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Environmentally-Friendly Pest Control Approaches for Invasive Insects

Edited by
Yibo Zhang, Hongbo Jiang and Ying Yan

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Article

The Effect of X-ray Irradiation on the Fitness and Field Adaptability of the Codling Moth: An Orchard Study in Northeast China

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Simple Summary: The codling moth, *Cydia pomonella* (L.), is an invasive agricultural pest species of pome fruits and walnuts that has developed resistance to many insecticides. Alternative eco-friendly approaches are warranted to reduce the dependence on insecticides for the sustainable management of *C. pomonella*. The sterile insect technique (SIT) is an autocidal strategy of control that is being trialed in experiments against various pests and that could reduce the risk of the development of insecticide resistance. In our previous study, we found that 366 Gy X-ray irradiation can effectively make the *C. pomonella* male sterile. In this study, we report on the investigation of the effect of X-ray irradiation on the fitness and adaptability of sterile insects, as well as the first pilot release of sterile male adults of *C. pomonella* in orchards in China. Results show that 366 Gy of X-ray irradiation significantly shortened the lifespan of the sterile male moths, reduced the mating competitiveness of males, and resulted in males being sterile in the orchards.

Abstract: The codling moth, *Cydia pomonella* (L.), is an invasive agricultural pest of pome fruits and walnuts in China that threatens the apple industry in the Loess Plateau and Bohai Bay; it has developed resistance to many insecticides. Sterile insect technique (SIT) combined with area-wide integrated pest management (AW-IPM) can reduce the risk of resistance to insecticides and effectively control some insect pest species. Our previous laboratory experiment found that irradiation with 366 Gy of X-ray caused the males of the codling moth to become sterile. However, the sterility and adaptability of males after being irradiated with 366 Gy X-ray in the field are still unclear. In this study, we investigated the effect of X-ray irradiation on the fitness of male adults that emerged from pupae irradiated with 366 Gy to explore their adaptability and mating competitiveness, and to examine the effect of releasing sterile male insects in orchards in northeast China on the fruit infestation rate of the Nanguo pear. The results showed that 366 Gy of X-ray irradiation significantly reduced the mating competitiveness of males and the hatching rate of the eggs laid by females pairing with sterile males. Meanwhile, the lifespan of the sterile male moths was significantly shorter than that of the normal ones in the field. A pilot test showed that the release twice of sterile male moths in the orchards had no significant effect on the fruit infestation rate. Our field experiments provide a scientific basis for the further optimization of the SIT technology program for controlling *C. pomonella*.

Keywords: *Cydia pomonella*; physical prevention and control; sterile insect technology; X-ray irradiation; mating competition

1. Introduction

The codling moth, *Cydia pomonella* (L.), is a devastating pest in pome fruits and walnuts worldwide [1,2]. This pest is also an invasive agricultural pest species in China [3]. The larvae of *C. pomonella* bore into the center of fruits to feed on the pulp and seeds, leaving frass on the surface and causing the abscission of fruits [4]. Generally, one larva may damage 3–4 fruits because of the characteristic of fruit switching [5]. As a result, it has been listed in the list of invasive alien species under key management issued by six departments, including the Ministry of Agriculture and Rural Affairs, the Ministry of Natural Resources, the Ministry of Ecology and Environment, the Ministry of Housing and Urban–Rural Development, the General Administration of Customs, and the State Forestry and Grass Administration of China (PRC). The codling moth originated from Eurasia and was first reported in the Xinjiang Uygur Autonomous Region of China in 1957 [6]. Since the insect's invasion of China, the distribution of *C. pomonella* has extended to 195 counties in nine provinces as of 2021, including Xinjiang, Gansu, Ningxia, Inner Mongolia, Heilongjiang, Jilin, Liaoning, Hebei, and Tianjin, with the occurrence area reaching 40,000 hm^2, which causes annual economic losses up to USD 605 million [7–9].

Currently, *C. pomonella* is controlled by agricultural, physical, biological, and chemical measures [10]. Generally, the egg and initial larval stages of *C. pomonella* are the key periods for chemical control. However, with the characteristics of generations overlapping and the short time that neonate larvae stay outside of the fruits after egg hatching, this pest is difficult to control using chemical insecticides. Moreover, the extensive use of insecticides has resulted in the development of *C. pomonella* resistance to a range of chemical insecticides [11,12], particularly organophosphates [13] and pyrethroids [14], and it may also cause biodiversity reduction and insecticide residues on fruits [15]. Therefore, alternative eco-friendly approaches are warranted to reduce the dependence on insecticides for the sustainable management of *C. pomonella*.

