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Mechanisms of premating isolation between *Helicoverpa armigera* (Hübner) and *Helicoverpa assulta* (Guenée) (Lepidoptera: Noctuidae)

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Abstract

Helicoverpa armigera and *Helicoverpa assulta* are sympatric sibling species, and in the laboratory they can interbreed and produce viable offspring. To assess the contributions of temporal barriers and sexual barriers to premating isolation, we investigated both the temporal rhythms of calling behavior and pheromone titers of *H. armigera* and *H. assulta* females and the behavioral responses of males to conspecific and heterospecific calling females in a wind tunnel. Both *H. armigera* and *H. assulta* females called throughout the scotophase, and there was more calling during the second half of the scotophase than during the first half. Maximal pheromone titer and maximal calling activity in *H. armigera* synchronously occurred at the sixth hour into the scotophase, whereas, in *H. assulta*, the maximal pheromone titer occurred 2 h before the peak of calling. Pheromone blend ratios of the two species were opposite and, within each species, changes in the ratio within the scotophase and at different ages were relatively small. Males of both *H. armigera* and *H. assulta* responded strongly to their conspecific calling females in the wind tunnel and completed the whole courtship sequence. In contrast, they did not land and had no copulation attempts in response to heterospecific calling females. These results show that the two species do not have obvious temporal differences in calling behavior and pheromone production, and the specificity of sex pheromone blend emitted by females plays a key role in their premating isolation. In addition, we summarized the potential isolation mechanisms of *H. armigera* and *H. assulta*.

Keywords: *Helicoverpa armigera*; *Helicoverpa assulta*; Sexual behavior; Sex pheromone; Premating isolation

1. Introduction

The two closely related species, the cotton bollworm, *Helicoverpa armigera*, and the oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae), are sympatric and serious crop pests in China and eastern Asia (Chen, 1999). It is common to come across mixed populations of *H. armigera* and *H. assulta* in the field, and thus, reproductively active adults of both species are very likely to meet at the same time. Moreover, in the laboratory, if confined in cages the two species can hybridize and produce viable offspring (Wang and Dong, 2001; Zhao et al., 2005). Therefore, behaviors that reduce interspecific mating mistakes may play a key role in maintaining reproductive isolation between the two species in nature.

Premating isolation can be caused by spatial, temporal, sexual and mechanical barriers (Dobzhansky, 1970), and they may act in concert. In order to understand the role of sexual communication systems in premating isolation, it is necessary to take into account both temporal barriers and sexual barriers that prevent contact between *H. armigera* and *H. assulta*. First, the species-specificity of sex pheromone component blends is the most common factor resulting in sexual isolation (Roelofs and Cardé, 1974). *H. armigera* and *H. assulta* both use (Z)-11-hexadecenal (Z11-16: Ald) and (Z)-9-hexadecenal (Z9-16: Ald) as their

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sex pheromone components but in opposite ratios, 100:2.5 and 6:100, respectively, with Z11-16: Ald being the major sex pheromone component in *H. armigera* (Piccardi et al., 1977; Nesbitt et al., 1979, 1980; Kehat et al., 1980; Kehat and Dunkelblum, 1990; Wu et al., 1997), whereas Z9-16: Ald is the major component in *H. assulta* (Sugie et al., 1991; Cork et al., 1992; Liu et al., 1994; Wang et al., 2005). Zhao et al. (2006) reported that some proportion of *H. armigera* males performed upwind flights and landed in response to the binary synthetic pheromone blends of *H. assulta* in wind-tunnel assays, and this suggested that partial cross-attraction between the two species might exist. However, whether cross-attraction between *H. armigera* and *H. assulta* exists or not should be proved by using live calling females as baits.

Second, differences in temporal patterns of sexual behavior could play an effective role in reproductive isolation (Haynes and Birch, 1986; Sawamura and Tomaru, 2002; Mazor and Dunkelblum, 2005). Kou and Chow (1987) studied the calling behavior of *H. armigera* at $25 \pm 1\,^{\circ}C$ under a 16:8 h light-dark photoperiod, and they found that maximum calling of *H. armigera* occurred between the third and eighth hour of scotophase. Kamimura and Tatsuki (1993) investigated the calling behavior of *H. assulta* at $23 \pm 0.5\,^{\circ}C$ under a 15:9 h light-dark photoperiod, and they reported that maximum calling of *H. assulta* occurred between the second and sixth hour of scotophase. It is unreliable to compare their results for understanding whether there is temporal difference in calling behavior between *H. armigera* and *H. assulta* because the insects they used were not sympatric (*H. armigera* from Taiwan, and *H. assulta* from Japan), and the environmental conditions (photoperiod, temperature) during rearing and experiments were also different. Therefore, to verify whether temporal isolation exists between the two species it is better to use the sympatric *H. armigera* and *H. assulta* under the same environmental conditions.

