Insect science

*Insect Science* (2023) 0, 1–12, DOI 10.1111/1744-7917.13171

ORIGINAL ARTICLE

# Pheromones emitted by both female and male moths regulate coordination between the sexes for *Agriphila aeneociliella* (Lepidoptera: Crambidae)

Yi-Di Zhan[1], Ying-Jie Liu[2], Jia-Hui Liu[1,3] and Yong Liu[1]

[1]*College of Plant Protection, Shandong Agricultural University, Taian, Shandong Province, China;* [2]*Staff Development Institute of China National Tobacco Corporation, Zhengzhou, China and* [3]*Functional and Evolutionary Entomology, Gembloux Agro-Bio Tech, Liège University, Gembloux, Belgium*

**Abstract** The complex and efficient sex pheromone communication system in insects is essential for reproduction and for reproductive isolation of species. In moths, sex pheromone communication starts with male attraction to compounds emitted by females; only a few species act in the reverse. However, how the pheromones that are emitted by both sexes co-regulate and coordinate mate finding and mating remains unknown. Here, we identified both the male and female pheromones of Eastern Grass Veneer moth, *Agriphila aeneociliella* (Lepidoptera: Crambidae), and demonstrated their efficiency in manipulating behavioral responses of the opposite sex. Combining data from analysis of gas chromatography-electroantennogram detection, gas chromatography-mass spectrometry, and olfactory behavior assays, the female pheromone of *A. aeneociliella* was identified as (*Z,Z,Z*)-9,12,15-octadecatrienal and (*Z*)-9-hexadecenyl acetate, while the male pheromone was determined to be 1-nonanal. Both the 2 individual components of the female pheromone and their binary mixture were significantly attractive to males, and the 1-nonanal male pheromone induced strong electrophysiological responses in females and induced attraction of females in a Y-tube olfactory test. Depending on the concentration of 1-nonanal, its addition to the binary mixture of the female pheromone either enhanced ($10^{-3}$ or $10^{-2}$ $\mu$g/$\mu$L) or reduced (1 $\mu$g/$\mu$L) the aphrodisiac effect of the mixture on males. In wind-tunnel bioassays, different concentrations of pheromones, including the binary mixture of female pheromone and the mixture of male and female pheromones, had significant effects on male behavior. Our findings suggested that the blend of both female and male pheromones plays a significant role in the sexual communication system in some moths.

**Key words** Crambidae; male pheromone; reproductive strategy; sexual reproduction; wheat pest

## Introduction

Pheromones are chemical signals used for communication between conspecific individuals. Behavioral and physiological activities are mediated by pheromones in many organisms (Stökl & Steiger, 2017). Sex pheromones induce sexual behaviors in the opposite sex, are often the primary signals in sexual reproduction, and support species reproductive isolation in many insects (Groot *et al.*, 2016; Henneken & Jones, 2017; Khallaf *et al.*, 2021). The discovery of insect sex pheromones not only expanded our understanding of the

Correspondence: Yong Liu, College of Plant Protection, Shandong Agricultural University, No. 61 Daizong Road, Taian, Shandong 271018, China. Email: liuyong@sdau.edu.cn

diversity of phenotypic traits and the formation of new species, but also provided new tools for pest management (Yew & Chung, 2015).

Research on moth sex pheromones began about 100 years ago, and the pheromones of ca. 700 species of moths have been identified so far (Naka & Fujii, 2020; Ando & Yamamoto, 2022; El-Sayed, 2022). Typically, sexual reproduction in Lepidoptera is preceded by long-distance attraction of males and short-range courtship of females, both mediated by pheromones (Blomquist & Vogt, 2003; Roelofs & Rooney, 2003; Wyatt, 2003). Intraspecific sex pheromone variation can have important effects on attractiveness, mating success, and mating behavior of individuals, regardless of the source (diet, age, mating status, or nearness to conspecifics) of this variation (De Pasqual e*t al*., 2021). Sex pheromones can be used as indicators of mate quality for sexual selection, and they can also be used to avoid low-fitness mating's and so reduce mating costs (De Pasqual e*t al*., 2021). Based on their distinctive structural and biosynthetic features, lepidopteran female sex pheromones are classified into 4 groups (Types I, II, III, and 0): Type I ($C_{10}-C_{18}$ straight chain with 0−3 double bonds, typically have a functional group at the terminal position), Type II (polyunsaturated hydrocarbons with $C_{17}-C_{25}$ straight chain and their corresponding epoxy derivatives), Type III (contain 1 or more methyl branches in the carbon chain), and Type 0 (short-chain secondary alcohols and their corresponding methyl ketones) (Ando *et al.*, 2004; Löfstedt *et al.*, 2016; Naka & Fujii, 2020). Type I and Type II pheromones have been detected in about 600 and 100 moth species, respectively (Naka & Fujii, 2020). Pheromone compounds may be similar because of common ancestry or as a result of independent, convergent evolution (Löfstedt *et al.*, 2016). The development of pheromone blends rather than a single component was another significant evolutionary event that had major consequences for speciation.

From the perspectives of animal behavior and evolution, male pheromones directly affect females, but may also have other effects. Male pheromones also enhance species recognition, help maintain reproductive isolation, and reduce hybridization and the loss of fitness associated with hybridization (Conner & Iyengar, 2016). Male pheromones likely mediate inter- and intra-sexual communication in moths, and they are essential for the close-range attraction of females in the mating process (Birch *et al.*, 1989, 1990) because they induce females to be more receptive to courting males (Baker & Cardé, 1979; Baker *et al.*, 1981; Teal *et al.*, 1981; Jacquin *et al.*, 1991). Females of the Oriental fruit moth, *Grapholitha molesta* (Busck), display overt movement toward male hair pencil compounds (Baker *et al.*, 1981). The components of the pheromone released by males of *Heliothis virescens* (F.) are not only important in mate acceptance by females, but they also play a role in mate choice and species isolation (Hillier & Vickers, 2004). Moreover, electrophysiological evidence suggests that males can also perceive male pheromones (Hirai *et al.*, 1978; Fitzpatrick *et al.*, 1989). The male pheromone of *Conogethes punctiferalis* (Guenée) (tiglic acid) is a signal of conspecific male recognition and also may be a pheromone that acts as an aphrodisiac (Takayoshi & Hiroshi, 1999), while the male pheromone of the armyworm *Pseudaletia unipuncta* (Haw.) prevents multiple males from competing for a single female (Hirai *et al.*, 1978). Studies on the chemistry of pheromones released by male moths, as well as associated intra- and inter-sexual behavior, advance understanding of chemical communication in moths (Teal & Tumlinson, 1989).

The Eastern Grass Veneer moth, *Agriphila aeneociliella* (Eversmann) (Lepidoptera: Crambidae), is a recently reported pest in China that feeds on the base of wheat stems, causing serious yield loss during pest outbreaks (Chi *et al.*, 2016). Previous study found that the calling females and males of *A. aeneociliella* exhibited typical pheromone-releasing behaviors, with females exposing their pseudo-ovipositor and males vibrating their everted abdominal hair pencils (Zhan *et al.*, 2020). By repeatedly extruding and retracting their abdominal hair pencils, males trigger excitatory responses from females and other males. The overt arousal behaviors of both sexes of this moth toward displaying males provide a rare opportunity to study a male moth pheromone that is recognized by both sexes. Therefore, we hypothesized that in this species there are specific male and female pheromones that effectively manipulate the recognition of both sexes during courtship and copulation. We identified the female and male pheromone compounds using gas chromatography–electroantennogram detection (GC-EAD) and gas chromatography–mass spectrometry (GC-MS), combined with olfactory behavioral assays. Subsequently, the responses of *A. aeneociliella* to a series of pheromone blends were examined to determine the possible effects of both female and male pheromones on the courtship behaviors of this moth.