Sterile insect technique (SIT) is an eco-friendly species-specific control method used in pest management, which is usually used as a part of the integrated pest management in large areas to control pests. SIT is not a stand-alone technique requiring the prior suppression of pest populations in most cases [16,17]. Exposure to ionizing radiation such as X-rays, electron beams, or γ-rays is a common treatment method for yielding sterile insects using SIT. After a large number of sterile insects are released into the field, they can mate with virgin females to produce infertile offspring and reduce the population density [18]. Unlike other control methods, releasing sterile males does not directly impact the pest population but reduces its offspring. Therefore, the number of pests can be reduced by the continuous release of sterile insects in the field to inhibit population expansion [19,20].

SIT has been successfully applied as a part of the AW-IPM program for the eradication of some pest species, such as *Cochliomyia hominivorax* (Coquerel) in America [21] and *C. pomonella* [22,23] in Canada. Many investigations have focused on producing sterile insects and on the competitiveness of irradiated insects, for example, *Ephestia elutella* [24], *Aedes aegypti* and *A. albopictus* [25], and *C. pomonella* [26]. However, radioactive materials are hazardous, and their usage is becoming increasingly limited. There is an urgent need to find alternative sources of radiation. Studies have shown that X-ray irradiation is a safer alternative to gamma radiation; it is easily obtained and operated, and it can fulfill SIT programs' requirements [27,28]. Jiang [29] found that the sterility ratio of *Spodoptera litura* was 84% at the most appropriate irradiation dose (71.26 Gy) and that it could achieve 91% sterility in an indoor mating competition experiment when the release ratio of irradiated males (75 Gy) to non-irradiated males reached 12.6:1.

Our previous study has documented that the 8-day-old male pupa was the most suitable for X-ray irradiation [28]. Also, the proportions of sub-sterile (20.93%) and completely sterile (100%) males were determined by using 183 Gy and 366 Gy, respectively. Laboratory experiments showed that the mating competitiveness of male moths decreased at 366 Gy of X-ray irradiation but still had potential in the SIT program for controlling the codling moth [28]. Therefore, the present study aims to explore whether the 366 Gy of

X-ray-irradiated males are sterile in the field. The effects of X-ray irradiation on the fitness and field adaptability of *C. pomonella* in an orchard from northeast China were assessed.

2. Materials and Methods

2.1. Insects

A *C. pomonella* strain [30] was reared on an artificial diet under laboratory conditions of 26 ± 1 °C, 60 ± 5% relative humidity (RH), and a 16:8 h (L:D) photoperiod for more than 50 generations without exposure to any ionizing radiation.

2.2. Irradiation

A new X-ray irradiator (JYK-001 type), independently developed by Hebi Jiaduoke Industry and Trade Co., Ltd., Hebi, China, was used in this study. The equipment used in this test is the same as in the previous test [28]. The irradiation dose used in this study is the average dose, and the dose rate was measured using a Radcal Accu-Dose+ digitizer with a 10 × 6–0.6 CT ion chamber. The 8-day-old male pupae were irradiated with 366 Gy X-ray, and their fitness was assessed in a Nanguo pear orchard.

2.3. Orchard Cage Experiments

2.3.1. Effect of X-ray Irradiation on the Lifespan, Fecundity, and Fertility of Male Adults of *C. pomonella*

A tree was installed with a stand and a high-density (40 mesh) insect net (3.5 m × 3.5 m × 3.5 m) (Figure 1) in the Nanguo pear orchard in Zhangwu County (42°28′42″ N, 122°7′5″ E), Liaoning Province, China. A small insect feeding net (60 cm × 60 cm × 90 cm) was fixed on the pear tree inside the high-density insect net, with a waxed paper-lined cage (25 × 12 × 15 cm) placed inside. The 8-day-old male pupae (8 days being the most suitable age for irradiation) were irradiated with 366 Gy of X-ray as described previously [28]. After the emergence of the moths, the irradiated males and non-irradiated females were randomly paired at a sex ratio of 1:1 in the waxed paper-lined cage. The moths were provided with a 10% honey solution on absorbent cotton. Three groups, each with 8 pairs, were formed, with the non-irradiated males instead of the irradiated males used as the controls. The cage was placed in the field, and the survival of each male adult was observed every day, and the lifespan of male adults was recorded. The emerging moths were paired with non-irradiated female adults to allow them to lay eggs on the waxed papers. The waxed papers were collected, and the number of eggs laid and hatched was recorded daily. The mate capsule of the dead female adults was dissected under a stereoscope with 1 × objective (TS-63X, Shanghai Shangguang New Optical Technology Co., Ltd., Shanghai, China) to check whether there was a spermatophore, in order to calculate the single female oviposition. During the field experiment, the weather conditions were also recorded every day.

