Pesticide Biochemistry and Physiology 204 (2024) 106033

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Regulation of three novel pepper thiothiazolidinones on the fecundity of *Spodoptera frugiperda*

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ARTICLE INFO

Keywords:
Pest control
Spodoptera frugiperda
Trehalose metabolism
Regulation of insect fertility

ABSTRACT

Spodoptera frugiperda has emerged as a major invasive pest worldwide. The utilization of chemical pesticides not only poses numerous ecological concerns but also fosters resistance in *S. frugiperda*. In this study, we designed and synthesized three novel thiothiazolidinone compounds (6a, 7b, and 7e) and incorporated innovative thiothiazolidinone structural elements into the piperine skeleton. Treatment with compounds 6a and 7e resulted in the blackening and agglomeration of oviduct eggs within the ovaries of certain female moths, impeding the release of normal eggs. The levels of vitellogenin and vitellogenin receptor, along with three trehalase inhibitors, exhibited a dynamic equilibrium state, leading to no discernible change in egg production but a notable increase in the generation of low-hatching-rate egg fragments. Compared with the injection of 2%DMSO, the eclosion rate of 6a injection was significantly decreased, as followed the spawning time and longevity were prolonged or significantly prolonged in the trehalase inhibitors of 6a, 7b, and 7e. We aimed to investigate the regulatory impacts of three new pepper thiothiazolidinone compounds on the reproduction of *S. frugiperda*, and to authenticate the efficacy of novel alginase inhibitors in inhibiting the reproduction of *S. frugiperda*. This research endeavors to aid in the identification of efficient and steadfast trehalase inhibitors, thereby expediting the research and development of potent biological pesticides.

1. Introduction

Insects represent one of the most pivotal life forms on Earth, with Lepidoptera standing out as one of the most prevalent insect orders (Wu et al., 2023). This kind of insects are globally invasive pests due to their extensive hosts, causing significant crop damage and developing resistance to conventional insecticides, a concern of global significance (Wang et al., 2022a; Agnihotri et al., 2016; Corrêa et al., 2019). Among them, *Spodoptera frugiperda*, commonly known as the fall armyworm, is a notorious agricultural pest native to North and South America (Tay et al., 2023). It was first detected on the African continent in 2016 (Kenis et al., 2023). Owing to its larval dispersal capability, adult migratory behavior, and high environmental adaptability (Roger et al., 2017), *S. frugiperda* has swiftly spread from the Americas to entire continents of Africa and Asia. With a broad spectrum of hosts, *S. frugiperda* feeds on approximately 350 native plant species in the Americas (Harrison et al., 2019). Over the past decade, it has emerged as a major invasive threat worldwide, seriously jeopardizing global food crop security (Luo et al., 2022; Tay et al., 2023). Most of the regions it infiltrates boast mild climates and abundant host plant resources, offering conducive conditions for its continuous proliferation (Goergen et al., 2016). Given its robust reproductive capacity, *S. frugiperda* is anticipated to pose a significant threat to vital crops such as maize (Midega et al., 2018). Chemical control has been an important measure for the prevention of *S. frugiperda* for decades (Wang et al., 2023a). However, the long-term and extensive use of insecticides can lead to insecticide resistance,

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https://doi.org/10.1016/j.pestbp.2024.106033
Received 26 May 2024; Received in revised form 9 July 2024; Accepted 15 July 2024
Available online 25 July 2024

also produces lethal or sublethal effects on beneficial arthropods in the environment (Desneux et al., 2007; Jia et al., 2022; Haddi et al., 2023). So further develop towards a green and sustainable control technology system (Pavela et al., 2023), such as agricultural control, and biological control (Hou et al., 2022; Liu et al., 2022; Li et al., 2023a; Karakkottil et al., 2024; Perumal et al., 2024), as well as base on RNAi technology (Wan et al., 2021; Yan et al., 2023; Su et al., 2023; Chao et al., 2023).

Energy metabolism plays a crucial role in insect development, with trehalose serving as a primary energy source for the reproductive cycle. Therefore, precise adjustment of energy storage anabolism and catabolism is essential for maintaining metabolic equilibrium throughout the insect life cycle (Foster, 2009; Huang and Lee, 2011). In the case of *Helicoverpa armigera*, lipid metabolism predominates during larval stages, aiding in the maintenance of stable hemolymph trehalose levels under starvation conditions, where triglycerides in adipose tissue are hydrolyzed to serve as energy reserves (Jiang et al., 2019). Trehalose, the primary sugar in insect hemolymph, fuels activities such as muscle contraction and ovarian function. In locusts, inhibition of trehalose metabolism leads to reduced blood sugar levels in flight muscles, thereby disrupting normal chitin synthesis during migration (Wegener et al., 2003; Tang et al., 2024). Throughout the reproductive cycle of *Blattella germanica*, hemolymph trehalose levels steadily increase, serving as energy fuel for oocyte maturation. Trehalose is metabolized into glycogen under prolonged starvation conditions, which can be converted back to trehalose and released into the bloodstream (Bede et al., 2007). In *Rhodnius prolixus*, carbohydrate metabolism and its hormonal regulation play a pivotal role in adapting to various physiological states, including reproduction and starvation. Oocytes store carbohydrates released from the fat body, and membrane-bound trehalase supplies glucose for carbohydrate accumulation. Trehalose released from the fat body is absorbed by the ovary to meet the physiological demands of egg formation, as the ovary lacks the capacity to synthesize trehalose (Santos et al., 2008; Leyria et al., 2021).

