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# Trehalase regulates ovarian maturation and egg hatchability of *Nilaparvata lugens*

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## Abstract

Trehalase (TRE) is an important enzyme that is responsible for trehalose hydrolysis. However, the effect of NLTRE on the reproduction of *Nilaparvata lugens* has not been clearly reported. To comprehensively evaluate the pest control potential of NLTRE, this study analyzed the effect of NLTRE on female reproduction of *N. lugens* by inhibiting TRE with dsTREs injection at mRNA level and validamycin injection at protein level, respectively. The results showed that validamycin not only significantly reduced the female body weight, but also extended the preoviposition time, but dsTREs had no significant effect on these phenotypes. Besides, validamycin significantly inhibited the ovarian development of females in the early stage, while dsTREs affected the ovarian development in the later stage. However, both two treatments have extremely significantly reduced the total number of eggs laid by female, and the egg hatchability also was extremely significantly decreased, likely due to the destruction of chitin components in egg shells. Therefore, TRE inhibition can decrease the fecundity of *N. lugens* female, which suggest that TRE is a potential pest control target.

**Keywords** *Nilaparvata lugens* · Trehalase · RNA interference · Validamycin · Fecundity

## Introduction

Trehalose is a non-reducing disaccharide formed from two glucose molecules linked by α, α-1, 1-glucoside bonds. It was first reported in 1832 in ergot and commonly found in bacteria, fungi, plants, and invertebrates (Elbein et al. 2003; Shukla et al. 2015). Trehalose is known as the "blood sugar" of insects, and it plays an important role in insect molting, metamorphosis, diapause, and development (Elbein et al. 2003; Mariano et al. 2009; Shukla et al. 2015). Due to its unique chemical properties, trehalose can be used to protect organisms from different environmental stresses, including hypoxia, dryness, high temperatures, low temperatures, or oxidative damage, in addition to being an energy source (Iordachescu and Imai 2008; Tang et al. 2008; Shukla et al. 2015; Liu et al. 2016; Shen et al. 2021). The trehalose synthesis pathway varies greatly among different classes, which the trehalose is synthesized at fat bodies in insects. It is mainly accomplished by two enzymes, trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). TPS is responsible for catalyzing the combination of glucose-6-phosphate and UDP-glucose to produce trehalose 6-phosphate, while TPP dephosphorylates the trehalose-6-phosphate and finally produces trehalose (Tang et al. 2018). After trehalose is synthesized in the fat body, it is transported to hemolymph and other tissues, where it can be hydrolyzed by trehalase (TRE) to meet the energy requirement of activities, such as flight.

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The biosynthetic pathway of trehalose may be different in different classes, but the way of trehalose degradation is unique. That is, trehalase hydrolyzes trehalose into two glucose molecules, which is irreversible (Avonce et al. 2006). Two forms of trehalase have been found in insects, soluble trehalase (TRE1), and membrane-bound trehalase (TRE2). TRE1 usually dissociates in the cytoplasm and is responsible for degradation of intracellular trehalose, while TRE2 is mainly responsible for extracellular trehalose (Mitsumasu et al. 2005; Lee et al. 2007; Tang et al. 2016, 2017, 2018). Multiple *TRE1* genes have been identified in insects such as *Nilaparvata lugens*, *Harmonia axyridis*, and *Tribolium castaneum*, while have not been found to have multiple coding genes (Tang et al. 2014, 2016; Zhao et al. 2016). TRE is indispensable for insect, and studies have shown that its inhibition can lead to increased mortality and infertility, abnormal morphology, and damaged flight ability (García and Argüelles 2021).

*N. lugens* is classified as a major rice pest, which lead to a reduction in rice production by sucking rice phloem with stinging mouthparts, laying eggs in rice tissue, and spreading plant virus such as grass dwarf virus and rice sheath blight virus. *N. lugens* exhibits wing plasticity, with long-winged individuals promoting population escaping from adverse environment, while short-winged individuals having strong reproductive ability (Xu and Zhang 2017). However, both two kinds of *N. lugens* adopt the R strategy in ecological adaptation, and its high fecundity aggravated its damage to rice production (Engel 2015). Therefore, it is the key to control the reproductive ability of *N. lugens* for inhibiting their population in rice field. Previous research has shown that the *TRE* silencing with RNA interference or inhibition of TRE activity with validamycin in *N. lugens* has changed sugar levels, which thus affected the chitin content and caused abnormal nymphal molting (Tang et al. 2017; Zhao et al. 2016). Therefore, *NLTREs* are potential targets for pest control. However, it is still unknown whether *NlTREs* can be used to inhibit adult reproduction. Further investigation into the impact of *NLTREs* on reproduction would be beneficial for a comprehensive assessment of the potential of *NLTREs* as targets for pest control. In this study, we analyzed the effect of NLTRE on female reproduction of *N. lugens* by inhibiting NLTRE with RNA interference (RNAi) and validamycin (a trehalase inhibitor) injection, respectively, which can help to evaluate the pest control potential of TRE as a biological target.

## Materials and methods

### Insect rearing

The rice variety used in the study was Taichung Native 1 (TN1). *N. lugens* was collected from the field area of China National Rice Research Institute and reared in the laboratory under controlled conditions more than 60 generations. The artificial climate chamber is set as follows: temperature 27 ± 1℃, relative humidity 65 ± 5%, photoperiod 18L:6D (light:dark).