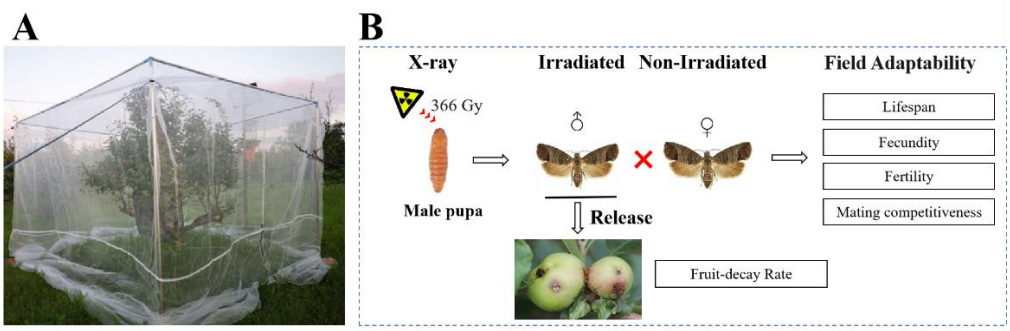

Figure 1. Experimental design for the effect of X-ray irradiation on the fitness and field adaptability of the codling moth in a Nanguo pear orchard. A tree was installed with a stand and a high-density (40 mesh) insect net, and a small insect feeding net (60 cm × 60 cm × 90 cm) was fixed on the tree

with a waxed paper-lined cage (25 × 12 × 15 cm) placed inside (**A**). After irradiating the 8-day-old male pupae with 366 Gy of X-ray, the moths that emerged were paired with non-irradiated females. The lifespan of the male adults that emerged, the fecundity and fertility of the females that mated with males irradiated with 366 Gy X-ray irradiation, and the non-irradiated (control), as well as the mating competitiveness of males, were recorded. Furthermore, the effect of the pilot release of sterile male adults of *C. pomonella* on the fruit infestation rate of the Nanguo pear was also determined (**B**).

2.3.2. Effect of X-ray Irradiation on the Mating Competitiveness of Male *C. pomonella*

Irradiated males (IM), non-irradiated males (NM), and non-irradiated females (NF) were introduced into waxed paper spawning cages (25 cm × 12 cm × 15 cm) at the ratio of 0:1:1, 1:0:1, and 1:1:1, respectively, for mating. Each mating ratio combination was repeated three times, and each contained 8 insects. For example, IM:NM:NF = 0:1:1 means that 0 irradiated males: 8 non-irradiated males: 8 non-irradiated females were introduced into a cage for mating. Then, the total number of eggs laid and hatched in each group was recorded. According to the method of [31], the competitive mating index (C) was calculated using the following formula:

$$\mathrm{E} = \frac{\boldsymbol{N}(\boldsymbol{Ha}) + \boldsymbol{S}(\boldsymbol{Hs})}{\boldsymbol{N} + \boldsymbol{S}}$$

$$\mathrm{C} = \frac{\boldsymbol{Ha} - \boldsymbol{Ee}}{\boldsymbol{He} - \boldsymbol{Hs}}$$

N is the number of NM. *S* is the number of IM. Ha is the egg-hatching rate (hatched/total eggs) of NM paired with NF, whereas *Hs* is the egg-hatching rate of IM paired with NF. *Ee* represents the observed hatching rate of mixed male adults with different S/N ratios paired with normal female adults. E represents the expected hatching rate of mixed male adults with different S/N ratios paired with normal female adults, respectively.