In the present study we examined the temporal rhythms of calling behavior and sex pheromone titers of *H. armigera* and *H. assulta* females, determined behavioral responses of males to conspecific and heterospecific calling females, and finally assessed the potential isolation mechanisms of the two sibling species.

2. Materials and methods

2.1. Insect rearing

H. armigera and *H. assulta* were originally collected in the field as larvae from the suburb of Zhengzhou, Henan province of China, and were separately maintained for successive generations in the laboratory at $26 \pm 1\,^{\circ}C$ and 55–65% relative humidity under a 16:8 h light-dark photoperiod regime. Larvae were reared on an artificial diet with wheat germ as the main ingredient (Wu and Gong, 1997). Pupae were separated by sex, and female pupae and male pupae were separately placed in cages. Moths were provided with a 10% honey solution. The emerged moths were collected daily to ensure that each cage contained moths of the same age and to calculate their age. The age of moths that emerged during the photophase and within the first 2 h of the scotophase and lived through the scotophase was designated as 1-day-old, 2-day-old, 3-day-old, etc. on subsequent days.

2.2. Calling behavior

Newly emerged virgin females of *H. armigera* and *H. assulta* were held individually in self-made mesh cages (17 cm long and 15 cm in diameter) with cotton balls containing a 10% honey solution under the photoperiod described above. Since preliminary observations indicated that all females did not call during photophase, calling behaviors were observed continuously during scotophase at a light intensity of approximately 0.6 lux with a red lamp in the room, from 1- to 6-day-old, or until female died. Calling behavior can be easily recognized by the extruded ovipositor beyond the abdomen tip. A penlight was used to see the extruded ovipositor and to observe the calling behavior. Observations were made every 5 min during scotophase and the time when a female extruded and retracted her ovipositor was recorded. The following calling parameters were calculated by using the detailed records of every individual female during scotophase: the percent calling, the mean onset time of calling, the mean duration of calling, and the mean number of calling bouts. 23 *H. armigera* females and 27 *H. assulta* females were observed respectively.

2.3. Pheromone extraction and analysis

Ovipositors and the associated pheromone glands of female moths were extruded by finger pressure, and the glands were dissected with a pair of fine scissors. For determination of pheromone components, a single pheromone gland was placed in a thin glass tube, extracted in 5 μl hexane containing 5 ng/μl of internal standard (Z)-10-hexadecenal (Z10-16: Ald), left hermetically at room temperature for 15 min, and last removed from the tube. Gland extracts were kept at $-20\,^{\circ}C$ until analysis with a Hewlett-Packard HP 5890 Series II Plus gas chromatograph (GC) equipped with a capillary column (50QC2/BPX70-0.25, SGE), a flame ionization detector (FID), and a splitless injector system. The column temperature was initially programmed to $210\,^{\circ}C$ from $80\,^{\circ}C$ at $4\,^{\circ}C$/min, and then programmed to $240\,^{\circ}C$ from $210\,^{\circ}C$ at $10\,^{\circ}C$/min and held for 5 min. Nitrogen was used as the carrier gas at an inlet pressure of 200 KPa. Pheromone components Z11-16: Ald and Z9-16: Ald were identified by comparing their retention times with those of authentic compounds, and were quantified by relating their peak areas to that of the internal standard Z10-16: Ald.

2.4. Pheromone titer

For *H. armigera* and *H. assulta* females, the effects of time into the scotophase and age on pheromone titer in the pheromone gland were studied. First, the hourly fluctuation pattern of pheromone titer was investigated by extracting individual pheromone glands of 3-day-old females at different times of the scotophase. Second, the daily changes in pheromone titer of the individual pheromone gland of 1- to 7-day-old female moth were examined for *H. armigera* at the sixth hour of the scotophase and for *H. assulta* at the fourth hour of the scotophase. More than 11 individuals for each species were analyzed for different times of the scotophase and for different ages.