The polysaccharide chitin is present in the extracellular matrix surrounding insect larvae, pupae, and adults, forming the outer skin and lining the midgut, and it serves to protect or provide structural support to the entire body (Zhu et al., 2016; Bouchebti et al., 2023). Deficiencies in chitin can result in fetal abnormalities, defects in epidermal structure, and growth arrest (Moussian, 2019; Muthukrishnan et al., 2020). In *R. prolixus*, a blood-feeding insect whose eggs rely on blood intake, the injection of CHS-dsRNA reduces the gene transcription of chitin synthase (CHS), consequently lowering the chitin content in treated eggs and impairing their normal development (Souza-Ferreira et al., 2014). Similarly, treatment of *Lygus lineolaris* with novaluron, a chitin synthesis inhibitor, for 48 h induces ultrastructural changes in the ovaries of 1-day-old adults, alters the development of follicular epithelial cells in oocytes, and results in a rapid decline in hatching rate (Catchot et al., 2020). The follicular epithelium, which actively synthesizes chitin and transports yolk and non-yolk proteins to developing oocytes, plays a crucial role in this process (Bouts et al., 2007; Mansur et al., 2010). CHS1 and CHS2 are responsible for chitin synthesis in different parts of the insect. CHS1 synthesizes chitin in the outer skin, whereas CHS2 synthesizes chitin in the peripheral extracellular matrix (Hogenkamp et al., 2005). In *Lepeophtheirus salmonis*, the expressions of LsCHS1 and LsCHS2 are observed in oocytes, ovaries, gut, and outer skin. Silencing LsCHS2 leads to significantly shorter egg strings compared to the control group, while silencing LsCHS1 expression disrupts development and growth (Harðardóttir et al., 2021). Vitellogenin (Vg) and vitellogenin receptor (VgR) are vital for insect reproduction, including Lepidoptera. Vg, a precursor of vitellin, is a phosphoglycoprotein that forms vitellin and provides essential nutrients for embryonic development. VgR acts as the carrier of immune activators from the mother to the egg (Salmela et al., 2015; Asad et al., 2020; Han et al., 2022). Changes in the relative gene expression levels of Vg and VgR were analyzed to further explore the effects of three novel trehalase inhibitors on the fecundity of armyworm.

In response to the growing global population, agriculture has expanded progressively, yet crop pests continue to cause significant annual losses worldwide. When addressing these losses, it is imperative to balance economic considerations with ecological sustainability, necessitating a thorough understanding of pest ecological characteristics (Hackett and Bonsall, 2019). While chemical insecticides have been employed for centuries to mitigate pest damage, mounting concerns over their adverse impacts on biodiversity underscore the urgent need for alternative approaches. Widespread chemical usage poses threats to human health, ecosystems, and non-target organisms (Desneux et al., 2007; Little et al., 2019; Mateos Fernández et al., 2022). Trehalase, a key enzyme highly specific to trehalose, catalyzes the irreversible hydrolysis of trehalose into glucose, crucial for cellular energy metabolism and chitin synthesis (Tevatiya et al., 2020; Luo et al., 2022). As the sole α-glucosidase responsible for endogenous trehalose hydrolysis, trehalase is competitively inhibited by most glucosidase inhibitors, offering valuable insights into insect trehalase function and glucose metabolism (Shukla et al., 2015; García and Argüelles, 2021). Given the biological linkage between trehalose and trehalase, trehalase inhibitors have emerged as potential insecticides (Asano, 2003), exhibiting inhibitory effects across various insect orders including Lepidoptera, Diptera, Hemiptera, and Coleoptera (Tatun et al., 2014; Tatun et al., 2008; Tang et al., 2017; Yu et al., 2021). They offer high efficacy, safety, environmental friendliness, and non-persistence, making them promising candidates for non-toxic insecticides in the era of sustainable development (Adhav et al., 2018; Matassini et al., 2020). MicroRNAs (miRNAs), which regulate the expression of target genes involved in crucial biological processes, play a pivotal role in insect development and insecticide resistance (Zhang et al., 2021). Inhibiting the expression of these key genes can have lethal effects on pests. Both plants and insects employ miRNAs to regulate their biological processes and modulate insect behavior and developmental signaling pathways (Li et al., 2018). Disrupting these regulatory mechanisms may exert significant effects on behavior, development, and phenotype, potentially leading to increased insect mortality.

In the past our studies, some trehalase inhibitors have been developed, for example piperine, thiazolidinone and its derivative (Han et al., 2021; Jiang et al., 2022), which can induce increased insect mortality, abnormal development, reduced flight capability, and decreased fecundity (Wang et al., 2022b; Zhong et al., 2023; Jiang et al., 2023a; Tang et al., 2024). This study aims to investigate the effects of three thiazolidinone, namely 6a, 7b, and 7e, on the fecundity of *S. frugiperda*. Following the injection of these novel inhibitors into *S. frugiperda* larvae, evaluations were conducted on pupal eclosion rate, eclosion deformity rate, adult armyworm fecundity, longevity, and anatomical observations of female ovaries to assess the impact of these inhibitors on both larvae and adults of *S. frugiperda*.

2. Materials and methods

2.1. Source and breeding of S. frugiperda

The *S. frugiperda* specimens utilized in this study were sourced from the Zhejiang Academy of Agricultural Sciences (Hangzhou, Zhejiang) and reared in our laboratory. Both adult and larval stages were maintained in an artificial climate chamber with a temperature of 26 °C ± 1 °C, relative humidity of 60% ± 10%, and a photoperiod of 16 h light and 8 h darkness (day/night).

Female pupae were treated in an artificial climate chamber and monitored daily until eclosion. The eclosion day recorded as day 0. Female pupae that emerged normally were selected and paired with untreated male pupae of the same eclosion day. These pairs were placed in plastic cups with dimensions of 7.5 cm diameter at the opening, 4.1 cm diameter at the base, and 8.3 cm height, within a cage measuring 38.5 cm in length, width, and height. One female and one male from each pair were designated as one adult pair. Subsequently, the opening of each plastic cup was covered with a 10 cm square piece of 120-mesh

nylon cloth, and eclosion time, group, and serial number were meticulously recorded. The worms were fed a diet of 10% honey water daily.