2.3.3. Effect of Pilot Release of Sterile Male Adults of *C. pomonella* on Fruit Infestation Rate of Nanguo Pear

A Nanguo pear orchard with a size of 500 acres was used in this study. Of this area, 25 acres of the orchard were used for the pilot release of sterile male adults of *C. pomonella*, and another 25 acres of the orchard were used for control. To ensure that the sterile males of the experimental group released in the orchard center and the wild males in the control group would not interfere with each other, a blank area of land of several hundred meters between the pilot release area and the control orchard was set. On the day before the release of the sterile males, 25 triangle traps (Beijing Zhongjie Sifang Biotechnology Co., Ltd., Beijing, China), each with a lure core containing female pheromones placed above a sticky board, were randomly arranged on the trees in the orchard. A total of 12 moths were trapped in these traps for 1 week, which represents the initial number of *C. pomonella* in the orchard. A total of 20 fruit trees were randomly selected, and a five-point sampling method was used to investigate the fruit infestation rate of the trees in the four corners (east, south, west, and north). The fruits with hanging insect droppings and wormholes were counted as rotten fruits, and this number was used to calculate the fruit infestation rate of the Nanguo pear in the control and the experimental orchards, respectively. When being released, irradiated males placed in Petri dishes were released at a height of about 1.5 m above the ground in the center of the orchard. Based on our previous study, the estimated mating competitiveness of sterile males was 0.0088 (0.01), which means that about 113 sterile males were as competitive as 1 normal male. Thus, we released twice (22 July 2022, and 2 September 2022), each time with 1250 sterile males, respectively, because the female codling moth had mated more than once based on our observation in the laboratory. Sterile moths were not released in the control orchard. The fruit infestation rate of each group was checked every 10 days post-release to evaluate whether there was a control effect between the two orchards.

2.4. Data Analysis

Significant differences in the mean value of the mortality and the fruit infestation rate were subjected to two-way ANOVA ($p < 0.05$), and the number of eggs laid (fecundity) and hatched (fertility) were subjected to one-way ANOVA with a *t*-test ($p < 0.05$) using SPSS 19 (IBM Inc, Chicago, IL, USA). The results were plotted using the GraphPad Prism 5 (PRISM 5.0, GraphPad Software, CA, USA) software. A detailed description of the data analysis is given in Supplementary Materials.

3. Results

3.1. Effect of X-ray Irradiation on the Lifespan of Male Adults of C. pomonella

In order to determine whether X-ray irradiation could influence the lifespan of male adults of *C. pomonella*, the between-subjects effects (treatments and days post-treatments) were analyzed using two-way ANOVA (Table S1). Compared with the control group, the irradiation of pupae with 366 Gy of X-ray significantly reduced the life span of *C. pomonella* ($F = 74.05$, $df = 1$, $p < 0.001$), with average lifespans of 12.29 ± 1.18 d (mean of triplicates ± SE) for the male adults in the control group and 9.13 ± 0.88 d for those in the irradiated group, respectively (Figure 2).

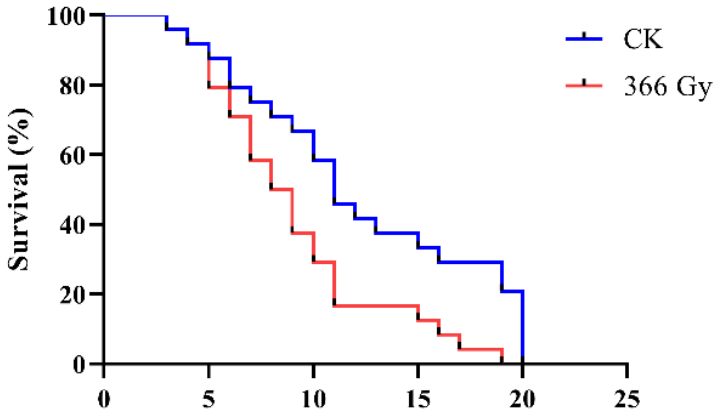

Figure 2. Survival curves of *C. pomonella* irradiated with 366 Gy X-ray irradiation in the field. Three replicates, each with eight males per group, were coupled with eight virgin females. Survival period data for each treatment were recorded and analyzed by means of a *t*-test ($p < 0.001$).

