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Rice Stripe Virus Infection Facilitates the Reproductive Potential of *Laodelphax striatellus*

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Abstract: The rice stripe virus (RSV) is transmitted by the small brown planthopper *Laodelphax striatellus* in a persistent and circulative–propagative manner. During the last few decades, RSV has caused serious disease outbreaks in rice fields in China. The results of long-term coevolution have led to complex and diverse relationships between viruses and vector insects, and understanding these interactions is critical for the management of diseases and vector organisms. In this study, three groups of comparative experiments were performed to investigate the effects of RSV infection on the reproductive parameters, *vitellogenin* (*Vg*), and *Vg receptor* (*VgR*) expressions of *L. striatellus*. The results showed that RSV infection promoted the fecundity, hatchability, and *Vg* and *VgR* expressions of *L. striatellus* but had no effect on the preoviposition period and oviposition period. Furthermore, the RNA interference of the nucleocapsid protein (*CP*) gene and the injection of the CP antibody in RSV-viruliferous *L. striatellus* reduced the fecundity, hatchability, and *Vg* and *VgR* expressions, further strengthening the hypothesis that RSV may manipulate the reproductive behavior of insect vectors to promote its own transmission. The results of this study can further reveal the interaction mechanism of virus–vector insects in reproduction and virus transmission, and provide new insights for the control of insect-borne viruses.

Keywords: rice stripe virus; *Laodelphax striatellus*; reproductive parameters; nucleocapsid protein; vitellogenin

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1. Introduction

Plant virus-induced plant diseases have caused devastating damage to sustainable agriculture, resulting in severe losses for the global economy annually [1,2]. Most plant viruses are insect-borne viruses that can be efficiently transmitted by hemipteran insects such as planthoppers, whiteflies, thrips, and aphids [3,4]. The transmission of insect-borne plant viruses is mediated by complex interactions between virus–host–insect vectors [5]. Among the complex triangular association, insect vectors are the only organisms that can be freely dispersed and interact closely with both host plants and plant viruses [6–8]. Recent studies have shown that viruses could modulate the behavior or performance of insect vectors, either indirectly or directly, thereby affecting the spread of viruses and the occurrence of viral diseases. The indirect regulation of insect vectors by plant viruses is often reflected in the influence of insect feeding or other behavioral responses by modifying the phenotype of host plants [9,10]. Cucumber mosaic virus (CMV) infected tobacco leaves, and the accompanying Y-satellite RNA caused the tobacco leaves to turn yellow, making them more attractive to the vector aphids, and the Y-satellite could alter aphid physiology to accelerate wing formation for spread [11]. Moeini et al. [12] found that maize Iran mosaic virus (MIMV) infection could modify the host plant pigment to manipulate the host

preference behavior of the insect vector *Laodelphax striatellus*, thereby affecting the spread of the virus. The vector-borne viruses also could directly regulate the physiological and behavioral processes of their vectors to facilitate their transmission. In *L. striatellus*, barley yellow striate mosaic virus (BYSMV) modulates its locomotor activity for transmission [13]. In *Bemisia tabaci*, infections of the tomato yellow leaf curl virus (TYLCV) [14] and the tomato chlorosis virus (ToCV) [15] could change the feeding preference or behavior, and then affect the spread of the viruses. In addition, some viruses even regulate the fecundity of vector insects to mediate the spread [16]. Wan et al. [17] found that the copulation time, fecundity, and the male ratio of progeny increased in tomato spotted wilt orthotospovirus (TSMV)-exposed *Frankliniella occidentalis*. Hence, understanding the interactions between plant viruses and vector insects is conducive to the subsequent control of plant virus transmission and insect vectors.

Rice stripe virus (RSV) is one of the most destructive rice viruses in East Asia. RSV is a single-stranded RNA virus that contains four RNA segments. Among them, the segment of RNA3 is ambisense, encoding NS3 (gene-silencing suppressor, p3) and nucleocapsid protein (CP), and CP is the smallest unit protein that constitutes the RSV ribonucleoproteins (RNPs), which are critical for viral genomic RNA assembled for replication and transmission [18,19]. In addition, CP can not only protect the viral genome from proteolytic enzymes and nucleases but also transfer the viral genome through specific binding to host cell receptors. The knockdown of *CP* by RNA interference significantly inhibits the replication of RSV genomes [20]. Recent studies have shown that CP and viral genomic RNA were partially transported to the nucleus of the vector and affected the immune response by utilizing the nuclear importin protein α transport system [21,22]. Therefore, RSV CP plays an important role in the process of virus transmission.