## Materials and methods

### *Insects*

A colony of *A. aeneociliella* was established at the Department of Entomology, Shandong Agricultural

University (in 2016), and later supplemented with insects from wheat fields in Jimo County (36.38° N, 120.45° E), Shandong Province, China (2016–2022). Larvae were fed wheat seedlings (Lumai No. 21, 10–15 cm height) and held at 24.0 ± 0.5 °C, 75% ± 5% relative humidity, under a 12 : 12 light : dark photoperiod in an artificial climate chamber. Final instar larvae were isolated individually in 30-mL plastic cups containing sterilized fine vermiculite (with 20% water content) for pupation and adult emergence.

### *Pheromone sampling*

Two approaches were used to collect pheromone components from *A. aeneociliella* moths. First, gland extracts of calling females were collected based on the mating rhythm of *A. aeneociliella* (Zhan *et al.*, 2020) to obtain pheromone from inside the gland. Pheromone glands of 1- to 3-d-old virgin females were excised and soaked in hexane for 15 min to extract pheromone. In total, 60 glands were extracted in 600 $\mu$L of hexane (high-performance liquid chromatography grade), and the supernatant was used for structural analysis and behavioral tests. Second, volatiles were collected from different combinations of moths (1M1F: 1 newly emerged virgin female and a newly emerged male; 2F: 2 newly emerged virgin females; 2M: 2 newly emerged virgin males) that were held in a clean 350-mL jar. The upstream end of the collection system consisted of an air pump and an activated carbon filter that pushed air into one end of the collection jar. Air was then moved out of the jar into a Porapak Q trap (20 mg, 50- to 80-mesh). Each treatment was allowed to release pheromones for 48 h, and the collection process was repeated 6 times. Trapped volatiles were desorbed by solvent extraction using hexane (1 mL), and then nitrogen was used to concentrate the solvent to about 300 $\mu$L.

### *Gas chromatography-electroantennogram detection*

Electroantennogram (EAG) responses of male and female antennae to gland extracts and to volatile collections were measured using a GC-EAD system with an HP7890 GC (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5MS UI column (30 m × 0.25 mm × 0.25 $\mu$m; Agilent Technologies). The effluent air (from the odor source) was divided in a 1 : 1 ratio between a flame ionization detector (FID) and the EAD. An air stimulus controller (CS-55; Syntech, Hilversum, the Netherlands) was used for gas delivery. Tips of antennae of 1- to 2-d-old virgin males were cut off, and the head of a male moth with antennae was attached to the fork electrode with an electrode gel (Parker Laboratories, Inc., Fairfield, NJ, USA). Helium was used as carrier gas at a flow rate of 1.0 mL/min, and the injector temperature was set at 250 °C. The oven temperature was maintained at 50 °C for 1 min after injection and then increased by 5 °C/min to 200 °C with a hold for 1 min, then increased by 10 °C/min to 300 °C, with a final hold of 1 min. Recordings were made using the GC-EAD Pro ver. 4.1 software (Syntech).

### *Chemical analysis*

Gland extracts and volatile collections were analyzed using an Agilent 7890B GC instrument interfaced with an Agilent 7000D MS detector. One microliter of the liquid concentrated by nitrogen gas was injected through the autosampler, and this process was repeated 3 times for each treatment. The column type and procedure conditions were the same as those used in the GC-EAD test. Mass spectra were compared with the NIST14 database. Putative pheromone structures were confirmed by comparing retention times and mass spectra with authentic standards.

### *Electroantennogram*

The electrophysiological responses of *A. aeneociliella* olfactory detection were tested with an EAG system. The potential activities of male and female antennae against gland extracts, volatile collections (1M1F, 2M, 2F), and the 3 putative pheromone compounds with different concentration gradients ($10^{-3}$, $10^{-2}$, $10^{-1}$, $10^{0}$ $\mu$g/$\mu$L, solvent: hexane) were tested. Putative female pheromones were combined in proportion to the chromatographic results from female gland extracts. The potential activities of male antennae to a binary mixture of the 2 putative female pheromones—0.16 $\mu$g/$\mu$L (*Z,Z,Z*)-9,12,15-octadecatrienal + $10^{-2}$ $\mu$g/$\mu$L (*Z*)-9-hexadecenyl acetate—when combined with different concentrations of the putative male pheromone (0–1 $\mu$g/$\mu$L) were tested. Filter paper strips (0.5 × 2.0 cm) loaded with 10.0 $\mu$L of each solution were inserted into glass Pasteur pipettes. One antenna per insect was stimulated 3 times for 0.5 s with an interval time of 10 s. Each sample was tested with 9 different antennae.

### *Y-tube olfactometer bioassay*

The preference differences for paired stimuli were determined in a Y-tube olfactometer for 4 groups of comparisons: (1) pheromone extracts versus hexane (solvent),

(2) different concentrations (0–1 $\mu$g/$\mu$L) of identified active components versus hexane, (3) the binary mixture of 2 female pheromone components versus hexane, and (4) the binary mixture of female pheromone versus a mixture of the female pheromone components and the male pheromone. We used a Y-tube olfactometer (stem, 14 cm; arms, 12 cm at 75° angle; internal diameter, 2.4 cm) for behavioral bioassays for tested compounds (Table 1). For each trial, 10 $\mu$L of the test solution was added to a filter paper strip (0.5 × 2.0 cm), which was placed in a glass jar. The flow rate of filtered air was maintained at 0.5 L/min with a flowmeter. The Y-tube olfactometer was placed in darkness and observed under a red light. At the beginning of each test, the moth was released from the holding tube at the downwind end of the Y-tube. Each moth was allowed 5 min to respond to the treatment, and each moth's first choice for 1 arm of the olfactometer was recorded. Thirty moths were tested for each treatment. Moths making no response to either arm for 5 min were recorded but discarded. After 5 moths had been tested, the stimuli in the 2 arms were reversed to avoid positional bias.

### *Wind tunnel experiment*

A 160 × 65 × 80 cm wind tunnel was used to assess moths in flight. It was held at 24.0 ± 0.5 °C and had an air flow of 30 cm/s. The wind tunnel was in a dark room, and moths were observed under red light for responses to test odors (see Table 1). Ten microliters of each test odor solution were spread evenly over the groove of a folded piece of filter paper (10 × 1 cm), which was then hung 10 cm from the wind source. Test males were placed in a cup for 30 min in the dark to adapt to the experimental environment. Each treatment was conducted with 30 individuals, and each male was tested only once. The following types of behavior were recorded: No response, Wing fanning, Taking flight, Oriented upwind flight, and Source contact. The time needed to reach the odor source was recorded.

### *Statistical analysis*

The EAG amplitudes for different concentrations were analyzed using one-way analysis of variance followed by Tukey's honestly significant difference test (SPSS 20.0; IBM, Armonk, NY, USA). Preference numbers between odors were analyzed using $\chi^2$ test. Crosstabs and Fisher's exact test were used to analyze the behavioral responses of males in different treatments.

