

Article

Feeding *dsSerp1* Enhanced *Metarhizium anisopliae* Insecticidal Efficiency against *Locusta migratoria manilensis*

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Abstract: RNA interference (RNAi) induces gene silencing in order to participate in immune-related pathways. Previous studies have shown that the *LmSerp1* gene upregulates the immune responses of *Locusta migratoria manilensis*, and that the infection of locusts with *Metarhizium anisopliae* can be enhanced through the injection of the interfering *dsSerp1*. In this study, dsRNA was synthesized from the *Serp1* gene and fed to the third instars of *L. m. manilensis* at concentrations of 5 µg, 10 µg, and 20 µg. Feeding *dsSerp1* increased the mortality of *L. m. manilensis*, and 20 µg *dsSerp1* had the highest lethality, followed by 10 µg *dsSerp1*, with the lowest being observed at 5 µg *dsSerp1*. *Serp1* silencing and temperature fever induction by feeding with *dsSerp1* had a strong synergistic effect on *M. anisopliae*, showing a dose-dependent response. When *L. m. manilensis* were fed on a diet containing 20 µg dsRNA and *M. anisopliae* (2.5×10^8 spores/g wheat bran), 3-day mortalities significantly increased. The *dsSerp1* plus *M. anisopliae* treatments resulted in a 6-day mortality of 60%, accelerating insect death. These results indicated that feeding with *dsSerp1* could be an effective way to control pests and that the control effect and dsRNA concentration were dose-dependent within the first seven days. In particular, 20 µg was an effective dosage to enhance the insecticidal efficiency of *M. anisopliae*.

Keywords: locust; mortality; serpins; feed; immune

1. Introduction

Locust plagues have caused direct economic losses as high as USD 3.5–10 million every year since the mid-20th century according to FAO statistics [1]. *Locusta migratoria manilensis* (Orthoptera: Locustidae) is the most widespread and damaging locust species in Southeast Asia and East Asia, including in China [2]. Historically, locust plagues were regarded as one of three main natural disasters in China, and there have been more than 900 recorded outbreaks since 707 BCE, over 90% of which have been caused by *L. m. manilensis* [3,4]. Currently, *Metarhizium anisopliae* is recommended by the FAO as the most major biological control measure against locusts [5]. *M. anisopliae* has been used as a biological control agent in South America [6], Africa, Australia, and China [7,8] and as a model for insect–fungus interaction studies. A major criticism of *M. anisopliae* is the relatively slow kill speed in field conditions [9]. Although *M. anisopliae* is a good biological control agent against locusts, locusts show resistance to foreign substances through their immune systems, such

as via thermoregulation and innate immunity [10,11]. Therefore, it may be beneficial to enhance the virulence of *M. anisopliae* to ensure enough virulence to kill locusts after initial feeding [12].

RNA interference (RNAi), also known as post-transcriptional gene silencing, refers to the introduction of specific homologous double-stranded RNA (dsRNA) into cells, resulting in the non-expression or a decrease in the expression level of the target gene [13]. RNAi technology has been used as a novel pest control strategy since the discovery of RNAi and its regulatory functions to genes [14]. Therefore, RNAi-based strategies should be explored to enhance the toxicity of *M. anisopliae* as an alternative to conventional management methods for controlling *L. m. manilensis*.

Serine protease inhibitors (serpins), an important member of the protein superfamily of endogenous inhibitors, are widely distributed in eukaryotes [15]. It has been confirmed that serpins can participate in several immune responses in many insects, including in *Chilo suppressalis* [16], *Helicoverpa armigera* [17], *Manduca sexta* [18], and *L. m. manilensis* [19]. Chen et al. [20] obtained seven non-redundant serpin genes (named *Serpin1*–*Serpin7*) through the transcriptome analysis of *L. m. manilensis*. Indeed, the injection of dsRNA-targeting *Serpin1* in locusts infected with *M. anisopliae* resulted in a significant increase in mortality from 67.78% to 94.31% [19]. Research indicates that *Serpin1* is a critical insect immune gene and a potential target for RNAi-mediated pest control. Limited studies on the role of *Serpin1* have been carried out using RNAi technology on *L. m. manilensis*. This study intended to estimate the efficiency of a field-applicable feeding approach for administering different doses of *dsSerpin1* together with *M. anisopliae* to *L. m. manilensis*. The mortality of *L. m. manilensis*, the RNAi efficiency of *dsSerpin1* feeding, and the temperature of *L. m. manilensis* were measured to find a suitable dose of *dsSerpin1* and *M. anisopliae* for insect control and to improve the biological control efficiency.