L. striatellus is widely distributed worldwide, causing serious harm to rice, barley, and maize fields. In addition to directly feeding on the phloem sap, it can also transmit various viruses [23]. RSV can be efficiently transmitted by horizontal and vertical transmission through *L. striatellus*, resulting in persistent and circulative–propagative transmission [24]. After ingestion, RSV occurs first in the midgut epithelium, and then the infection spreads to adjacent epithelial cells, and finally to various tissues including the salivary glands and reproductive system via the hemolymph. The virus particles accumulated in the salivary glands are ingested into uninfected plants by *L. striatellus*, thus completing horizontal transmission [25–27]. RSV transmitted to the ovary binds to the vitellogenin (Vg) in the hemolymph and is transported to the ovarian reproductive area through the endocytosis mediated by the Vg receptor (VgR). Finally, they enter the oocytes with the nutrient filaments and are transmitted to the offspring through the maternal generation, thus completing the vertical transmission [28–30]. Recent studies have shown that RSV could modulate the feeding preference of *L. striatellus* from the stems to the leaves of rice plants to promote RSV infection [31], and manipulate the long-winged morph of *L. striatellus* males [32], further suggesting the co-evolution of plant viruses and insect vectors.

As is well known, for insect-borne plant viruses, the fecundity of vector insects is closely related to the transmission ability of viruses [12,17]. Although some studies have reported on the relationships between RSV and the reproduction of *L. striatellus* [33–35], there was still a lack of investigations on the regulation and related mechanisms of RSV on reproduction. In this study, we set up three groups of comparative experiments: RSV-viruliferous *L. striatellus* vs. non-viruliferous *L. striatellus*, *dsGFP* (double-stranded RNA of *green fluorescent protein*) treatment group vs. *dsCP* (double-stranded RNA of *CP*) treatment group, and PBS (phosphate buffered saline) treatment group vs. CP antibody treatment group. This study will contribute to the understanding of the effects of RSV infection on the reproduction of *L. striatellus*, and provide insight into the control of insect vectors and plant virus transmission.

2. Materials and Methods

2.1. Insects

The *L. striatellus* used in this experiment are laboratory strains that have been continuously reared for many generations. They were reared on rice seedlings, Wuyujing 3, in the laboratory with the environmental conditions of 26 ± 1 °C, relative humidity 75 ± 5%, and photoperiod 16L: 8D [36,37]. The original strains were obtained from rice fields infected with RSV in Yangzhou, Jiangsu Province, China.

One female and one male were selected from the newly emerged *L. striatellus* which were continuously reared in the laboratory for several generations. They were paired into the glass tube with 2–3 rice seedlings at three-leaf stage. About 7 days after oviposition, the female was examined for RSV by DIBA (dot immunobinding assay) [38]. On the membrane, the color reaction could be observed in the sample of the viruliferous *L. striatellus*, while the samples without RSV had no color reaction. The F1 generation of the infected female was used as RSV-viruliferous *L. striatellus* strains, and the F1 generation of the uninfected female was used as non-viruliferous *L. striatellus* strains. The two populations were raised separately for subsequent experiments. In addition, a female was selected from the infected population every month for DIBA detection to ensure that the RSV infection frequency was maintained at more than 80%.

2.2. RNA Interference

Double-stranded RNA of *CP* (*dsCP*) was generated using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA), and *dsGFP* was used as the control. The synthesis and purification of *dsRNAs* were performed according to the instructions [37]. Specific primers with the T7 RNA polymerase promoter used in the synthesis of *dsRNAs* are listed in Table S1. After evaluating the purity and concentration of *dsRNAs* using a NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA), the concentration was diluted to around 6000 ng/μL. A volume of 48 nL *dsCP* or *dsGFP* was dispensed over each newly emerged viruliferous female (within 24 h) through a side trimmed glass needle using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific, Broomall, PA, USA). The *dsCP* treatment group and the *dsGFP* control group contained 150 treated viruliferous females, respectively, and then transferred them to the healthy rice seedlings. After 24 h, they were mated with the newly emerged viruliferous *L. striatellus* males, and then used for subsequent experiments.

2.3. Injection of CP Antibody

The anti-RSV CP monoclonal antibody used in this experiment was obtained from Zhejiang University. Newly emerged viruliferous females were anesthetized with carbon dioxide for 5 s, and then CP antibody (48 nL) was injected using the Nanoliter Injector [39]. PBS was used as a control. Both CP antibody treatment group and PBS control group contained 150 infected *L. striatellus* females. As in Section 2.2, the treated females were paired with virgin males and reared together for subsequent test.