2.5. Wind tunnel behavioral assays

Tests were carried out in the plexiglass wind tunnel (2.4 m long, 0.9 m wide, 1 m high) under the following conditions: $26 \pm 1\,^{\circ}C$, 55–65% relative humidity, 0.6 lux of red light and 0.5 m/s of wind speed. Prior to test, males and females were moved to the flight-tunnel room at the beginning of scotophase to acclimate to the new conditions. When needed for an experiment, a mesh cage (17 cm long and 15 cm in diameter) containing one 2–3-day-old female that was calling was selected and placed on a sheet-metal platform elevated 40 cm above the tunnel floor. A 2–3-day-old male was then placed in a mesh cage (10 cm long and 5 cm in diameter) 30 cm above the tunnel floor and released downwind 1.8 m from the calling female moth. Males were tested between the fourth and seventh hour of the scotophase. The responses of *H. armigera* and *H. assulta* virgin males to conspecific and heterospecific calling virgin females were tested, and more than 38 males were tested for each of the four combinations. We chose four phases of the behavioral response to describe male attraction towards calling females: initiating flight; upwind flight, zigzagging in the pheromone plume; flying close to the calling female within 10 cm; landing and attempting copulation. Males that did not take off from the release cage within 5 min were replaced by new males.

2.6. Data analysis

The effects of age on four parameters of calling behavior of *H. armigera* and *H. assulta* and the effects of age or time into the scotophase on the quantity and ratio of two pheromone components of the two insect species were estimated with two-way ANOVA and differences among the means were compared with Student–Newman–Keuls multiple range test at the $P = 0.05$ level of significance. The percentage of male responses to calling females was calculated for each of the four behavioral phases as the number of males exhibiting a given behavior phase divided by the number of tested males. Differences in the male behavioral responses of *H. armigera* and *H. assulta* to conspecific and heterospecific calling females were analyzed by using Chi-square 2×2 tests for each behavioral phase with the threshold of significance set at $P = 0.01$. All the above analyses were carried out with SPSS 11.01 (2001) software package.

3. Results

3.1. Calling behavior

Calling of *H. armigera* and *H. assulta* females occurred throughout the scotophase except for 1-day-old females, and there were more females calling during the second half of the scotophase than the first half (Fig. 1). The percentage of *H. armigera* and *H. assulta* females calling at any given time into the scotophase usually increased with age.

Age had a significant effect on calling percentage, calling onset time and calling duration, and had a marginally significant effect on the number of calling bouts, but there was no significant effect of species on these parameters (Table 1). The interaction between age and species was found to be significant.

The calling percentages of *H. armigera* and *H. assulta* females during their first scotophase were low, but became higher on subsequent scotophases (Table 2), suggesting that most females of *H. armigera* and *H. assulta* become reproductively mature from 2-day-old. The percentage of

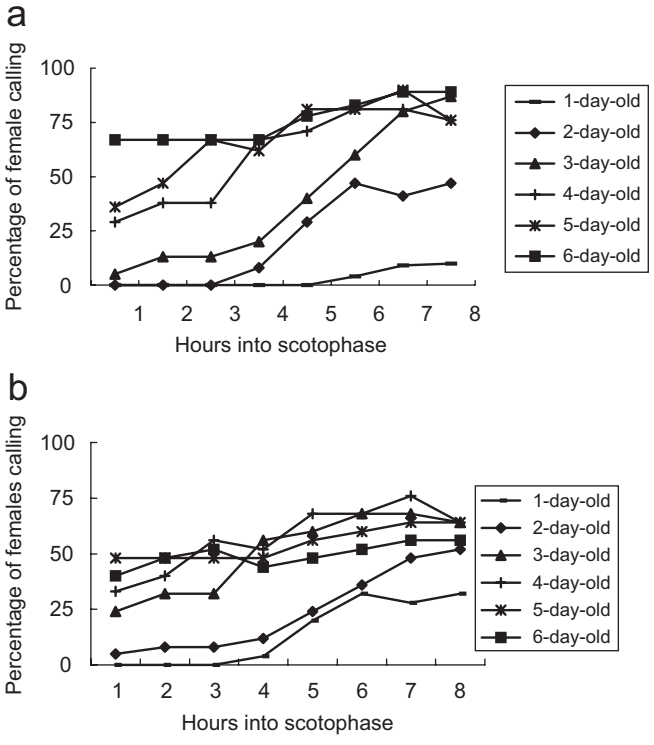

Fig. 1. Effects of age and time into scotophase on percentage of virgin female calling. (a) *H. armigera* ($N = 23$), (b) *H. assulta* ($N = 27$).

