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Unprecedented oviposition tactics avoid plant defences and reduce attack by parasitic wasps

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Abstract

Female insects oviposit in sites suitable for the development of their offspring. The Oriental armyworm, *Mythimna separata* is a serious pest of various crops including wheat and prefers to oviposit on withered leaves rather than on fresh plant material, which is surprisingly different from other insects. Studies here showed that this oviposition tactic enables avoidance of wheat defence against eggs and emerged larvae. Intact plants responded to *M. separata* egg deposition by releasing oviposition-induced plant volatiles including acetophenone, tetradecene and pentadecane after 24 h. Acetophenone was identified as quantitatively accounting for the attraction of the egg parasitoid wasp (*Trichogramma chilonis*). Leaf jasmonic acid levels significantly increased after *M. separata* laid eggs, and primed the plant against emerging larvae. In addition, newly emerged *M. separata* larvae adopted a fast crawling behaviour and starvation tolerance compared with other noctuid larvae, which enhanced the survival of larvae on the withered leaves. The elucidation of this complex and surprising plant-insect interaction provides the first explanation for a herbivore laying eggs on withered leaves to avoid natural enemies and live-plant defence against emerging larvae.

KEYWORDS

hormone, insect herbivore, *Mythimna separata*, plant-insect interaction, preference-performance hypothesis, *Trichogramma chilonis*, volatiles

1 | INTRODUCTION

Herbivores avoid predation in various ways at vulnerable stages of development. However, when host plants emit signals associated with attack that serve to recruit carnivores at the third trophic level, the causative herbivores can be highly vulnerable. As the first stage of attack, insect eggs can experience various dangers, especially from predators and parasitoids (Hilker & Fatouros, 2015). Female insects often attempt to locate an appropriate environment for their offspring when laying eggs, referred to as the preference-performance hypothesis (PPH) or ‘mother knows best’ hypothesis (Mayhew, 2001).

Positive development conditions, that is, sufficient food and a low risk of being preyed upon or parasitized, are necessary for offspring survival, which largely determine oviposition and thereby larval performance (Griese et al., 2020; Hilker & Meiners, 2008).

However, plants have evolved a series of defence mechanisms with negative effects on insect eggs (Fatouros et al., 2012; Hilker & Meiners, 2010; Meiners & Hilker, 2000). Among these defence mechanisms, the plant hormones jasmonic acid (JA) and salicylic acid (SA) play important roles in mediating plant responses to insect egg deposition, and result in direct or indirect defence against eggs (Fatouros et al., 2014; Reymond, 2013). Plant direct defence includes

desiccation, dropping, crushing or accumulating toxic secondary metabolites to block egg hatching (Balbyshev & Lorenzen, 1997; Doss et al., 2000; Griese et al., 2017; Petzold-Maxwell et al., 2011), while indirect defence is mediated by oviposition-induced plant volatiles (OIPVs) (Blenn et al., 2012; Hilker & Meiners, 2008). Plant volatiles play a vital function in plant-herbivore-natural enemy interactions (Beyaert et al., 2012; Rering et al., 2018; Tamiru et al., 2011). To defend against oviposition by herbivores, plants emit complex blends of OIPVs that attract parasitic wasps against the herbivores (Blenn et al., 2012; Hilker & Meiners, 2008). Interestingly, even single volatile compounds can attract egg parasitoids (Büchel et al., 2011; Takabayashi et al., 1991; Whitman & Eller, 1992).

Egg deposition on plants represents the first stage at which plants could detect the herbivorous insect, and consequently also provide warning signals for future larval herbivory (Altmann et al., 2018; Beyaert et al., 2012; Geuss et al., 2018; Lortzing et al., 2020). Insect oviposition not only triggers plant defence against eggs but also reinforces plant defence against the emerging larvae (Altmann et al., 2018; Beyaert et al., 2012; Lortzing et al., 2020). Previous studies showed that egg-mediated plant anti-herbivore defence reduces the performance of the hatching larvae. For example, oviposition by *Spodoptera exigua* primes inducible defence of *Nicotiana attenuate* and exerts a negative effect on larval survival (Bandoly et al., 2015). *Vicia faba* plants recognise *Halyomorpha halys* oviposition and impair the development of the nymphs (Rondoni et al., 2018). *Pieris brassicae* larvae gain less biomass when feeding on egg-deposited plants (*Arabidopsis thaliana*) than on egg-free plants (Geiselhardt et al., 2013). In another study, the entire egg incubation time is needed for a plant to mount defence against larval herbivory (Valsamakis et al., 2020).

Oriental armyworm, *Mythimna separata* (Walker) (Lepidoptera: Noctuidae), is an important migratory pest in eastern Asia, which attacks various grain crops (Oku & Koyama, 1976; Sharma & Davies, 1983). Strikingly, females of *M. separata* preferentially lay eggs on withered leaves rather than on fresh ones, which is different from other insects. In addition, the eggs encounter low parasitism in the field (Zhang, 1980). For more than half a century Chinese farmers use withered leaves as a trap for egg-laying, and then burned this material to kill the eggs (Yang, 1980). According to the PPH, we hypothesise that this peculiar oviposition preference is a strategy for female *M. separata* by helping its eggs or larvae escape plant defence.

In this study, a series of assays were performed in the laboratory and field conditions to evaluate induced plant defence against eggs after female *M. separata* oviposit on fresh leaves. Egg hatching rate of *M. separata* and the preference of *Trichogramma chilonis* (Ishii) (Hymenoptera: Trichogrammatidae) (a generalist parasitoid wasp) were tested to evaluate plant direct and indirect defence against eggs, respectively. The OIPVs were identified and tested to determine the active volatile compounds in attracting *T. chilonis*. The latter part of this study determined the effects of oviposition-induced defence on the development of the emerged larvae. Plant hormones (JA and SA) were tested to explore plant defence signalling against oviposition and larval feeding behaviour. Furthermore, the

crawling speed and survival ability without available food of newly emerged larvae were compared to other noctuid larvae (*Helicoverpa armigera*, *Spodoptera exigua*, and *Spodoptera litura*). This study provides the first insights into the phenomenon of *M. separata* preferring to lay its eggs on withered leaves to avoid induced plant defence and enhance egg survival and subsequent larval development.