2.4. Sample Preparation and RNA Extraction

After RSV screening, 100 newly emerged females of the F1 generation of infected *L. striatellus* were randomly selected and raised in fresh rice cup seedlings. After 24 h of feeding, an equal number of virgin males were added. At 3-, 5-, and 7-days after emergence, ten females were randomly selected and transferred to a 2 mL tube containing 500 μL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for subsequent RNA extraction. The newly emerged females of F1 generation of non-viruliferous *L. striatellus* were sampled by the same method. In the RNAi experiments, the interference efficiency was detected at 3-, 5-, and 7-days post injection. In the CP antibody injection experiments, at 3-, 5-, and 7-days after treatment with PBS or CP antibody, the whole body was sampled for gene expression analysis. Ten *L. striatellus* females were contained in one replicate; three replicates were prepared for each treatment. The total RNA of all the above samples

was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions [37]. Subsequently, a HiScript®III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) was used to reversely transcribe 1 μg RNA into cDNA.

2.5. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was used to detect the interference efficiency and explore the effects of RSV infection on the expressions of *Vg* and *VgR* in *L. striatellus*. Primer3 (http://bioinfo.ut.ee/primer3/ (accessed on 13 June 2023)) was used to design specific primers listed in Table S1. Each reaction contained 3 μL of cDNA template, 1 μL of each forward and reverse primer (10 μM), 10 μL of ChamQ™ SYBR qPCR Master Mix (Vazyme, Nanjing, China), and 5 μL of ddH_2O. The qRT-PCR detection was run on a CFX 96™ Real-Time Detection System (Bio-RAD Laboratories Inc., Hercules, CA, USA) under the following conditions: a preheating step for enzyme activation at 95 °C for 3 min, followed by 39 cycles of 95 °C for 10 s and 58 °C for 30 s. *β-actin* was selected to normalize the expression of candidate genes [40]. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative transcription levels [41].

2.6. Reproduction Assays

To explore the effects of RSV infection on the reproduction of *L. striatellus*, three groups of comparative experiments were established, including RSV-viruliferous *L. striatellus* vs. non-viruliferous *L. striatellus*, *dsGFP* treatment group vs. *dsCP* treatment group, and PBS treatment group vs. CP antibody treatment group. For each group, one newly emerged female and one contemporaneous untreated virgin male were paired in a glass tube containing healthy rice seedlings. Each group comprised at least forty mating pairs. To determine the preoviposition, the rice seedlings were replaced and dissected by a microscope daily until the female *L. striatellus* began to lay eggs. During the oviposition period, the seedlings were replaced every 5 days until the female died. The number of hatched nymphs in the replaced rice stems was recorded, and unhatched nymphs in the rice seedlings were dissected and counted. The fecundity of *L. striatellus* female refers to the sum of the number of hatched nymphs and unhatched eggs. The hatchability refers to the ratio of the number of hatched nymphs to the fecundity.

2.7. Statistical Analyses

Data presented in this study were analyzed and graphs were generated using the GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). The significance levels of gene expressions and reproductive parameters between the two groups were determined by Student's *t*-test ($p < 0.05$). All data are expressed as means ± SEMs.

3. Results

3.1. Effects of RSV Infection on Reproductive Parameters of L. striatellus

To better understand the effects of RSV infection on the reproduction of *L. striatellus*, the preoviposition period, oviposition period, fecundity, and the egg hatchability were evaluated in RSV-viruliferous and non-viruliferous *L. striatellus*. The results showed that RSV infection had no impact on the preoviposition (Figure 1A) and oviposition period (Figure 1B) of *L. striatellus*. However, the number of eggs laid by RSV-viruliferous *L. striatellus* female (Figure 1C) and the hatching rate of corresponding eggs (Figure 1D) were significantly higher than non-viruliferous *L. striatellus*.