3.2. Effect of X-ray Irradiation on the Fecundity of C. pomonella

The females mated with the males irradiated with 366 Gy of X-ray irradiation (23.68 ± 1.80) laid a significantly less ($F = 7.86$; $df = 2,6$, $p = 0.021$) total number of eggs (fecundity) compared to females mated with the non-irradiated control (45.25 ± 9.92) (Figure 3).

3.3. Effect of X-ray Irradiation on the Fertility of C. pomonella

The hatching rate of eggs (fertility) was significantly influenced by X-ray irradiation in the field, with a hatching rate of 1.91 ± 2.59% for the females mated with the males irradiated with 366 Gy X-ray irradiation, which was significantly lower ($F = 89.375$, $df = 2,6$, $p < 0.001$) than the rate for the females mated with the non-irradiated males (64.15 ± 10.45%) (Figure 4).

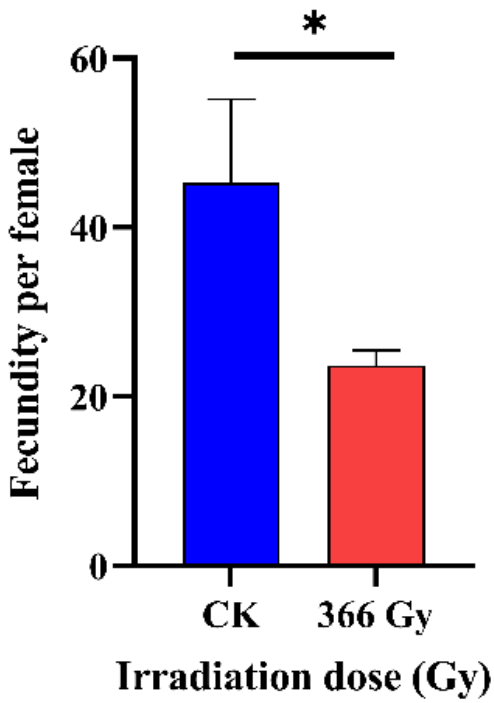

Figure 3. Effect of 366 Gy X-ray irradiation on the fecundity of the *C. pomonella*. Male pupae from 1 day before emergence (8-day-old) were irradiated with 0 Gy (CK) or 366 Gy X-ray, and mated with non-irradiated virgin females, respectively. Three replicates, each with eight males per treatment, were coupled with eight virgin females. The results are shown as the mean ± SD. Error bars represent the standard errors calculated from three replicates. Asterisk (*) on the error bars indicate significant differences analyzed by means of *t*-test ($p < 0.05$).

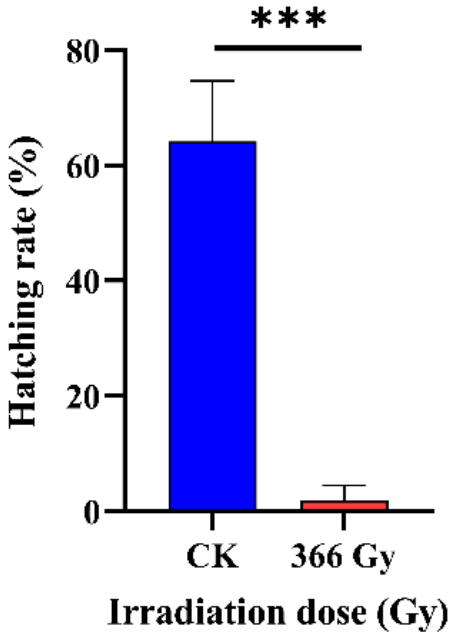

Figure 4. Effect of 366 Gy X-ray irradiation on hatching rate of *C. pomonella* in the orchard. Male pupae from 1 day before emergence (8-day-old) were irradiated with 0 Gy (CK) or 366 Gy, and the eclosed adults were crossed with non-irradiated females. Three replicates, each with eight males per group, were coupled with eight virgin females. The results are shown as the mean ± SD. Error bars represent the standard errors calculated from three replicates. Asterisk (***) on the error bars indicate significant differences analyzed by means of *t*-test ($p < 0.001$).