**Table 1** Compounds for Y-tube olfactometer and wind-tunnel bioassays (F and M for virgin female and male, respectively).

| No. | Components |
|---|---|
| A | Hexane |
| B | Gland extracts |
| C | Volatile collections (1M1F) |
| D | Volatile collections (2F) |
| E | Volatile collections (2M) |
| F | (*Z,Z,Z*)-9,12,15-octadecatrienal ($10^{-3}$ $\mu$g/$\mu$L) |
| G | (*Z,Z,Z*)-9,12,15-octadecatrienal ($10^{-2}$ $\mu$g/$\mu$L) |
| H | (*Z,Z,Z*)-9,12,15-octadecatrienal ($10^{-1}$ $\mu$g/$\mu$L) |
| I | (*Z,Z,Z*)-9,12,15-octadecatrienal (1 $\mu$g/$\mu$L) |
| J | (*Z*)-9-hexadecenyl acetate ($10^{-3}$ $\mu$g/$\mu$L) |
| K | (*Z*)-9-hexadecenyl acetate ($10^{-2}$ $\mu$g/$\mu$L) |
| L | (*Z*)-9-hexadecenyl acetate ($10^{-1}$ $\mu$g/$\mu$L) |
| M | (*Z*)-9-hexadecenyl acetate (1 $\mu$g/$\mu$L) |
| N | 1-nonanal ($10^{-3}$ $\mu$g/$\mu$L) |
| O | 1-nonanal ($10^{-2}$ $\mu$g/$\mu$L) |
| P | 1-nonanal ($10^{-1}$ $\mu$g/$\mu$L) |
| Q | 1-nonanal (1 $\mu$g/$\mu$L) |
| R | (*Z*)-9-hexadecenyl acetate ($10^{-2}$ $\mu$g/$\mu$L) + (*Z,Z,Z*)-9,12,15-octadecatrienal (0.16 $\mu$g/$\mu$L) |
| S | (*Z*)-9-hexadecenyl acetate ($10^{-2}$ $\mu$g/$\mu$L) + (*Z,Z,Z*)-9,12,15-octadecatrienal (0.16 $\mu$g/$\mu$L) + 1-nonanal ($10^{-3}$ $\mu$g/$\mu$L) |
| T | (*Z*)-9-hexadecenyl acetate ($10^{-2}$ $\mu$g/$\mu$L) + (*Z,Z,Z*)-9,12,15-octadecatrienal (0.16 $\mu$g/$\mu$L) + 1-nonanal ($10^{-2}$ $\mu$g/$\mu$L) |
| U | (*Z*)-9-hexadecenyl acetate ($10^{-2}$ $\mu$g/$\mu$L) + (*Z,Z,Z*)-9,12,15-octadecatrienal (0.16 $\mu$g/$\mu$L) + 1-nonanal ($10^{-1}$ $\mu$g/$\mu$L) |
| V | (*Z*)-9-hexadecenyl acetate ($10^{-2}$ $\mu$g/$\mu$L) + (*Z,Z,Z*)-9,12,15-octadecatrienal (0.16 $\mu$g/$\mu$L) + 1-nonanal (1 $\mu$g/$\mu$L) |

1M1F, a newly emerged virgin female and a newly emerged male; 2F, 2 newly emerged virgin females; 2M, 2 newly emerged virgin males.

## Results

### *Identification and determination of female and male pheromones*

GC-EAD analysis of female gland extracts identified 2 compounds that stimulated males in EAG tests,

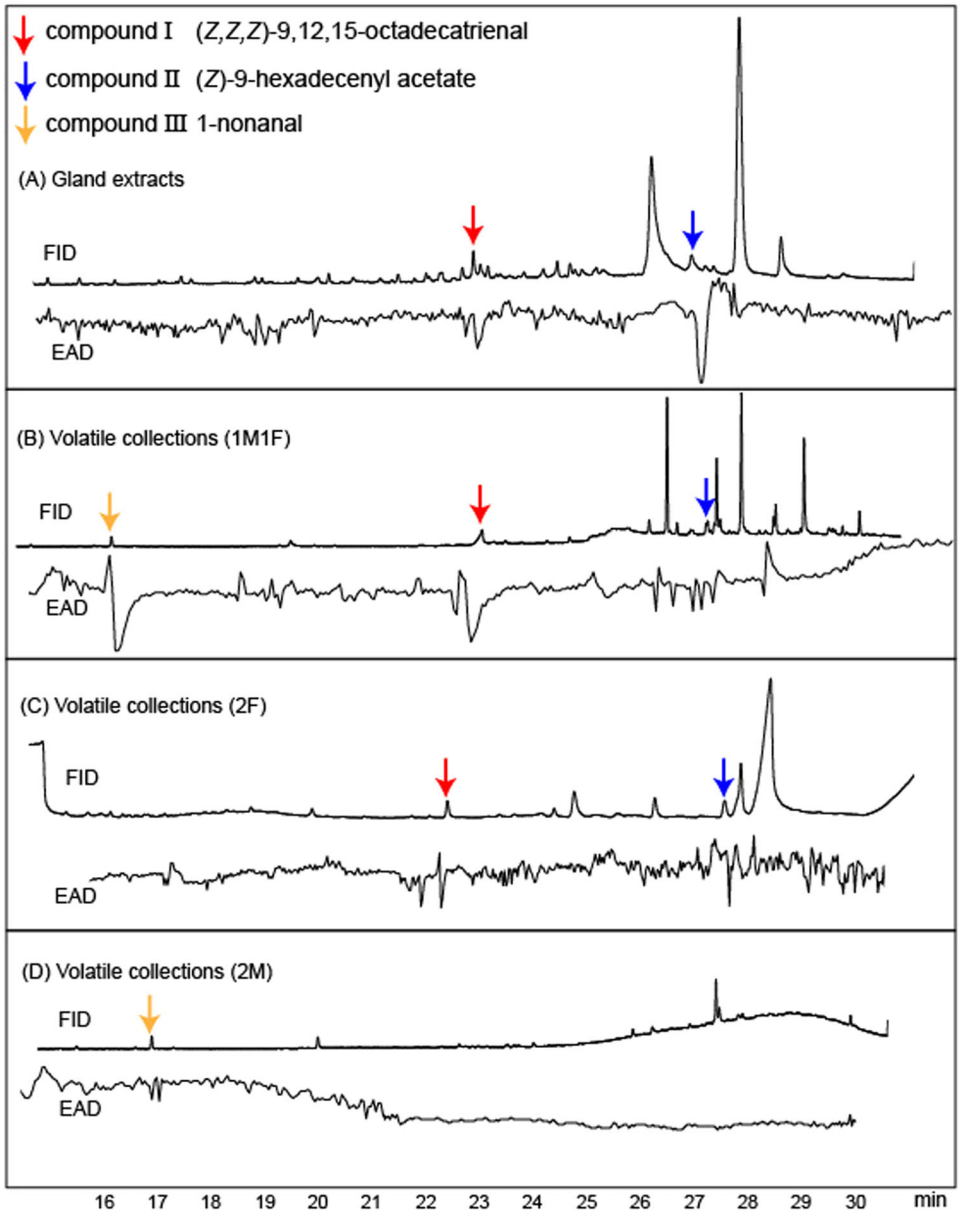


**Fig. 1** Coupled GC-EAD analysis of gland extracts and volatile collections of *Agriphila aeneociliella* (A) Gland extracts of female *A. aeneociliella*. (B) Volatile collections (1M1F): a newly emerged virgin female and a newly emerged male. (C) Volatile collections (2F): 2 newly emerged virgin females. (D) Volatile collections (2M): 2 newly emerged virgin males. (A)−(C) Upper trace: simultaneous recording of FID; and lower trace: antennal response from male *A. aeneociliella*. (D) Upper trace: simultaneous recording of FID; and lower trace: antennal response from female *A. aeneociliella*. FID, flame ionization detector; GC-EAD, gas chromatography-electroantennography.