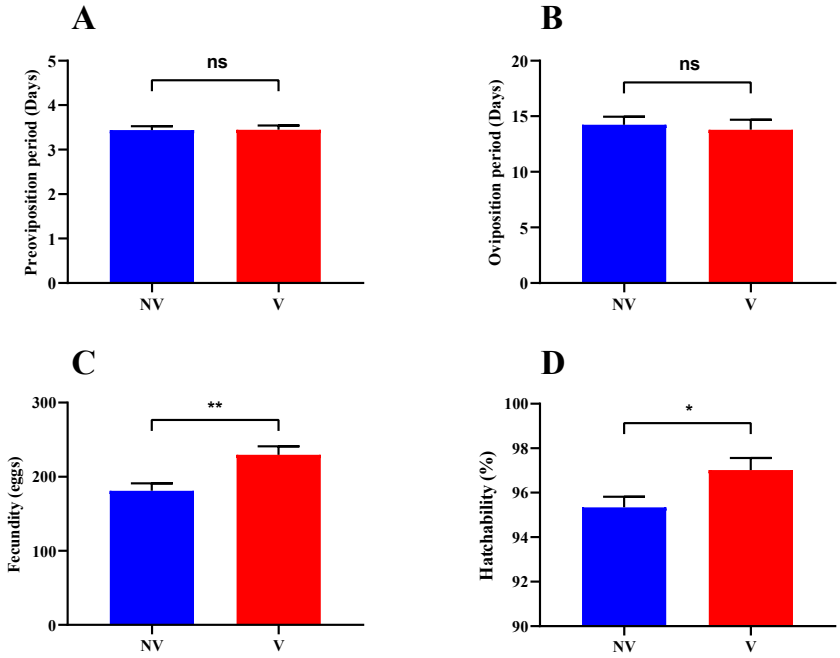

Figure 1. The reproductive parameters in RSV-viruliferous and non-viruliferous *L. striatellus*. (**A**) Preoviposition period; (**B**) Oviposition period; (**C**) Fecundity; (**D**) Hatchability. NV represented non-viruliferous *L. striatellus*, and V represented viruliferous *L. striatellus*. Student's *t*-test was used to determine the statistical differences between the samples (ns: no significant differences, * $p < 0.05$, ** $p < 0.01$) (n = 20–34).

3.2. Effects of CP Silencing on Reproductive Parameters of L. striatellus

To detect the interference efficiency, we quantified the transcription levels of *CP* in viruliferous *L. striatellus* at 3-, 5-, and 7-days after injection with *dsCP* and *dsGFP*. At 3-, 5-, and 7-days after *dsCP* injection, the expression levels of *CP* decreased by more than 50%, and the gene interference effect was the best on the 7th day, which decreased by 80% (Figure 2). Furthermore, we counted the reproductive parameters of the two groups. The results indicated that compared with the control *dsGFP*, *CP* knockdown had no significant effect on the preoviposition and oviposition period of the infected females (Figure 3A,B). However, the number of eggs laid was significantly suppressed (Figure 3C), and the hatching rate also decreased significantly (Figure 3D).

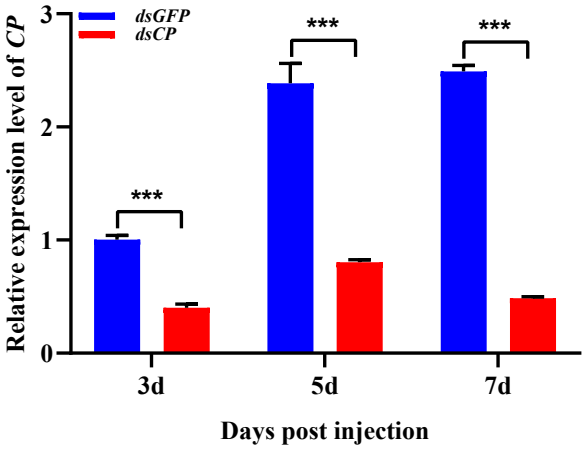

Figure 2. Detection of *CP* gene interference efficiency. The transcript levels of *CP* in viruliferous *L. striatellus* at 3-, 5-, and 7- days post-injection of dsRNA. Student's *t*-test was performed to analyze the significant differences (*** $p < 0.001$).