Table 1
ANOVA for the effects of age on calling behavior of *H. armigera* and *H. assulta*

Source of variation	Calling percentage				Onset time of calling				Calling duration				No. of calling bouts			
	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Model	6	0.120	5.998	0.034	11	85072.271	6.743	<0.001	11	71032.720	7.535	<0.001	11	7.430	2.523	0.005
Age	5	0.138	6.945	0.027	5	145167.878	11.506	<0.001	5	91589.637	9.715	<0.001	5	6.437	2.186	0.057
Species	1	0.025	1.265	0.312	1	2.771	0.000	0.988	1	15896.834	1.686	0.196	1	7.408	2.516	0.114
Age × Species					5	39161.530	3.104	0.010	5	57577.094	6.107	<0.001	5	10.481	3.559	0.004
Error	5	0.020			192	12616.790			192	9427.419			192	2.945		

Table 2
Effect of age on calling behavior of *H. armigera* and *H. assulta*

Species	Age (days)	No. of females	% calling at least once	Mean onset time of calling X ± SE (min)	Mean duration of calling X ± SE (min)	Mean no. of calling bouts X ± SE
H. armigera	1	23	13	422 + 29a	26 + 9c*	1.3 + 0.3c*
	2	23	88	272 + 28b	31 + 9c*	2.3 + 0.3bc*
	3	23	91	228 + 48bc	114 + 35b	2.7 + 0.4b*
	4	21	95	179 + 29c	202 + 32ab	3.3 + 0.4ab
	5	21	95	94 + 25d*	258 + 22a*	3.7 + 0.4a
	6	18	100	81 + 24d*	227 + 31a*	4.4 + 0.4a
H. assulta	1	27	33	317 + 39a	103 + 32a	3.3 + 0.9a
	2	27	85	242 + 22b	97 + 14a	3.3 + 0.4a
	3	27	85	221 + 20bc	146 + 16a	4.3 + 0.4a
	4	27	93	209 + 22bc	134 + 16a	3.0 + 0.4a
	5	27	63	189 + 24cd	139 + 21a	3.2 + 0.3a
	6	25	68	154 + 23d	116 + 18a	3.4 + 0.4a

For each species, means in the same column followed by different letters are significantly different (Student–Newman–Keuls multiple range test, $P<0.05$); *denotes a significant difference (Student–Newman–Keuls multiple range test, $P<0.05$) from *H. assulta* females of the same age.

H. armigera females calling increased with age, and the percentage of *H. assulta* females calling increased gradually from 1- to 4-day-old and then decreased. The percentage of *H. armigera* calling at 5-day-old and 6-day-old was higher than that of *H. assulta* females.

For both *H. armigera* and *H. assulta* females, the mean onset time of calling varied with age, and the older females called earlier than the younger ones (Table 2). From 1- to 3-day-old, the onset time of calling was not significantly different between *H. armigera* and *H. assulta* females of the same age, though *H. assulta* females usually called about 7–105 min earlier than *H. armigera* females of the same age. In contrast, *H. armigera* females of 5-day-old and 6-day-old called about 73–95 min earlier than *H. assulta* females of the same age.

For *H. armigera* females, the mean duration of calling was clearly influenced by age with 1-day-old and 2-day-old moths calling for a significantly shorter total duration than older moths. However, for *H. assulta* females, the mean duration of calling did not significantly vary with age (Table 2). Additionally, while younger *H. armigera* females (1- and 2-day-old) called significantly shorter, by about 66–77 min, than *H. assulta* females of the same age, older *H. armigera* females (5-day-old and 6-day-old) called significantly longer, by about 111–119 min, than *H. assulta* females of the same age.

H. assulta females made significantly more calling bouts than *H. armigera* females from 1- to 3-day-old but not from 4- to 6-day-old (Table 2). For *H. assulta* females, the mean number of calling bouts did not significantly vary with age, whereas for *H. armigera* females, the mean number of calling bouts varied significantly with age.

3.2. Pheromone titer and ratio

Age, time into scotophase, and species all showed significant effects on the titer and ratio of sex pheromone components Z9-16:Ald and Z11-16:Ald (Tables 3 and 5). A greater portion of total variation was related to species than to age or to time into scotophase. Both Age × Species and Time into scotophase × Species interaction were significant. Therefore, the main effects of species and age or the main effects of species and time into scotophase need to be interpreted together.

