




## ORIGINAL ARTICLE

Reproduction does not impede the stopover departure to ensure a potent migration in *Cnaphalocrocis medinalis* mothsJiawen Guo<sup>1,2</sup> , Fan Yang<sup>1,3</sup>, Haiyan Zhang<sup>4</sup>, Peijiong Lin<sup>1,5</sup>, Baoping Zhai<sup>1</sup>, Zhongxian Lu<sup>2</sup>, Gao Hu<sup>1,6</sup>  and Pengcheng Liu<sup>1,6,7</sup> 

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**Abstract** Despite the importance of reproduction in insects, its relation with multi-stop flight remains poorly understood in migratory species. To clarify whether reproductive maturation commences during the multi-stop flight or after the completion of migration, we conducted physiological and behavioral assays in the rice leaf roller *Cnaphalocrocis medinalis* with laboratory-simulated conditions and field-captured populations. We found that the ovarian development was significantly promoted by tethered flight treatment for 1–2 nights when compared to the unflown group, while the flight muscle development was not impaired. There was no significant difference in flight duration, flight distance and flight velocity between mated and virgin female moths, indicating that mated moths remained competent for the subsequent flights as did the virgins. Using an integrated field assay, we identified that over 60% of the female moths in the migrating populations captured by high-altitude searchlights in the Immigration period of a season had completed the ovarian development and mating. Sexually mature and mated moths collected in the rice field in the Emigration period were found capable of engaging in migratory take-off, as observed using an indoor monitoring platform. Overall, our findings point out that *C. medinalis* managed to complete reproductive maturation to a large extent during the multi-stop migratory flight without compromising the migration performance. Such a cost-effective strategy ensures a successful migration for the moths. These findings advance our understanding of the relationship between reproduction and migration, thus shedding light on the development of novel control measures for the outbreak of migratory insect pests.

**Key words** mating; multi-stop flight; ovarian development; pest control; rice leaf roller

## Introduction

Many insects utilize migration as an adaptive strategy to evade the adverse environment and settle in habitats that enhance survival and reproduction, which ultimately promotes the persistence of populations

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(Johnson, 1963; Johnson, 1969; Southwood, 1977; Roff & Fairbairn, 2007; Dingle, 2014). This strategy is shaped by the antagonism between life-history traits associated with reproduction and flight, including the developmental trade-off between the reproductive and the flight organs (Johnson, 1969). The relationship between reproduction and migration, however, is regarded as rather complex and remains largely unexplored.

Reproduction and migration are energy-demanding processes in most insects (Beenackers *et al.*, 1985; Arrese & Soulages, 2010). Nonmigratory individuals tend to allocate more energy to reproduction than migratory ones, which often allocate more energy to flight relative to other processes (Zera & Denno, 1997; Kent & Rankin, 2001). The trade-off between reproduction and migratory flight had been referred to as “oogenesis-flight syndrome” (Johnson, 1969), which describes the general pattern wherein migratory activity in female insects occurs during the adult preoviposition stage when ovarian development and mating behavior are suppressed; rapid ovarian maturation then ensues following the end of migration (Dingle, 2014; Stefanescu *et al.*, 2021). This central concept of insect migration theory has been shown to apply to various wing-polymorphic insects, including *Nilaparvata lugens* and *Gryllus rubens* (Walters & Dixon, 1983; Mole & Zera, 1993; Zera & Denno, 1997), as well as wing-monomorphic insects such as *Helicoverpa armigera*, *Chrysoperla sinica* and *Plutella xylostella* (Colvin & Gatehouse, 1993; Muhamad *et al.*, 1994; Shirai, 1995; Begum *et al.*, 1996; Khuhro *et al.*, 2014).

Migratory insects with wings not undergoing morphological differentiation can fly on consecutive nights to complete their long-distance migration, and this is referred to as multi-stop migration (Chapman *et al.*, 2010; Wang *et al.*, 2017). Although reproduction is inhibited before migratory flight, an increasing number of studies have shown that migration tends to promote the ovarian development of the female moths, shortens the preoviposition period and actuates synchronized oviposition (Cheng *et al.*, 2012; Zhang *et al.*, 2015; Cheng *et al.*, 2016; Ge *et al.*, 2021). Migrating moths captured by high-altitude searchlights on the island of Bohai Strait, China, in spring, such as *Mythimna separata*, *Agrotis ipsilon* and *Plutella xylostella*, possess well-developed ovaries and have already mated (Zhao *et al.*, 2009; Fu *et al.*, 2014; Liu *et al.*, 2015; Vargas *et al.*, 2018). Migratory moths with such multi-stop migration pose a challenge to the “oogenesis-flight syndrome” theory. A systematic literature search and meta-analysis conducted by Tigreros and Davidowitz (2019) revealed that the cessation of reproduction is not a prerequisite for migratory flight in monomorphic insects, because there is little evidence for

a trade-off between migration and reproduction across studies.

Long-distance flight performance is not necessarily impaired by mating in certain wing-monomorphic moths such as *Cydia pomonella*, *Agrotis ipsilon* and *Spodoptera exigua* (Sappington & Showers, 1992; Schumacher *et al.*, 1997; Han *et al.*, 2008; Jiang *et al.*, 2010). These moths usually take off at dusk, stop flying at next dawn and then resume the take-off at next dusk (Chang *et al.*, 1980; Chapman *et al.*, 2010; Wang *et al.*, 2017). It is surmised that the moths manage to mate during the migration because opportunities for securing a mate after completion of the entire migration are scarce. Moreover, the moths with multi-stop flight pattern often travel downwind for up to hundreds of kilometers per night (Gatehouse, 1997; Chapman *et al.*, 2010; Alerstam *et al.*, 2011; Hu *et al.*, 2021). Such long-distance flights are thought to increase the dispersal distance between individual moths and be a source of mortality, which decreases the probability of successfully mating after the migration (Johnson & Gaines, 1990; Clobert *et al.*, 2012). Moths might achieve sexual maturity and complete mating and oviposition during their multi-stop migration, but there is currently little empirical evidence to support this speculation.