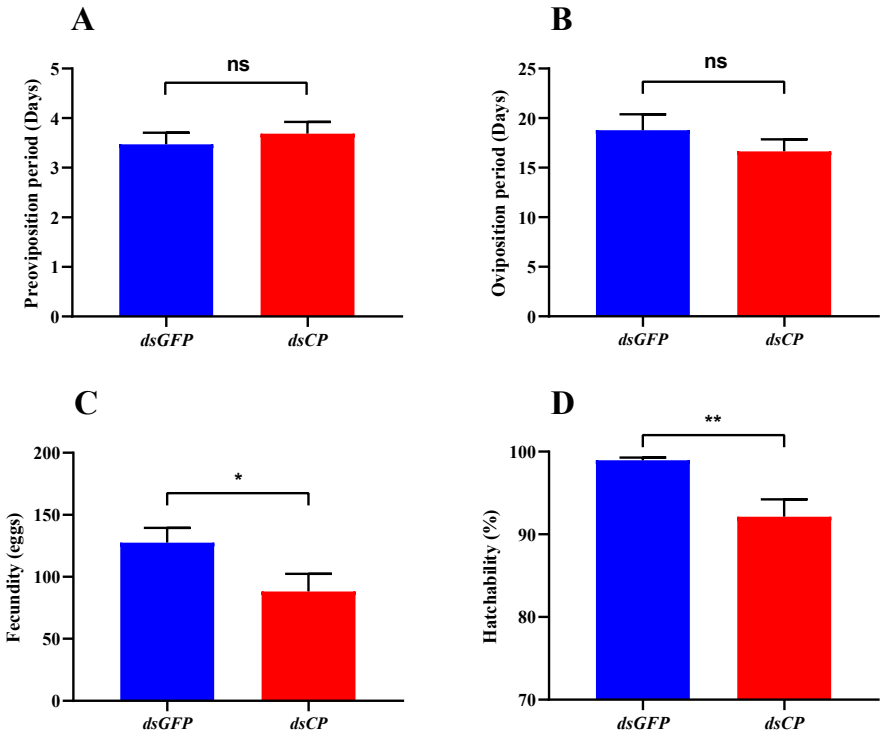

Figure 3. Effects of *CP* silencing on the reproductive parameters of *L. striatellus*. (**A**) Preoviposition period; (**B**) Oviposition period; (**C**) Fecundity; (**D**) Hatchability. Student's *t*-test was used to determine the statistical differences between the samples (ns: no significant differences, * $p < 0.05$, ** $p < 0.01$) (n = 14–23).

3.3. Effects of CP Antibody Injection on Reproductive Parameters of L. striatellus

To further understand whether the CP gene was involved in the changes to reproduction in *L. striatellus* regulated by RSV infection, we injected a CP antibody into the viruliferous *L. striatellus* females. Compared with PBS injection, the transcription levels of *CP* were significantly up-regulated at 3 days but significantly inhibited at 5 and 7 days after injection with the CP antibody (Figure 4). Although the CP antibody injection had no significant effect on the preoviposition period (Figure 5A) and oviposition period (Figure 5B) in RSV-viruliferous *L. striatellus* females, it could significantly suppress egg laying (Figure 5C) and hatchability (Figure 5D).

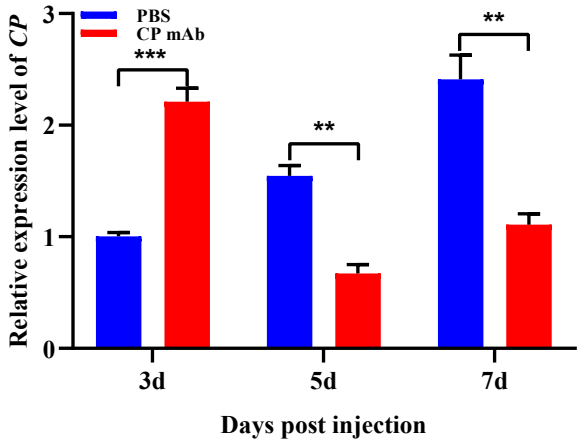

Figure 4. The transcript levels of *CP* in viruliferous *L. striatellus* at 3-, 5-, and 7-days post-injection of CP antibody. Student's *t*-test was performed to analyze the significant differences (** $p < 0.01$, *** $p < 0.001$). CP mAb: CP monoclonal antibody.