2 | MATERIALS AND METHODS

2.1 | Plants and insects

The wheat (*Triticum aestivum* L.) variety used in this study was HuaMai 1168. Seedlings were grown and maintained under conditions of $24 \pm 2^\circ\text{C}$, 40%-60% relative humidity (RH) and 14/10 h of light/dark photoperiod. Wheat plants of 25 ± 2 days old were used for all experiments. Withered leaves are commonly found on plants or on the ground during plant field growth. To avoid the effect of leaf position on female oviposition, withered leaves on withered plants (dead and dried in the greenhouse naturally) with the same number of leaves (from fresh plant material) were used as controls.

The *M. separata*, *T. chilonis*, *H. armigera*, *S. exigua*, and *S. litura* eggs were obtained from Zhongke Baiyun Green Bio-technology Co. Ltd (Beijing, China). Larvae of *M. separata* were reared on an artificial diet (Zhongke Baiyun Green Bio-technology Co. Ltd) in an incubator until pupation at 28°C , 75% RH and 14/10 h of light/dark photoperiod. In the experiment of female *M. separata* oviposition preference, larvae were fed on wheat plants until pupation. The *T. chilonis* was maintained under similar controlled conditions as above. Adults of *T. chilonis* were fed on 4% honey solute.

2.2 | Oviposition preference of *M. separata*

Ten males and 10 females of newly emerged *M. separata* adults fed on the wheat plant or artificial diet during their larval stage were placed together in a Perspex cage ($50 \times 30 \times 30$ cm) and allowed to mate for 3 days, respectively. Then, eight wheat plants with fresh leaves and eight wheat plants with withered leaves were introduced into the cage interlaced to avoid the preference of position. Usually, female insects lay eggs during darkness. Thus, 12 h of darkness period was provided for the females to make oviposition choices. To provide adequate space for oviposition, plants or withered leaves with eggs were replaced with corresponding unspawned material every 2 h during the experiment. The total number of deposited eggs and egg clusters were counted on wheat plants with fresh leaves or withered leaves. The number of eggs deposited on fresh leaves and withered leaves was calculated as a proportion of the total number of eggs, respectively. The experiment was repeated 12 times, using different *M. separata* adults, wheat plants with fresh leaves and withered leaves each time.

2.3 | Egg hatching rate of *M. separata*

Before the experiment, the total number of eggs laid by *M. separata* on each wheat plant with fresh leaves or withered leaves was recorded. Then, wheat plants were transferred to an incubator at 28°C, 75% RH and 14/10 h of light/dark photoperiod. To avoid the migration of emerged larvae, wheat plants with fresh leaves or withered leaves were placed in glass tubes (height: 40 cm, diameter: 10 cm). The opening of the tubes was covered with gauze. The number of hatched eggs was recorded on each wheat plant with fresh leaves or withered leaves. The experiment included 12 biological replicates.

2.4 | Y-tube olfactometer

A Y-tube olfactometer (total arm length: 18 cm and internal diameter: 1.5 cm) was used to test the preference of *T. chilonis* females (Supporting Information: Note 1). Clean air flowed from the two different odours. A 60-W lamp provided illumination. The *T. chilonis* was tested against odours of (1) healthy and oviposition-treated plants after 12, 24, and 36 h; and (2) withered leaves and oviposition-treated withered leaves after 24 h. To test the effect of volatile compounds in attracting parasitoids, commercial authentic compounds (Sigma-Aldrich) were diluted in liquid paraffin and 10 µL of the solution was added to a filter paper (1×1 cm). Liquid paraffin (10 µL) was used as the control. Before testing, *T. chilonis* was allowed to adapt to the environment of the bioassays for at least 20 min. Every parasitoid was given 5 min to make a choice. To avoid positional bias, the stimulus was changed after testing three insects. The experiment was conducted 35–50 times using different mated female parasitoids.

2.5 | Cage experiment

In the cage experiment, 20 *T. chilonis* mated females were released to search for hosts in a cage (80 × 30 × 30 cm). The parasitic differences between *M. separata* eggs laid on (1) wheat plants with fresh leaves or withered leaves and (2) withered leaves treated with acetophenone or liquid paraffin were tested. All *M. separata* eggs were laid within 1 day. After 1 day of parasitism, wheat plants and withered leaves were transferred to an incubator (28°C, 75% RH and 14/10 h of light/dark) and the number of parasitized eggs on wheat plants and withered leaves were counted. The number of parasitized eggs on plants and withered leaves was calculated as a proportion of the total number of parasitized eggs for each replicate, respectively. The experiment was repeated 10 times.

2.6 | Field experiment

After 1 day of oviposition on wheat plants with fresh leaves or withered leaves, 20 fresh plants and 20 withered leaves with eggs (800–900) were placed in adjacent rows of wheat fields at Huazhong Agricultural University (114.36 °N, 30.47 °E), China. The experiments

were carried out on sunny days (12–29°C). To prevent eggs from being fed upon by predators, leaves were covered with gauze (bore diameter: 0.15 cm). Two days later, wheat plants were returned to the laboratory and the parasitized egg number on fresh leaves and withered leaves was calculated.

2.7 | Collection and analysis of plant or egg volatiles

A closed-loop dynamic headspace collection system was used to collect volatiles from fresh wheat plants at 12, 24, or 36 h following egg deposition by *M. separata* as previously described (Shi et al., 2019). To avoid damaging the plant, the eggs were not removed from the plant. Wheat plants without eggs were used as controls. To quantify volatiles, a graded series of standard volatile compounds in liquid paraffin was used to establish the standard curve (Supporting Information: Table S1). Plants were placed in clean collection glass chambers (10 L) immediately after treatment. Clean air flowed at a rate of 400 mL/min. Volatiles were absorbed by Super Q trap (100 mg) and eluted with 1 mL of hexane. Each collection lasted 8 h (Li et al., 2020). The internal standard was 10 µL of nonyl acetate (0.1 mg/mL) in hexane. To confirm the source of induced volatiles, volatiles from *M. separata* eggs and the blank glass chambers were extracted and compared with the identified egg-induced volatiles. Each treatment included four biological replicates.