which had retention times of 23.15 min (compound I) and 27.18 min (compound II) and a ratio of 16.04 : 1 (Fig. 1A). These 2 active compounds also were detected in volatile collections from either 1M1F or 2F (Figs. 1B, C). The relative molecular weight of compound I was 262, and compound I showed characteristic ions at *m/z* 247, 233, 219, 193, 163, 149, 135, 121, 108, 93, 79, 67, and 55 (Fig. S1). The mass spectrum of compound II had diagnostic fragment ions at *m/z* 222 ($M^+$-$CH_3COOH_2^+$) and 61 ($CH_3COOH_2^+$) (Fig. S1). The

 

mass spectra of compounds I and II closely matched the spectra for (*Z,Z,Z*)-9,12,15-octadecatrienal (I) and (*Z*)-9-hexadecenyl acetate (II), according to diagnostic ions and peak enhancement.

All 4 crude extracts induced electrophysiological responses in both female and male antennae (Fig. 2A). In the dose–response tests, the EAG amplitude of male antennae was enhanced significantly with increase in the concentrations of both (*Z,Z,Z*)-9,12,15-octadecatrienal ($F = 119.502$, df = 3, $P < 0.001$) and (*Z*)-9-hexadecenyl acetate ($F = 52.894$, df = 3, $P < 0.001$) (Fig. 2B,C). In the Y-tube assay, gland extracts and volatiles collected from treatments 1M1F and 2F both caused significant behavioral responses by males (Fig. 3A). Three concentrations ($10^{-3}$, $10^{-2}$, $10^{-1}$ $\mu$g/$\mu$L) of (*Z*)-9-hexadecenyl acetate and (*Z,Z,Z*)-9,12,15-octadecatrienal induced significant levels of attraction in males compared with the solvent in the Y-tube olfactometer tests (Fig. 3B,C). In the wind-tunnel measurements, both gland extracts and volatile collections induced excitatory activities of males (Fig. 4A). The wind-tunnel bioassays showed that different concentrations of (*Z,Z,Z*)-9,12,15-octadecatrienal had significant effects on male behavioral responses ($\chi^2 = 21.112$, df = 12, $P = 0.049$), whereas the concentration of (*Z*)-9-hexadecenyl acetate had no significant effect ($\chi^2 = 15.298$, df = 12, $P = 0.226$). When the odor source was (*Z*)-9-hexadecenyl acetate ($10^{-2}$ $\mu$g/$\mu$L) or (*Z,Z,Z*)-9,12,15-octadecatrienal ($10^{-1}$ $\mu$g/$\mu$L), 10% of the tested individuals reached the odor sources. In addition, compared with the other 3 concentrations of (*Z*)-9-hexadecenyl acetate or (*Z,Z,Z*)-9,12,15-octadecatrienal, the number of males flying against the wind was higher for both $10^{-2}$ $\mu$g/$\mu$L and $10^{-1}$ $\mu$g/$\mu$L (Fig. 4A).

Compound III, identified as 1-nonanal (according to NIST, Fig. S1) was present in both volatile collections from 2 treatments (1M1F and 2M), and it elicited electrophysiological responses in both males and females (Figs. 1B, D). Both males and females produced strong electrophysiological responses to this candidate pheromone in the EAG assay (Figs. 2B, C, D). Females of *A. aeneociliella* selected the olfactometer arm baited with the lower concentrations ($10^{-3}$, $10^{-2}$, $10^{-1}$ $\mu$g/$\mu$L) of 1-nonanal significantly more than the blank arms. No significant difference (versus the control) was observed in the response of females to the highest tested concentration (1 $\mu$g/$\mu$L) of 1-nonanal (Fig. 3E).

### *Pheromones co-regulate behavioral and electrophysiological responses*

In the dose–response assays, the EAG responses of males to 1-nonanal were significantly higher at higher doses (Fig. 2D) ($F = 471.267$, df = 3, $P < 0.001$). However, in Y-tube olfactory bioassays, differences in dosage of male pheromone did not induce any significant selective responses by males (Fig. 3D). Interestingly, the ternary mixture of both female pheromones and the male pheromone induced stronger EAG responses in males than did the binary mixture of female pheromones alone (Fig. 2E). Additions of $10^{-3}$ or $10^{-2}$ $\mu$g/$\mu$L of 1-nonanal into the binary mixture of female pheromones significantly increased the attractiveness to males (Fig. 3F). When concentration of 1-nonanal in the ternary mixture was 1 $\mu$g/$\mu$L, the attractancy of the mixture to males was significantly reduced (Fig. 3F), and no males contacted the source (Fig. 4A). The number of males that engaged in oriented upwind flight and contacted the odor source in the wind tunnel increased when the odor source included either $10^{-3}$ or $10^{-2}$ $\mu$g/$\mu$L of 1-nonanal in the binary mixture of female pheromones (Fig. 4A). Most males showed excitement (taking flight and oriented upwind flight), but none reached the odor source when the binary female mixture also included 1 $\mu$g/$\mu$L of 1-nonanal. When odor sources were either volatile collections from the treatment 1M1F or the ternary mixture of the 2 female pheromones and $10^{-2}$ $\mu$g/$\mu$L of 1-nonanal, males took less time to reach the odor source (Fig. 4B).

## Discussion

In moths, sex pheromones are most often released by females and perceived by males, and this pheromone communication system has been extensively studied as a model for sexual attraction based on pheromones (Karlson & Butenandt, 1959; Zhang *et al.*, 2015). However, male pheromones may also play an important role in the mating process in moths. Until now, no studies have investigated both male and female pheromones of a moth simultaneously. This study identified the pheromones of both sexes of *A. aeneociliella* and demonstrated that adding the male pheromone component 1-nonanal to the female pheromone altered the male's response up to a certain threshold. *Agriphila aeneociliella* is a potential model for investigating the functional significance of female and male pheromones in lepidopteran species.