The amount of Z11-16: Ald, the main pheromone component of *H. armigera*, reached a peak at 2-day-old and remained unchanged until 5-day-old; the amount of the minor component Z9-16: Ald reached a peak on the second night after emergence and remained unchanged on subsequent nights (Table 4). The ratio of Z9-16: Ald to Z11-16: Ald in the sex pheromone gland of *H. armigera* was nearly constant from 2- to 7-day-old with

a significantly low ratio at 1-day-old ($P<0.05$). For *H. armigera*, Z11-16: Ald titer peaked during the sixth hour of scotophase and remained unchanged for the rest of the scotophase, whereas the minor component Z9-16: Ald peaked during the fifth hour of scotophase and remained unchanged until the end of scotophase (Table 6). The proportion of Z9-16: Ald to Z11-16: Ald increased from 1 to 2 h after lights-off, and this ratio was nearly constant on the subsequent 3 h and then decreased (Tables 3–6).

The amount of the major sex pheromone component of *H. assulta*, Z9-16: Ald, increased dramatically after lights-off, peaked 4 h later, remained at this level for 4 h, and then decreased (Table 6). Quantification of the minor sex component of *H. assulta*, Z11-16: Ald, showed similar temporal patterns to that of Z9-16: Ald, and the maximal titer was detected from 3 to 6 h after lights-off. The titer of Z9-16: Ald in the sex pheromone gland of *H. assulta* significantly increased from 1- to 2-day-old, but there were no significant differences in the titers at 2-day-old and 3-day-old (Table 4). After that, the titer decreased. The titer of Z11-16: Ald in the sex pheromone gland of *H. assulta* showed similar temporal patterns to that of Z9-16: Ald, and the maximal titer was detected at 2-day-old. For *H. assulta*, there was a gradual increase and decrease in pheromone ratio, with the highest ratio being found during the third to sixth hour of scotophase and from 2- to 5-day-old.

3.3. Male behavioral response to calling virgin females

The percentages of sequential behavioral responses of *H. armigera* and *H. assulta* males to conspecific and heterospecific calling virgin females in wind tunnel assay are shown in Table 7. Both *H. armigera* and *H. assulta* males responded strongly to their conspecific calling females with about half of them having completed the

Table 3
ANOVA for the effects of age on titer and ratio of sex pheromone components of *H. armigera* and *H. assulta*

Source of variation	Z9-16:Ald				Z11-16:Ald				Z9-16:Ald/Z11-16:Ald			
	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Model	13	1389.486	17.440	<0.001	13	305.794	25.299	<0.001	13	666.619	20.089	<0.001
Age	6	691.875	8.684	<0.001	6	155.622	12.875	<0.001	6	135.688	4.089	0.001
Species	1	8375.308	105.119	<0.001	1	2415.208	199.819	<0.001	1	6987.364	210.573	<0.001
Age × Species	6	661.611	8.304	<0.001	6	122.941	10.171	<0.001	6	135.694	4.089	0.001
Error	146	79.674			146	12.087			146	33.183		

Table 4
Effect of age on titer (ng/female, mean ± SE) and ratio (mean ± SE) of sex pheromone components Z9-16:Ald and Z11-16:Ald in pheromone glands of *H. armigera* and *H. assulta* females

Age (days)	*H. armigera*				*H. assulta*			
	N	Z9-16:Ald	Z11-16:Ald	Ratio	*N*	Z9-16:Ald	Z11-16:Ald	Ratio
1	10	0.01 ± 0.01b	1.09 ± 0.32c	0.005 ± 0.005b	11	12.02 ± 3.99bc	1.14 ± 0.39b	10.33 ± 1.82b
2	8	0.51 ± 0.11a	14.22 ± 3.09a	0.039 ± 0.004a	12	39.25 ± 6.42a	2.97 ± 0.69a	15.02 ± 2.01a
3	13	0.32 ± 0.05a	13.45 ± 1.42a	0.024 ± 0.003a	12	17.37 ± 3.31ab	1.02 ± 0.18b	17.85 ± 1.78a
4	10	0.23 ± 0.04a	11.63 ± 2.00a	0.021 ± 0.002a	11	11.36 ± 3.57bc	0.63 ± 0.12b	19.89 ± 4.54a
5	11	0.22 ± 0.04a	10.93 ± 1.47a	0.020 ± 0.002a	13	12.05 ± 2.46bc	0.74 ± 0.09b	14.84 ± 1.87a
6	11	0.21 ± 0.04a	7.73 ± 1.13b	0.026 ± 0.003a	13	7.52 ± 1.76bc	0.83 ± 0.11b	8.58 ± 1.72b
7	13	0.12 ± 0.03a	3.71 ± 0.71bc	0.030 ± 0.005a	12	4.20 ± 1.12c	0.65 ± 0.13b	6.96 ± 1.40b

Means in the same column followed by different letters are significantly different (Student–Newman–Keuls multiple range test, $P<0.05$).

