panels show the activated areas superimposed on the gray-scale images separately. (B) MGC activities (Mean ± SE) when potential pheromone components stimulated at the dose of 100 μg. Different letters indicate significant differences (ANOVA & Tukey HSD multiple comparison, $P < 0.05$, n = 5). (C) Glomerular activities (Mean ± SE) induced by *Z*11-16:Ald at different dosages (the left panel). Different letters indicate significant differences (ANOVA & Tukey HSD multiple comparison, $P < 0.05$, n = 8). The right panel shows dose response patterns. (D) Images of an AL at different depths under a confocal microscope. D, dorsal; L, lateral; AN, antenna. (E) The volume of the cumulus (CU), dorso-anterior part (DA), and dorso-posterior part (DP) in the macroglomerular complex (MGC). n = 13. (F) Reconstruction of the AL including the MGC (CU, green; DA, red; DP, purple) and ordinary glomeruli. (G) The relative position of glomeruli activated by *Z*11-16:Ald (green), *Z*11-16:OH (red) and *Z*11-16:Ac (purple).

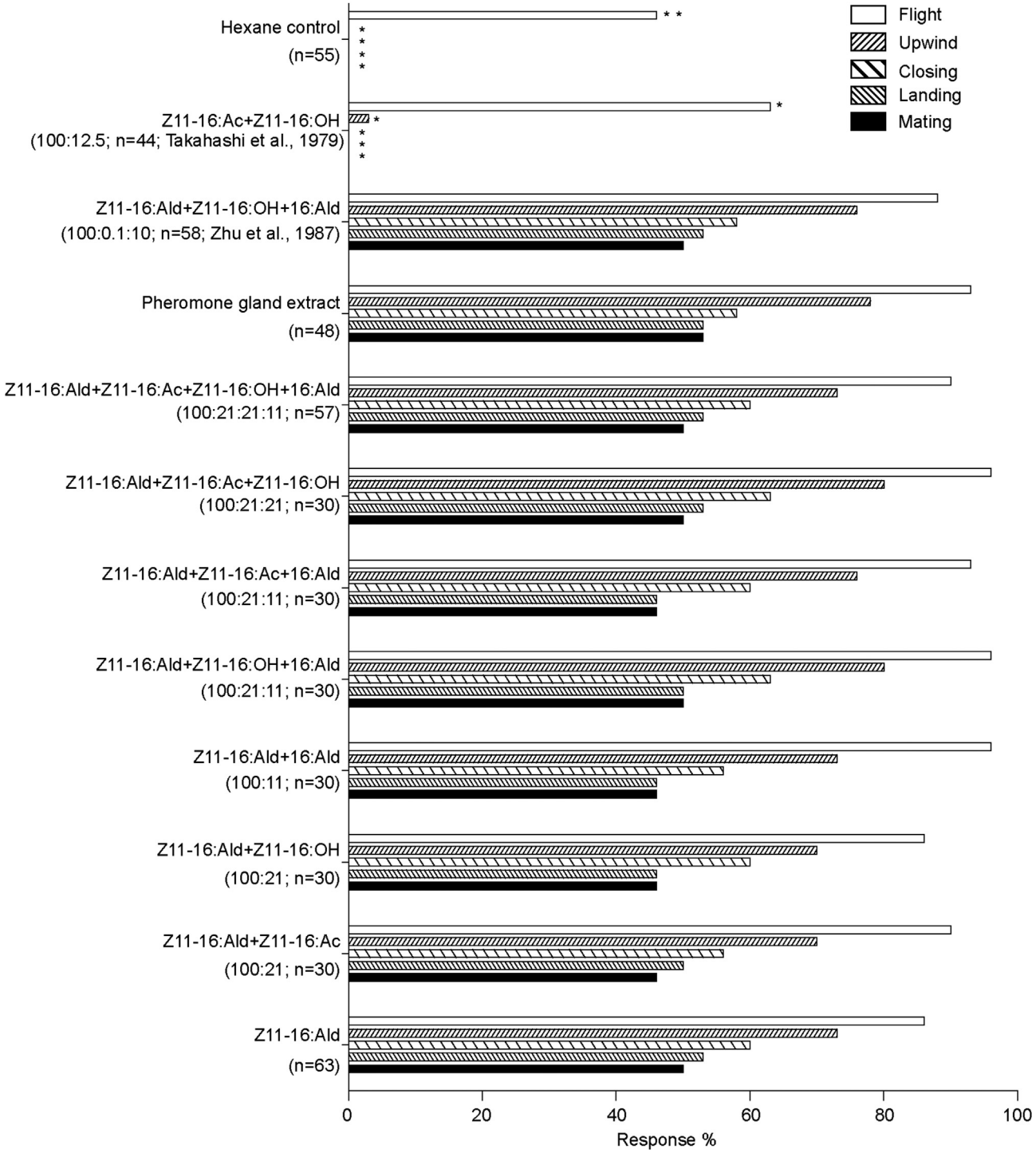


Fig. 6. Behavioral responses of *M. separata* males to sex pheromone lures in the wind tunnel. One star indicates significant difference with no star labeled treatments in the same behavior category, and two stars indicate significant difference with all the other treatments in the same behavior category (Chi-square test, $P < 0.05$).

(2008) found that MsOR1 is specially tuned to *Z*11-16:Ac, the major pheromone component of the Tokyo population, while the present study shows that MsepOR3 is specially tuned to *Z*11-16:Ald, the major pheromone component of the Baoding population. The amino acid sequences of MsOR1 and MsepOR3 only share 40% identity. Recently several studies on transcriptome analyses of *M. separata* antennae or heads were carried out (Chang et al., 2017; Du et al., 2018; He et al., 2017; Liu et al., 2017), but most of the sequences of putative PRs have not been made public. Chang et al. (2017) reported the sequence of MsepOR3.1 is closely related to that of MsOR3 identified by Mitsuno et al. (2008), and then we compare the amino acid sequence of our MsepOR3 with MsepOR3.1, and find they share 98% identity. Du et al. (2018) showed MsepPR3 has the highest expression level among 6 putative PRs in the male antennae through q-PCR experiments, which is consistent with the expression pattern of MsepOR3 in the present study. Based on the phylogenetic analyses, we speculate that *MsOR3* (Mitsuno et al., 2008), *MsepOR3.1* (Chang et al., 2017), *MsepPR3* (Du et al., 2018), and *MsepOR3* in this study sharing high identity, should be