The volatiles emitted from 3 different moth groupings, namely, 1M1F, 2F, and 2M were collected and identified. Two EAG-active components, (*Z*)-9-hexadecenyl acetate and (*Z,Z,Z*)-9,12,15-octadecatrienal, were identified both in the *A. aeneociliella* female crude glandular extracts and in volatile collections with females (1M1F and 2F). Three concentrations ($10^{-3}$, $10^{-2}$, $10^{-1}$ $\mu$g/$\mu$L) of (*Z*)-9-hexadecenyl acetate and of (*Z,Z,Z*)-9,12,15-octadecatrienal significantly attracted males in Y-tube

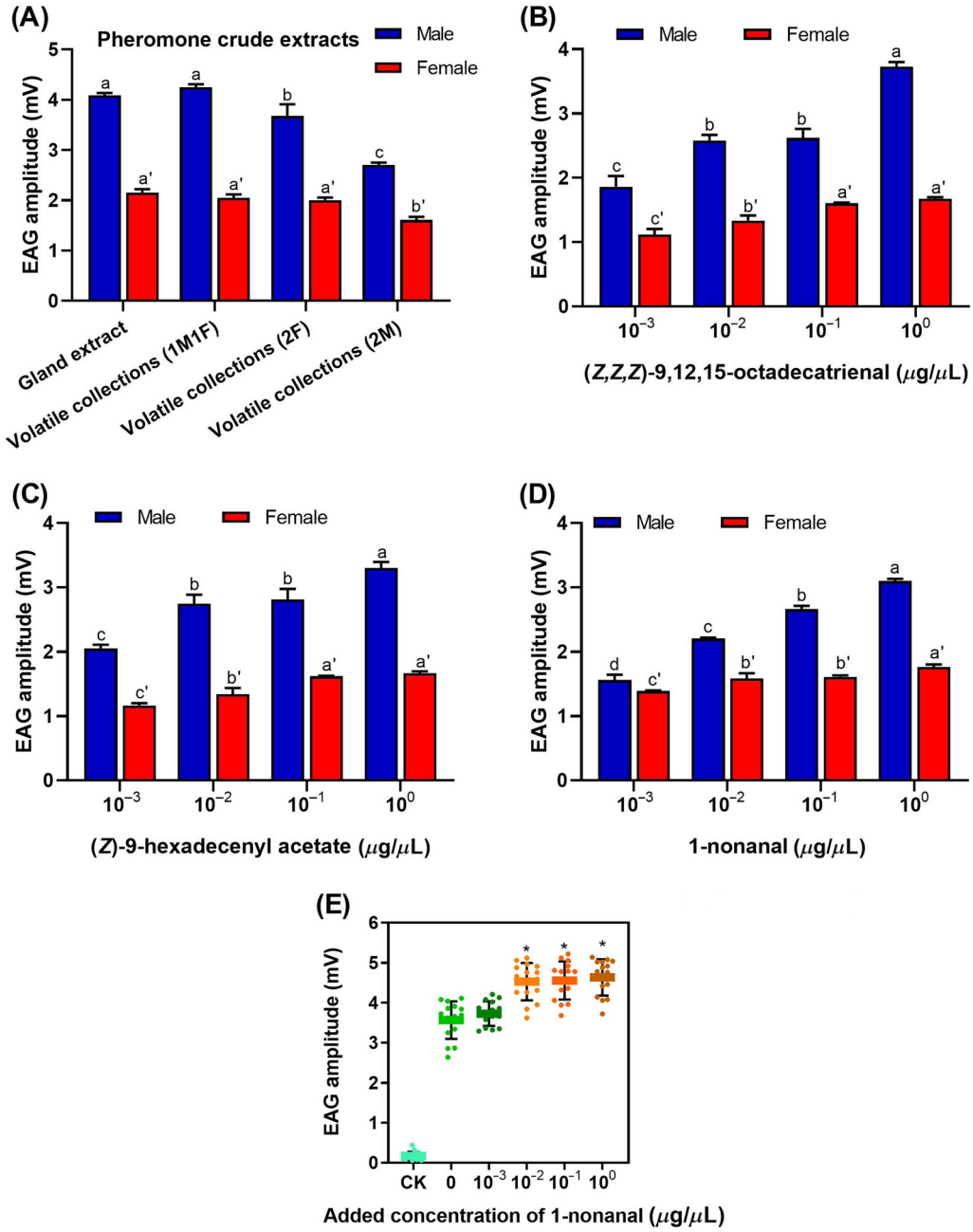


**Fig. 2** EAG responses of *Agriphila aeneociliella* female and male antennae to 4 pheromone collections (gland extracts and volatile collections) (A) and concentration gradients ($10^{-3}$ to 1 $\mu$g/$\mu$L) of 3 synthetic pheromone chemicals (B−D). *A. aeneociliella* male EAG responses to putative male pheromone with concentration gradient ($10^{-3}$ to 1 $\mu$g/$\mu$L) added in putative female pheromones mixture ($10^{-2}$ $\mu$g/$\mu$L (*Z*)-9-hexadecenyl acetate + 0.16 $\mu$g/$\mu$L (*Z,Z,Z*)-9,12,15-octadecatrienal) (E). Data are presented as means ± SE. Different lowercase letters indicate the significance of the difference in EAG response values of males (a, b, c) or females (a′, b′, c′) to different pheromone concentrations. The asterisks indicate significance differences at $P < 0.05$. EAG, electroantennogram; SE, standard error.