### RNA interference (RNAi) and trehalase inhibitor injection

Five *N. lugens* adults were collected to extract total RNA with TRIzol reagent (Takara, Kyoto, Japan). *NLTRE1-1*, *NLTRE1-2*, and *NLTRE-2* were screened, and specific primers were synthesized as Table 1 (Zhang et al. 2017). First-strand complementary DNA (cDNA) was synthesized with the Prime Script RT reagent Kit With gDNA Eraser (Takara, Kyoto, Japan). The dsRNA synthesis was conducted following the T7 RiboMAX Express RNAi System (Promega, Madison, USA). A green fluorescence protein (GFP) gene amplicon was used as a control. Four injection groups were set up: (1) dsGFP; (2) the synthesized dsTRE1-1, dsTRE1-2, and dsTRE2 were mixed into dsTREs (dsTRE1-1: dsTRE1-2: dsTRE2 = 1:1:1); (3) $ddH_2O$, labeled as ddWater; (4) validamycin (a TRE inhibitor). The injection concentration of dsRNA was 4000 ng/μL, and the validamycin concentration was 0.5 μg/μL, with 100 nL injected into each group. The concentrations of dsRNA and validamycin were selected

**Table 1** Primers for dsRNA synthesis

| Primer name | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| NLTRE1-1 | GATGCAATCAAGGAGGTGTTATGGC | CGTATTCACCTCCACCTCCGT |
| NLTRE1-1-T7 | T7-GATGCAATCAAGGAGGTGTTATGGC | T7-CGTATTCACCTCCACCTCCGT |
| NLTRE1-2 | AGATGAAGGCATGTGGTTCG | CATCGATTCGCCAACTGGTAAGC |
| NLTRE1-2-T7 | T7-AGATGAAGGCATGTGGTTCG | T7-CATCGATTCGCCAACTGGTAAGC |
| NLTRE2 | CCAACTGCTATGACACCGACAAG | GGGTTCAGATCCTGCCGTCGCT |
| NLTRE2-T7 | T7-CCAACTGCTATGACACCGACAAG | T7-GGGTTCAGATCCTGCCGTCGCT |
| GFP | AAGGGCGAGGAGCTGTTCACCG | CAGCAGGACCATGTGATCGCGC |
| GFP-T7 | T7-AAGGGCGAGGAGCTGTTCACCG | T7-CAGCAGGACCATGTGATCGCGC |

T7 sequence: 5′-GGATCCTAATACGACTCACTATAGG-3’

based on preliminary experiments demonstrating optimal inhibition of TRE activity (Zhao et al. 2016; Tang et al. 2017). Newly emerged long-winged females were selected to inject with TransferMan 4r microinjector (Eppendorf, Hamburger, Germany).

### Insect sampling and gene expression quantification

Females were sampled at 48 h and 72 h after injection. In addition, the injected female was mated with normal male in a 1:1 ratio. Five females were transferred into a culture cup containing fresh rice seedlings after 3 days to lay eggs. After 1 day, the adults were removed and eggs were collected from each group after 2 days. Each treatment group was repeated three times or more, and five to six *N. lugens* or 60–140 eggs were sampled for RNA extraction and cDNA synthesis. The primers for quantitative real-time polymerase chain reaction (qRT-PCR) are shown in Table 2, and *actin* gene was used as internal reference gene. The qRT-PCR was conducted with TB Green Premix Ex Taq™ (Takara, Kyoto, Japan). QRT-PCR was performed using a Bio-Rad CFX96TM system following standard protocols. Amplification curve and melting curve were used to determine the specificity of amplification reaction, and the relative gene expression was calculated according to $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

### Fecundity measurement

Females injected with dsRNA or validamycin were mated with untreated males in a 1:1 ratio. Five females were weight and repeated three groups on the 3rd day, and ovaries were dissected on the 3rd, 4th, 5th, and 6th day under a stereomicroscope (Leica, Wetzlar, Germany). The number of mature eggs in the ovaries on 3rd day was counted, and phenotype of ovaries was observed according to Lu et al (2011). In addition, one female after injection was paired with normal male, and fifteen or more biological replicates were conducted. Then the number of eggs laid by females was counted every 24 h until the 10th day. Finally, treated females and normal males were fed in a 1:1 ratio for 3 days and 1–3 females were transferred to a new culture cup with fresh rice seedlings. Adults were removed after 3 days, and the newly hatched nymph was counted until no nymphs. Rice seedlings were dissected and the number of unhatched eggs was counted to calculate the eggs hatchability. Nine or more biological replicates were conducted to analyze the egg hatchability.

### Chitin staining

Chitin was firstly present in the 72 h eggs of *N. lugens*; thus, the 72–96-h eggs laid by females were selected to chitin staining (Lu et al. 2022). Eggs were soaked in 10% paraformaldehyde fixative and washed in 30% sucrose solution, following being embedded in optimal cutting temperature compound (OCT). Samples were cut into 5-μm-thick sections using a frozen slicer (Epredia, Shanghai, China) and stained at room temperature in the dark for 15 min with 10% KOH and Calcofluor white (Coolaber, Beijing, China). Then, sections were washed with 1 × PBS and sealed with MM24. Finally, the sections were observed and photographed using a laser confocal microscope (Zeiss, Oberkochen, Germany).