To isolate and analyse volatile compounds, GC-mass spectrometry (GC-MS) (QP-2010, Shimadzu) with an HP-5 MS fused-silica column (30 × 0.25 × 0.25 µm) (Agilent Technologies) was used. Helium (1 mL/min) acted as the carrier gas. Volatile compounds were identified by comparison with NIST17 library mass spectra and Kovats retention indices with relative peak areas of internal standards used to quantify volatile amounts. The enantiomeric composition of chiral compounds was not determined. Although acetophenone can be a contaminant of volatile absorption it has been previously fully authenticated as a volatile compound produced by wheat plants (Birkett et al., 2004; Shibamoto et al., 2007).

2.8 | Larval development of *M. separata*

On the fourth day after *M. separata* eggs were deposited on fresh wheat plants, all eggs were removed and five newly emerged larvae were introduced to feed on each plant. Eggs were hatched four to 5 days after deposition. Five newly emerged larvae were also introduced onto the healthy plant without egg deposition. Larval weight was recorded after 3 days. The mean weight of the larvae on each plant was considered as one biological replicate, and eight replicates were conducted.

2.9 | Extraction and analysis of JA and SA

To induce JA and SA production, four treatments were established including (a) control plant (egg-free plant without larvae feeding) (C),

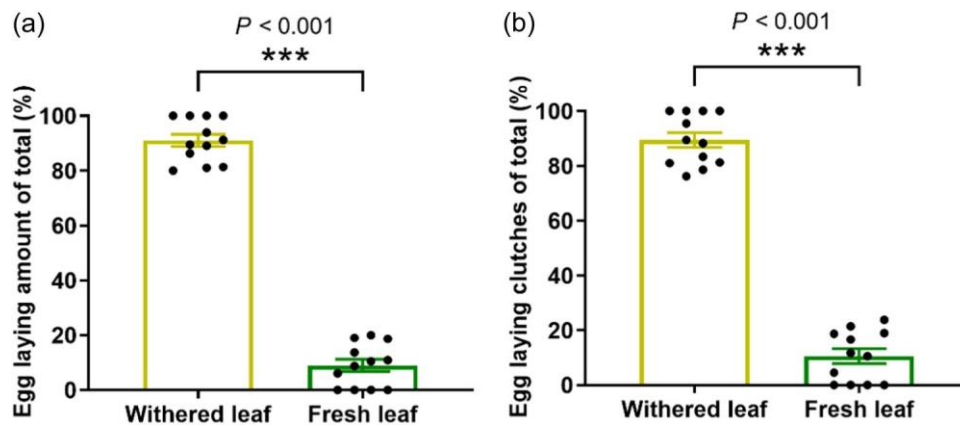


FIGURE 1 Oviposition preference of *Mythimna separata* raised on artificial diets between plant with withered leaves and fresh leaves. (a) egg laying amount (of total) between plant with withered leaves and fresh leaves. (b) egg laying clutches (of total) between plant with withered leaves and fresh leaves. Data are presented as mean values \pm SEM. *** $p < 0.001$, Wilcoxon signed-rank test ($n = 12$). [Color figure can be viewed at wileyonlinelibrary.com]

(b) *M. separata* egg-deposited plant (O), (c) larvae feeding on egg-free plant (F), and (d) larvae feeding on egg-deposited plant (OF). For OF treatment, all eggs were removed on the fourth day after *M. separata* oviposition and five emerged larvae were introduced to feed. After larvae feeding for 6, 12, or 24 h, plant leaves from damaged sites were collected. Plant hormones were extracted and analysed on liquid chromatography-mass spectrometry (LC-MS) system (Xevo G2-XS Qtof) as previously described (Pan et al., 2010) (Supporting Information: Note 2). The peak area of internal standards was used to quantify hormone amounts. Each treatment included four biological replicates.

2.10 | MeJA treatment

Methyl jasmonate (MeJA) was dissolved in diluted water to 0.5 or 2 mM, containing 0.25% (v/v) ethanol and 0.01% (v/v) Tween 20. Wheat plant was sprayed from above with 10 mL MeJA solution. Control plants were sprayed with 10 mL of 0.25% (v/v) ethanol and 0.01% (v/v) Tween 20 and placed separately from MeJA-treated plants. After three and a half days, five newly emerged larvae were introduced to feed on each treated plant. Larval weight was recorded after 3 days. The mean weight of the larvae on each plant was considered as one biological replicate, and eight replicates were conducted.

2.11 | Crawling speed of newly emerged larvae

Newly emerged larvae of *M. separata*, *H. armigera*, *S. exigua*, and *S. litura* were used to test the crawling speed of insects. White filter paper (diameter of 15 cm) was placed under the video camera. Individual insects were placed in the centre of the white filter paper. Before recording, larvae were allowed to adapt to the environment for 5 min. The recording duration lasted for 1 min. The video was analysed using Ethovision software. Every treatment included 50 biological replicates.

2.12 | Survival rate of newly emerged larvae

Newly emerged larvae of *M. separata*, *H. armigera*, *S. exigua* and *S. litura* were individually placed in a glass tube (height: 5 cm; diameter: 1 cm) without a food source. The number of dead and live insects was recorded every 12 h until all insects were dead. Every treatment included 50 biological replicates.

2.13 | Data analysis

A chi-squared test was used to analyse data from field experiments and parasitoid two-way choices. Wilcoxon signed-rank test was used to analyse data from oviposition preference and parasitism rate. Student's *t*-test (two-tailed) was applied to test differences in egg hatching rate and larval weight. One-way analysis of variance and least significant difference test were used to test differences in plant volatiles content, hormones level, larval weight after MeJA application, and larval crawling speed. The normality of data was checked by Shapiro-Wilk normality test. If data were not normally distributed, data were root-transformed to fulfil the criteria for parametric test procedures. Otherwise, the nonparametric test was employed. The survival curve was analysed by the log-rank (Mantel-Cox) test. All data were analysed using R software (Version 4.0.3) and GraphPad Prism (Version 8.0). The results were considered statistically significant at $p < 0.05$.