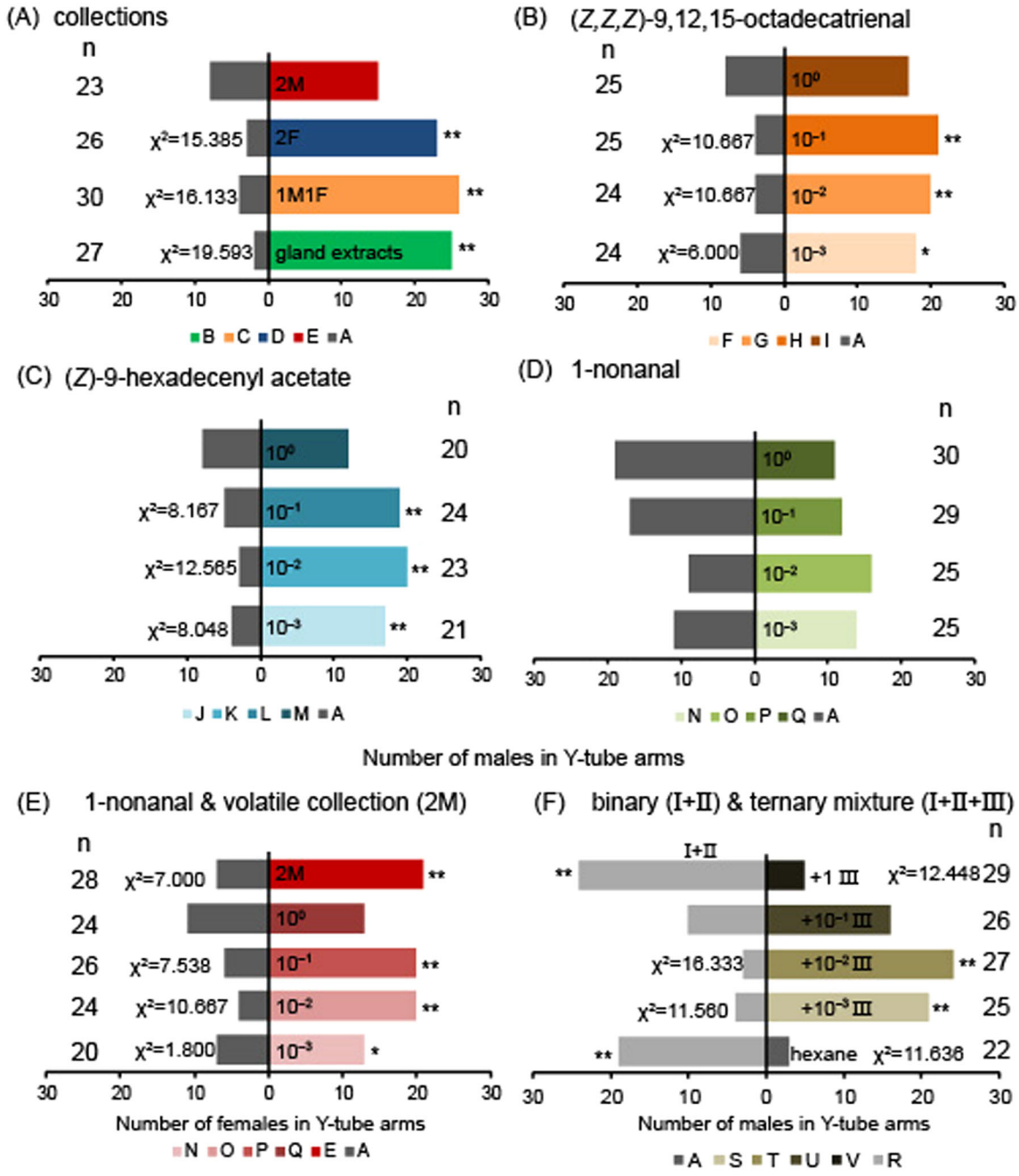


**Fig. 3** Behavioral responses to sex pheromone binary chemical mixtures in Y-tube olfactometer. (A) Olfactory responses of male *Agriphila aeneociliella* to gland extracts/volatile collections (collections) versus solvent. (B) Olfactory responses of male *A. aeneociliella* to concentration gradients ($10^{-3}$ to 1 $\mu$g/$\mu$L) of (*Z,Z,Z*)-9,12,15-octadecatrienal (I) versus solvent. (C) Olfactory responses of male *A. aeneociliella* to concentration gradients ($10^{-3}$ to 1 $\mu$g/$\mu$L) of (*Z*)-9-hexadecenyl acetate (II) versus solvent. (D) Olfactory responses of male *A. aeneociliella* to concentration gradients ($10^{-3}$ to 1 $\mu$g/$\mu$L) of 1-nonanal (III) versus solvent. (E) Olfactory responses of female *A. aeneociliella* to volatile collections (2M)/concentration gradients ($10^{-3}$ to 1 $\mu$g/$\mu$L) of 1-nonanal versus solvent. (F) Olfactory responses of males to putative female pheromones binary mixture and mixtures of male and female pheromone candidates. A: hexane; B−V: collected samples and synthetic pheromone chemicals (listed in Table 1); *n*: number of responders. The asterisks indicate significance differences at $P < 0.05$ (*) and $P < 0.01$ (**).

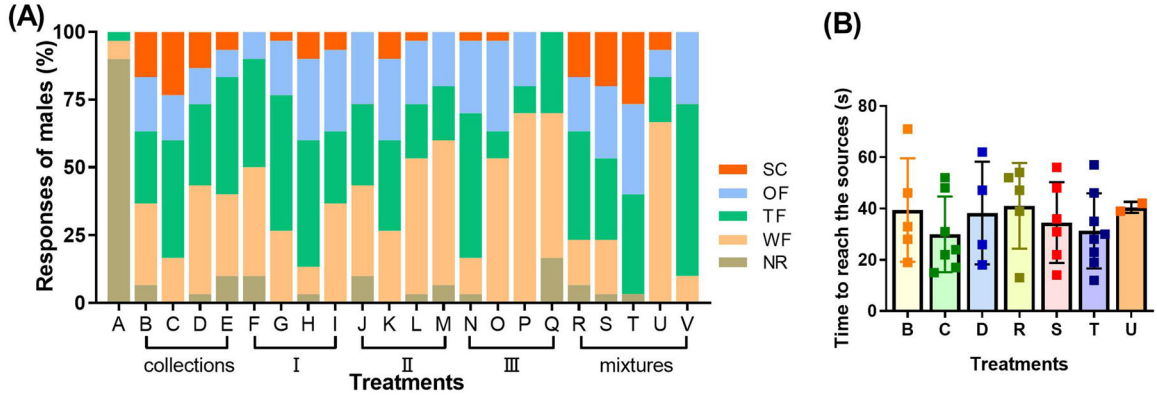


**Fig. 4** Behavioral responses to sex pheromone mixtures in wind tunnel (A) Responses of male *Agriphila aeneociliella* to collected pheromones (collections), different concentrations of pheromones (I: (*Z,Z,Z*)-9,12,15-octadecatrienal; II: (*Z*)-9-hexadecenyl acetate; III: 1-nonanal), and combinations of female and male pheromones (mixtures) (listed in Table 1). NR, No response; OF, Oriented upwind flight; SC, Source contact; TF, Taking flight; WF, Wing fanning. (B) Time of males reach the source.

bioassays and elicited excitatory responses in wind-tunnel assays, suggesting that these compounds are the 2 components of the female pheromone of *A. aeneociliella*. Higher concentration (1 $\mu$g/$\mu$L) of female pheromone components did not affect the male response. Our previous observations showed that male behavioral responses were inhibited when presented with 3 females (Zhan *et al.*, 2020), which may also be related to the higher concentration of female pheromone likely to be present. However, 1-nonanal (the male-produced pheromone) was only detected from treatments with males (1M1F and 2M), and this compound induced electrophysiological responses in the antennae of both males and females. Male pheromones such as 1-nonanal are considered to be aphrodisiacs for females (Birch, 1970, 1974; Baker *et al.*, 1981; Royer & McNeil, 1992; Hillier & Vickers, 2004; Hillier & Vickers, 2011; Xiao *et al.*, 2020). Y-tube behavioral observations showed that *A. aeneociliella* females were attracted to 1-nonanal (at $10^{-2}$ and $10^{-1}$ $\mu$g/$\mu$L). Although 1-nonanal did not induce significant selective behavior of males in the Y-tube, it did stimulate almost all males to produce excitatory behaviors and a few males to show oriented upwind flight behavior in the wind tunnel. These results suggested that 1-nonanal is an active pheromone component released by *A. aeneociliella* males. Furthermore, we found that male pheromone had multiple functions. In addition to its aphrodisiac effect on females, it also worked with female pheromones to manipulate male courtship behaviors. Researchers have suggested that *P. unipuncta* males are not inhibited or repelled by male pheromones, although they were able to perceive them (Fitzpatrick *et al.*, 1988, 1989). Indeed, *A. aeneociliella* males showed neither significant preference nor avoidance behavior to 1-nonanal, but the electrophysiological activity results did show that males could perceive this male pheromone. However, the male pheromone may work together with the female pheromone in regulating the competition among courting male moths.

In *A. aeneociliella*, a certain degree of male competition (2M : 1F) can increase the mating success rate, which could be called "male cooperation". However, higher levels of male competition (e.g. 3M : 1F) did not result in successful mating, even though it can induce male excitatory behavior (Zhan *et al.*, 2020). The ternary pheromone mixture may play a dual role in the regulation of sexual coordination in moths. It elicits cooperation under a certain concentration but switches to inducing an avoidance behavior when present at high concentration. Our study showed that adding different concentrations of 1-nonanal to the binary mixture of female pheromones produced significant changes in male responses. The EAG responses of males to the ternary mixture with different concentrations of 1-nonanal were significantly higher than those to the binary female pheromone. Also, the ternary mixture with a lower concentration ($10^{-3}$ and $10^{-2}$ $\mu$g/$\mu$L) of 1-nonanal attracted significantly more males than the binary mixture of female pheromones. Although the EAG amplitude induced by the ternary mixture with a high concentration (1 $\mu$g/$\mu$L) of 1-nonanal was higher, the ternary mixture lost its attractiveness to males in the Y-tube and caused the males to produce excitatory responses in the wind tunnel without reaching the odor source.