2. Materials and Methods

2.1. Insect Culture

All of the test insects used in the experiments were 3rd instars of *L. m. manilensis* reared in the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (IPPCAAS), Beijing, China. The eggs were collected from Jianshui, Yunnan Province, China (23°63' N, 102°82' E, 1323 m above sea level) and hatched under 30 °C ± 1 °C, 60% ± 5% RH, and a 14 h:10 h = L:D photoperiod in an incubator (PRX-250 B-30; Saifu Experimental Instrument Factory, Jiangsu Province, China). The locusts were reared in cages made of wooden frames and metallic mesh (50 cm × 60 cm × 60 cm) that could hold 800 to 1000 first instar nymphs per cage regulated at 27 °C ± 1 °C and at 60% ± 5% RH under a 14 h:10 h = L:D photoperiod [19].

2.2. dsRNA Preparation

The verified plasmid (1040 bp) for the *Lmserpin1* gene sequence was obtained from IPPCAAS. A fragment of the *Lmserpin1* gene was amplified by PCR using the following method: 94 °C for 10 min; 35 cycles of 94 °C for 30 s; 58 °C for 30 s; 72 °C for 90 s; and 72 °C for 10 min with *Lmserpin1* gene-sequence-specific primers containing T7 promoters (*dsSerpin1*-F: TAATACGACTCACTATAGGATCAGCACAGCCAGGAAAC and *dsSerpin1*-R: TAATACGACTCACTATAGGCGGCATCGGAGAAGTATTG). Next, the PCR products (561 bp) were resolved on 1.5% agarose gel and purified using a DNA Gel Extraction Kit (TsingKE, Beijing, China). The purified products (561 bp) were then maintained at −20 °C before the subsequent dsRNA synthesis. Later, the purified products were used as a DNA template for dsRNA synthesis using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA). The concentration and quality of *dsSerpin1* was verified using a NanoDrop-2000 spectrophotometer (Thermo Scientific™, Waltham, MA, USA) and electrophoresis on a 1.5% agarose gel. *DsSerpin1* was stored at −80 °C until it was required for further tests.

2.3. Bioassays through Feeding *M. anisopliae*

An artificial diet was prepared according to the methods of Mullen et al. [21] and Li et al. [19], with the addition of 2.5×10^8 spores *M. anisopliae* /1 g wheat bran containing 5% of vegetable oil. There were three different feeding treatments: 5, 10, and 20 μg *dsSerp1*. Each bioassay had an artificial diet in a sterile Petri dish. Positive controls were only fed with 1 g of wheat bran containing 5% vegetable oil with 2.5×10^8 spores *M. anisopliae* and ddH₂O. The negative controls were only fed 1 g of wheat bran containing 5% vegetable oil and ddH₂O. Each treatment was replaced by fresh wheat seedlings after 24 h.

The healthy and active 3rd instars of *L. m. manilensis* were starved for 12 h, and 25 individuals were placed into a bioassay plastic basket (30 cm $\times$ 12 cm $\times$ 9 cm) under the same rearing conditions. Each plastic bioassay basket was covered with a transparent glass cover plate to prevent the *L. m. manilensis* from escaping. After addition of the locusts, a Petri dish (90 mm) containing one of the different artificial diets was added into the container. Each treatment was replicated 5 times. The number of alive, dead, and lost locusts were counted daily, and these numbers were recorded for 10 days.

2.4. Quantitative Real-Time PCR Analysis of *Serp1* Expression

The locust treatment protocol was similar to the protocol mentioned in 2.3, with 4 treatments, 5 replications, and 25 individuals per treatment. Ten individuals from each different feeding treatment were taken at 6 h, 18 h, 24 h, 48 h, and 72 h after the administration of the treatment. Then, the digestive tracts were dissected, and samples were taken for testing. Total RNA was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's instructions. The RNA concentration (OD₂₆₀/OD₂₈₀ = 2.0–2.5) was measured using a NanoDrop-2000 spectrophotometer (Thermo Scientific TM, Waltham, MA, USA). Then, the first strand of cDNA was synthesized using 200 ng of total RNA using PrimeScript™ One Step RT-PCR Kit Ver. 2 (Takara, Dalian, China.). cDNA samples were diluted to 100 ng/ μL before being used in RT-qPCR according to the instructions for Bestar™ qPCR MasterMix (SYBR Green) (DBI® Bioscience, Germany). The qPCR reactions were prepared at a total volume of 20 μL with 1 μL of cDNA and 0.5 μL 100 $\mu\text{mol/L}$ of each primer. qPCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, United States). The parameters for qPCR amplification were 95 °C for 30 s followed by 40 cycles of 95 °C for 10 s, 52 °C for 34 s, and 72 °C for 30 s. All of the RT-qPCR assays were carried out in three biological replicates, including two or three technical replicates. The sequences of the primers used for RT-qPCR followed those used in Li et al. [19].