3 | RESULTS

3.1 | Female *M. separata* preference for laying eggs on withered leaves

Female *M. separata* oviposition preference was determined by counting the amounts of eggs and egg clusters on withered or fresh leaves

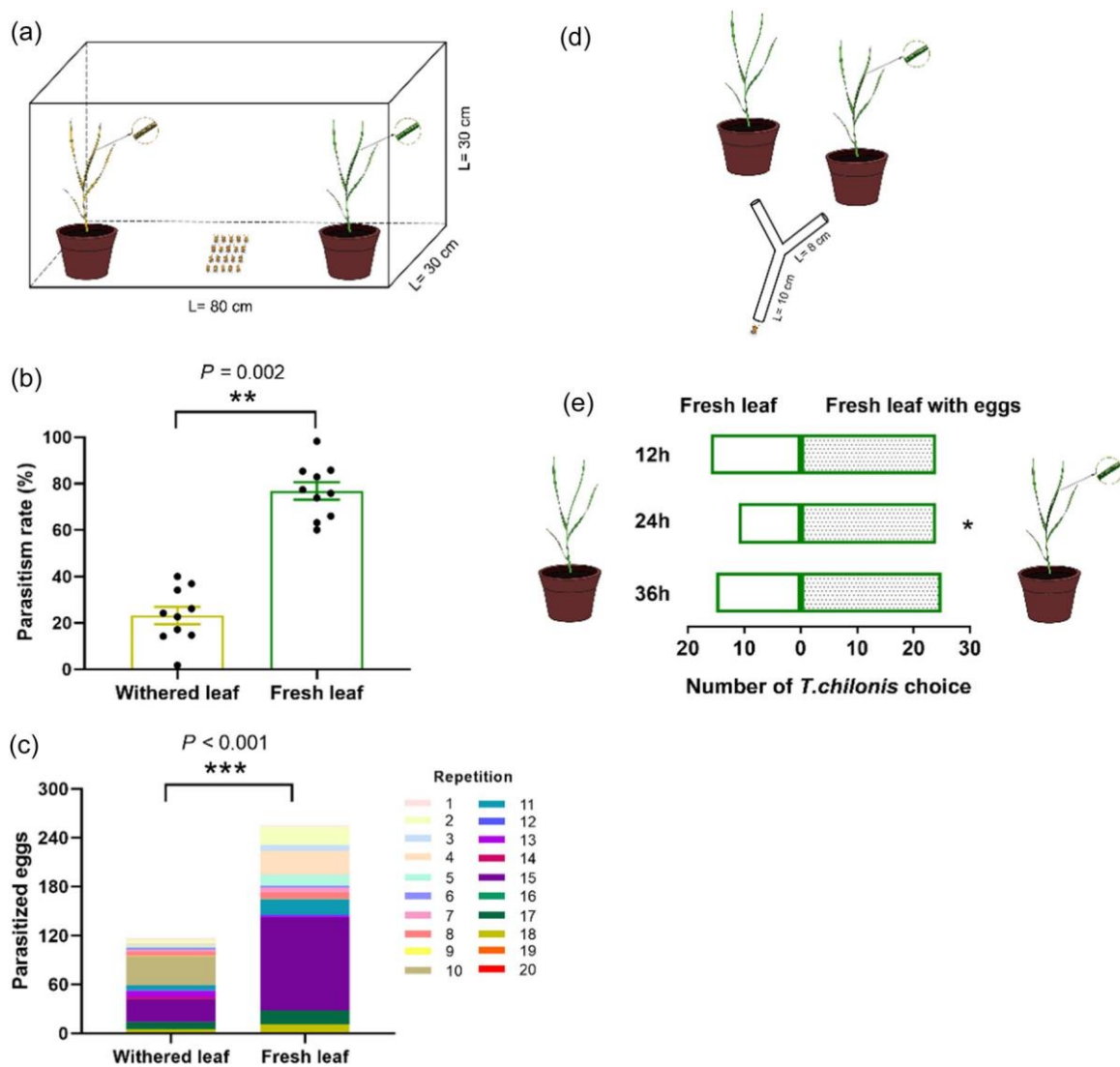


FIGURE 2 Behaviour of *Mythimna separata* associated egg parasitoid wasp. (a) Cage experiment equipment. (b) *Trichogramma chilonis* responds to signals from fresh wheat leaves and withered leaves. (c) The number of parasitized eggs in the field. (d) Y-tube equipment. (e) Preference of *T. chilonis* behaviour towards fresh wheat leaves with *M. separata* eggs (after 12, 24 and 36 h) and without eggs. Wilcoxon signed-rank test in b (data are presented as mean values \pm SEM, $n = 10$). Chi-squared test in c ($n = 20$) and e ($n = 35$ to 40). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 1). The result showed that *M. separata* raised on artificial diets laid more than 90% of the eggs ($p < 0.001$; Figure 1a) and egg clusters ($p < 0.001$; Figure 1b) on the withered leaves. Consistent with the behaviour of those raised on artificial diets, *M. separata* raised on wheat plants prefer to lay eggs on withered leaves. Both egg numbers and clusters laid on withered leaves were significantly more than on fresh leaves ($p < 0.001$; Supporting Information: Figure S1).

3.2 | Egg-induced wheat defence against *M. separata* eggs

Wheat direct defence and indirect defence were determined by testing *M. separata* egg hatching and *T. chilonis* preference, respectively. The result showed that no significant difference in

hatching rate occurred between eggs on wheat plants with fresh leaves and withered leaves ($t = 1.20$, $p = 0.257$; Supporting Information: Figure S2). The preference of *T. chilonis* associated with *M. separata* eggs was tested in a cage experiment (Figure 2a). The results showed that there was a significant difference in the parasitism rate of eggs on wheat plants with fresh leaves compared to withered leaves ($p = 0.002$; Figure 2b). Results of field experiments were in line with those obtained by cage experiments indicating that the parasitism rate of eggs on wheat plants with fresh leaves was more than twice as high as on wheat plants with withered leaves ($\chi^2 = 51.194$, $p < 0.001$; Figure 2c). Thereafter, the two-way choice olfactometer was used to test *T. chilonis* response to wheat plants with eggs against wheat plants without eggs (Figure 2d). It was found that *T. chilonis* had a significant preference for wheat plants with eggs after 24 h, compared to healthy plants ($\chi^2 = 4.829$, $p = 0.028$;