2.2. Preparation and microinjection of trehalase inhibitors

The novel trehalase compounds 6a, 7b, and 7e were provided by the PMDD Laboratory of China Agricultural University (Beijing) (Tang et al., 2024). Trehalase inhibitor of three thiazolidinone compounds (6a, 7b, 7e) powders were dissolved to prepare 1×10^{-3} mmol/mL solutions with 2% dimethyl sulfoxide (DMSO). Microinjections of the three new trehalase inhibitors were conducted on the first day of pupation. Pupae were positioned on a clean petri dish atop a microinjection table (Eppendorf TransferMan® 4r), and 300 nL of the prepared solution was injected into the junction of the 5th and 6th ventral segments by gently turning the pupae over with a small tip brush. Pupae from the first day of pupation (CK group), which did not undergo injection during the same period, served as the control group and were cultured in the artificial climate chamber in a 6-well plate. Trehalase inhibitors were injected into the cultured pupae using the same method, with 2% DMSO injected as the control.

2.3. Pupal eclosion rate and eclosion deformity statistics

Methods for judging eclosion were as follows: complete eclosion of the adult from the pupal shell was considered as complete eclosion, whereas a pupa that did not display any movement or twisting of the abdomen upon gentle pressing of the head with a small tip brush was deemed a dead pupa, indicating incomplete eclosion. Any adult that only partially emerged from the pupal shell, retaining part of its body attached to the shell, was categorized as incomplete. Treated pupae were divided into three groups, each consisting of 20–30 pupae. Eclosion of pupae was observed daily from the 5th day post-treatment, with the number of eclosions, dead pupae, and incomplete eclosions recorded on a data sheet. Using the recorded data, eclosion rate, dead pupa rate, and incomplete eclosion success rate were calculated as follows:

$$\text{Eclosion rate} = \text{Number of eclosion}/\text{Total number of pupae after treatment} \times 100\%.$$

$$\text{Dead pupae rate} = \text{Number of dead pupae}/\text{Total number of pupae after treatment} \times 100\%.$$

$$\text{Incomplete eclosion rate} = \text{Number of incomplete eclosion}/\text{Total number of pupae after treatment} \times 100\%.$$

The eclosion of adult insects is considered normal if their wings are fully extended and they can crawl and fly without impediment. Conversely, the presence of abnormal wing folding, wherein wings cannot fully extend, resulting in immobility, signifies an eclosion deformity. Post-eclosion phenotypes of pupae were observed, and photographs were captured using a Canon EOS 50D camera. The number of eclosion deformities was meticulously recorded on a data sheet, and the eclosion deformity rate was calculated based on the recorded data. The calculation method is as follows:

$$\text{Eclosion deformity rate} = \text{Number of eclosion deformity}/\text{Number of eclosion} \times 100\%.$$

2.4. Statistics of female oviposition period, oviposition amount, and lifespan of S. frugiperda

From the day of eclosion until death, female were monitored daily to determine if they laid eggs. Females that did not lay eggs until death were deemed infertile, and such individuals were excluded from subsequent experimental data analysis. The oviposition behavior and lifespan of fertile females were recorded daily until the death of the adult female. Oviposition quantity, pre-oviposition period, oviposition period, and lifespan of fertile females were calculated based on the collected data. Oviposition quantity was calculated only within 7 days of eclosion. Each treatment was replicated three times biologically, with 10–20 pairs of adults per group.

2.5. Statistics on hatchability of S. frugiperda eggs

Oviposition from day 2 to day 7 after eclosion were collected to determine the hatching rate. Eggs produced by 10 pairs of adult moths were collected daily from each treatment group, and one egg was randomly selected from each pair. A total of 10 egg masses were recorded, with each mass placed in a feeding box measuring 3.8 cm in diameter at the base and 2.3 cm in height. The number of eggs, treatment group, and serial number were marked, and the egg masses were incubated in an incubator. The hatching rate was calculated after the larvae hatched. Egg masses that did not hatch after 7 days were considered inactive. The calculation method for the hatching rate was as follows:

$$\text{Hatching rate} = \text{Number of larvae hatched}/\text{Number of eggs} \times 100\%.$$

2.6. Anatomy and observation of S. frugiperda ovaries

Females mated on the 2nd, 4th, and 6th days after eclosion were selected for ovarian dissection under a Lycra microdissection microscope. Ovaries were photographed using a Canon EOS 50D camera for observation. At least three females were dissected from each treatment group, and ovarian development was assessed according to the criteria established by Zhao et al. (2018).

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

On the 2nd, 4th, and 6th days after eclosion, mated females were selected for ovarian dissection, and fat body tissues and ovarian tissues were collected to detect changes in the expression levels of Vg and VgR. Five females were dissected from each treatment group, with each female serving as a biological replicate, resulting in 5 biological replicates.

Total RNA was extracted from larvae collected 48 h after injection using Trizol reagent according to the manufacturer's instructions. Three larvae were collected from each group and five biological replicates were performed. The concentration and quality of the extracted RNA were determined using a NanoDropTM 2000 spectrophotometer. The RNA integrity was verified through 1% agarose gel electrophoresis. The RNA samples were stored at −80 °C for further analysis. Subsequently, cDNA was synthesized using the PrimeScript® RT reagent kit with gDNA Eraser (NARISHIGE, Japan). The procedure was performed on ice. The primers were synthesized by Hangzhou Shangya Biotechnology Corporation and the RPL10 gene of *S. frugiperda* was used as the internal reference gene (Gurusamy et al., 2020). Each 10 μL reaction volume contained 5 μL SYBR Green master mix, 3.2 μL deionized water, 0.4 μL of both forward and reverse primers, and 1 μL cDNA sample. The qRT-PCR reaction procedure consisted of pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, extension at 60 °C for 20 s, and 40 cycles. Primer sequences are listed in Table 1.