### Statistical analyses

All data obtained were presented as the means ± standard errors (SEs) of biological replicates. The difference analysis was carried out with IBM SPSS Statistics 23.0 and Student's *t* test. When $P < 0.05$, there was a significant difference between the treatment group and the control group, which was represented by *; when $P < 0.01$, there was an extremely significant difference between the treatment group and the control group, which was represented by **. When $P > 0.05$, there was no significant difference between the treatment group and the control group, which was indicated by "ns."

## Results

The following results highlight the impact of NLTRE inhibition on various reproductive parameters of *N. lugens*.

**Table 2** Primers for qRT-PCR

| Primer name | Forward primer (5′–3′) | Reverse primer (5′–3′) | Length (bp) |
|---|---|---|---|
| QNLTRE1-1 | GCCATTGTGGACAGGGTG | CGGTATGAACGAATAGAGCC | 132 |
| QNLTRE1-2 | GATCGCACGGATGTTTA | AATGGCGTTCAAGTCAA | 178 |
| QNLTRE2 | TCACGGTTGTCCAAGTCT | TGTTTCGTTTCGGCTGT | 197 |
| QNLCHS1 | CCGCAAACGATTCCTACAGA | AGGTCCTTGACGCTCATTCC | 222 |
| QNLCHS1a | TGTTCTTGCTACAACTCAATAAA | ACACCAATCCGATAGGCTC | 141 |
| QNLCHS1b | GCTGTCTTTGCTTTCTTCAT | ACACCAATCCGATAGGCTC | 187 |
| Actin | TGGACTTCGAGCAGGAAATGG | ACGTCGCACTTCATGATCGAG | 200 |

## Determination of RNAi efficiency

As shown in Fig. 1, the relative expression levels of *NLTRE1-1*, *NLTRE1-2*, and *NLTRE2* were significantly decreased both on 3d and 6d after dsTREs injection. Compared with control group, the relative expression levels of *NLTRE1-1* ($t=5.404$; $P=0.0057$) and *NLTRE1-2* ($t=7.577$; $P=0.0016$) were significantly decreased. Moreover, the relative expression level of *NLTRE2* ($t=10.71$; $P<0.0001$) exhibited an even more pronounced decrease at the same time point. Additionally, with dsTREs injection on 6d, the relative expression levels of *NLTRE1-2* ($t=4.384$; $P=0.0071$) and *NLTRE2* ($t=4.366$; $P=0.0073$) were also significantly downregulated, while *NLTRE1-1* ($t=10.80$; $P=0.0001$) demonstrated an extremely significant reduction. These indicated that dsRNA inhibited target genes effectively, and the inhibition effect lasted for a long time (Fig. 1a, b).

## Effect of trehalase on body weight of female *N. lugens*

The weight of female showed no significant difference compared to control group on 3rd day after dsTREs injection ($t=1.621$; $P=0.1803$; Fig. 2a). The significant reduction in body weight observed in validamycin-treated females suggests a critical role of TRE in maintaining normal physiological functions ($t=3.221$; $P=0.018$; Fig. 2b).

## Effects of trehalase on ovarian development

Compared with the dsGFP injection group, the ovary area of *N. lugens* injected with dsTREs on 5th and 6th days was smaller, and the oocyte number was also lower (Fig. 3a). Similarly, the ovarian development was also delayed on 3rd and 4th day after validamycin injection, with smaller area and decreased number of oocyte (Fig. 3a). In addition, the ovaries were graded and the number of mature oocytes was counted at 3d after injection. Results showed that females injected with dsTREs had the similar ovarian development as those in the control group, with most of them reaching the III grade (Fig. 3b). In addition, there

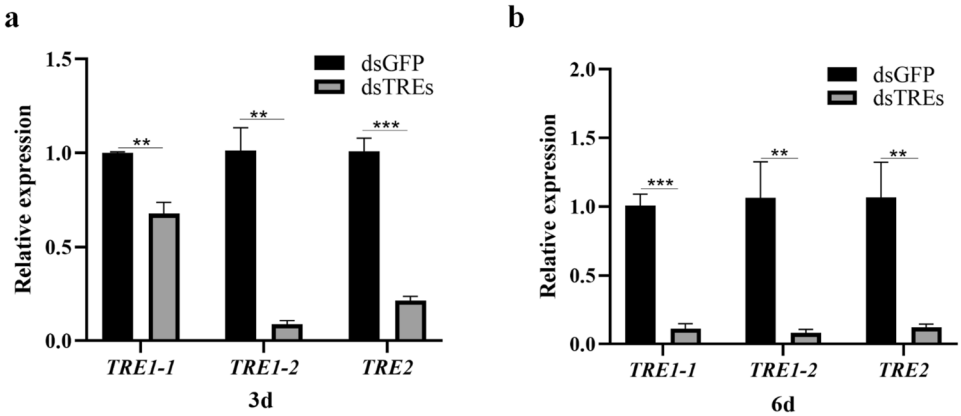


**Fig. 1** Relative expression of *TRE1-1*, *TRE1-2*, *TRE-2* at 3d (**a**) and 6d (**b**) after dsGFP and dsTREs injection in *Nilaparvata lugens*. The relative expression of *TRE1-1*, *TRE1-2*, *TRE-2* was determined by quantitative real-time PCR with *actin* gene as the internal control. The data were shown as mean + standard errors ($n \geq 3$) and analyzed using Student's *t* test. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