2.5. Surface Body Temperature

The external body temperatures of the 3rd instars nymphs from 2.3, were measured using a Fluke Ti401 PRO infrared thermal imaging camera (Fluke, Everett, WA, USA) at 6 h, 18 h, 24 h, 48 h, and 72 h after the administration of the artificial diet treatments in the experimental room. None of the treatments had a thermal gradient. For each treatment at least 10 individuals were tested. The infrared camera focal point was directed at the thorax area of each locust to take temperature readings [22]. Smartview software was used to capture and annotate the locust's body surface temperature data.

2.6. Statistical Analysis

The mortality of the 3rd instar locusts was adjusted for the corrected mortality; that is, the mortality in the treatment group was adjusted for the natural mortality in the control group. Corrected mortality = (number of dead insects in the treatment group – number of dead insects in the control group)/(1 – number of dead insects in the control) $\times$ 100%. All statistical analyses were analyzed by one-way ANOVA followed by Duncan's test at a significance ($p < 0.05$) using SPSS version 23 (IBM Corp., Armonk, NY, USA). Data were expressed as mean $\pm$ standard error of the mean (SE).

3. Results

3.1. RNAi Efficiency by Feeding Different Dosages of *dsSerp1* and *M. anisopliae*

The relative expression of *Serp1* with 20 µg and 10 µg *dsSerp1* added into the diet was significantly lower than that of the treatment with only *M. anisopliae* for all time periods ($p < 0.05$), and the relative expression of *Serp1* with 5 µg *dsSerp1* added into the diet was significantly lower than that of the treatment with *M. anisopliae* only from 18 h to 24 h ($p < 0.05$, Figure 1). The relative expression of *Serp1* with 20 µg, 10 µg, and 5 µg *dsSerp1* stayed between 0.15–0.28, 0.48–0.73, and 0.72–0.96, respectively, and higher amounts of *dsSerp1* indicated a stronger RNAi efficiency.

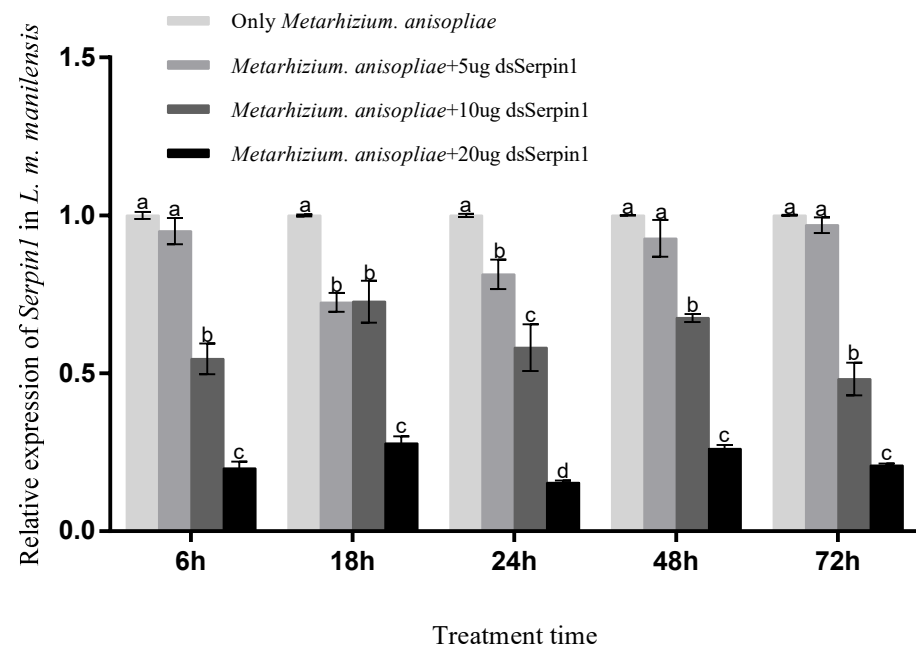


Figure 1. Relative expression of *Serp1* in *L. m. manilensis* showing the effect of feeding different dosages of *dsSerp1* with *M. anisopliae* at five points in time after treatment administration. (There were differences between a–d representatives and other groups).