Table 5
ANOVA for the effects of time into scotophase on titer and ratio of sex pheromone components of *H. armigera* and *H. assulta*

Source of variation	Z9-16:Ald				Z11-16:Ald				Z9-16:Ald/Z11-16:Ald			
	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Model	15	1080.906	15.341	<0.001	15	328.132	15.517	<0.001	15	455.565	25.335	<0.001
Time	7	385.646	5.473	<0.001	7	174.896	8.271	<0.001	7	71.018	3.949	0.001
Species	1	10729.901	152.284	<0.001	1	2320.980	109.759	<0.001	1	5807.572	322.967	<0.001
Time × Species	7	380.374	5.398	<0.001	7	168.393	7.963	<0.001	7	70.556	3.924	0.001
Error	167	70.460			167	21.146			167	17.982		

Table 6
Effect of time into scotophase on titer (ng/female, mean ± SE) and ratio (mean ± SE) of sex pheromone components Z9-16:Ald and Z11-16:Ald in pheromone glands of *H. armigera* and *H. assulta* females

Hour of scotophase	*H. armigera*				*H. assulta*			
	N	Z9-16:Ald	Z11-16:Ald	Ratio	*N*	Z9-16:Ald	Z11-16:Ald	Ratio
1	11	0.03 ± 0.03c	1.69 ± 0.73d	0.007 ± 0.004b	11	4.59 ± 1.26b	0.75 ± 0.12b	6.86 ± 2.27b
2	13	0.08 ± 0.04bc	2.71 ± 0.98d	0.016 ± 0.005b	10	9.83 ± 1.98b	0.94 ± 0.17b	9.94 ± 1.08b
3	11	0.17 ± 0.04b	5.75 ± 1.62cd	0.033 ± 0.002a	11	21.31 ± 3.27a	1.71 ± 0.25a	12.87 ± 1.24a
4	11	0.15 ± 0.02b	6.35 ± 1.36c	0.029 ± 0.004a	11	29.02 ± 5.09a	2.19 ± 0.48a	16.18 ± 2.39a
5	10	0.27 ± 0.07a	9.28 ± 2.24bc	0.028 ± 0.001a	11	21.74 ± 5.37a	1.30 ± 0.19ab	15.47 ± 2.07a
6	12	0.32 ± 0.05a	17.94 ± 2.62a	0.018 ± 0.002b	12	18.52 ± 3.23a	1.43 ± 0.19ab	12.58 ± 1.27a
7	13	0.30 ± 0.06a	12.26 ± 1.91ab	0.023 ± 0.003ab	12	9.57 ± 3.21b	1.09 ± 0.23b	6.91 ± 1.39b
8	14	0.22 ± 0.04ab	11.59 ± 2.16ab	0.018 ± 0.005b	10	10.02 ± 3.80b	0.93 ± 0.25b	9.91 ± 2.58b

Means in the same column followed by different letters are significantly different (Student–Newman–Keuls multiple range test, $P<0.05$).

Table 7
Responses percentage (%) of *H. armigera* (AR) and *H. assulta* (AS) males to conspecific and heterospecific virgin females in wind tunnel

Response of male to female	Number of males	Initiating flight	Upwind flight	Close to calling female within 10 cm	Landing and having copulation attempts
AR to AR	45	73a	64a	51a	49a
AR to AS	40	33b	10b	3b	0b
AS to AS	43	81a	72a	60a	58a
AS to AR	38	34b	3b	3b	0b

Within each column, percentages followed by the same letter are not significantly different at $P<0.01$ according to the χ^2 test of independence.

whole courtship sequence. However, in responses to heterospecific calling females, both *H. armigera* and *H. assulta* males did not land and no copulation attempts were made, though a small percentage of them showed the early three behaviors of the response sequence. For each of the four behavioral phases of the response sequence, significant differences were observed between male responses of *H. armigera* or *H. assulta* to conspecific and heterospecific calling virgin females ($P<0.01$). But no significant differences were observed between male responses of *H. armigera* and *H. assulta* to their conspecific or heterospecific calling virgin females ($P>0.01$).