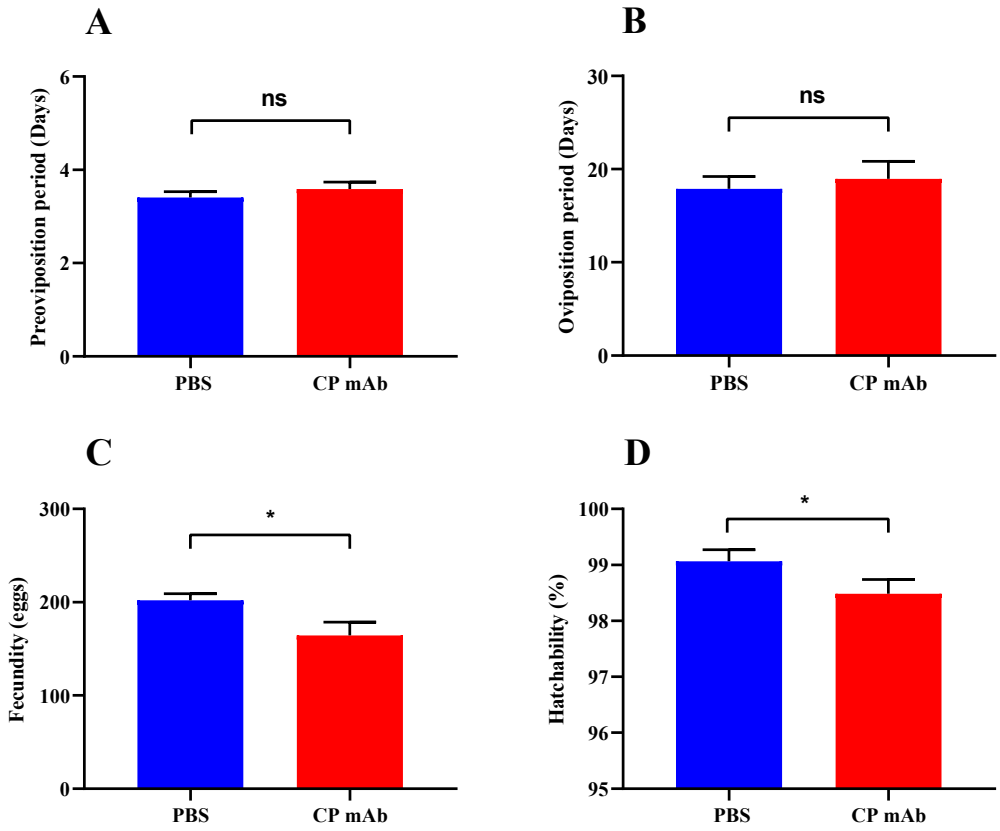

Figure 5. Effects of CP antibody injection on the reproductive parameters of *L. striatellus*. (**A**) Preoviposition period; (**B**) Oviposition period; (**C**) Fecundity; (**D**) Hatchability. Student's *t*-test was used to determine the statistical differences between the samples (ns: no significant differences, * $p < 0.05$) (n = 14–26). CP mAb: CP monoclonal antibody.

3.4. Effects of RSV Infection on Vg/VgR Expressions of L. striatellus

To clarify the mechanism of RSV infection affecting the reproduction of *L. striatellus*, we further detected the relative expressions of *Vg* and *VgR* in three experimental groups: RSV-viruliferous *L. striatellus* vs. non-viruliferous *L. striatellus*, *dsGFP* treatment group vs. *dsCP* treatment group, and PBS treatment group vs. CP antibody treatment group.

Compared with the non-viruliferous *L. striatellus*, the expression levels of *Vg* and *VgR* in the viruliferous *L. striatellus* were significantly increased at 3-, 5-, and 7-days after emergence (Figure 6A,B). Silencing *CP* led to reduced *Vg* expression in females compared with *dsGFP*-treated controls at 3-, 5-, and 7-days post injection (Figure 6C). In addition, the transcript level of *VgR* was lower than in the *dsGFP* controls 5 days after *dsCP* injection, while no significant variations were seen on days 3 and 7 (Figure 6D). Compared with the PBS injection group, the transcription levels of *Vg* (Figure 6E) and *VgR* (Figure 6F) were up-regulated at 3 days after CP antibody injection, but were significantly reduced at days 5 and 7.