Here, we evaluated whether sexual maturity and mating affected the initiation of multi-stop flights during long-distance migration in rice leaf roller *Cnaphalocrocis medinalis*, an economically significant agricultural pest in Asia. We first studied the ovarian development, mating status and flight muscle development of the laboratory-reared *C. medinalis* moths after they were subjected to successive nocturnal flights in a flight mill system. We then investigated the natural populations of the moths captured with high-altitude searchlights at different migration periods of the peaked season in Jiangyan, China, with a special focus on the immigrant individuals. Last, we examined the relationship between the mating status and migration propensity of emigrating individuals collected in rice fields. The results of this study enhance our understanding of the mechanism by which reproduction regulates migration behavior in *C. medinalis*, and will aid the development of measures to control multi-stop migratory insect pests.

## Materials and methods

### Laboratory insect rearing

Larvae of *C. medinalis* were originally collected from rice fields in Nanning, Guangxi Zhuang Autonomous Region (108.33°E, 22.84°N), China, and reared using maize

seedlings. Pupae were removed from the seedlings and transferred into a transparent plastic box (16 × 24 × 22 cm in length, width and height, respectively), the bottom of which was filled with moist cotton wool to maintain high relative humidity (RH). Pairs of newly emerged male and female adults were transferred to 500-mL transparent cups with absorbent cotton wool soaked in 10% honey solution as a supplemental nutrient. The cups were covered with plastic film, and the adults oviposited on the film. All the insects were reared in intelligent artificial climate chambers (RXZ-380C-LED, Ningbo Jiangnan Instrument Factory) at 26 ± 1 °C and 80%–90% RH, and with a photoperiod of 14 h light : 10 h darkness.

#### Nocturnal successive flight tests

Nocturnal successive flight tests were conducted on a 24-channel computer-interfaced flight mill system (Jiaduo Science, Industry and Trade) that automatically recorded flight duration, distance and average velocity. *C. medinalis* adults generally take off at dusk with a peak for 20–40 min after the sunset, and land before sunrise (Chen & Wang, 1980; Riley *et al.*, 1995; Yang *et al.*, 2013). Take the city Guangxi Zhuang Autonomous Region (108.33°E, 22.84°N) for example, time of the sunrise and the sunset in August or September is from 06:00 to 07:00 Beijing Time (BJT) and from 19:00 to 20:00 BJT, respectively. Therefore, the flight tests starting time were set at 19:00 BJT. Radar observations show a significant reduction in the number of *C. medinalis* in the air after 23:00 BJT (Gao *et al.*, 2008), and flight activity was significantly reduced after 4 h of continuous flight (see supplementary Fig. S1), causing most migrants to descend and land. So, flight duration was set for 4 h (19:00–23:00 BJT). Each adult was tethered according to a technique described in previous studies (Sun *et al.*, 2013; Guo *et al.*, 2019a). The moths were lightly anesthetized with ether before the test. The scales of the adults at the junction between the metathorax and abdomen were brushed off using a soft brush pen, and the metathorax of each adult was glued onto a hollow plastic tether (diameter of 1 mm and length of 2 cm) with 502-superglue (Nianba Adhesive). Then, the tethered moth was attached to the flight arm, with the pipe perpendicular to the horizontal direction. A small piece of tissue was provided to the moth for tarsal contact, which greatly reduced trivial flight before test. Dusk was simulated by setting the light intensity to gradually decrease from 1 000 to 0.1 lx over a 45-min period after the onset of test, ending with complete darkness throughout the rest of the assay, which is similar to the take-off behavior of *C. medinalis* in fields.

Details on the lighting system have been described previously (Yang *et al.*, 2013; Guo *et al.*, 2019b). The flight tests were performed in a climate chamber with temperature and humidity maintained at 26 ± 1 °C and 70%–80% RH, respectively. After flight test on the first night, moths were carefully removed from the tether, transferred individually to a plastic cup and provided with honey solution. Then, the same process was repeated until the insect died. In order to maximally lower the damage to the insect body, the contact area of superglue was made small enough to detach easily from the arm.

#### Determination of flight muscle dry mass

After flight treatment, the virgin female moths were flash frozen and stored in liquid nitrogen. The whole body was placed in a 60 °C oven for 48 h to a constant weight. The flying muscles near the thorax was separated using the forceps from the dorso-longitudinal muscle of the mesothorax, and were then weighed on an electronic balance with a precision range of 10<sup>-3</sup> mg (Mettler-Toledo AG, XP6). The dry muscle weight of the virgin females at the same age without flight treatment was used as a control. The number of repetitions per process was no less than 15.

#### Characterization of ovarian development

At least 12 females after 4 h tethered flight on each night were dissected immediately to determine the levels of ovarian development based on the criteria by Zhang *et al.* (1979): (I) no follicular differentiation, (II) distinct follicular development, (III) well developed ovaries with a few chorionated eggs, (IV) most eggs matured and laid, and (V) ovarian atrophy stage. More than 24 virgin females at the same age without flight treatment were used as controls. Female moths with ovarian development level I–II are considered “sexually immature individuals”, while females with level III–V are considered “sexually mature individuals”.