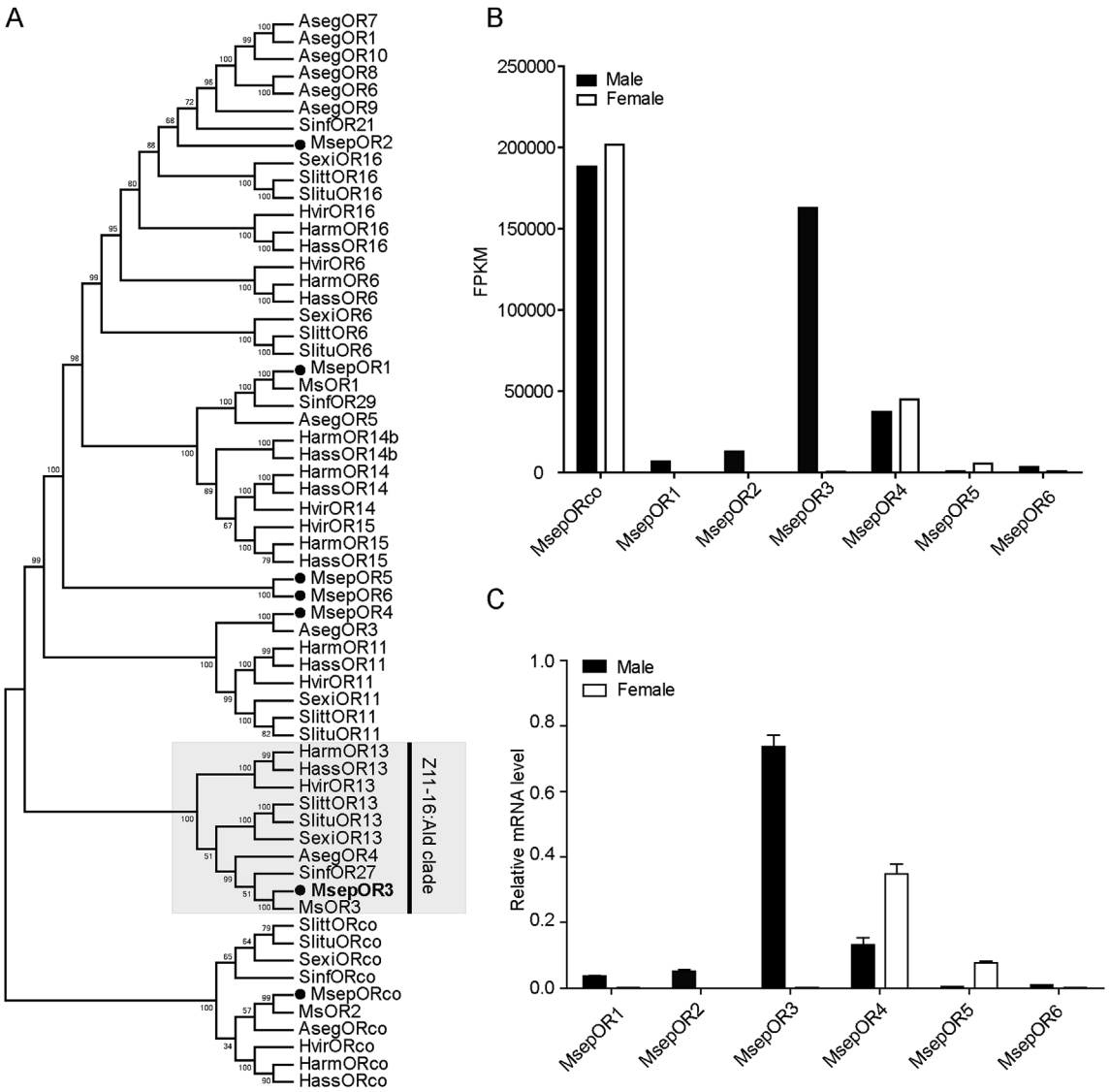


Fig. 7. Phylogenetic relationships and expression of pheromone receptor genes. (A) Phylogenetic tree of the PRs in Noctuidae. Bootstrap values are based on 1000 replicates, and values over 50 are shown at corresponding nodes. Harm, *Helicoverpa armigera*; Hass, *Helicoverpa assulta*; Msep, *Mythimna separata* in this study (indicated by a black circle); Ms, *M. separata* from reported from a Japanese population (Mitsuno et al., 2008); Hvir, *Heliothis virescens*; Sinf, *Sesamia inferens*; Aseg, *Agrotis segetum*. (B) Expression of all six PRs and ORco in male and female antennae based on the antenna transcriptome of *M. separata* in North China. FPKM, fragments per kilobase of exon per million fragments. (C) Relative mRNA expression levels of PRs in male and female antennae of *M. separata* in North China by q-PCR. Data are presented as Mean ± SE, n = 3.

identical or homologous genes.

Based on the differences in the major pheromone components and their tuning receptors, we speculate that the geographic populations investigated under the name of *M. separata* may belong to two different species, one uses *Z*11-16:Ald as the major pheromone component, and another uses *Z*11-16:Ac as the major pheromone component. The latter one may be more closely related to the true armyworm moth, *Mythimna* (*Pseudaletia*) *unipuncta* (Haworth) (Mcdonough et al., 1980), for both of them use *Z*11-16:Ac as the major component in their sex pheromones although the minor components are different.

4.2. *Z11-16:Ald alone induces the entire sequence of male sexual behaviors*

Most of moth species use multiple components as their sex pheromone (Roelofs et al., 1977; Silverstein et al., 1977). However, some species such as *Bombyx mori*, *Cydia pomonella* use only one component to attract males (Butenandt et al., 1962; Kasang et al., 1978; Mcdonough et al., 1995; Witzgall et al., 2001, 2008). In our study, *Z*11-16:Ald alone is comparable to the pheromone gland extract in attractiveness to males in wind tunnel.