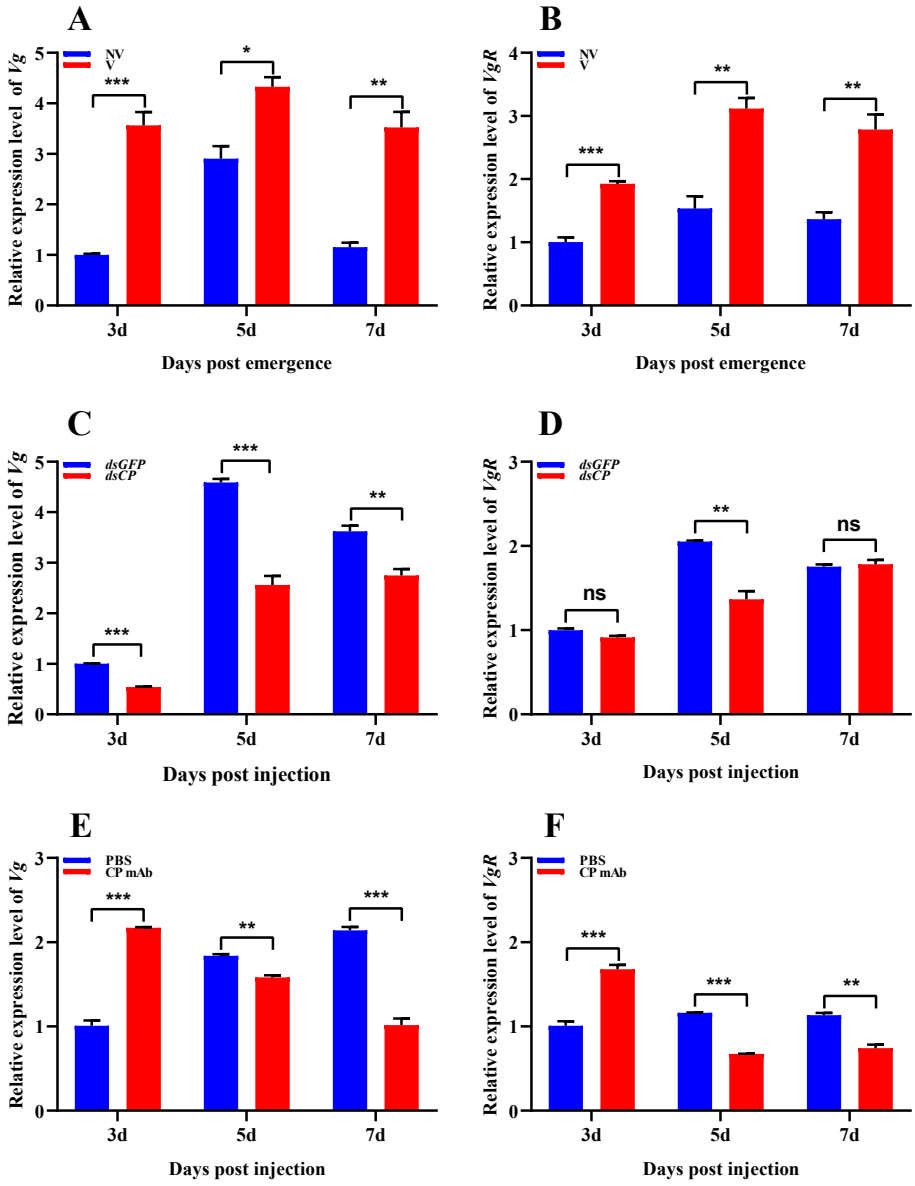

Figure 6. Effects of RSV infection on *Vg* and *VgR* expressions of *L. striatellus*. The transcript levels of *Vg* (**A**) and *VgR* (**B**) in RSV-viruliferous and non-viruliferous *L. striatellus* at 3-, 5-, and 7-days after emergence. The transcript levels of *Vg* (**C**) and *VgR* (**D**) in viruliferous *L. striatellus* at 3-, 5-, and 7-days post-injection of *dsCP*. The transcript levels of *Vg* (**E**) and *VgR* (**F**) in viruliferous *L. striatellus* at 3-, 5-, and 7-days post-injection of CP antibody. Student's *t*-test was performed to analyze the significant differences (ns: no significant differences, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4. Discussion

The process of plant virus disease outbreak and transmission is inseparable from the role of vector insects. Many plant viruses have evolved the ability to manipulate the reproduction, physiology, and behavior of vector insects, thereby facilitating their fitness or transmission [6]. In the present study, three groups of comparative experiments including RSV-viruliferous *L. striatellus* vs. non-viruliferous *L. striatellus*, *dsGFP* treatment group vs. *dsCP* treatment group, and PBS treatment group vs. CP antibody treatment group were executed to explore the relationships between RSV infection and the reproduction of *L. striatellus*.

The results showed that the number of eggs laid by RSV-viruliferous *L. striatellus* females and the hatching rates of the corresponding eggs were significantly increased

compared with the non-viruliferous *L. striatellus* (Figure 1). In fact, the interactions between viruses and insect vectors have become complex and diverse due to long-term evolutionary development. The relationships between them may be beneficial, neutral, or antagonistic, depending on the species involved [34]. Studies have shown that a Southern rice black-streaked dwarf virus (SRBSDV) infection significantly inhibited the fecundity of *Sogatella furcifera* [42,43]. Liang et al. [44] showed that the fecundity of *Nephotettix cincticeps* was increased by a rice dwarf virus (RDV) infection. In *F. occidentalis*, exposure to TSMV could promote the fecundity of females [17]. In *B. tabaci*, the fecundity was increased by ToCV infection [45]. Moreover, Moeini et al. [46] found that MIMV infection increased the fecundity of *L. striatellus* females, which was consistent with the results of this study, indicating that plant viruses may manipulate the fecundity of their vectors to promote their own transmission.