#### Effect of mating on flight capability

A laboratory assay was designed to analyze the flight capability of the virgin and the mated female moths after successive nocturnal flights. Approximately 80 adult females were numbered and randomly divided into 2 groups: (1) virgin group, where female moths were reared alone; and (2) mated group, where each female was paired with 2 unflown male moths. After flight treatment,

the tether attached to adult pronotum was carefully cut away and each female was returned to its original cup with or without its male partners. The same procedure was repeated each day until insects ceased flying. Flight duration, distance and average velocity were recorded every day. Female moths were dissected after the treatment to confirm the mating status. Individuals with  $\geq 1$  spermatophores in bursa copulatrix were deemed to be mated.

#### *Examination of the reproductive capacity in the migrating population*

*Cnaphalocrocis medinalis* used for the observation of ovarian development and mating status was obtained in the area Jiangyan, Jiangsu Province, China (32.52°N, 120.15°E). The data were collected from July 18 to October 21 in 2019. Migrating populations were caught by a time-partition automatic trapping device based on insect searchlights that capture high-altitude immigrants (up to 500 m above the ground) (Wan *et al.*, 2016). Field populations were manually collected from the rice fields in the same area.

The rotation of the turntable on the trapping device is controlled at a constant interval as the prescribed start and end time. The lamp on the device is automatically turned on at 19:00 and turned off at 07:00 (BJT) the next day. The trapped insects were automatically separated every hour and the females were collected daily for dissection. In August or September, the sunset time is between 19:00 and 20:00 (BJT) and the sunrise time is between 05:00 and 06:00 (BJT) in Jiangyan. Since we were interested in immigrant populations to study the relation between ovarian development and migration, we removed the female moths that entered take-off phase for 3 h, and focused on those captured between 22:00 and 07:00 (BJT) for the study of ovarian development level and mating frequency. A total of 1 920 migratory female moths were collected and dissected. As to the field population, 959 female moths were collected from rice fields with a procedure described by Wang *et al.* (2017). The female moths in each group were dissected, and the ovarian development level and the number of spermatophore in the bursa copulatrix denoting mating frequency were analyzed. The ovarian development level was determined as described above, and the proportion of sexually mature individuals was calculated subsequently. According to the criteria developed by Zhang *et al.* (1979), the migration occurrence season of *C. medinalis* was divided into the Immigration period (from July 18 to July 24, 2019), Immigration and Local Breeding period (from July 25 to September 9, 2019) and Emigration period (from September 10 to

October 21, 2019), based on the ovarian development level of the field population and the population dynamics monitored by searchlights and field surveys (Table S4).

#### *Observation of take-off behavior*

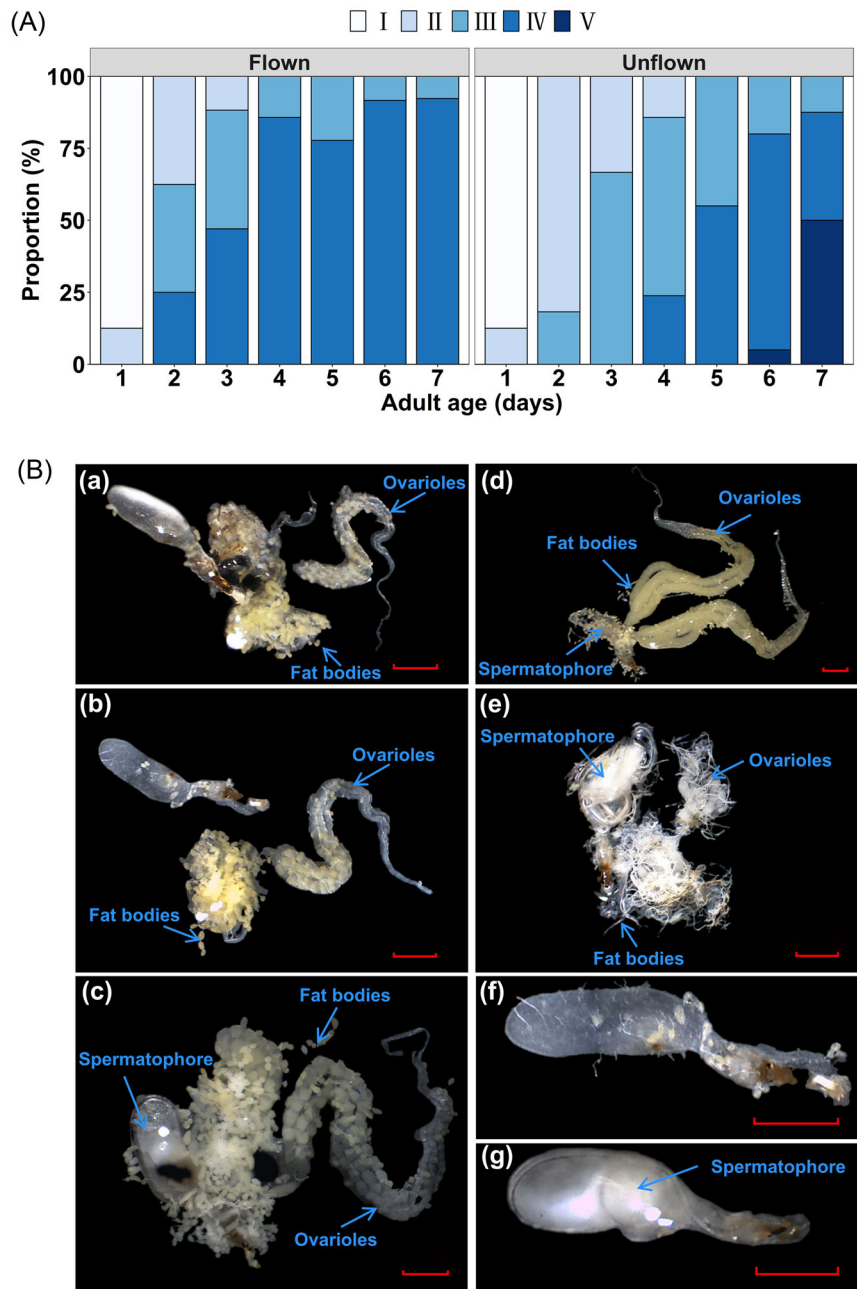
Twenty female *C. medinalis* moths were collected from the rice field in Jiangyan at 06:00 each day, with a total of 960 from August 28 to October 14, 2019. Those collected before and after September 9 were deemed at the Immigration and Local Breeding period and Emigration period, respectively. The moths were then used for the analysis of take-off behavior in a laboratory condition near the local sites.