4. Discussion

In moths, the diel rhythm of sexual activity, such as calling activity and sex pheromone production and release, usually depends upon endogenous (neural, hormonal) and exogenous factors (photoperiod, temperature) (Baker and Cardé, 1979; Hollander and Yin, 1982; Raina and Klun, 1984; Schal and Cardé, 1986; Delisle and Mcneil, 1987). Therefore, although the calling behaviors of *H. armigera* and *H. assulta* were, respectively, studied by Kou and Chow (1987) and Kamimura and Tatsuki (1993), their results concerning the two species were incomparable because the insect species they used were not sympatric and environmental conditions were also different. Kou and Chow (1987) reported that no *H. armigera* individuals called during the first scotophase following emergence, but we found that there were actually low proportions of calling during the first scotophase (Fig. 1). Kamimura and Tatsuki (1993) reported that calling behavior and sex pheromone titer in *H. assulta* were synchronous, but by our results, the peak of pheromone production occurred about 2 h before the peak of calling. These discrepancies may be attributed to differences between insect populations used in their studies and ours. Both calling behavior and sex pheromone titers of *H. armigera* and *H. assulta* showed distinct diel periodicity during the scotophase (Fig. 1 and Table 6). Maximal pheromone titer and maximal calling activity in *H. armigera* both synchronously occurred at the sixth hour during the scotophase, whereas, in *H. assulta*, they did not occur at the same time.

Since not all females initiated calling at the same chronological age, it is better to compare calling rhythm as a function of calling age instead of chronological age if there was a considerable degree of individual variability in the age at which females initiate calling for the first time following emergence (Turgeon and McNeil, 1982). More than 85% of both *H. armigera* and *H. assulta* females called during the second night following emergence (Table 2), suggesting that most females are reproductively mature at age 2 and there was not a considerable degree of individual variability in reproductive maturation. In this study we analyzed temporal rhythms of calling behavior and sex pheromone titer in function of chronological age, and the results indicated that all age-related trends were statistically significant. Because females of the same calling age are more consistent in reproductive maturation rates than those of the same chronological age, we speculate that

the calling age-related trends of calling rhythm and sex pheromone titer may be more remarkable than the chronologically age-related trends.

Older females of *H. armigera* and *H. assulta* called sooner and longer after onset of the scotophase than younger females (Table 2), and this was consistent with the results of other researchers (Kou and Chow, 1987; Kamimura and Tatsuki, 1993). This phenomenon was explained as the adaptation of older females to improve chances of mating by being the first to attract males over younger females (Swier et al., 1977).

Most moths use specific mixtures of several compounds as their specific sex pheromone blend, and the ratios of those compounds are critical for attracting conspecific males. We investigated the temporal fluctuation patterns of two sex pheromone components in glands of *H. armigera* and *H. assulta*, and the results showed that their blend ratios were opposite, and changed little at different ages and at different time into the scotophase (Tables 4 and 6). Opposite ratios between species reduced the likelihood of interspecific mating mistakes and relatively small ratio variation within species fell within the range variation of conspecific male responses.

Mayr (1963) defined species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (biological species concept), and Dobzhansky (1970) classified reproductive isolation into two general categories: premating isolation (habitat isolation, temporal isolation, sexual isolation and mechanical isolation) and postmating isolation (gametic mortality, zygotic mortality, hybrid inviability, hybrid sterility, hybrid breakdown). For any particular case, reproductive isolation is not likely to be due to any one particular factor, and premating and postmating mechanisms may all contribute to it.

Here, we discuss potential isolation mechanisms of *H. armigera* and *H. assulta* according to Dobzhansky's classification as previously mentioned:

(1) *Habitat isolation*. *H. armigera* is a polyphagous species that feed on more than 60 crops such as cotton, corn, tobacco and soybean; host plants of *H. armigera* belong to 47 families, including Malvaceae, Solanaceae, Gramineae, and Leguminosae (Jallow et al., 2004). *H. assulta* is an oligophagous species feeding upon some Solanaceous plants such as tobacco, hot pepper, and several *Physalis* species (Fitt, 1989; Chen, 1999). It is obvious that, although their host plant ranges are quite different, overlapping host plants, such as tobacco, and other plants in the family Solanaceae, still exist. Therefore, habitat isolation between *H. armigera* and *H. assulta* is incomplete.