As the main component of RNPs, CP is involved in many processes, including transcription initiation and genome replication, cap-snatching mechanism, and cellular protein interaction [47]. In addition, the amount of CP protein or the RNA level of the *CP* gene is usually considered to reflect the viral load [48]. Studies have shown that RNAi-mediated knockdown of *CP* could significantly inhibit RSV replication [20]. Therefore, in this study, RNAi technology was used to knockdown the expression of *CP* in RSV-viruliferous *L. striatellus*, and the effect on the reproductive behavior was studied. The results demonstrated that *CP* knockdown in viruliferous *L. striatellus* could significantly suppress the fecundity and egg hatchability (Figure 3). It is well known that viral proteins are required for attachment or entry into insect vectors. Therefore, using the specific binding affinity of these proteins to vector insect tissues is an important way to block the acquisition and transmission of viruses [49]. For instance, through the CP of plant viruses, insect neurotoxins were transferred into the hemocoel of vector aphids, the virus transmission was blocked, and the plants were provided with resistance to aphids [39]. Thus, an RSV CP antibody was injected to block the spread of RSV in *L. striatellus* in this study. The results indicated that the expression of *CP* in viruliferous *L. striatellus* was significantly down-regulated after 5 and 7 days of injection (Figure 4), indicating that the expected blocking effect was achieved. Likewise, *B. tabaci* transmitted TYLCV in a similar circular manner, and it was proved that the binding of a CP antibody to the midgut could reduce the amount of virus in *B. tabaci* [50]. In addition, we measured the reproductive parameters of the infected *L. striatellus* after injection of the CP antibody, which was consistent with the results of RSV-viruliferous *L. striatellus* vs. non-viruliferous *L. striatellus* and interference with *CP*. The CP antibody treatment group showed a lower egg production and hatching rate than the control (Figure 5). These three groups of reproductive experiments indicated that RSV may stimulate the reproductive behavior of *L. striatellus* to promote its transmission.

As an indispensable protein for embryonic development and a key element of vitellogenesis, Vg can be taken up by oocytes through endocytosis regulated by VgR, and provide key nutrients and functional substances for developing embryos [51]. Studies have shown that Vg and VgR are key factors for microorganisms including viruses and bacteria to break through the insect ovarian barrier for maternal transmission [52–54]. In RSV-*L. striatellus*, RNPs, composed of CP, RdRp, and genomic RNA, can bind with Vg produced by haemocyte and enter ovarian germarium nurse cells through VgR-mediated endocytosis to complete vertical transmission [29,30]. At the same time, Xu et al. [55] showed that the expression level of *Vg* in viruliferous *L. striatellus* was significantly higher than that in non-viruliferous *L. striatellus* at 4 days after emergence. Our results further verified the above study, and we found that *Vg* and *VgR* exhibited up-regulation of expressions in RSV-viruliferous *L. striatellus* (Figure 6). Correspondingly, the expressions of *Vg* and *VgR* were significantly inhibited in viruliferous *L. striatellus* after an injection of *dsCP* and CP antibodies. These results were similar to a study in which RDV infection significantly up-regulated the expression of *Vg* in the salivary glands of *N. cincticeps* [56]. Notably, Vg and VgR are important molecular indicators for predicting insect reproductive adaptability. Huang et al. [45] revealed that ToCV infection increased the fecundity of *B. tabaci* by in-

creasing the expression of *Vg*. Thus, we hypothesize that RSV might enhance the fecundity of *L. striatellus* by regulating *Vg* expression, thereby promoting its transmission.

5. Conclusions

The relationships between plant–virus–vector insects are complex, especially for plant viruses that can circulate permanently in vector insects. Understanding the interactions between viruses and vector insects is helpful to predict and control plant viruses. In this study, the effects of RSV infection on the reproduction of *L. striatellus* were explored from multiple dimensions. The results showed that RSV infection promoted the fecundity and hatching rate of *L. striatellus*, which may be a mechanism to promote its own transmission by regulating *Vg* and *VgR*. The results further revealed the interaction mechanism of virus–vector in reproduction and virus transmission. In addition, RSV is one of the most important plant viruses that replicate in insect vectors; the study of its interaction with *L. striatellus* can be used as an ideal model system for investigating the relationships between plant viruses and their vectors.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy14040714/s1, Table S1: Primers used in the present study.

Author Contributions: Conceived and designed the research, G.X. and G.Y.; Conducted the experiments, Y.Z., Y.Y., J.L. and M.X.; Analyzed the date, Y.Z., C.S., L.F. and M.Q.; Wrote the manuscript, Y.Z.; Reviewed and edited the manuscript, Y.Z., G.X. and G.Y.; Funding acquisition, G.X. and Y.Z.; Software, G.X. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article or Supplementary Material.

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Conflicts of Interest: The authors declare no conflicts of interest.

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