 

Any imbalance of the operational sex ratio is predicted to lead to stronger competition among individuals of the more abundant sex for access to mates (Janicke *et al.*, 2018). The electrophysiological and behavioral characteristics of the male *A. aeneociliella* responses to ternary mixtures containing different concentrations of 1-nonanal are consistent with the predicted mating behaviors under different male-biased operational sex ratio scenarios, as previously observed (Zhan *et al.*, 2020). The results of our current study indicated that the "quantity effects" (i.e. functional concentration) of male pheromone determined the outcome of competition between courting males during the mating process. Also, in this system the male pheromone usually works through coordination with the components of the female sex pheromone. One caveat is that the male pheromone functions that we observed in *A. aeneociliella* occurred under laboratory conditions. Therefore, we suggest that systematic studies should be conducted in a more natural laboratory or field setting to confirm our interpretations.

The moth *A. aeneociliella* is typical of species with a Type I pheromone. Several lepidopteran species have been identified that employ 1 of these 3 chemicals—(*Z,Z,Z*)-9,12,15-octadecatrienal, (*Z*)-9-hexadecenyl acetate, or 1-nonanal—as the female or male pheromone. In addition to *A. aeneociliella*, 5 species in Arctiidae use (*Z,Z,Z*)-9,12,15-octadecatrienal as the female pheromone, and 10 moth species use (*Z*)-9-hexadecenyl acetate as the female pheromone, including 7 species in the Noctuidae. *Elasmopalpus lignosellus* Zeller, in the same family as *A. aeneociliella*, also uses (*Z*)-9-hexadecenyl acetate as 1 of its female pheromone components. Two species of moths and 4 species of butterflies used 1-nonanal as the male pheromone (Fig. S2). Noctuoidea insects commonly use (*Z,Z,Z*)-9,12,15-octadecatrienal and (*Z*)-9-hexadecenyl acetate as components of their pheromones. Most Type I pheromones are biosynthesized from a common saturated fatty-acyl CoA, while most Type II pheromones are biosynthesized from dietary linoleic or linolenic acid (Naka & Fujii, 2020). The compound (*Z,Z,Z*)-9,12,15-octadecatrienal is classified as a Type I pheromone based on the presence of a terminal functional group, but it is derived from $\alpha$-linolenic acid, as are other Type II pheromones, according to the positions of double bonds and carbon chain length (Kiyota *et al.*, 2011; Naka & Fujii, 2020). We conclude that *A. aeneociliella* has both Type I and Type II pheromone synthesis pathways. The pheromone synthesis pathway of this moth is evolutionarily similar to that of the more advanced Noctuoidea. However, only 1 species (*E. lignosellus*) in the Pyraloidea superfamily shares the same pheromone component of (*Z*)-9-hexadecenyl acetate with *A. aeneociliella*. Selection will favor emitters that reshuffle enzymatic pathways to produce new pheromone blends and receivers with a broader ability to detect novel components (Symonds & Elgar, 2008; Allison & Cardé, 2016).

The results of our study show that the pheromones emitted by females and males co-regulate mate and species recognition in *A. aeneociliella*. We infer that under natural conditions, males of our study species could evaluate local moth sex ratios based on titers of male and female sex pheromones in the pheromone plume and then make their decision to pursue or avoid the odor source.

Compared with female structures, the scent-emitting structures and pheromone components of males exhibit striking diversity across moths, and evidence suggests that strong sexual selection acting on males has led to the evolution of male pheromones (Phelan & Baker, 1987; Weiss *et al.*, 2017). The precise release of pheromones and the specific perception mechanism of insects in nature enable them to manipulate their mating behaviors by subtle changes in pheromone release. The response mechanism of male *A. aeneociliella* moths to female and male sex pheromones provides a case study supporting the competitive signal evolution model that male courtship signals can increase male mating success relative to conspecifics (West-Eberhard, 1983, 1984). We simulated the male selection response under different intraspecific densities according to the response of males to sexual pheromones. The role of male signal and the variation in composition and proportion of female chemical signals result in differential male courtship behaviors. The sensitivity of males to both female and male pheromones and the concentration differences in the pheromone plumes indicate the evolution of a broader range of sensory systems with precise functional differentiation. To gain more insight into the role of male pheromones under the pressure of sexual selection, future studies are needed on more male pheromones and their role in the mating behaviors in moth species.

## Acknowledgments

This work was supported by grants from the Natural Science Foundation of Shandong Province (ZR2020MC121) and the National Key R&D Program of China (2017YFD0201705).

## Disclosure

The authors declare that they have no conflict of interest.

## References


Allison, J.D. and Cardé, R.T. (2016) Pheromones: reproductive isolation and evolution in moths. In: *Pheromone Communication in Moths* (eds. Allison, J.D. & Carde, R.T.), p. 414. University of California Press, Berkeley, California.

Ando, T. and Yamamoto, M. (2022) Internet database: https://lepipheromone.sakura.ne.jp/pdb_top.html

Ando, T., Inomate, S.I. and Yamamoto, M. (2004) Lepidopteran sex pheromones. *The chemistry of pheromones and other semiochemicals I* (ed. S. Schulz), pp. 51–96. Springer Berlin, New York.

Baker, T.C. and Cardé, R.T. (1979) Courtship behavior of the oriental fruit moth (*Grapholitha molesta*): experimental analysis and consideration of the role of sexual selection in the evolution of courtship pheromones in the Lepidoptera. *Annals of the Entomological Society of America*, 72, 173–188.

Baker, T.C., Nishida, R. and Roelofs, W.L. (1981) Close-range attraction of female oriental fruit moths to herbal scent of male hairpencils. *Science*, 214, 1359–1361.

Birch, M., Lucas, D. and White, P. (1989) The courtship behavior of the cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae), and the role of male hair-pencils. *Journal of Insect Behavior*, 2, 227–239.

Birch, M.C. (1970) Pre-courtship use of abdominal brushes by the nocturnal moth *Phlogophoru mticulosa* (L.) (Lepidoptera: Noctuidae). *Animal Behaviour*, 18, 310–316.

Birch, M.C. (1974) Aphrodisiac pheromones in insects. In: *Pheromones* (ed. Birch, M.C.), pp. 115–134. American Elsevier, New York.

Birch, M.C., Poppy, G.M. and Baker, T.C. (1990) Scents and eversible scent structures of male moths. *Annual Review of Entomology*, 35, 25–58.

Blomquist, G.J. and Vogt, R.G. (2003) Biosynthesis and detection of pheromones and plant volatiles—introduction and overview. In: *Insect pheromone biochemistry and molecular biology* (eds. Blomquist, G.J. & Vogt, R.G.), pp. 3–18. Academic Press, Elsevier.

Chi, B., Zheng, X., Liang, X. and Liu, Y. (2016) Temperature-dependent demography of *Agriphila aeneociliella* (Lepidoptera: Crambidae), a new insect pest of wheat in China. *Agricultural and Forest Entomology*, 18, 189–197.