The observation of take-off behavior was carried out in the following setting. Two 500-mL clear plastic cups with 10 female moths were placed in 2 take-off platforms and then were covered with a transparent PVC cage with a diameter of 60 cm and a height of 120 cm. An effective migratory take-off was defined if the moth spiraled with a vertical distance greater than 100 cm after taking off (Guo *et al.*, 2019b). If a moth stayed still or hovered at a height less than 100 cm, its action was regarded as nonmigratory take-off (Guo *et al.*, 2019b).

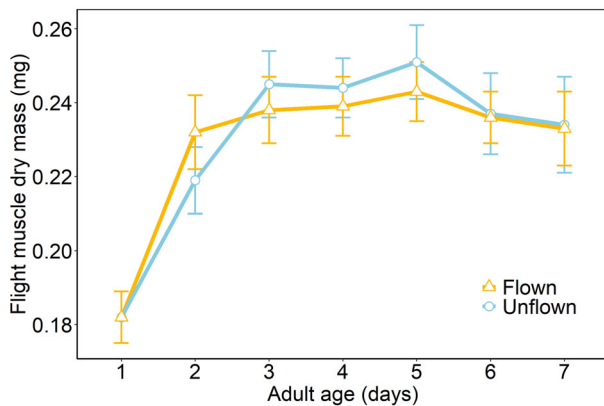
The field-collected moths were transferred into a climate chamber ( $26 \pm 1$  °C, 80%–90% RH) 1 h before the observation of take-off behavior at dusk (Yang *et al.*, 2013). A light source composed of 20 rows of fluorescent lamps (36 V/40 W) and 2 incandescent lamps (12 V / 40 W) was placed 200 cm above the take-off platform to simulate the light condition of the sunset, with minimizing the effect of the heat inside the PVC cage. The light intensity was changed by gradually extinguishing 20 parallel fluorescent lamps (2 every 3 min) and connecting the incandescent lamp with a potentiometer in order to create artificially simulated evening light. The indoor light intensity was gradually decreased from 1 000 lx to 0.1 lx within 45 min. The changes in light intensity during the observation period were simultaneously monitored with a TES-1330A illuminometer.

#### *Data analysis*

Data were tested for normal distribution and variance homogeneity using Shapiro–Wilk and Levene tests, respectively, before the analysis. Data not fitting a normal distribution were transformed by taking the root value or logarithmic value before performing variance analysis. Data failing to conform to the normal distribution after conversion were analyzed by nonparametric tests. Differences in proportion of sexually mature individuals



**Fig. 1** Ovarian development after flight treatment in *Cnaphalocrocis medinalis* moths. (A) Distribution of ovarian developmental stages in the flown and unflown groups shown as the percentages. More than 12 females after 4-h flight treatment each night were dissected to determine the stage of ovarian development, as described in Materials and Methods. (B) Ovaries classified as level I to IV. (a), (b), (c), (d) and (e) denote level I, II, III, IV and V, respectively, I and II indicate undeveloped ovaries, and III through V indicate developed ovaries. (f) and (g) denote the bursa copulatrix of unmated and mated female adults, respectively. The red bar denotes 1 000  $\mu\text{m}$ . Note that the presence of spermatophores in the bursa copulatrix indicates that moths had mated.



**Fig. 2** Flight muscle development and ovarian development of *C. medinalis* moths in tethered flights. The flight dry mass was presented in average milligrams per insect from more than 15 moths at each time point in either flown or unflown group. The ovaries were inspected simultaneously to help define the process of sexual maturity throughout the development post-adult emergence. Two-way ANOVA test was used to determine the difference throughout the developmental stages. Tukey's honestly significant difference test and *t*-test at  $\alpha = 0.05$  were used to determine the difference in the averaged mass between the 2 groups. Note that there was significant difference between day 1 and day 2 through day 7. However, there was no significant difference between flown and unflown groups at any of the stages.

between flown and unflown female moths and the effects of adult age on proportion of sexually mature individuals of flown and unflown female moths were analyzed by chi-square test. Flight muscle dry mass of *C. medinalis* treated with 2 flight status (flown, unflown) at different ages were subjected to a 2-way ANOVA test. Flight status, adult age and their interactions were included as categorical variables in the analysis. Subsequently, means of flight muscle dry mass (for flight status and adult age) were segregated with a Tukey's honestly significant difference test and *t*-test at  $\alpha = 0.05$ . Scheirer–Ray–Hare test was used to analyze flight parameters (flight duration, flight distance and flight velocity). Mating status (mated and virgin) and adult age (1–7-d-old) and their interactions were used as factors. Mann–Whitney *U*-test were used at  $\alpha = 0.05$  to separate the means. Mating percentage and the composition of ovarian development level and mating frequency of female moths captured by field and the searchlight, as well as the composition of ovarian development level and mating status of the migratory take-off female moths were all compared by chi-square test. All statistical analyses were performed using R (version 4.0.5, <https://www.r-project.org/>).