2.8. Data analysis

Statistical significance of the data was assessed using IBM SPSS Statistics 20 software, with normality and homogeneity of variance

Table 1
The primers for qRT-PCR.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Gene ID
Vg	CGAAGAACCTCAAATACGAAACTGT	TGGTGCTGGAGTGGGTAGATAA	MT505383
VgR	CGACGAGTGCACTGAAGATG	GAGGCGTCAGTATVGGTGTA	XM_035595777.1
RPL10	GACTTGGGTAAGAAGAAG	GATGACATGGAATGGATG	

evaluated. Differences between control and treatment groups were compared using one-way analysis of variance (ANOVA) or independent sample *t*-test. Post hoc tests were performed using the Tukey method for one-way ANOVA, with different letters indicating significant differences between groups ($P < 0.05$). For the independent sample t-test, "*" denoted a significant difference when $P < 0.05$, "**" indicated an extremely significant difference when $P < 0.01$, and "ns" indicated no significant difference. Data are presented as mean ± SD. GraphPad Prism version 9.0 software was used for data visualization.

The Chi-squared test was employed to compare differences in deformity rates between the control group and treatment group, with $P < 0.05$ considered indicative of a significant difference between the two groups, denoted by different letters (Li et al., 2023b).

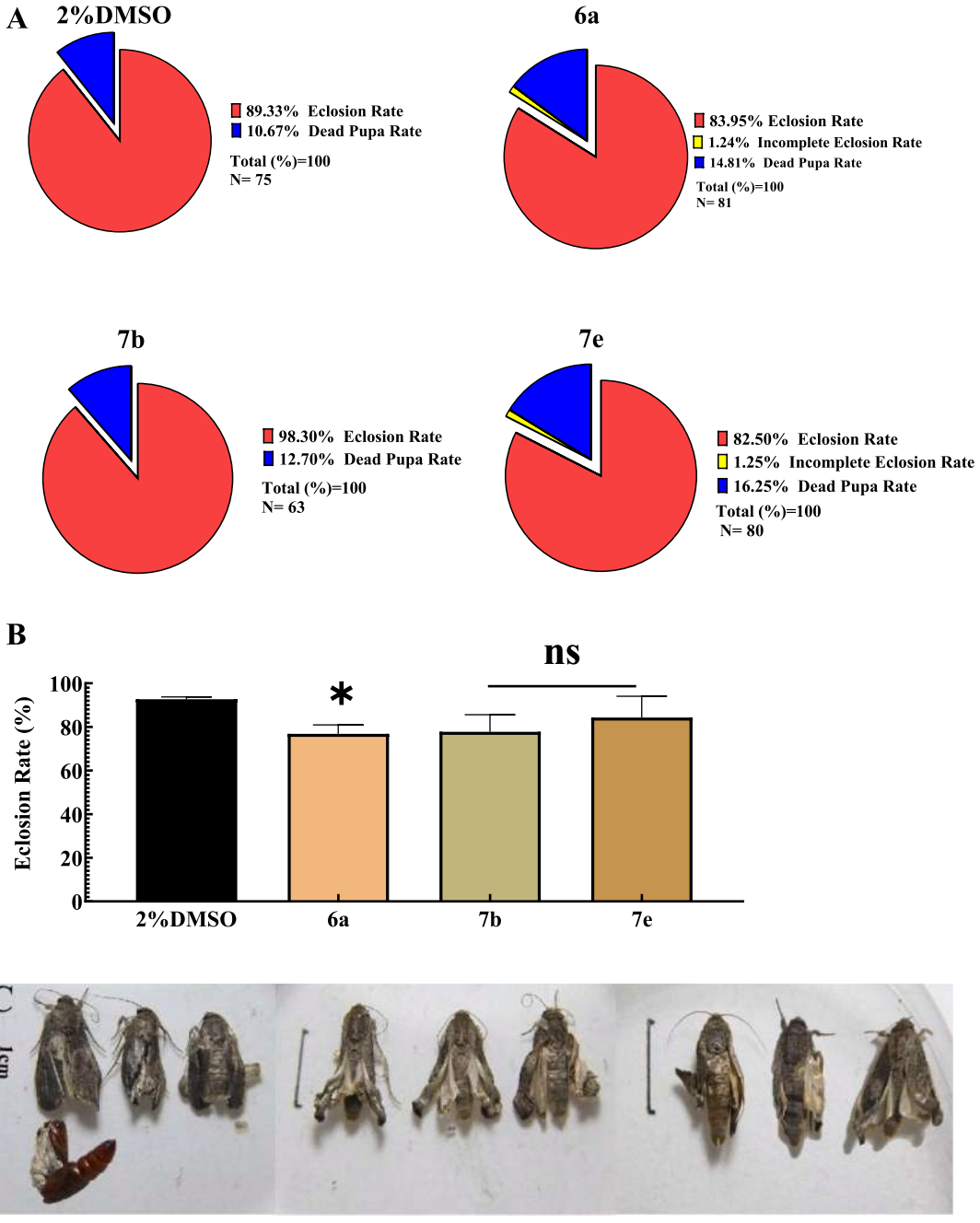

Fig. 1. Eclosion rate, incomplete eclosion rate, dead pupa rate (A), and significant differences in eclosion rates (B) of *S. frugiperda* following the injection of novel trehalase inhibitors. Abnormal phenotypes of adults after the injection of novel trehalase inhibitors (C).

3. Results

3.1. Eclosion rate, incomplete eclosion rate and dead pupa rate after injection of novel trehalase inhibitors

Compared to the 2% DMSO group, the eclosion rate and dead pupa rate in groups 6a, 7b, and 7e showed no significant difference, with a small number of incomplete eclosion adults observed in groups 6a and 7e (Fig. 1). The eclosion malformation rates in groups 6a, 7b, and 7e were all higher than those in the 2% DMSO group, with only group 6a showing a significant difference (Fig. 1B). The eclosion phenotype of pupae treated with the three trehalase inhibitors exhibited wing folding malformation in groups 6a, 7b, and 7e. While this phenotype did not generally affect the normal physiological activities of adult *S. frugiperda*, it posed certain obstacles to their normal flight (Fig. 1C).

3.2. Probability of fertile females after inhibitor treatment of S. frugiperda

All females in the 2% DMSO group and 7b group were fertile, whereas 6a and 7e treatments affected the fecundity of females. Some females treated with these three inhibitors did not lay eggs (Fig. 2A). Further dissection of the ovaries of non-oviposition females in groups 6a and 7e revealed normal development of ovarian tubes with full eggs, but the ovum in the middle fallopian tube appeared blackened and agglomerated, obstructing the output of normal eggs (Fig. 2B), which was the main reason for female infertility.