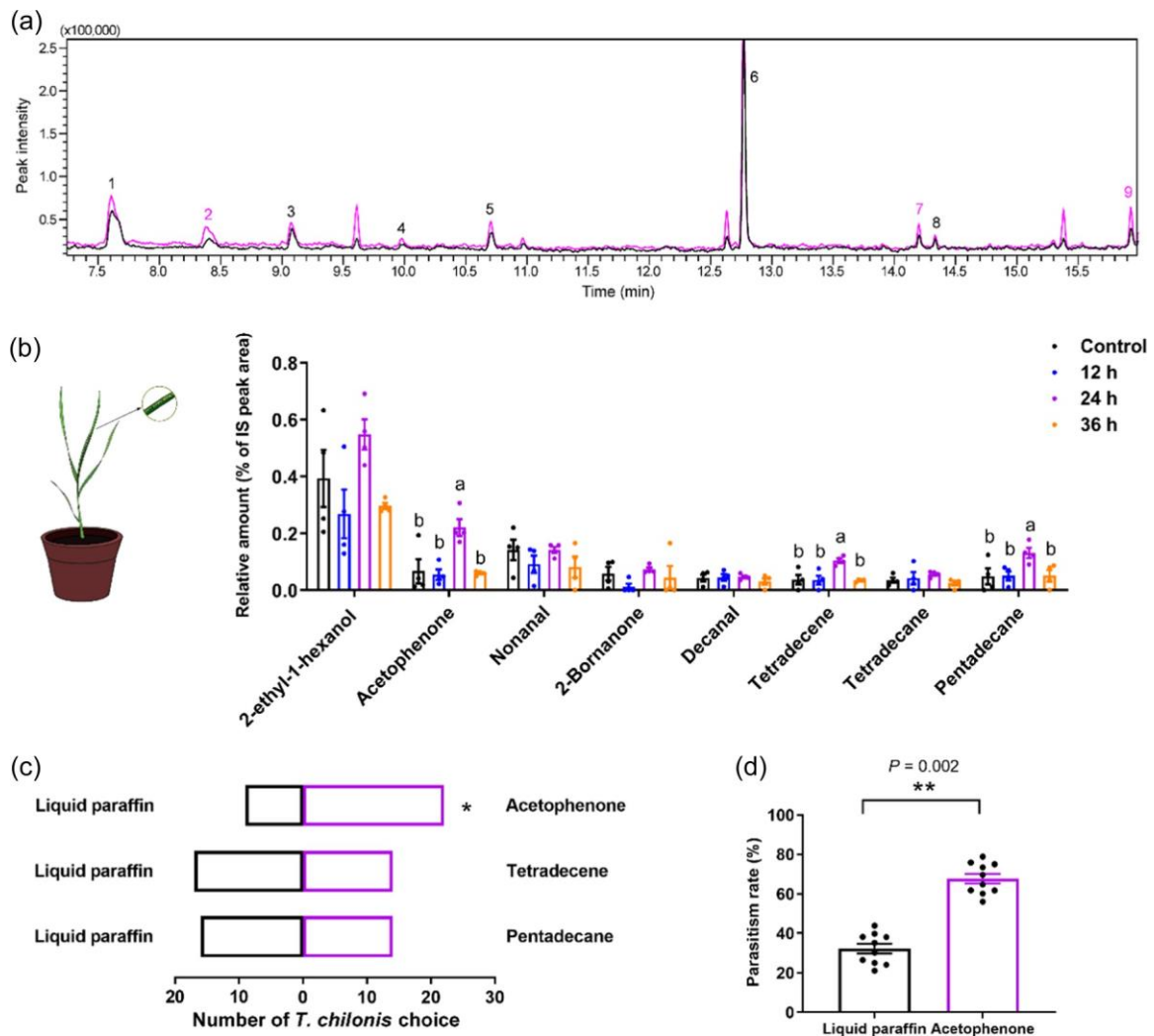


FIGURE 3 Relative amount (% of internal standard compound) of volatile compounds emitted from wheat plants and their representative chromatograms. (a) Representative chromatograms of head-space volatile compounds from wheat plants on which eggs were laid by *Mythimna separata* after 24 h (red peaks) compared to wheat plants without eggs (black peaks). 1: 2-ethyl-1-hexanol; 2: acetophenone; 3: nonanal; 4: 2-bornanone; 5: decanal; 6: nonyl acetate (internal standard); 7: tetradecene; 8: tetradecane; 9: pentadecane. Other peaks are from contaminants. (b) Volatile compounds from control and wheat plants with eggs after different time courses (12, 24 and 36 h). (c) Preference of *Trichogramma chilonis* between the compounds and liquid paraffin in Y-tube experiment. (d) Preference of *T. chilonis* between acetophenone and liquid paraffin in cage experiments. Data are presented as mean values \pm SEM in b and d. One-way ANOVA and least significant difference (LSD) in b ($n = 4$), chi-squared test in c ($n = 35$) and Wilcoxon signed-rank test in d ($n = 10$). Bars marked with different letters indicate significant differences ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 2e). However, parasitoids showed no response to eggs after 12 ($\chi^2 = 1.600$, $p = 0.206$) and 36 h ($\chi^2 = 2.500$, $p = 0.114$) compared to healthy plants. The preference of *T. chilonis* also showed no significant difference between withered leaves without eggs and withered leaves with eggs after 24 h ($\chi^2 = 1.280$, $p = 0.258$; Supporting Information: Figure S3).