3.2. Temperature Due to Feeding Different Dosages of *dsSerp1* and *M. anisopliae*

The average body surface temperature of *L. m. manilensis* with *dsSerp1* added into the diet and the addition of *M. anisopliae* only was significantly higher than the surface body temperature observed in *L. m. manilensis* (blank control, Figure 2). There was a significant increase in the temperature of *L. m. manilensis* after the administration of the RNAi treatments compared to when only *M. anisopliae* was administered at each measured treatment time ($p < 0.05$). The temperature of *L. m. manilensis* that had only been fed *M. anisopliae* was 24.95 ± 0.31 °C on average, and their temperature did not change much over time. Higher *L. m. manilensis* temperatures were strongly evident after the administration of the RNAi treatments after 6 h, and their temperatures peaked at 48 h. At 48 h, *L. m. manilensis* that had been fed with 5 µg, 10 µg, and 20 µg *dsSerp1* had mean temperatures of 27.73 ± 0.04 °C, 28.22 ± 0.14 °C, and 28.95 ± 0.11 °C, respectively. This high temperature was maintained until 72 h had passed. At 48 h and 72 h, the temperature increased with the increase in the *dsSerp1* dose, and there were significant differences among the different doses administered in the *dsSerp1* treatment groups.

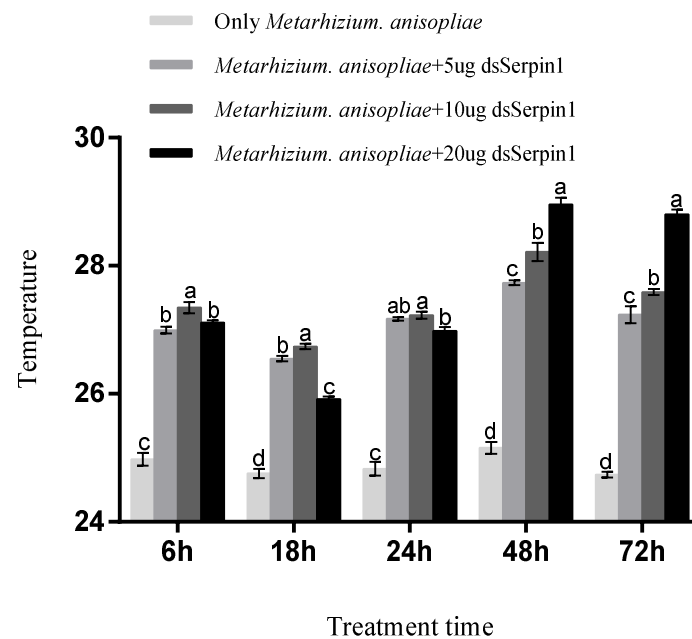


Figure 2. Temperature response of *L. m. manilensis* towards *M. anisopliae* at feeding different doses of *dsSerp1*. (There were differences between a–d representatives and other groups).

3.3. Corrected Mortality by Feeding Different Doses of *dsSerp1* and *M. anisopliae*

The statistical analysis showed that the corrected mortality of *L. m. manilensis* continuously increased across the different treatments and reached over 80% without significant differences being observed regardless of *dsSerp1* dosage at day 8 ($p > 0.05$, Figure 3). At day 3, the corrected mortality of the 20 μg *dsSerp1* and *M. anisopliae* treatment was significantly higher than that of the treatment with *M. anisopliae* only ($25.3\% \pm 3.17\%$ vs. $4.07\% \pm 1.83\%$). There was a significant difference up to day 7. The corrected mortalities of the 10 μg *dsSerp1* and *M. anisopliae* treatment were significantly higher than that of the treatment with only *M. anisopliae* ($50.83\% \pm 6.63\%$ vs. $25.09\% \pm 6.19\%$) at day 5.