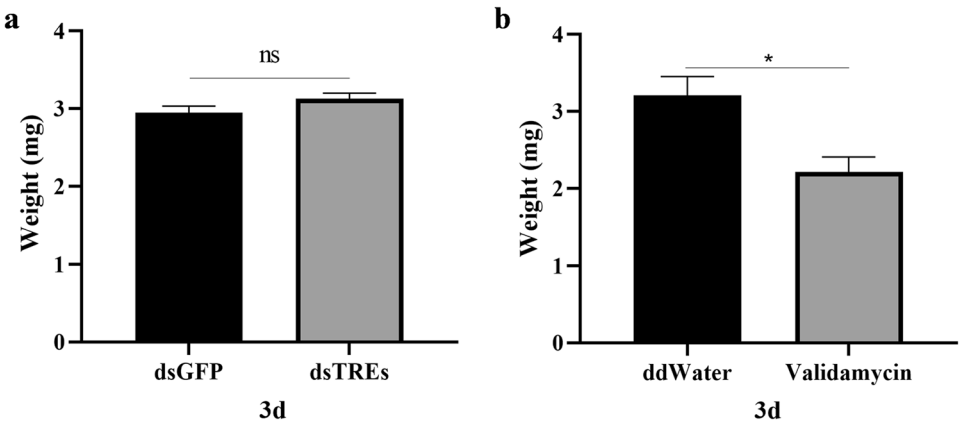


**Fig. 2** The weight of *Nilaparvata lugens* female at 3d after dsTREs (**a**) and validamycin (**b**) injection. Females injected with dsRNA or validamycin were mated with untreated males in a 1:1 ratio. Five females were weight and repeated three groups at 3rd day. The data were shown as mean + standard errors ($n \geq 3$) and analyzed using Student's *t* test. $^{*}P<0.05$; *ns* not significant