3.3 | Acetophenone plays an important role in attracting *T. chilonis*

Based on the behaviour of *T. chilonis*, wheat plant volatile profiles were extracted and compared between treatment and control. In total, eight

volatile compounds were detected. Among these, the content of acetophenone ($F = 7.05$, $p = 0.006$), tetradecene ($F = 7.86$, $p = 0.004$), and pentadecane ($F = 3.76$, $p = 0.041$) was significantly increased after 24 h of *M. separata* egg laying (Figure 3a,b; Supporting Information: Table S2). These compounds were not detected in egg volatiles (Supporting Information: Figure S4, S5). According to the standard curve of volatiles between relative amount and actual amount, 10 μ L of 0.36 mg/mL acetophenone, 0.34 mg/mL tetradecane, or 1.1 mg/mL pentadecane was used to test the preference of parasitic wasps, respectively (Supporting Information: Table S1). The most remarkable observation to emerge from the data comparison was that the wasps showed an attractive response to only acetophenone ($\chi^2 = 5.452$, $p = 0.020$; Figure 3c). As a result of the fact that acetophenone

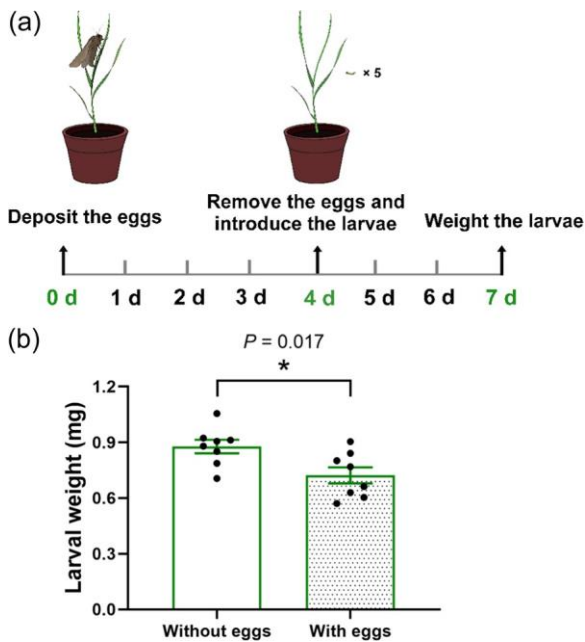


FIGURE 4 Egg-induced wheat direct defence to larvae of *Mythimna separata*. (a) Larval weight was determined after 3 day of feeding on egg-deposited wheat plants. (b) Weight of larvae feeding on egg-free or egg-deposited wheat plants. Data are presented as mean values \pm SEM. * $p < 0.05$, two-tailed Student's *t*-test ($n = 8$). [Color figure can be viewed at wileyonlinelibrary.com]

attracted *T. chilonis*, liquid paraffin or acetophenone was applied to withered leaves with eggs of *M. separata* and the parasitism rate was tested in a cage experiment. As expected, the parasitism rate was significantly higher when acetophenone was added compared to when liquid paraffin was added ($p = 0.002$; Figure 3d).

3.4 | Egg-induced wheat anti-herbivore defence to *M. separata* emerged larvae

An investigation as to whether eggs could subsequently trigger plant defence against the emerged larvae was conducted (Figure 4a). The results showed that newly emerged larvae fed on healthy plants gained more weight compared to those fed on wheat plants on which eggs had been deposited ($t = 2.73$, $p = 0.017$; Figure 4b).

3.5 | Wheat hormone in response to *M. separata* oviposition and feeding

Hormone signals in wheat plants were monitored in response to *M. separata* oviposition and feeding. There was a significant increase in JA at 6 h after oviposition and reached the maximum after 12 h compared to healthy plants ($F = 181.77$, $p < 0.001$; Figure 5a). However, there was no such response for SA ($F = 1.20$, $p = 0.351$; Figure 5b). After larvae feeding, JA level was higher than undamaged plant at 6 and 12 h but showed no significant difference at 24 h ($F = 10.89$, $p < 0.001$;

Figure 5c). No significant change in SA level was detected at all time points compared to undamaged plant ($F = 2.07$, $p = 0.158$; Figure 5d). We further determined JA and SA levels after larvae fed on plants that had previously been deposited by eggs for 4 days. The result showed that JA level was higher after larvae fed on egg-deposited plants than on egg-free plants (Figure 5c,e). Besides, JA level increased over time and was highest at 24 h ($F = 6.78$, $p = 0.006$; Figure 5e). Larvae feeding on egg-deposited plants at all time points also induced an increase in SA level ($F = 15.14$, $p < 0.001$; Figure 5f). To investigate whether eggs mediate wheat anti-herbivore defence against *M. separata* larvae, exogenous MeJA was applied to mimic egg-induced JA signalling in wheat plant (Supporting Information: Figure S6a). The results showed that both 0.5 and 2 mM MeJA decreased larval weight compared to control plant ($F = 47.30$, $p < 0.001$; Supporting Information: Figure S6b).

3.6 | Newly emerged larva of *M. separata* possesses a high crawling speed and survival rate

To explore the adaptability of *M. separata* larvae to the environment after emerging on withered leaves, its crawling speed and survival rate under starvation conditions were determined in comparison with that of other noctuid larval species (*H. armigera*, *S. exigua*, and *S. litura*), whose adults customarily lay their eggs on fresh plants. The results showed that *M. separata* newly emerged larva achieved the fastest crawling speed than other species ($F = 80.93$, $p < 0.001$; Figure 6). Additionally, the newly emerged larva of *M. separata* had a higher survival rate under starvation compared to other noctuids ($p < 0.001$; Figure 6).

4 | DISCUSSION

Females attempt to select a safe and favourable oviposition site and use multiple strategies to protect, in this way, their offspring (Gilbert & Manica, 2010; Mayhew, 2001; Wong et al., 2013). Here, a herbivorous insect is shown to avoid parasitism by laying eggs on withered, i.e. senescent, leaves and thereby not signalling for defence from the third trophic level (Figure 7). This tactic also avoids activating egg-induced plant defence against the emerging larvae by the living plant tissue. It explains the reason for field observations of *M. separata* females showing a clear oviposition preference for withered leaves over the years (Yang, 1980). The benefit is now confirmed to be that, oviposition on withered leaves helps the pest insect to avoid stimulating plant defences thereby obviating any resultant increase in parasitism of *M. separata* eggs and negative effects on emerging larvae. This egg-laying behaviour was similar between the adults fed on artificial diet and those fed on wheat plants during their larval stage. To our knowledge, this is the first such behaviour that has been elucidated and shown to be consistent with PPH.