3.3. Preoviposition period, oviposition period and lifespan of female S. frugiperda

After excluding the interference of non-fertile females as described in 2.2, changes in the pre-oviposition, oviposition, and lifespan of females in each treatment group were observed. There was no significant change in the pre-oviposition period among females in each group. Upon comparing the lifespan of females in each group, it was observed that females in group 7b exhibited the longest lifespan at 10.48 days, whereas females in other treatment groups exhibited significantly longer lifespans than those in the 2% DMSO group. There was no significant difference in lifespan compared to the 2% DMSO group in group 6a, whereas the lifespan of females in groups 7b and 7e increased significantly (Table 2).

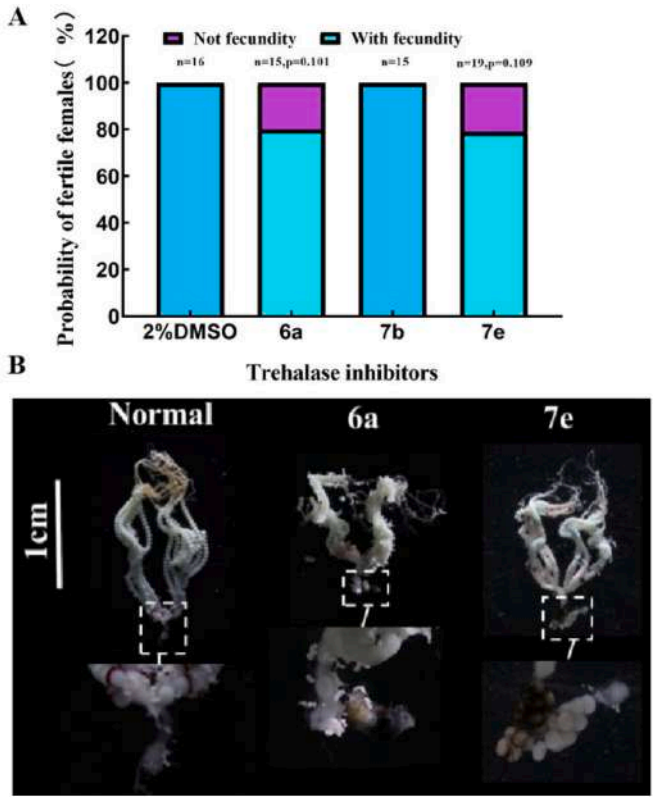

Fig. 2. Probability of fertile females after inhibitor injections (A) and the infertile ovaries of *S. frugiperda* female adults (B).

3.4. Changes in the number of oviposition by female S. frugiperda and the hatchability of oviposition by female S. frugiperda over 7 days

Egg production in groups 6a, 7b, and 7e was higher on the 2nd day of eclosion, with groups 6a and 7b significantly higher than the 2% DMSO group (Fig. 3A). Notably, egg production in the 2% DMSO group peaked on the 3rd day of eclosion, and subsequently, the daily egg production was slightly lower than that of the other three treatment groups, with the 7e group significantly surpassing the 2% DMSO group on the 6th day of eclosion. There was no significant difference in the total production of eggs within 7 days among all groups, but there was a tendency to increase (Fig. 3B). The daily number of oviposition by a single female was selected as one of the primary physiological indices to measure the fecundity of female moths, along with observing the hatching rate of oviposition. In the 2% DMSO group, the median hatching rate of oviposition on the remaining days remained around 80%, except for the hatching rate on day 7 (Fig. 3C).

3.5. Development of ovaries and changes in Vg and VgR expression in ovaries of S. frugiperda

On the 2nd day of eclosion, female adult armyworms in each group predominantly exhibited ovarian grades ranging from II (yolk deposition stage) to III (maturity waiting for delivery), with fully visible eggs. By the 4th day after eclosion, ovarian grades in all groups were primarily distributed between III (maturity waiting for perinatal period) and IV (prime perinatal period). On the 6th day after eclosion, ovaries in all groups except group 7e had reached the end of ovulation, while ovaries in group 7e remained in the full perinatal period, containing a significant number of mature eggs in the ovarian tube (Fig. 4A). In comparison with the 2% DMSO group, the three treatment groups exhibited no significant effect on Vg expression in the ovaries on the 2nd day after eclosion. However, they significantly or extremely significantly upregulated the expression of VgR. By the 4th day after eclosion, group 7b exhibited a significant downregulation of Vg expression, whereas groups 7b and 7e showed a significant increase in VgR expression. On the 6th day after eclosion, the expression of Vg was significantly downregulated in groups 7b and 7e, whereas the expression of VgR was significantly upregulated in group 6a (Fig. 4B).

4. Discussion

Insect wing length, body size, and forewings are significant predictors of flight distance and speed, whereas female reproduction can be relatively easily estimated by counting the number of oviposition. For Lepidoptera, energy and nutrients a cquired during the larval stage are redistributed in later stages to facilitate the formation of adult body

Table 2
Pre-oviposition period, spawning time, and longevity in female adults after pupal injections.