Comparing with the sex pheromone blend of *M. separata* identified in previous studies, our results are most relevant to that of the Jintan population in China (Zhu et al., 1987), which demonstrates a pheromone blend of *Z*11-16:Ald, 16:Ald and *Z*11-16:OH with a ratio of 100: 10: 0.1. However, Zhu et al. observed some conflicts in field trapping experiments. One field trapping experiment in Jintan, Jiangsu Province during 29th March to 23rd April in 1984 revealed that the single component lure of *Z*11-16:Ald is the best in total captures, while another field trial in Beijing during 3rd to 15th June in 1984 showed that a blend of the ternary components *Z*11-16:Ald, *Z*11-16:OH and 16:Ald (with a ratio of 100: 10: 0.1) got the highest total captures, but the best in the daily maximum male capture was still the single *Z*11-16:Ald lure. Another field trap work in Jiangsu and Hebei, China also showed that single *Z*11-16:Ald attracted males well (Wei and Pan, 1985). A most recent field trap work in Zhejiang and Yunnan, China also indicated that single *Z*11-16:Ald is the most attractive to males (Chen et al.,

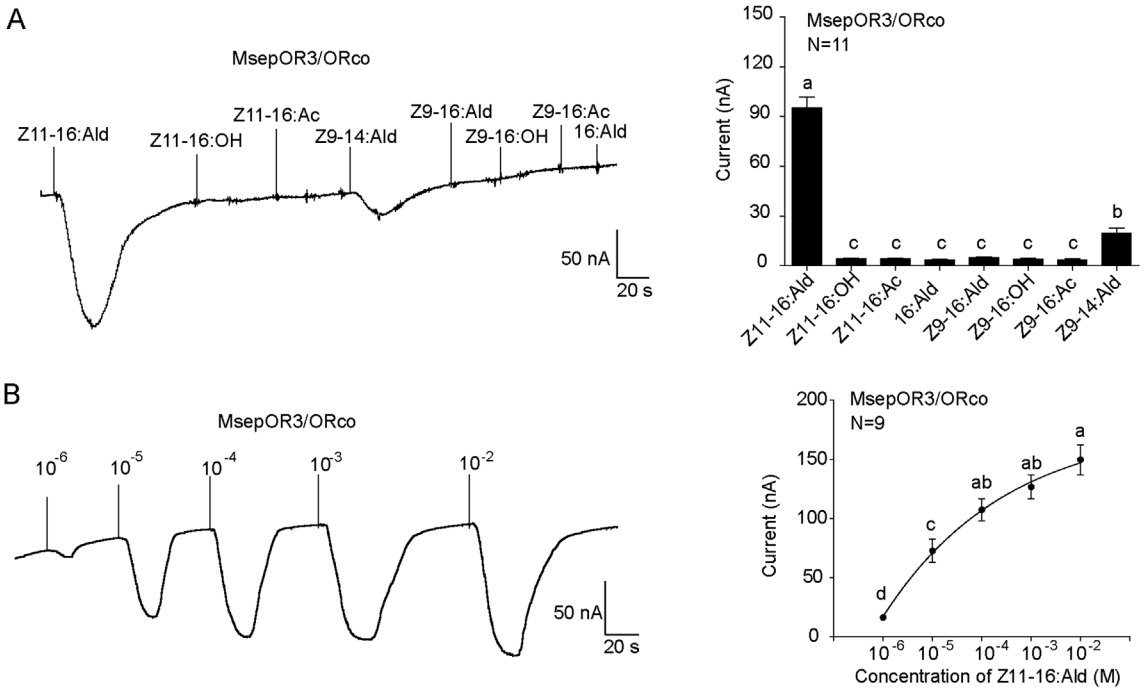


Fig. 8. Inward current responses of *Xenopus laevis* oocytes co-expressing *MsepOR3/MsepORco* stimulated with pheromone components and related compounds. (A) Inward current responses (left) and response profiles (right) to pheromone components and related compounds. The pheromone components were at 10^{-4} M concentrations. (B) Inward current responses (left) and response profiles (right) to a range of *Z*11-16:Ald concentrations. The EC_{50} value is 4.66×10^{-5} M. Bars indicate SE. Columns with different letters are significantly different (ANOVA & Tukey HSD multiple comparison, $P < 0.05$).