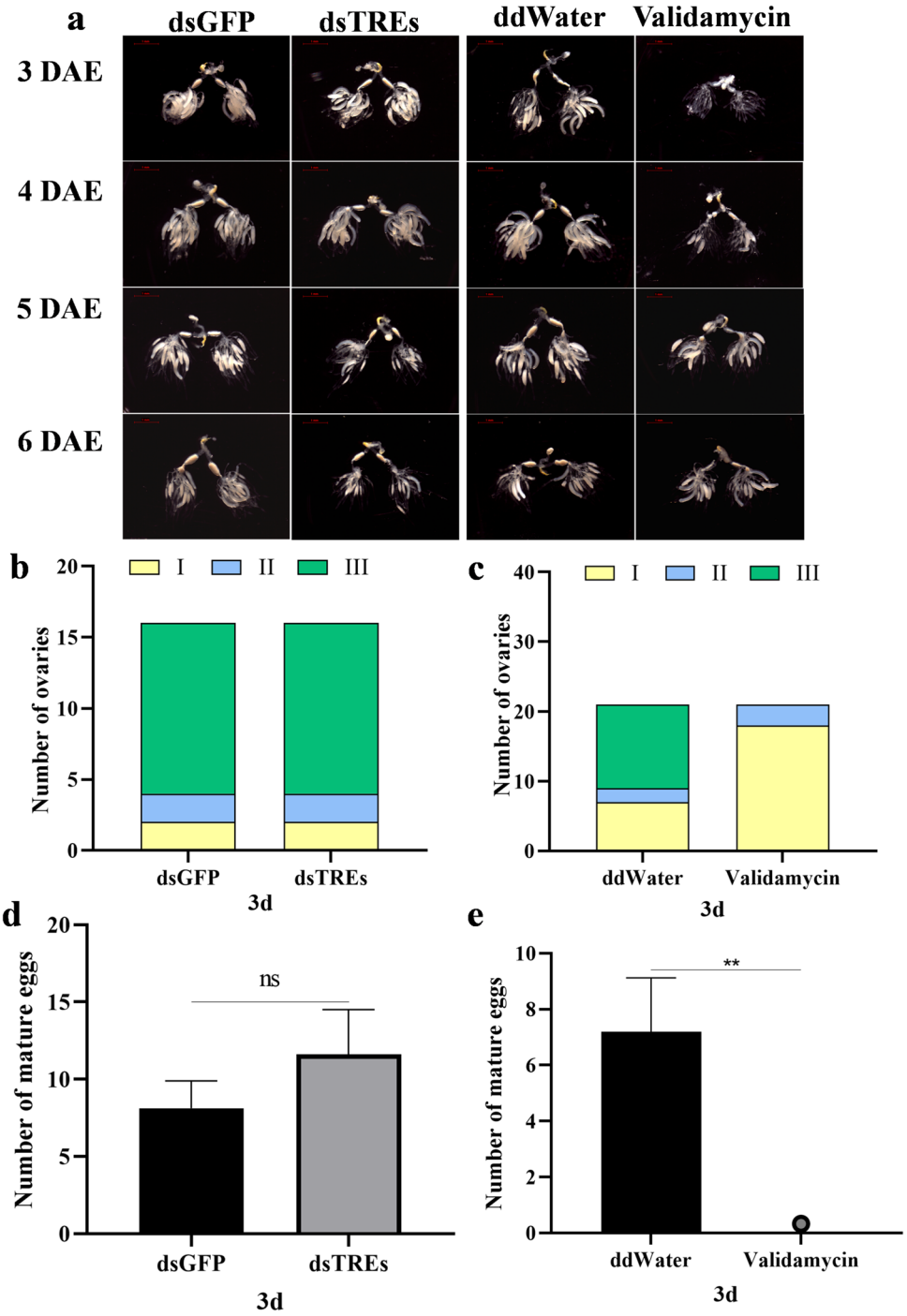


**Fig. 3** Ovarian development in *Nilaparvata lugens* after dsTREs and validamycin injection. **a** The change in ovarian development of *N. lugens* on 3rd, 4th, 5th, 6th day after dsGFP, dsTREs, ddWater, and validamycin injection (×16); **b** ovarian grading of *N. lugens* on 3rd day after dsTREs injection ($n=16$); **c** Ovarian grading of *N. lugens* on 3rd day after validamycin injection ($n=21$); **d** the number of mature eggs in ovary on 3rd day after dsTREs injection ($n=16$); **e** the number of mature eggs in ovary on 3rd day after validamycin injection ($n=21$), and the circle represents zero. The data were shown as mean + standard errors and analyzed using Student's *t* test in **e** and **f**. $^{**}P<0.01$; *ns* not significant

was no significant difference in the number of mature eggs of females on 3rd day after injection of dsGFP and dsTREs ($t=1.035$; $P=0.3090$; Fig. 3d). However, most of ovaries were still in Igrade on 3rd day after validamycin injection, with no mature oocytes ($t=3.722$; $P=0.0013$; Fig. 3c, e).

## Effects of trehalase on fecundity of females

The preoviposition period of *N. lugens* was 2.64d and 2.63d after dsGFP and dsTREs injection, respectively, with no significant difference between them ($t=0.034$; $P=0.973$; Fig. 4a). While the preoviposition period of