(2) *Temporal isolation*. Calling activity of *H. armigera* and *H. assulta* females both occurred throughout the scotophase (Fig. 1), and there is no sharp temporal separation, suggesting that temporal differences cannot result in their premating isolation. Generally, calling periods have shown partial or substantial overlaps between closely related, co-occurring moth species, such as *Autographa gamma* and *Cornutiplusia circumflexa* (Mazor and Dunkelblum, 2005), *Spodoptera latifascia* and *S. descoinsi* (Monti et al., 1995), *Euxoa campestris* and *E. rockburnei* or *E. declarata* (Teal et al., 1978), etc. However, in only a few studied sibling moth species has temporal partitioning in calling periods been great enough to be responsible for isolation. For example, the calling period of *Euxoa declarata* was exclusive from that of *E. rockburnei* (Teal et al., 1978); calling behavior of *Platyptilia carduidactyla* was confined to the first half of the scotophase, whereas *P. williamsii* was active in the second half (Haynes and Birch, 1986). Furthermore, in China, the phenologies of *H. armigera* and *H. assulta* overlap from mid-May to mid-October, during which period five generations occur (Chen, 1999; Zhao et al., 2005). Therefore, the two species have continuous and broadly overlapping generations, and this suggests that seasonal isolation between them does not exist.

(3) *Sexual isolation*. Both *H. armigera* and *H. assulta* males responded strongly towards their conspecific calling females and completed the whole courtship sequence, whereas males of neither species land or attempted copulation in response to heterospecific calling females (Table 7). This indicates that species-specific sex pheromones play a key role in their mate finding and sexual isolation. Zhao et al. (2006) reported that some proportion of *H. armigera* males showed landing response to the binary synthetic pheromone blends of *H. assulta* in a wind-tunnel, and this could be due to lower specificity of binary synthetic pheromone attractants compared to an entire suite of pheromone component-related volatiles emitted by live calling females. Therefore, complete sexual isolation between *H. armigera* and *H. assulta* may not be expected to develop only based on opposite ratios of their two main sex pheromone components. We suggest that there are additional minor pheromone components and/or behavioral antagonists of heterospecific males which may play an important role in increasing the specificity of male responses to conspecific females.

(4) *Mechanical isolation*. In the laboratory, *H. armigera* and *H. assulta* hybridized and produced fertile and sterile offspring, whereas successful mating in both reciprocal crosses was very low (Wang and Dong, 2001). Moreover, some cross pairs could not separate after copulation. The low mating success and failure to separate are likely linked to genital incompatibilities between the two species. It is obvious that mechanical isolation between the two species is present, but not complete.

(5) *Gametic mortality and zygotic mortality*. Male *H. armigera* and female *H. assulta* adults interbred and produced males and females, while female *H. armigera* and male *H. assulta* also hybridized but only yielded males (Wang and Dong, 2001). It is clear that gametic mortality does not exist. But, because the absence of F_1 females might be due to either lethality of heterogametic female

zygote or hybrid inviability in larvae stage, we do not know for sure whether zygotic mortality exists.

(6) *Hybrid inviability and hybrid sterility*. In F_1 hybrids, derived from female *H. armigera* crossed with male *H. assulta*, there were normal fertile males and abnormal sterile individuals but no females (Wang and Dong, 2001; Zhao et al., 2005). Zhao et al. (2005) speculated that hybrid sterility might result from the incompatibility of the Z-chromosome from *H. assulta* and autosomes from *H. armigera* and that hybrid inviability might be caused by cytoplasmic factors from *H. armigera* conflicting with the Z-chromosome from *H. assulta*.

(7) *Hybrid breakdown*. Zhao et al. (2005) interbred *H. armigera* and *H. assulta* and produced F_2 hybrids and six combinations of backcross hybrids. They found that F_2 hybrids and four combinations of backcross hybrids were normal males and females with 1:1 sex ratio, but two combinations of backcross hybrids were both fertile and sterile individuals with a skewed sex ratio and male bias (1:2). The skewed sex ratio may be due to the inviability of some female backcross hybrids. According to the inviability and sterility of partial backcross hybrids, hybrid breakdown exists to a certain extent.

In summary, the combination of several mechanisms is involved in the reproductive isolation of *H. armigera* and *H. assulta*: (1) difference in host plant range; (2) species-specific sex pheromones; (3) incomplete compatibility of genital tracts; (4) inviability and sterility of partial F_1 and backcross hybrids.

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