An overwhelming amount of evidence has shown that OIPVs play an important role in plant-herbivore-natural enemy

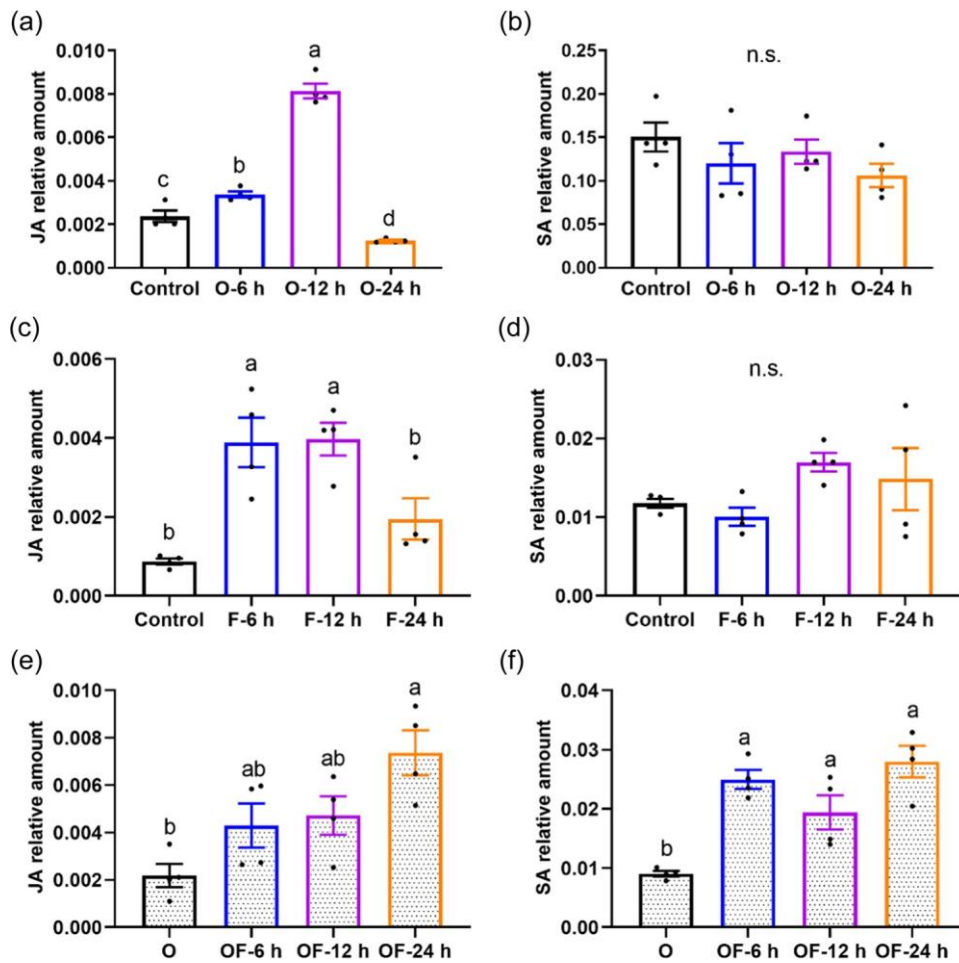


FIGURE 5 Impact of *Mythimna separata* oviposition and feeding on wheat plant phytohormones. (a) Jasmonic acid (JA) amount in egg-deposited plant for 6, 12 or 24 h. (b) Salicylic acid (SA) amount in egg-deposited plant for 6, 12 or 24 h. (c) JA amount in larvae feeding on egg-free plant for 6, 12 or 24 h. (d) SA amount in larvae feeding on egg-free plant for 6, 12 or 24 h. (e) JA amount in larvae feeding on egg-deposited plant for 6, 12 or 24 h. (f) SA amount in larvae feeding on egg-deposited plant for 6, 12 or 24 h. O: egg-deposited wheat plant. F: larvae feeding on egg-free plant. OF: larvae feeding on egg-deposited plant. Data are presented as mean values \pm SEM. Bars marked with different letters indicate significant differences ($p < 0.05$), one-way ANOVA and least significant difference (LSD) test ($n = 4$). [Color figure can be viewed at wileyonlinelibrary.com]

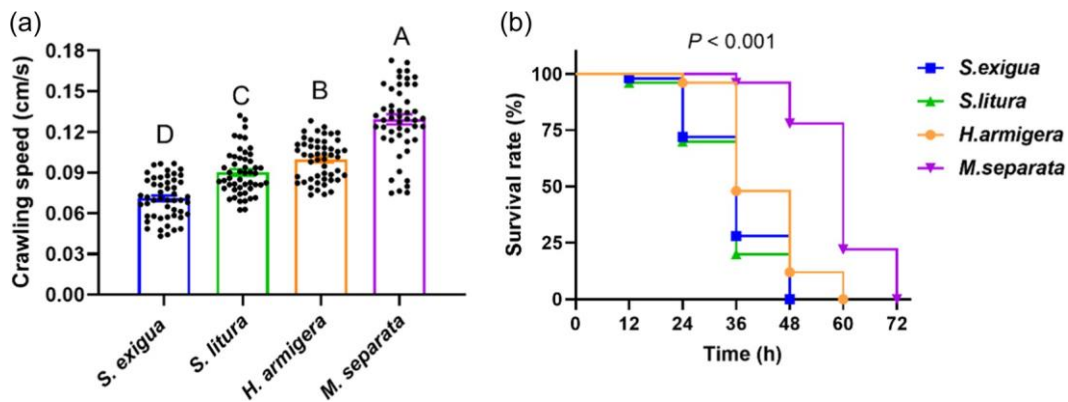


FIGURE 6 Newly emerged larval performance of *Mythimna separata*, *Helicoverpa armigera*, *Spodoptera exigua* and *Spodoptera litura*. (a) Crawling speed. (b) Survival rate under starvation. Data are presented as mean values \pm SEM. One-way ANOVA and least significant difference (LSD) test in a ($n = 50$). Log-rank (Mantel-Cox) test in b ($n = 50$). Bars marked with different letters indicate significant differences ($p < 0.001$). [Color figure can be viewed at wileyonlinelibrary.com]