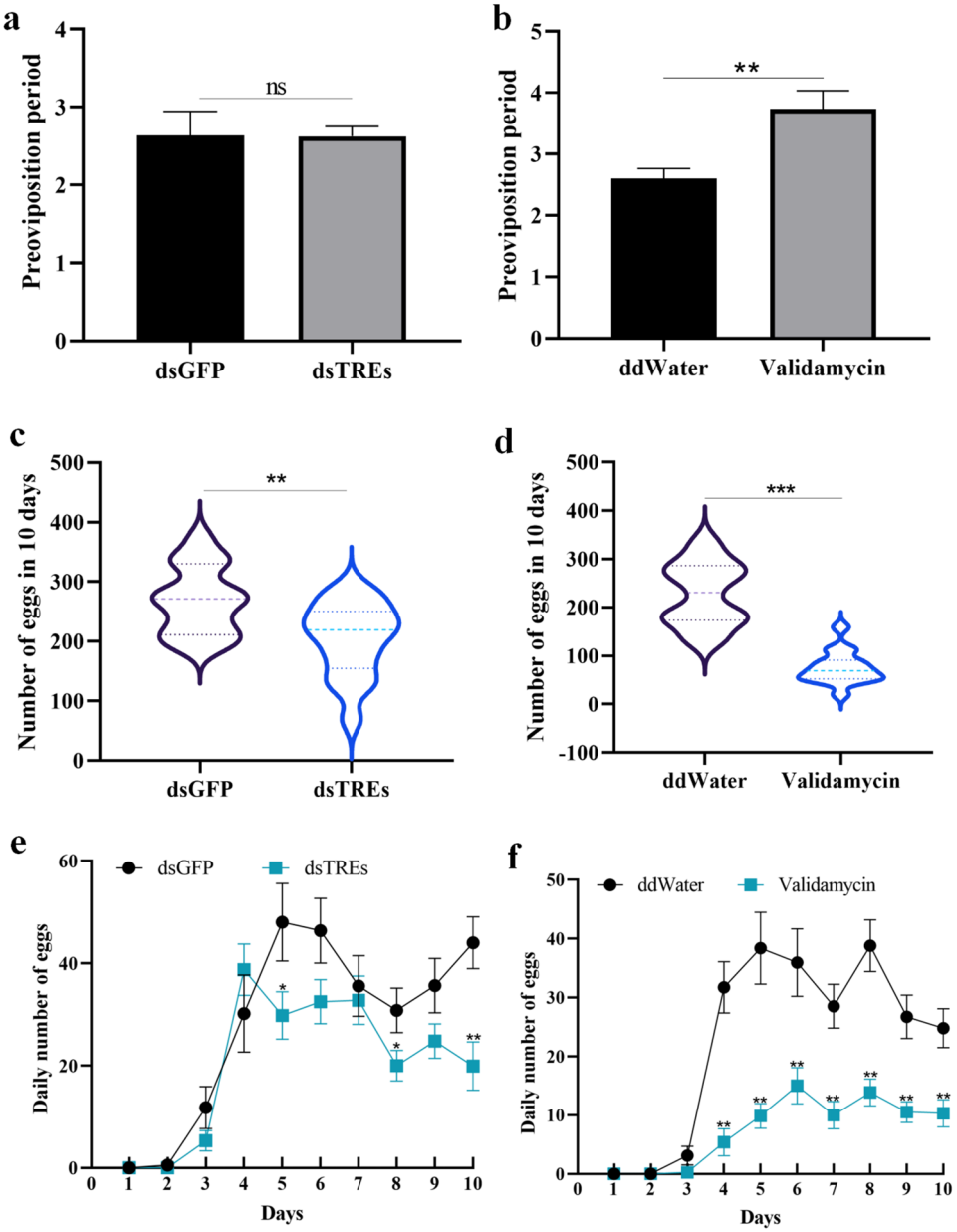


**Fig. 4** **a**, **b** Effects of trehalase on fecundity of females. Preoviposition, total number of eggs in 10 days (**c**, **d**), and daily number of eggs (**e**, **f**) of *Nilaparvata lugens* after dsTREs and validamycin injection. One female after injection was paired with normal male, and fifteen or more biological replicates were conducted. The data were shown as mean + standard errors ($n \geq 15$) and analyzed using Student's *t* test. $^{**}P < 0.01$; $^{***}P < 0.001$; *ns* not significant

females injected with validamycin was 3.73d, which was significantly prolonged compared to control group ($t = 3.316$; $P = 0.003$; Fig. 4b). Besides, the total number of eggs laid in 10 days by *N. lugens* was significantly decreased after dsTREs and validamycin injection ($t = 3.029$; $P = 0.0051$; $t = 8$; $P < 0.0001$; Fig. 4c, d). Moreover, the number of daily laid egg had multiple peak periods, and it was significantly or extremely significantly decreased after dsTREs treatment on 5th, 8th, and 10th day (Fig. 4e). Validamycin had a more obvious inhibitory effect on the fecundity, and the daily number of laid eggs was significantly lower than that of the control group from 3rd day (Fig. 4f).

## Effect of trehalase on egg hatchability

The egg hatchability was extremely decreased after dsTREs and validamycin injection ($t = 4.182$; $P = 0.001$; $t = 4.357$;

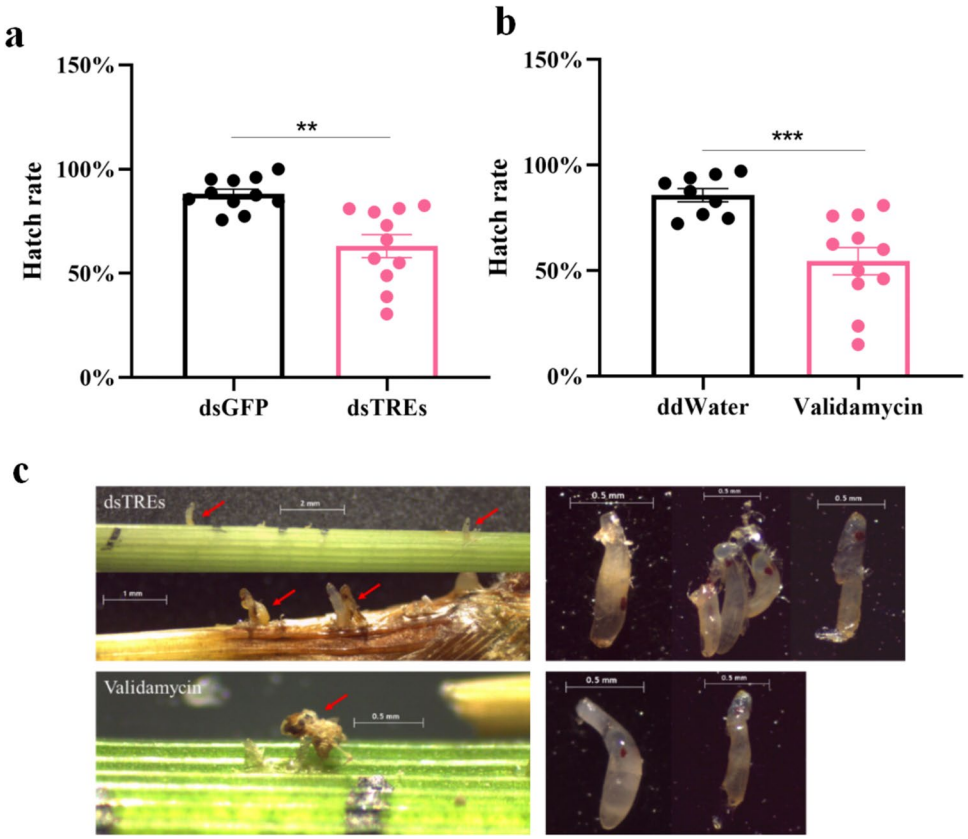


**Fig. 5** Hatching rate and non-hatching phenotypes of *Nilaparvata lugens* offsprings after dsTREs and validamycin injection. **a** Hatching rate of *N. lugens* offsprings after dsTREs injection; **b** hatching rate of *N. lugens* offsprings after validamycin injection; **c** non-hatching phenotypes of *N. lugens* offsprings after dsTREs and validamycin injection. The data were shown as mean + standard errors ($n \geq 9$, each treatment containing 1–3 individuals) and analyzed using Student's *t* test. $^{**}P < 0.01$; $^{***}P < 0.001$

$P = 0.0006$; Fig. 5a, b). Meanwhile, it was found that the unhatched eggs usually showed the phenotype of ecdysis failure in dsTREs and validamycin group, and even there was eye-point inversion after dsTREs treatment (Fig. 5c).