Unit: days	Pre-oviposition period	Spawning time	Longevity
2%DMSO	1.88 ± 0.08 a	5.20 ± 0.53 a	8.00 ± 0.20 b
6a	1.33 ± 0.19 a	6.67 ± 1.01 a	9.25 ± 1.32 b
7b	1.64 ± 0.20 a	7.22 ± 0.03 a	10.48 ± 0.45 a
7e	1.94 ± 0.28 a	6.36 ± 0.84 a	9.69 ± 0.67 ab

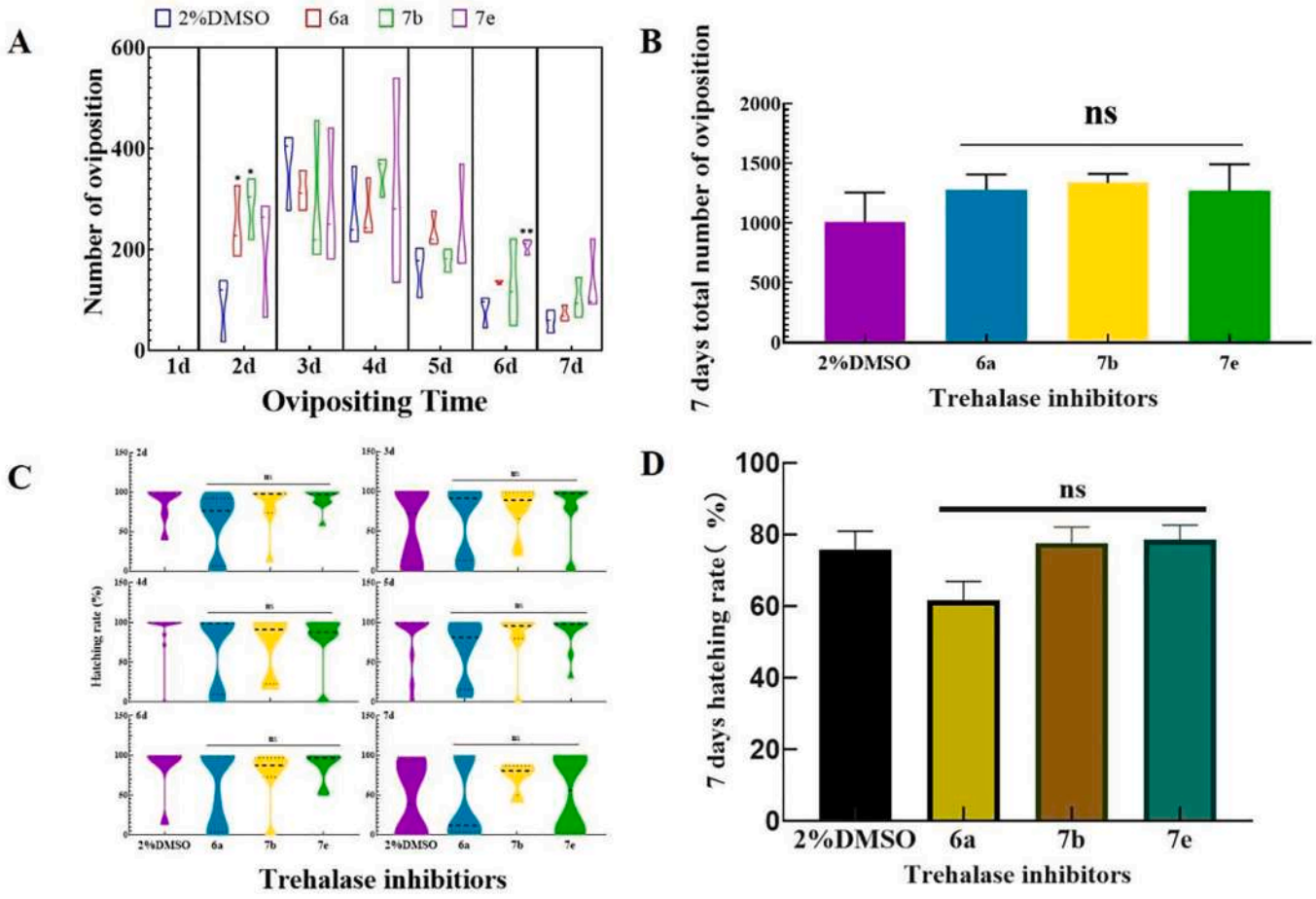

Fig. 3. Changes in the number of eggs in a single female adult within 7 days of treatment with three novel trehalase inhibitors (A) (2% DMSO represents the control group; 6a, 7b, and 7e represent the treatment groups). Changes in total number of eggs in a single female adult within 7 days (B), hatchability of eggs within 7 days in a female adult (C), and the hatching rate of 7 days (D).

structures in the pupal stage and meet metabolic and reproductive needs in adulthood, which is also reflected in the quantity of oviposition (Jahant-Miller et al., 2022). Therefore, the effect of three new trehalase inhibitors on armyworm fertility can be studied by observing pupal phenotypes, egg laying quantities, and ovary changes after injection of these inhibitors.

In recent studies, *Haemophilus oryzophilus* soluble trehalase (*LoTRE1*) shares similarities with some known insect trehalases. Feeding *LoTRE1* dsRNA to adult worms silenced *LoTRE1* transcription, thereby reducing trehalase activity and increasing trehalose content, resulting in a 12% mortality rate (Wang et al., 2022c). Trehalase dynamically controls trehalose and glucose content in insects. Soluble trehalase accounts for the majority of overall trehalase activity, primarily decomposing trehalose in cells (Tatun et al., 2008; Fraga et al., 2013). Daily stress application led to a significant decrease in fertility, longevity, weight, and triglyceride content, but a notable increase in trehalose and glucose content (Gruntenko et al., 2021). In our experiment, after trehalase inhibitor injection, the eclosion rate of groups 6a, 7b, and 7e was lower than that of the 2% DMSO group, with observed wing wrinkling (Fig. 1C). Silencing of trehalase in *Nilaparvata lugens* affects chitin synthesis and degradation, resulting in phenotypic deformities (Zhao et al., 2016), whereas *Leptinotarsa decemlineata* larvae die from underdevelopment due to trehalase silencing (Yu et al., 2021). This aligns with previous studies indicating trehalase silencing causing developmental malformations and insect death (Tang et al., 2017). Trehazolin, a potent alginase inhibitor, was injected into the hemolymph of locusts, and trehalase activity in flight muscles was monitored over a 30-day period. Total trehalase activity in flight muscles was notably inhibited during the first half of this period (Liebl et al., 2010; Wegener et al., 2010). Meanwhile, validamycin A has been shown to reduce glucose and increase trehalose levels during the development of *Aedes aegypti*. This delay in larval and pupal development prevents adult mosquitoes from flying. Offspring adult larvae from treated larvae displayed significantly shorter tibia lengths compared to those of host larvae treated with ddH_2O, suggesting that validamycin A delays offspring development, leading to a higher proportion of abnormal adults. Many larvae exhibited slow growth, abnormal molting, and pupation following validamycin treatment (Marten et al., 2020; Shao et al., 2021; Song et al., 2023). Therefore, it is inferred that trehalase inhibitors hinder the synthesis of trehalase, causing trehalose to accumulate excessively in the body. This accumulation may result in malformations or even death in adult worms.