## Results

### *Effect of flight on ovarian development and flight muscle development*

Laboratory-reared *C. medinalis* were subjected to a 4-h flight treatment every night for 1–6 nights using flight mills. On day 1 following adult emergence, approximately 87.5% of the dissected ovaries of the female moths in both flown and unflown groups showed no signs of follicular differentiation, indicating that ovaries were at level I (Figs. 1A and 1Ba). Ovarian development advanced over subsequent nights in both flown and unflown groups. However, ovaries in the flown group began to develop more rapidly than those in the unflown group on day 2. Fewer ovaries at level II and more ovaries at level III and IV were observed in the flown group compared with the unflown group; specifically, there were 37.5%, 37.5% and 25% ovaries at level II, III and IV in the flown group, respectively, and 81.2%, 18.85% and 0% ovaries at level II, III and IV in the unflown group, respectively ( $\chi^2 = 7.819$ ,  $df = 1$ ,  $P = 0.005$ ) (Figs. 1A and 1Bb). On day 3 following adult emergence, most of the ovaries were at level III (41.18%) and IV (47.06%) in the flown group, whereas those in the unflown group were mostly at level II (66.67%). The acceleration of ovarian development by the flight treatment was also observed on day 4 and the following few days compared with untreated moths (Figs. 1A and 1B). These findings indicate that flight for 3 nights following adult emergence accelerated ovarian development in female *C. medinalis*.

Ovarian development was also accompanied by the morphological changes in bursa copulatrix. In unmated female moths, the bursa copulatrix was transparent and lacked spermatophores (Fig. 1Bf). After mating, it was filled with 1–2 spermatophores (Fig. 1Bg). Consistent with the accelerated ovarian development in the flown group relative to the unflown group, condensed spermatophores were present in the flown moths with ovaries at level III to V (but not at level I and II) earlier than those in the unflown group (Fig. 1). This indicated that mating took place during flight and did not compromise flight activity.

We then examined the flight muscle development of female moths after the tethered flight treatment for 7 consecutive days. There was no significant difference in the dry mass of the flight muscles between the flown and the unflown groups each day of the treatment (Fig. 2). However, the dry mass of the flight muscles increased from day 1 to day 3 and was correlated with moth age (Table S1). The dry mass of the flight muscle was at lowest on day 1, slightly increased on day 2, peaked on day 3



**Table 1** Effect of mating on flight capability in tethered successive flights.

Parameters	Night flown	Virgin	Mated	Z	P
Flight duration (h)	1	1.020 ± 0.201	0.906 ± 0.370	-0.024	0.981
	2	0.736 ± 0.153	0.836 ± 0.341	-0.242	0.809
	3	0.519 ± 0.119	1.030 ± 0.515	-0.527	0.598
	4	0.457 ± 0.173	0.247 ± 0.142	-0.914	0.360
	5	0.080 ± 0.036	0.386 ± 0.223	-0.300	0.764
	6	0.108 ± 0.062	0.092 ± 0.053	-1.528	0.127
Flight distance (km)	1	3.570 ± 0.701	4.320 ± 1.760	-0.266	0.791
	2	2.170 ± 0.452	1.650 ± 0.675	-0.862	0.389
	3	1.380 ± 0.317	0.945 ± 0.472	-1.095	0.273
	4	1.560 ± 0.589	0.280 ± 0.161	-0.570	0.569
	5	0.336 ± 0.150	1.180 ± 0.684	-0.447	0.655
	6	0.435 ± 0.251	0.021 ± 0.012	-1.964	0.050
Flight velocity (km/h)	1	0.741 ± 0.145	1.010 ± 0.413	-0.217	0.828
	2	0.517 ± 0.108	0.269 ± 0.110	-0.915	0.360
	3	0.499 ± 0.115	0.532 ± 0.266	-0.771	0.441
	4	0.485 ± 0.183	0.609 ± 0.352	-0.114	0.909
	5	0.299 ± 0.134	0.515 ± 0.298	-0.447	0.655
	6	0.104 ± 0.060	0.080 ± 0.046	-1.964	0.050

and did not change thereafter, and there was no significant difference in the dry mass of the flight muscles between the flown and the unflown groups ( $F_{6,141} = 8.81$ ,  $P < 0.001$ ) (Fig. 2). Thus, the flight muscle development of female *C. medina* was not affected by the tethered flight treatment.

#### Effect of mating on the competency for tethered successive flights

We next sought to determine whether mating decreased the flight capacity of *C. medinalis*. Although Scheier-Ray-Hare tests indicated that flight duration, flight distance and flight velocity decreased gradually with flight treatment and moth age, there was no significant difference in flight capacity between mated and virgin moths for all successive flights (Table 1 and Table S2). This finding indicated that mated moths were capable of completing subsequent flights.