### Effects of trehalase on chitin metabolism of eggs

Results showed that dsTREs had no significant effect on the expression of *CHS1* and its two alternatively variants, *CHS1a* and *CHS1b* (Fig. 6a), while validamycin significantly inhibited the relative expression of *CHS1* ($t = 3.354$; $P = 0.028$; Fig. 6b). Meanwhile, the thickness of chitin in the egg from dsTREs group was thinner than that in the control group, while the abnormal distribution or chitin structure was occurred after validamycin injection (Fig. 6c, d). These results indicate that trehalase may affect the chitin component, which is an essential component in the embryo development.

## Discussion

RNAi is often used as a tool to gene silencing and usually mediated by dsRNA, which has a promising application as pest control (Scott et al. 2013; Zhao et al. 2016; Han 2018; Zhu and Palli 2020; Lu and Shen 2024). In this study, the dsTREs injection significantly reduced the transcription levels of *NLTRE1-1*, *NLTRE1-2*, and *NLTRE2* in female, and the inhibition time lasted for 6 days, which was consistent with the inhibitory effect in fifth instar nymphs (Zhao et al. 2016). As a commercial TRE inhibitor, the inhibitory effect of validamycin on the *NL*TRE1 and *NL*TRE2 enzyme activities of *N. lugens* has been studied and confirmed in previous research (Tang et al. 2017).

Enough nutrients and energy are required for supporting the insect growth, and the reduction of nutrients significantly reduces the body weight of *N. lugens* (Kang et al. 2022a, b). Carbohydrates provide a large amount of energy for the growth of *N. lugens*, and reduced carbohydrate transport leads to a significant decrease in body weight of *N. lugens* (Ge et al. 2015). In previous studies, dsTREs injection in *N. lugens* resulted in a decrease in trehalose content, and inhibition of TRE activity by validamycin *N. lugens* also resulted in a significant decrease in trehalose, glucose, and glycogen content (Zhao et al. 2016; Tang et al. 2017). However, in this study, only validamycin significantly reduced the body weight, which may be due to the more effective inhibition of validamycin to TRE enzymes activities in female. Similarly, validamycin inhibited the ovarian development, which was more obvious than dsTREs treatment. Trehalose accumulation in insect oocytes has been increased during egg