2018). Therefore, we suggest that *Z*11-16:Ald alone can be used for monitoring the population dynamics of *M. separata* in China because it is sufficient to attract males, and also simple and economical in term of practical application. Nevertheless, it is worth to mention that several other noctuid species, *H. armigera, Helicoverpa zea, Heliothis virescens,* and *Heliothis subflexa,* also use *Z*11-16:Ald as the major component in their sex pheromone blends, but their minor pheromone components are different (Klun et al., 1980; Nesbitt et al., 1979; Roelofs et al., 1974; Teal et al., 1981). The whole blend rather than a single compound plays a crucial role in intraspecific communication and interspecific isolation in these species (Groot et al., 2006).

4.3. MGC response patterns and pheromone receptor functions is conservative among noctuid species

Our *M. separata* population shares similar MGC structures with *Heliothis* and *Helicoverpa* species. In *H. virescens*, *H. zea*, and *H. armigera*, CU as the largest subunit of MGC is dedicated to processing the major sex pheromone component *Z*11-16:Ald (Berg et al., 2002, 2005; Lee et al., 2006). In *H. assulta*, CU is activated by the major sex pheromone component *Z*9-16:Ald (Wu et al., 2015; Zhao and Berg, 2010). In our *M. separata* population, CU is also activated by the essential pheromone component *Z*11-16:Ald, and other two smaller subunits, DA and DP, are activated by *Z*11-16:OH and *Z*11-16:Ac, respectively. However, the wind tunnel tests showed that *Z*11-16:Ac and *Z*11-16:OH in the tested pheromone blends have neither synergetic nor antagonistic effects. The role of the two compounds in the sex pheromone communication of *M. separata* in North China needs to be further studied in the field condition.

Pheromone receptors show remarkable conservation on both amino acid sequences and protein functions among noctuid species. The phylogenetic analysis showed that MsepOR3 shares 63% identity with HarmOR13 and HvirOR13, and 65% with HassOR13. MsepOR3 has the same ligand selectivity as HvirOR13, HarmOR13, and HassOR13, which are specifically tuned to *Z*11-16:Ald (Grosse-Wilde et al., 2007; Jiang et al., 2014; Liu et al., 2013; Wang et al., 2011). This implies that the pheromone receptor of *Z*11-16:Ald are under a high negative evolutionary selection pressure in evolution progress of noctuid species (de Fouchier et al., 2017; Hansson and Stensmyr, 2011).

In sum, we examined the female sex pheromone of *M. separata* in North China and found that *Z*11-16:Ald is an essential pheromone component and induce the entire sequence of male sexual behaviors. *Z*11-16:Ald is specifically tuned by MsepOR3 and elicits electrophysiological responses of the antennae, and then activates the cumulus of the MGC in male ALs. These olfactory features share with other noctuid species of *Heliothis* and *Helicoverpa*, indicating that general patterns of pheromone detection and processing are conserved within these moth species.

Table 4
Identified components and their proportions in the sex pheromone gland of *Mythimna separata* in different geographic regions.

Geographic population	Z11-16:Ac	Z11-16:OH	Z11-16:Ald	16:Ac	16:OH	16:Ald	References
Kyoto	100	12.5	–	–	–	–	Takahashi et al. (1979)
Jintan	–	0.1	100	–	–	10	Zhu et al. (1987)
Taipei	100	48.8	–	14.9	15.5	–	Kou et al. (1992)
South eastern Russia	5	–	100	–	–	–	Lebedeva et al. (2000)
Tokyo	100	61.9	–	34.4	28.8	–	Fónagy et al. (2011)
Baoding	21	21	100	–	–	11	This study

-: no detection.

Author contributions

Conceptualization: CZW and NJJ; Data curation: NJJ, RT, CZW; Investigation: NJJ, RT, HW, MX, CN, LQH, CZW; Experiment design and writing-original draft: NJJ and CZW; Resources: CZW, LQH; Funding acquisition and supervision: CZW.

Ethics

Animal experimentation: All procedures in this study were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences for the care and use of laboratory animals (protocol number IOZ17090-A). The *Xenopus laevis* was anesthetized 30min by bathed in the ice, the wounds were carefully treated to avoid infection. Every effort was made to minimize suffering.

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