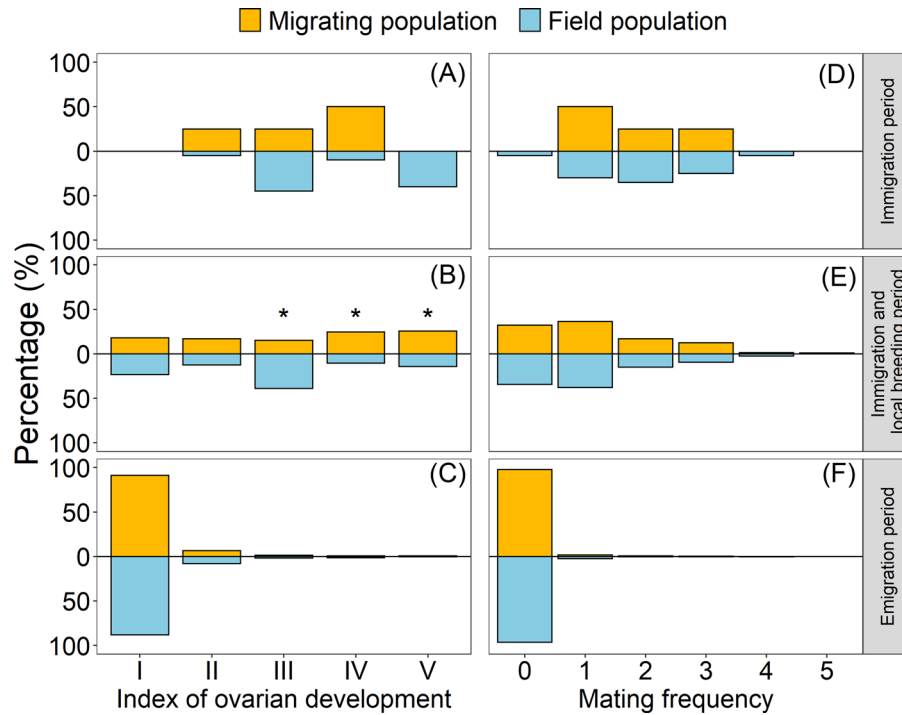
#### Ovarian development and mating status of long-distance immigrants

In light of the above findings from the laboratory assay, we characterized the relationship between the reproductive maturity and the migratory flight of wild-caught moths (Fig. 3). We captured moths in the vicinity of Jiangyan in Jiangsu Province, China, which is an area

where insect migration surveys have been performed for several years. According to the criteria of Zhang *et al.* (1979) developed on the basis of samples collected in this rice field, the migration season in 2019 in this area was divided into the Immigration period from July 18 to July 24, the Immigration and Local Breeding period from July 25 to September 9, and the Emigration period from September 10 to October 20. The *C. medinalis* moths captured by high-altitude searchlights were designated as the migrating population, and those collected manually in the rice fields were designated as the field population.

During the Immigration period where many of the searchlights-captured moths were in the process of completing long-distance and multi-stop flight, the ovaries were at level III to IV (50%), and the mating frequency evidenced by the presence of the spermatophores in the bursa copulatrix was greater than 1, implying successful mating (Figs. 4A, 4D). This was consistent with the findings in the field population (ovarian development level:  $\chi^2 = 6.720$ ,  $df = 3$ ,  $P = 0.081$ ; mating frequency:  $\chi^2 = 0.900$ ,  $df = 4$ ,  $P = 0.925$ ), as well as the results of Zhang *et al.* (1979). In the migrating population, the sexual maturity and mating percentage were as high as 75% and 100%, respectively, indicating that most *C. medinalis* moths making stopover departures were sexually mature (Table S3).

During the Immigration and Local Breeding period, the moths captured by searchlights included individuals that had made both long-distance and short-distance flights.



**Fig. 3** Ovarian development and mating frequency of the migrating populations of *C. medinalis*. Migrating moths were captured by searchlights, and the field populations were collected in the rice fields in Jiangyan, China, during 3 consecutive migration periods: the Immigration period (July 18 to August 24), the Immigration and Local Breeding period (August 25 to September 9), and the Emigration period (September 10 to October 21). Ovarian development during the Immigration period (A), Immigration and Local Breeding period (B), and Emigration period (C) was determined according to the 5-grade standard. Mating frequency during the Immigration period (D), Immigration and Local Breeding period (E), and Emigration period (F) was calculated based on the number of spermatophore in the bursa copulatrix. The data were shown as percentages, and \* indicates significant differences in the percentage of field populations and migrating populations at each stage of ovarian development and mating frequency during each period (chi-square test,  $P < 0.05$ ).

The field-collected moths included immigrants and the offspring of first-generation immigrants. Although there was no significant difference in the sexual maturity and mating frequency between the migrating population and the field population in this period, ovaries in the former group were at level IV and level V, whereas those in the latter group were mostly at level III (Figs. 3B, 3E and Table S3). The sexual maturity and mating percentage of the migrating population remained high at 64.88% and 67.89%, respectively, 4 females mated 4 times, and 3 females for 5 times.

During the Emigration period when most individuals are the offspring of the previous generation, ovaries in both the migrating and field populations were at level I (Fig. 3C). The mating frequency was nearly 0, indicating that these moths had not previously mated. The sexual maturity and mating percentage were found around 2%–3% in both populations (Figs. 3C, 3F, Table S3). This suggests that moths deciding to migrate during the Emigration period were largely immature.