The observation that there was no significant difference in the lifespan of group 6a compared with the control group, whereas the lifespans of females in groups 7b and 7e increased significantly (Table 2), is consistent with the phenomenon observed in experiments where long-lived fly populations are often selected based on lower fecundity. This is because flies with lower fecundity tend to live longer (Toivonen and Partridge, 2009). Similarly, in *C. elegans*, the DAF-2 pathway, involving the insulin receptor, is known to regulate aging, reproduction, and diapause independently, and longevity through this pathway often accompanies impaired growth or reproduction (Dillin et al., 2002). In the case of *S. frugiperda*, increased fertility has been associated with reduced longevity. Studies with trehalase inhibitors such as ZK-PI-5 and ZK-PI-9

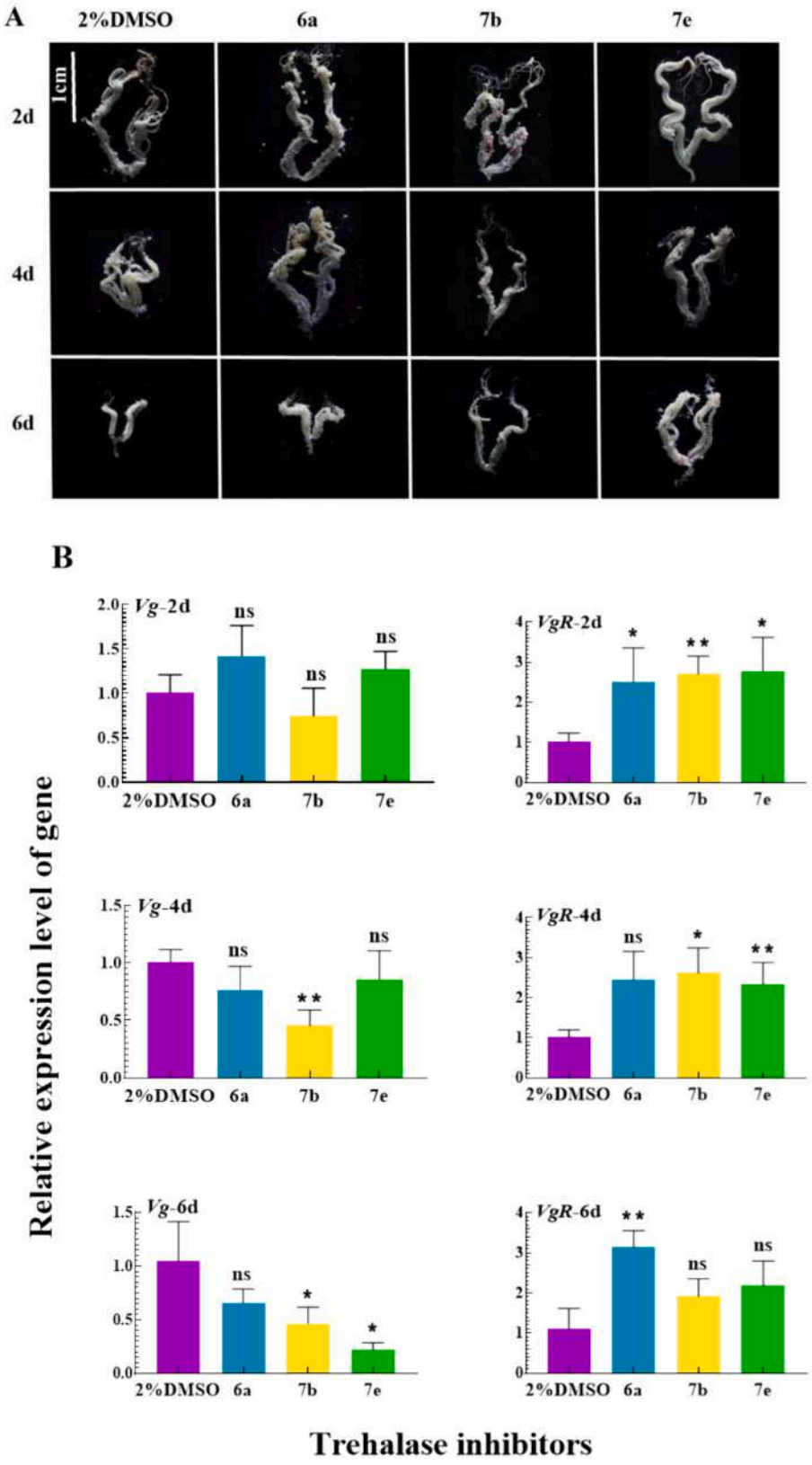

Fig. 4. The ovary of *S. frugiperda* female adults on the 2nd, 4th, and 6th days (A). Changes in relative expression of Vg and VgR genes in female adults on the 2nd, 4th, and 6th days of eclosion after the injection of novel trehalase inhibitors (B) (2% DMSO represents the control group; 6a, 7b, and 7e represent the treatment groups).