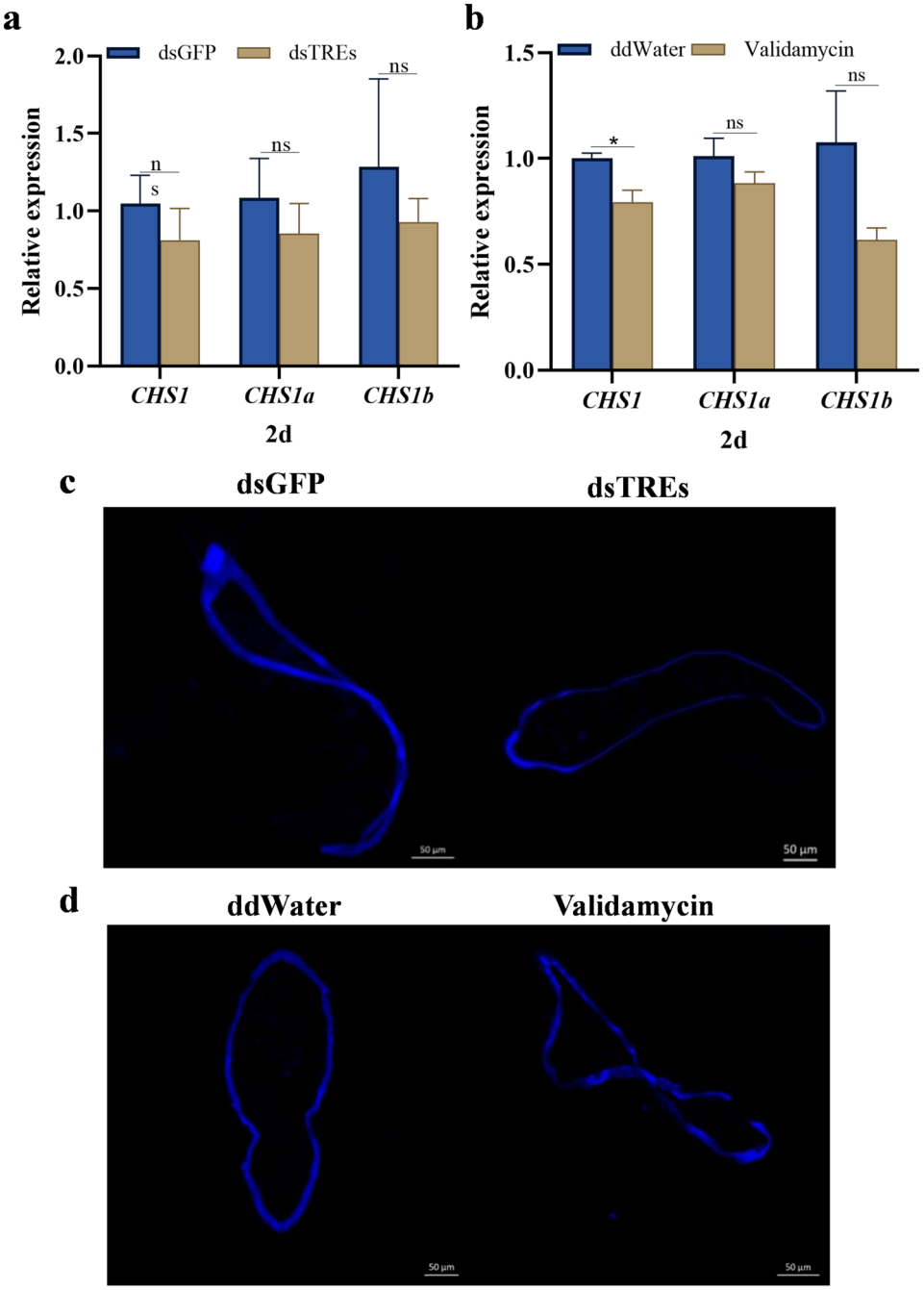


**Fig. 6** Effects of trehalase on chitin metabolism of eggs in *Nilaparvata lugens*. **a** Relative expression of *CHS1*, *CHS1a*, and *CHS1b* after dsGFP and dsTREs injection on the 2nd day; **b** relative expression of *CHS1*, *CHS1a*, *CHS1b* after ddWater and validamycin injection on the 2nd day. **c**, **d** present distribution of chitin in eggshell of *N. lugens* after dsTREs and validamycin injection. The data were shown as mean + standard errors ($n \geq 3$) and analyzed using Student's *t* test in **a**, **b**. $^{***}P < 0.001$, *ns* not significant. The blue fluorescence in **c**, **d** represents chitin

development, and the addition of exogenous trehalose has promoted the insect ovarian development (Santos et al. 2008; Li et al. 2020). Therefore, these results still demonstrated the importance of trehalose utilization to oocyte mature, which is consistent to previous studies that injection of trehalase inhibitors into *Periplaneta americana* also inhibited oocyte development (Kono et al. 2001).

Our study demonstrates that inhibiting TRE significantly impacts the reproductive capacity of *N. lugens*, providing insights into its potential as a target for pest control.

When trehalase metabolism is interfered by TRE inhibitory, the fecundity is also influenced (Jiang et al. 2023). In this study, the number of eggs laid by females injected with dsTREs and validamycin was also extremely significantly deceased, though only validamycin prolonged the preoviposition period, which was consistent with the ovarian development of females. This result is also similar to previous study that *TRE* silencing in *Anopheles stephens*i decreased the fecundity, which provide new insights into its specific impact on *N. lugens* (Tevatiya et al. 2020). Studies show that trehalose has a repairing effect on abnormal cumulus cells not only in insects, but also in mammals, indicating that it may play an indispensable role in the reproductive process (Kang et al. 2022a, b). Our results also demonstrated the indispensability of trehalose utilization in fecundity. Interruption of trehalose degradation not only affected the egg production, but also affects the egg quality. Both the *TRE* silencing and the inhibition of TRE activity resulted in a decrease in hatching rate. Similar results were found in silkworm, in which the injection of trehalase inhibitors resulted in decreased hatching rate (Katagiri et al. 1998). The significant reduction in fecundity and egg hatchability upon TRE inhibition suggests that targeting trehalase could be an effective strategy for managing *N. lugens* populations in rice fields.

In this study, it was observed that the unhatched eggs mainly showed molting failure. Previous research showed that chitin was present in embryos of *N. lugens*, and the trehalose metabolism significantly interfered with the chitin synthesis in nymph, so it was speculated that the inhibition of trehalase in this study also affected the chitin metabolism in the egg shells (Tang et al. 2017; Zhang et al. 2017; Lu et al. 2022). Results showed that egg thickness in the dsTREs group was relatively thinner than that in the control group, while the egg chitin in the validamycin group was unevenly distributed, indicating that TRE influenced the composition of embryo chitin. However, the mechanism may be different, since validamycin reduced the relative expression of the chitin synthase *CHS1*, while dsTREs had no significant effect on its expression. Similar result was also found in the study of *Diaphorina citr*, in which the expression levels of genes related to chitin metabolism were significantly decreased in validamycin treatment (Yu et al. 2021). Chitin in insects requires chitin-binding proteins to form ordered construction that are not readily degraded by chitinases (Moussian 2010). Therefore, the effect of dsTREs on chitin in egg shell may be caused by chitin-binding protein or chitinase, which needs further study. Further studies are needed to elucidate the specific pathways through which TRE influences chitin metabolism and embryo development, which could reveal additional targets for pest control interventions.

In conclusion, the inhibition of NLTRE significantly reduces the reproductive potential of *N. lugens*, highlighting its viability as a target for developing novel pest control strategies. Future research should focus on optimizing TRE inhibitors and understanding their broader ecological impacts.

## Author contributions

Resources were provided by L.Y., S.S. and B.T. designed the experiments; L.Y., S.W., X.W., L.G., C.X., and B.X. performed the experiments; L.Y., B.T., and S.W. performed the data analysis; Y.L. and Y.Z. wrote the original manuscript; B.T. and S.S. reviewed and revised the manuscript. All authors commented on previous versions of the manuscript, and all authors read and approved the final manuscript.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Competing interests** The authors declare no competing interests.

**Ethics approval** This is an observational study. The Hangzhou Normal University Research Ethics Committee has confirmed that no ethical approval is required.

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