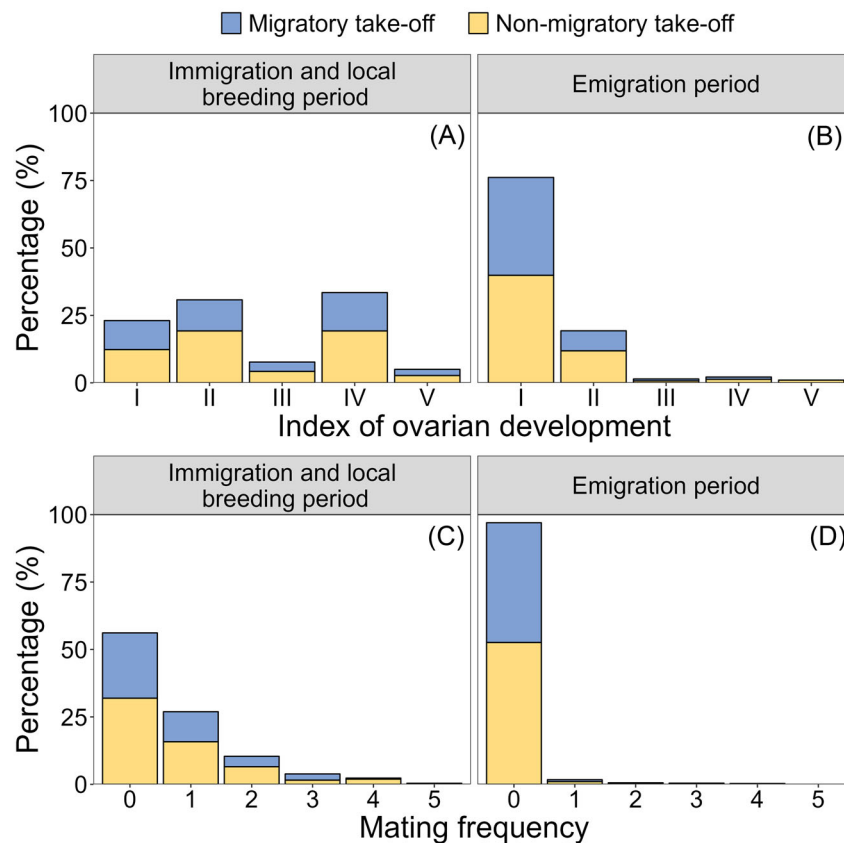
Overall, we found that *C. medinalis* moths making long-distance and multi-stop flights completed ovarian development and mated by the end of their migration.

#### *Relationship between reproductive maturity and migratory flight propensity*

To address whether reproductive maturity impeded the migratory flight propensity, we collected *C. medinalis* moths from the rice field in Jiangyan during the Immigration and Local Breeding period and Emigration period, and then investigated their take-off behavior in the laboratory. Under a light condition simulating sunset, the moths showed 2 distinct flight patterns in the cage: a migratory take-off, in which tested moths spiraled vertically at distances greater than 100 cm, and nonmigratory take-off, in which moths flew to heights less than 100 cm.

Moths collected in the field during the Immigration and Local Breeding period included immigrants and the





**Fig. 4** Sexual maturity and migratory take-off behavior of *C. medinalis* moths throughout the migration season. Moths collected during the Immigration and Local Breeding period (August 28 to September 9) and Emigration period (September 10 to October 14) were subjected to an observation assay, wherein take-off behavior was evaluated for approximately 1 h under simulated sunset. The proportion of migratory take-off moths and nonmigratory take-off moths is shown as the percentage of the total number at each stage. [Correction added on 12 Sep 2022, after first online publication: “Index of ovarian development” was amended to “Mating frequency” in the x-axis of Figure 4.]

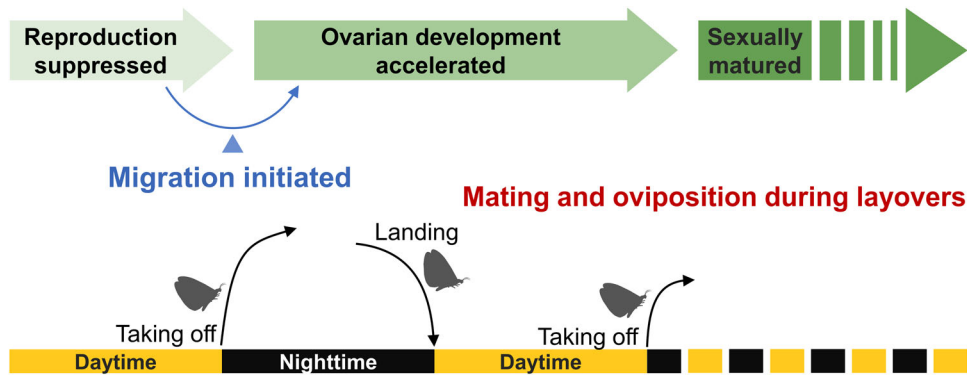
offspring of the previous generation. Consistent with the above results, the developmental progress of the ovaries of these moths varied greatly and ranged from level I to level V. In the migratory take-off group, 47.27% of the ovaries were at level III or above, and the rest were below level II (Fig. 4A). The number of ovaries at level III or above was similar in the nonmigratory take-off moths. The mating frequency ranged from 0 to 4, and there was no significant difference between the migratory and nonmigratory take-off groups (Fig. 4C). This demonstrated that reproductively mature *C. medinalis* moths were capable of migratory take-off.

Moths collected during the Emigration period largely consisted of individuals with the intent to migrate. In the migratory take-off group, the ovaries of 96.53% of individuals were immature (level I and II), and the percentage of immature ovaries was similar in the non-

migratory take-off group (Fig. 4B). The mating percentage was 1.89% and 3.92% in the migratory and nonmigratory take-off group, respectively, and there was no significant difference in the mating percentage between these 2 groups (Fig. 4D). This indicated that the emigrant population largely consisted of sexually immature moths that engaged in migratory take-off behavior.