Conner, W.E. and Iyengar, V.K. (2016) Male pheromones in moths: reproductive isolation, sexy sons, and good genes. In: *Pheromone Communication in Moths* (eds. Allison, J.D. & Carde, R.T.), pp. 191–208. University of California Press, Berkeley, California.

De Pasqual, C., Groot, A.T., Mappes, J. and Burdfield-Steel, E. (2021) Evolutionary importance of intraspecific variation in sex pheromones. *Trends in Ecology & Evolution*, 36, 848–859.

El-Sayed, A.M. (2022) Pherobase: http://www.pherobase.com/

Fitzpatrick, S.M., McNeil, J.N. and Dumont, S. (1988) Does male pheromone effectively inhibit competition among courting true armyworm males (Lepidoptera: Noctuidae)? *Animal Behaviour*, 36, 1831–1835.

Fitzpatrick, S.M., Mcneil, J.N. and Miller, D. (1989) Age-specific titer and antennal perception of acetic acid, a component of male *Pseudaletia unipuncta* (Haw.) hairpencil secretion. *Journal of Chemical Ecology*, 15, 641–648.

Groot, A.T., Dekker, T. and Heckel, D.G. (2016) The genetic basis of pheromone evolution in moths. *Annual Review of Entomology*, 61, 99–117.

Henneken, J. and Jones, T.M. (2017) Pheromones-based sexual selection in a rapidly changing world. *Current Opinion in Insect Science*, 24, 84–88.

Hillier, N.K. and Vickers, N.J. (2004) The role of heliothine hairpencil compounds in female *Heliothis virescens* (Lepidoptera: Noctuidae) behavior and mate acceptance. *Chemical Senses*, 29, 499–511.

Hillier, N.K. and Vickers, N.J. (2011) Hairpencil volatiles influence interspecific courtship and mating between two related moth species. *Journal of Chemical Ecology*, 37, 1127–1136.

Hirai, K., Shorey, H.H. and Gaston, L.K. (1978) Competition among courting male moths: male-to-male inhibitory pheromone. *Science*, 202, 644.

Jacquin, E., Nagnan, P. and Frerot, B. (1991) Identification of hairpencil secretion from male *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae) and electroantennogram studies. *Journal of Chemical Ecology*, 17, 239–247.

Janicke, T., Morrow, E.H. and Hosken, D. (2018) Operational sex ratio predicts the opportunity and direction of sexual selection across animals. *Ecology Letters*, 21, 384–391.

Karlson, P. and Butenandt, A. (1959) Pheromones (ectohormones) in insects. *Annual Review of Entomology*, 4, 39–58.

Khallaf, M.A., Cui, R., Weissflog, J., Erdogmus, M., Svatos, A., Dweck, H.K.M. *et al.* (2021) Large-scale characterization of sex pheromone communication systems in *Drosophila*. *Nature Communications*, 12, 4165.

Kiyota, R., Arakawa, M., Yamakawa, R., Yasmin, A. and Ando, T. (2011) Biosynthetic pathways of the sex pheromone components and substrate selectivity of the oxidation enzymes working in pheromone glands of the fall webworm, Hyphantria cunea. *Insect Biochemistry and Molecular Biology*, 41, 362–369.

Löfstedt, C., Wahlberg, N. and Millar, J.G. (2016) Evolutionary pattern of pheromone diversity in Lepidoptera. In: *Pheromone Communication in Moths* (eds. F.D. Allison & R.T. Cardé), pp. 43–78. University of California Press, Berkeley, California.

Mitter, C., Davis, D.R. and Cummings, M.P. (2017) Phylogeny and evolution of Lepidoptera. *Annual Review of Entomology*, 62, 265–283.

Naka, H., and Fujii, T. (2020) Chemical divergences in the sex pheromone communication systems in moths. In: *Insect Sex Pheromone Research and Beyond* (ed. Ishikawa, Y.), pp. 3–17. Entomology monographs, Springer, Singapore.

Phelan, P.L. and Baker, T.C. (1987) Evolution of male pheromones in moths: reproductive isolation through sexual selection? *Science*, 235, 205–207.

Roelofs, W.L. and Rooney, A.P. (2003) Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proceedings of the National Academy of Sciences USA*, 100, 14599–14599.

Royer, L. and McNeil, J. (1992) Evidence of a male sex pheromone in the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae). *Canadian Entomologist*, 124, 113–116.

Stökl, J. and Steiger, S. (2017) Evolutionary origin of insect pheromones. *Current Opinion in Insect Science*, 24, 36–42.

Symonds, M.R. and Elgar, M.A. (2008) The evolution of pheromone diversity. *Trends in Ecology & Evolution*, 23, 220–228.

Takayoshi, K. and Hiroshi, H. (1999) Identification and possible functions of the hairpencil scent of the yellow peach moth, *Conogethes punctiferalis* (Guenée) (Lepidoptera: Pyralidae). *Applied Entomology and Zoology*, 34, 147–153.

Teal, P.E.A. and Tumlinson, J.H. (1989) Isolation, identification, and biosynthesis of compounds produced by male hairpencil glands of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae). *Journal of Chemical Ecology*, 15, 413–427.

Teal, P.E.A., McLaughlin, J.R. and Tumlinson, J.H. (1981) Analysis of the reproductive behavior of *Heliothis virescens* (F.) under laboratory conditions. *Annals of the Entomological Society of America*, 74, 324−330.

Weiss, K., Herzner, G. and Strohm, E. (2017) Sexual selection and the evolution of male pheromone glands in philanthine wasps (Hymenoptera, Crabronidae). *BMC Evolutionary Biology*, 17, 128.

West-Eberhard, M.J. (1983) Sexual selection, social competition, and speciation. *Quarterly Review of Biology*, 58, 155–183.

West-Eberhard, M.J. (1984) Sexual selection, competitive communication, and species-specific signals in insects. In: *Proceedings of the 12th Symposium of the Royal Entomological Society of London* (ed. Lewis, T.), pp. 283–324. Academic Press, London.

Wyatt, T.D. (2003) *Pheromones and animal behavior*. Cambridge University Press, New York.

Xiao, Y., Liu, K., Elgar, M.A., Cheng, Y., Jiang, X., Zhang, L. *et al.* (2020) Male ventroposterior brush display increases the sexual receptivity of females in the gregarious beet webworm *Loxostege sticticalis* (Lepidoptera: Crambidae). *Journal of Insect Behavior*, 33, 184–192.

Yew, J.Y. and Chung, H. (2015) Insect pheromones: an overview of function, form, and discovery. *Progress in Lipid Research*, 59, 88−105.

Zhan, Y., Liu, J. and Liu, Y. (2020) The mating strategy and reproductive performance of *Agriphila aeneociliella* (Lepidoptera: Crambidae), a new insect pest of wheat in China. *Agricultural and Forest Entomology*, 22, 203–211.

Zhang, J., Walker, W.B. and Wang, G. (2015) Pheromone reception in moths: from molecules to behaviors. *Progress in Molecular Biology and Translational Science*, 130, 109–128.

Manuscript received May 8, 2022
Final version received November 28, 2022
Accepted December 16, 2022

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Mass spectra of components of female and male pheromones.

**Fig. S2** Pheromone recruitment in moths and butterflies.