have shown that they significantly inhibit trehalase activity, with ZK-PI-9 also significantly inhibiting chitinase activity in female pupae. Interestingly, female adults treated with low concentrations of ZK-PI-9 and ZK-PI-5 laid significantly more eggs compared to the control group (Jiang et al., 2023b). Previous research on *Spodoptera exigua* has indicated a linear positive correlation between the expression of the VgR gene and the number of oviposition by female moths (Zhao et al., 2018). Consistent with this, the experimental results of this study showed significant or extremely significant upregulation of VgR expression compared to the 2% DMSO group (Fig. 4B). Furthermore, oviposition in groups 6a, 7b, and 7e increased after the second day of eclosion, with groups 6a and 7b showing significantly higher oviposition rates than the 2% DMSO group, and group 7e displaying significantly higher oviposition rates than the 2% DMSO group after the sixth day of eclosion (Fig. 3A). Given the low concentration of the new trehalase inhibitor used in this experiment, it is speculated that these inhibitors may increase the number of oviposition by female adults. Additionally, the expression of membrane-bound trehalase in *S. frugiperda* ovaries is regulated by diapause hormone. An increase in diapause hormone levels stimulates trehalase expression and glucose content in the ovaries, providing more energy to the ovaries and leading to ovary diapause (Kamei et al., 2011). Furthermore, when *H. axyridis* egg cells uptake insufficient trehalose, it may inhibit the expression of Vg, leading to delayed egg laying. Supplementation with glucose or trehalose has been shown to increase the reproductive rate of ladybugs, highlighting the importance of adequate energy sources for insect fertility (Li et al., 2020). Defects in ovarian development have also been associated with higher mortality rates (Zalucki et al., 2002; Li et al., 2021).

The findings in *N. lugens* and *Locusta migratoria*, where silencing of the adipokinetic hormone receptor impedes the action of triacylglycerols, leading to decreased trehalose content in the hemolymph and increased levels of trehalase in fat bodies, resulting in impaired Vg uptake and reduced levels of VgRs in the ovaries, leading to delayed oocyte maturation, prolonged preoviposition, reduced oviposition, and decreased fertility (Santos et al., 2012; Lu et al., 2019), provide insights into the potential mechanisms underlying the effects observed in this study. In this experiment, the injection of trehalase inhibitors (6a, 7b, and 7e) into the pupae of *S. frugiperda* resulted in various developmental defects in the adult worms. Specifically, eggs in the median oviduct of groups 6a and 7e exhibited blackening and clumping (Fig. 2B). Similar observations have been made in mosquitoes, where silencing of the *CpCHSA* gene, encoding chitinase synthase A, significantly reduced mosquito fecundity. Microinjection of short interfering RNA targeting *CpCHSA* resulted in abnormal follicle phenotypes, ranging from complete atrophy to truncation, along with changes in nuclear size, staining intensity, and fragmentation, ultimately affecting cytoplasmic material deposition into the oocyte (Wang et al., 2023b). Moreover, RNA interference targeting the glutamine synthase gene significantly reduced ovarian size in female adults, leading to ovarian hypoplasia and reduced egg development, thereby regulating ovarian development by controlling the accumulation of Vg in the ovaries (Zhai et al., 2013). Given that trehalase is the first gene in the chitin biosynthesis pathway, trehalase inhibitors have been shown to regulate the chitin synthesis and degradation pathway, thereby controlling chitin metabolism (Tang et al., 2017). Therefore, it is reasonable to speculate that trehalase inhibitors could hinder ovarian chitin synthesis, affecting egg morphology, with black clumps potentially obstructing the output of normal eggs, consequently reducing the reproductive ability of *S. frugiperda*.

Eggs, being the product of ovarian follicles in mature female Lepidoptera insects, undergo a rapid formation process. As oocyte volume increases, the contents of proteins, lipids, and sugars accumulated during larval stages and stored in pupae decrease, particularly Vg synthesized by the fat body, which is then transported and chelated to developing eggs and transported to the ovary via the hemolymph to provide nutrients for developing oocytes (Cole et al., 1987; Swevers and Iatrou, 2003; Telfer, 2009). Studies in cockroaches have shown that after injection of validamycin A, the vitellogenin (Vn) content barely increased until day 8, remaining at the day 2 control level, before sharply decreasing (Kono et al., 2001). As Vg is a precursor of Vn, experimental results in this study were consistent with this finding, where Vg content remained unchanged on the 2nd day but decreased significantly on the 4th and 6th days (Fig. 4B). The levels of Vg and VgR in response to the three trehalase inhibitors were in a state of dynamic equilibrium, leading to no change in spawning quantity (Fig. 3B). However, there was a significant increase in the production of eggs with low hatching rates. This suggests that trehalase inhibitors may inhibit the trehalase activity of eggs, resulting in oocytes being unable to absorb the required Vg, thereby reducing Vg accumulation by the ovary and ultimately lowering the hatching rate of eggs, thus inhibiting the reproduction of *S. frugiperda*. In insects, most vitellogenin is synthesized outside the ovary in the fat body. Vg is secreted into the hemolymph and taken up by oocytes through receptor-associated endocytosis, where it can be converted into Vn, the final form that is essential for embryonic development, survival, and reproduction (Tufail and Takeda, 2009; Mitchell 3rd et al., 2019). Stimulation of Vg biosynthesis or induction of follicles to promote Vg uptake by growing oocytes can promote ovarian development in insects (Abdullah Al Baki and Kim, 2019).

CRediT authorship contribution statement

Bin Tang: Writing – original draft, Resources. **Ye Han:** Writing – original draft, Methodology, Data curation. **Hongxia Duan:** Writing – original draft, Resources. **Yan Wu:** Writing – original draft, Resources. **Nicolas Desneux:** Writing – review & editing. **Shigui Wang:** Conceptualization, Supervision, Writing – review & editing. **Gao Hu:** Methodology, Validation, Writing – review & editing. **Busheng Liu:** Validation, Methodology. **Liyuhan Hua:** Writing – review & editing, Resources. **Yujia Luo:** Investigation, Methodology, Writing – review & editing. **Haoyu Fu:** Conceptualization, Writing – review & editing. **Qixuan Mao:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgment

This work was supported by the National Key Research and Development Program of China (Grant No. 2023YFC2605200), the Sixth Batch of Guizhou Province High-level Innovative Talent Training Program [2022], the Guiyang Science and Technology Personnel Training Project [2022]43-16, the Program for Natural Science Research in Guizhou Education Department QJJ[2023]024, Guiyang Training Project for “Thousand Level” Talents in Guizhou Province GCC[2022] 005.

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