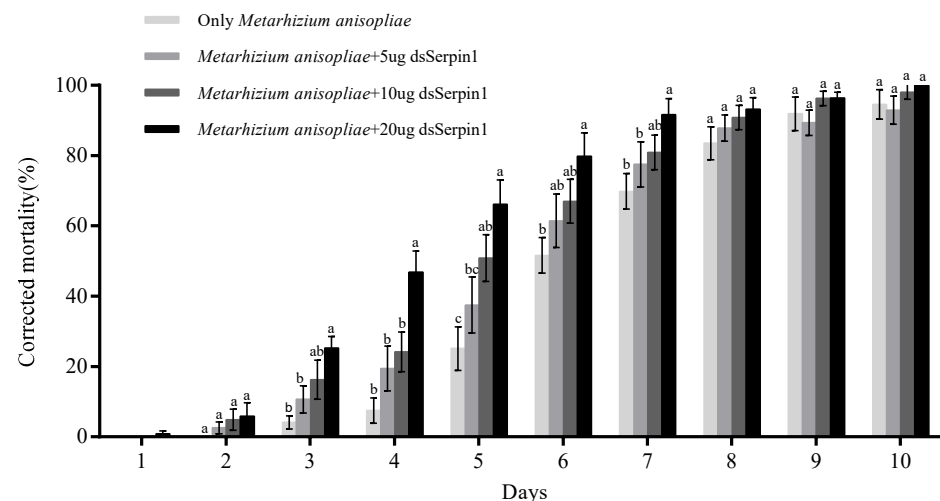


Figure 3. Corrected mortality of *L. m. manilensis* showing the effects of feeding different doses of *dsSerp1* with *M. anisopliae* for up to 10 days. (There were differences between a–c representatives and other groups).

4. Discussion

RNAi technology has been used for insect control. Xie et al. [23] revealed that applying RNAi knockdown of the *Im-nxf1* gene reduced the survival of *L. m. manilensis*. Bacteria

expressing immune-suppressive dsRNA caused a remarkable enhancement in the Bt killing activity in both the fourth and fifth instar larvae of *Spodoptera littoralis* [24]. The mortality of *Ostrinia furnacalis* reached 100% by day 5 when treated with dsCTP16, and the mortality induced by Bt treatment reached 100% at day 5, showing the enhanced insecticidal efficiency of Bt in *O. furnacalis* [25]. These studies demonstrated that the specific dsRNA of insect response-associated genes serve as a potential tool to facilitate the application of RNAi for pest control.

The most effective way to achieve dsRNA transmission for RNAi is mainly by its microinjection and feeding to insects [26]. Microinjection can cause damage to individuals in laboratory tests, and it is more difficult in smaller insects. Moreover, microinjection is impractical for large-scale pest control applications in the field. The direct feeding and soaking of insects in dsRNA [14] have been studied, and the effects were not clear or active. Previously, we showed that the immune impairment of *L. m. manilensis* was induced by injecting *dsSerp1*, causing an increase in susceptibility to *M. anisopliae* and resulting in *L. m. manilensis* dying more quickly [19]. However, the efficiency of dsRNA feeding was not clear. Feeding needs to be efficient, safe, and economically sustainable for field applications. Specific feeding details, such as the feeding dose and changes after feeding, need to be explored further.

Biological control using entomopathogenic fungi, which helps to mitigate serious environmental problems and promotes sustainable development, has attracted an increasing amount of attention and shown clear advantages compared to the use of chemical control methods to control locust populations [27]. However, the results based on field experiments indicated that the mortality caused by fungal formulations such as *M. anisopliae* was relatively slow and variable [28]. In addition, insects can overcome pathogens through immune responses such as thermoregulation and other aspects of innate immunity [29]. Therefore, compared to chemical control, its use for the large-scale control of locusts in the field is limited due to the low insecticidal efficiency, its insecticidal effect is limited [30].

Our results clearly demonstrate that the immunosuppression induced by feeding *dsSerp1* decreased the average time required to kill the insect with *M. anisopliae*. There was a dose-dependent response with 20 µg *dsSerp1* and *M. anisopliae*, which killed 80% of the locusts by day 6 compared to 50% of the those that were treated only with *Metarhizium* (Figure 3). Similarly, feeding with 20 µg *dsSerp1* induced the highest level of RNAi efficiency among the tested times compared to 5 µg *dsSerp1* and 10 µg *dsSerp1* (Figure 1). There was a positive correlation between the dose of *dsSerp1* and RNAi efficiency; the higher the dose of *dsSerp1* significantly increased the mortality of *L. m. manilensis*. Song [31] demonstrated that the activity of dsRNases was limited in *L. m. manilensis* midguts, leading to low RNAi efficiencies by feeding, but it could extend persistence of dsRNases by microinjection in the hemolymph. This may be one possible reason why a large amount of dsRNA is required to supplement consumption. In a larval RNAi experiment with *Tribolium castaneum*, 5 pg dsRNA could inhibit the expression of the *gfp* gene [27], and the dose of *T. castaneum* parental RNAi was only 0.03–0.3 µg dsRNA [28]. *Gryllus bimaculatus* effectively inhibited dsRNA with an injection amount of 1.2 µg [29]. Compared to the above insects, the amount of dsRNA required by RNAi of *L. m. manilensis* is several times higher. We speculate that the reason for this may be that *L. m. manilensis* has a larger body size, and hence contains more hemolymph. For example, a healthy locust contains around 200 µL of hemolymph, and the dsRNA is able to be diluted many times in the locust [30]. Therefore, a larger dose is required to produce RNAi.