## Discussion

Migration occurs when the local habitat of insects becomes unfavorable for survival and reproduction (Dingle & Drake, 2007), and the need for resources is thought to motivate such decisions. To clarify the relationship between migration and reproduction, we investigated the ovarian development, mating status and migratory



**Fig. 5** Relationship between reproduction and multi-stop migratory flight in *C. medinalis* moths. Migration is activated during the sexually immature stage (reproduction is temporarily suppressed). After a few stopovers, ovarian development is initiated. Sexually matured moths engage in mating during multi-stop flights when mates are available, and oviposition occurs thereafter. Given that such activities do not compromise their ability to complete subsequent migratory flights, the delay of sexual maturation following the first flight and mating during layovers enhance the reproductive success of individuals and minimize inbreeding.

take-off behaviors in *C. medinalis* moths under a simulated flight environment and in their natural habitats. Migration of *C. medinalis* was initiated during the sexually immature stage. Ovarian development and mating occurred during multi-stop flights on subsequent nights. Sexual maturation and mating did not suppress the migratory take-off propensity; thus, multi-stop migration in *C. medinalis* moths provides both survival and reproductive benefits.

A large number of previous studies have suggested that there is a trade-off in energy allocation between migration and reproduction (Dingle, 2014). Reproductive activities in migratory insects, including ovarian maturation, mating and oviposition, are thought to commence immediately following the completion of migration, which is accompanied by the degradation of the flight muscle. In *C. medinalis*, however, ovarian development was promoted after flight for 1–2 nights, and flight for 4–5 nights had little effect on ovarian development. These findings are consistent with the results of Zhang *et al.* (2015). The development of the ovaries did not affect the development of the flight muscles in *C. medinalis*, and the mating status of females did not impair flight performance as has been demonstrated in other migratory insects (e.g., *S. exigua* and *Agrotis ipsilon*) (Sappington & Showers, 1992; Jiang *et al.*, 2010). This indicates that the development of the ovaries and flight muscles occurs simultaneously and that sexually mature moths are capable of completing long-distance and multi-stop migratory flights while being reproductively active.

When migration was initiated, *C. medinalis* moths are often sexually immature and unmated. This temporary lack of investment in reproduction might benefit long-

term population persistence because it might reduce the frequency of inbreeding. However, we found that the ovarian development of several migrating populations of *C. medinalis* proceeded during their multi-stop flights and many individuals mated during this period, often several times. Although these findings do not meet the predictions of “oogenesis-flight syndrome”, they are consistent with the results of previous studies of *Plutella xylostella* (Fu *et al.*, 2014) and *Danaus plexippus* (Vargas *et al.*, 2018). Achieving reproductive maturity and mating during multi-stop flights can enhance reproductive success. Given that the reproductive performance of female moths decreases with age, excessive delays in mating can potentially decrease reproductive success (Kawazu *et al.*, 2014). The development of sexual maturity during the migration allows moths to locate favorable habitats for oviposition immediately after or even prior to completing their migration, which promotes local population expansion.

Most of the female *C. medinalis* moths captured during the Immigration period in our study were found to have mated more than twice. Considering that *C. medinalis* prefer to mate at midnight and typically once per night (Lu *et al.*, 1981), we suspect that they had travelled for several successive nights before being caught at our sampling site. This finding, coupled with the observation that mated individuals captured from the field were capable of migratory take-off, indicated that mating status does not inhibit migratory flight and that *C. medinalis* employ multi-stop flights to complete their migration. The moths are thought to refuel themselves by feeding on nectar during each layover, and the nutrients acquired during these

layovers support the moths during their subsequent flights.

Several searchlight-captured *C. medinalis* immigrants partially completed oviposition by the end of migration. Mating and oviposition during each layover of multi-stop flights can effectively promote population expansion. Furthermore, distributing the offspring across disparate sites can greatly reduce intraspecific competition (McAnelly & Rankin, 1986). Female moths thus might hedge their reproductive bets by spreading their breeding efforts in space and time, which distributes offspring across various environmental conditions (Holland *et al.*, 2006).

In sum, our findings shed new light on the relationship between reproduction and migration in the rice leaf roller *C. medinalis*. Moths are typically sexually immature when making their first flight. Ovarian development is activated thereafter and is nearly complete after 3 or more nights of flight. Multiple mating and oviposition occur during the layovers until the end of the migration, and these reproductive activities do not compromise flight capacity (Fig. 5). Additional field studies and laboratory assays are needed to identify the factors underlying the maintenance of migrating populations.

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## Disclosure

The authors declare that they have no competing interests.

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## Supporting Information

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

**Fig. S1** Nocturnal flight activity of migratory *Cnaphalocrocis medinalis*. A total of 110 healthy moths at 2-d-old were selected and tested. Flight tests were performed on a 24-channel computer-interfaced flight mill system (Jiaduo). A total of 66 of these moths were migrants with an accumulative flight duration greater than 130 min (Wang et al., 2010). Flight activity was represented by flight distance in a 5-min interval. Light-yellow shaded area indicates significant differences in average flight activity within each hour compared with other test durations (Tukey's HSD test:  $F = 3480$ ;  $df = 1, 7883$ ;  $P < 0.001$ ).

**Fig. S2** Number of *C. medinalis* captured by search-light traps and counted at experimental sites in rice field in Jiangyan, China, in 2019. The abundance of moths in the field was recorded based on a survey that was carried out at 06:00 (BJT), and the specific method was based on the description by Wang et al. (2017).

**Table S1** Two-way ANOVA for the effects of flight status and adult age on flight muscle dry mass in female *C. medinalis*.

**Table S2** Scheirer–Ray–Hare test for the effects of flight status and adult age on flight capability of female *C. medinalis*.

**Table S3** Sexual maturity and mating status of female *C. medinalis* in migrating population and field population in different migration periods in Jiangyan, China.

**Table S4** Abundance and female ovarian development of *C. medinalis* in Jiangyan, China.