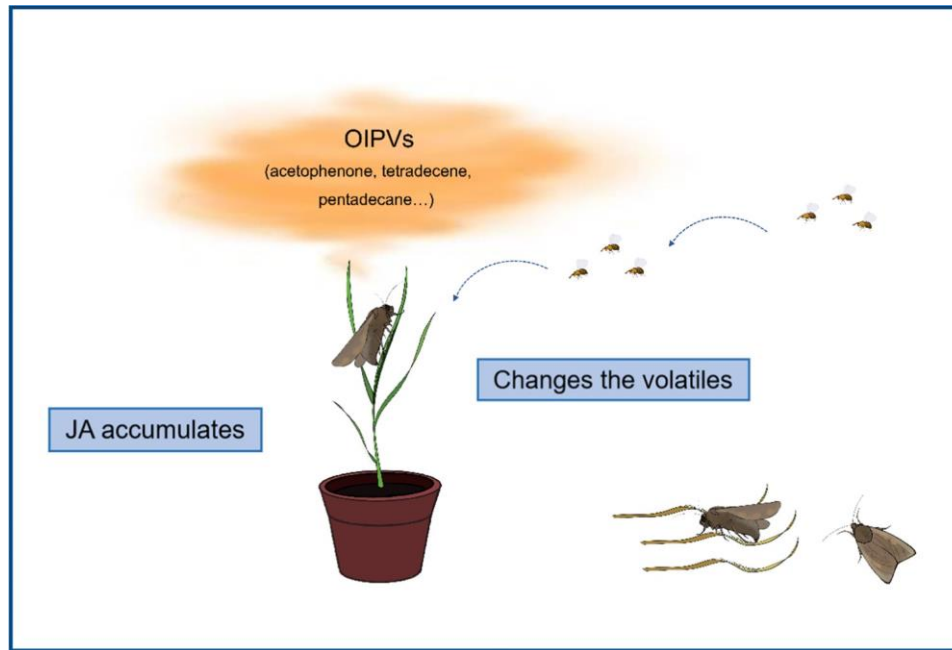


FIGURE 7 *Mythimna separata* prefers to lay eggs on withered leaves rather than fresh plants. This tactic can avoid plant defence and reduce the attack of egg parasitic wasps. Acetophenone, among other OIPVs, plays the dominant role in attracting egg parasitoid wasps. JA, jasmonic acid; OIPVs, oviposition-induced plant volatiles. [Color figure can be viewed at wileyonlinelibrary.com]

relationships. This study demonstrates that OIPVs produced by fresh plants in response to *M. separata* eggs attracted egg parasitoids in laboratory and field experiments. Chemical analysis shows that the volatiles were changed by *M. separata* egg laying, especially after 24 h, which is consistent with the preference for egg parasitoids. OIPVs, including acetophenone, tetradecane, and pentadecane, were quantified by GC-MS. Of the enhanced volatiles following 24 h *M. separata* egg deposition, acetophenone played a key role in attracting *T. chilonis*. Previous studies have noted the importance of acetophenone in attracting the wheat pest, *Sitodiplosis mosellana*, to wheat plants (Birkett et al., 2004). Acetophenone also mediates other plant-insect interactions (Erbilgin et al., 2007; Rohrig et al., 2008; Tang et al., 2012). Our findings demonstrate the role of acetophenone in controlling *M. separata*, by affecting the attraction of natural enemies, specifically

T. chilonis, and thereby aiding the search for hidden eggs of *M. separata*. This approach could make an important contribution to raising the value of egg parasitoids in biological control and integrated pest management programmes. However, to what extent acetophenone influences the attraction of natural enemies in the field remains to be determined. Additionally, further investigations are needed to explore the effect of OIPVs on other natural enemies.

Eggs-induced plant defence also showed a significant negative effect on subsequently emerged *M. separata* larval growth and development. The result revealed that eggs laid on wheat plants induced anti-herbivore defence against future larval feeding, which is similar to other studies (Bandoly et al., 2015; Valsamakis et al., 2020). Thus, plant hormones (JA and SA) were tested to investigate the

associated pathways in plant defence. The observed elevation of JA level provides evidence of this plant hormone stimulating plant defence when *M. separata* lays eggs on fresh leaves, compared to an unchanged level of SA in fresh leaves without oviposition. This finding is similar to a report of an elevated JA level following *Helicoverpa zea* egg laying (Kim et al., 2012). The phytohormone JA was widely reported to mediate plant defence against lepidopteran pests (Malook et al., 2019; Mao et al., 2017; Senthil-Nathan, 2019). In our study, larvae feeding also increased the amount of JA. Applying exogenous MeJA exerted a negative effect on larval weight gain. These results showed that egg-induced plant anti-herbivore defence against the hatching larvae was mediated by JA signalling. *Tetra-nyctus urticae* egg extract reduced its female fertility by altering JA and ethylene pathways in *Arabidopsis thaliana* (Ojeda-Martinez et al., 2021). In addition, although JA and SA are antagonistic in most cases, both significantly accumulate when larvae fed on egg-deposited plants compared to egg-free plants. The cross-talk between plant hormones is complex with evidence of JA and SA being synergistically involved in defence against herbivores (Jia et al., 2018; Shi et al., 2019; Valsamakis et al., 2020).

In the process of evolution, arthropods have acquired characteristics that are conducive to their survival, for example, the larval stage of lepidopteran transfer between plants via ballooning using silk threads. It is shown here that the newly emerged *M. separata* larvae possess a great advantage of mobility by crawling faster and surviving longer during starvation when compared to other noctuid larvae. It is possible that these characteristics can help newly emerged larvae in the essential step of moving to the healthy parts of the host plant. However, other potential strategies and behaviour of newly emerged

M. separata larvae in overcoming adverse environmental conditions in searching for suitable fresh feeding sources would need further investigation. Additionally, more species need to be investigated to explore the larval survival advantages.

In conclusion, we provide the first statistically significant evidence of the preference for *M. separata* females to oviposit on withered leaves. This enables the eggs and emerging larvae to overcome plant defence. Although the co-evolutionary nature of the arms race between plants and herbivores needs to be studied and explained further (Griese et al., 2021), the particular behaviour presented here offers new insights for explaining frequent pest outbreaks of *M. separata*, and perhaps other related species.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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