Interestingly, a time lag phenomenon was found in the mortality of *L. m. manilensis*, with its mortality often occurring after gene silencing. For instance, feeding with 20 µg *dsSerp1* induced the highest level of gene knockdown at 24 h, but a significantly corrected mortality of *L. m. manilensis* was observed on day 3. Similarly, feeding with 10 µg *dsSerp1* induced the highest level of gene knockout at 72 h, and a significant corrected mortality was determined 2 days later. This phenomenon may be caused by the *Serp1* protein, which is synthesized by the residual mRNA to perform its normal cellular immune function in *L. m.*

manilensis and does not immediately respond. Jew [32] showed that, when synthesized by mRNA, the protein can temporarily maintain its function. Alternatively, the subsequent reductions in the corrected mortality after feeding with *dsSerp1* may be attributed to the mRNA turnover and the half-life of the protein.

After *L. m. manilensis* were inoculated with *M. anisopliae*, the surface temperature response began at 6 h and peaked at 48 h. Hunt et al. [33] showed that the fever duration was longer than 48 h, which was relatively consistent with our results. The intensity of the fever varied slightly at five time points, showing that the fever was often the highest at 48 h. The peak fever temperatures at 48 h were significantly positively related to the *dsSerp1* dose, with more severe infections inducing more intense fever responses.

Temperature directly affects the growth of pathogens. When locusts thermoregulate, they try to increase their body temperature to slow the growth of the fungus, thus extending the lifespan of the host [34]. In this study, feeding with *dsSerp1* caused intense fever responses; therefore, further research into the detailed time courses of such fevers may help to reveal the extent to which fever development is determined by the build-up of immune elicitors [35]. The peak temperatures at 48 h were significantly positively related to the *dsSerp1* dose, with higher doses of *dsSerp1* inducing higher temperatures. An explanation for the dose dependency of the fever responses has been proposed by Anderson et al. [36], who suggested that the intensity of fever responses is related to the number of immune elicitors presented by the invading pathogen. Feeding with 20 µg *dsSerp1* induced the highest level of gene knockout at 24 h, so it is speculated that the number of immune elicitors subsequently increased and that the temperature reached its highest peak at 48 h. However, the corrected mortality significantly increased on day 3, possibly because the fever response and related immune response did not overcome the RNAi and *M. anisopliae*. Such an explanation is consistent with experimental data for the relative expression of *Serp1*, which demonstrated that temperature responses were triggered by *dsSerp1*. This study proved that feeding with *dsSerp1* could down-regulate the immune responses of *L. m. manilensis* and enhance the *M. anisopliae* infection. However, the relationship between *M. anisopliae* and *dsSerp1* has not been disclosed. It is necessary to determine how to use RNAi technology against locusts as a new control method.

5. Conclusions

This study tried to develop a sustainable approach for the biological control of locusts. *Serp1* RNAi and *M. anisopliae* previously proved to be useful for increasing locust mortality via *Serp1* injection. The experiments in this study were carried out from the perspective of potential treatments used in field applications, as the use of microinjection is not possible in those situations. The results indicated that when *L. m. manilensis* were fed with 20 µg *dsSerp1*, mortality significantly increased at day 3. *Serp1* silencing was induced by feeding the locusts different dosages of *dsSerp1*, and there was a strong synergistic effect on *M. anisopliae*. These results showed that feeding with 20 µg *dsSerp1* could enhance the virulence of *M. anisopliae* and control *L. m. manilensis*, making it an effective pest control method.

Author Contributions: Conceptualization: H.L. and G.Z.; methodology: H.L., J.W. and G.W.; data curation and analysis: J.W., Y.C. and X.N.; writing—original draft preparation: J.W. and H.L.; supervision: H.L. and G.Z.; writing—reviewing and editing: B.L. and U.D. All authors have read and agreed to the published version of the manuscript.

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

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