3.4. Effect of X-ray Irradiation on the Male Mating Competitiveness of C. pomonella

The egg hatching rate of IM:NM:NF at a ratio of 1:0:1 was 1.91 ± 2.59%, which was significantly (F = 89.375, *df* = 2,6, $p < 0.001$) lower than the ratio of 0:1:1 (egg hatching rate of 64.15 ± 10.45%) and 1:1:1 (egg hatching rate of 63.49 ± 3.57%), with an expected hatching value (E) of 33.03% when male adults were mixed with different matching ratios and paired with normal female adults. The competitive mating index (C) is 0.0088, indicating that 366 Gy of irradiation reduced the mating competitiveness of *C. pomonella* male moths (Table 1).

Table 1. Effect of X-ray irradiation on the mating competitiveness of *C. pomonella* adults in the field.

Matching Ratio (IM:NM:NF) [1]	Total Egg Production	Number of Eggs Laid/Female	Hatching Rate %		Competitive Capacity (C)
			Observed Value	Expected Value (E)	
0:1:1	300.33 ± 147.70 ab	45.25 ± 9.92 ab	64.15 ± 10.45 a		
1:0:1	102.33 ± 12.01 b	23.68 ± 1.80 b	1.91 ± 2.59 b	33.03	0.0088
1:1:1	534 ± 165.76 a	66.75 ± 20.72 a	63.49 ± 3.57 a		

[1] Irradiated males (IM), non-irradiated males (NM), and non-irradiated females (NF) were introduced into the cage in the ratios of 0:1:1, 1:0:1, and 1:1:1, respectively, for mating. Three replicates were assessed, each with 0 (at the ratio of 0:1:1) or 8 (at the ratio of 1:0:1 or 1:1:1) IM. The data in the table are the mean ± standard deviation (SD). Letters behind indicate significant differences analyzed by the two-way analysis of variance (ANOVA) using Duncan's test ($p < 0.05$).

3.5. Effect of Pilot Release of Sterile Male Adults of C. pomonella on Field Fruit Infestation Rate

In order to determine whether the pilot release of sterile male adults of *C. pomonella* could influence the fruit infestation rate of the Nanguo pear, the between-subjects effects (treatments and days post-treatments) were analyzed using two-way ANOVA (Table S2). The results showed that the fruit infestation rate of the orchard with a pilot release of sterile male adults of *C. pomonella* was 0.82 ± 0.37%, and the value in the control group was 0.85 ± 0.37%. There was no significant difference in the fruit infestation rate between the orchard with the released sterile males and the control area during a field investigation from 22 July to 3 October (F = 0.011, $p > 0.05$) (Figure A1; Table S2).

4. Discussion

Laboratory results are not fully representative of the fecundity, hatching rate, survival, and mating competitiveness of *C. pomonella* in the field [32,33]. Therefore, we explored the adaptability of the sterile codling moth under natural conditions in a pear orchard. According to our previous studies, the appropriate radiation dose for *C. pomonella* should be ≤366 Gy, and the sterile male insects irradiated by 366 Gy can effectively reduce the mean egg-hatching rate of a target population and lead to its suppression. Our results are in line with the results reported by other researchers, who found that the fertility of the codling moth was close to zero when it was irradiated with 400 Gy by a ^{60}Co source [34]. However, the suitable irradiation dose used for South African and Canadian populations was 150 Gy, which is not a dose for complete infertility [35]. These results indicate that in the SIT plan for codling moth control, the radiation dose chosen has gone from 350–400 Gy for sterility to a lower dose for sub-sterility, whereas some scholars still insist on choosing a dose above 300 Gy [23]. Similarly, the results of the field cage experiment in this study show that after mating with the males irradiated with 366 Gy X-ray irradiation, the hatching rate of the females was significantly less than that of the control group; this would cause population depression via sterility. At the same time, mating competitiveness has also decreased. Indeed, an optimal radiation dose employed should raise the levels of sterility induced in the insects, reduce their damage-producing potential without sterility, and compromise their competitiveness in mating with wild moths [36]. The above results indicated that sterile males irradiated with 366 Gy X-ray might be at a disadvantage in competition with wild males. The number of eggs laid by the non-irradiated females after mating with the irradiated males did not decrease significantly, but the mating competitiveness dramatically

decreased. These results indicate that when sterile males are released into orchards with more complex environmental conditions than in the laboratory, their competitiveness will be even lower in competing with wild males.

The life span of irradiated males is a crucial parameter in evaluating the quality of sterile insects [37]. A higher irradiation dose may also affect the somatic cells of insects, resulting in a shortened life span [38]. To increase their chance of mating with females, sterile males need to live a long life in the wild [39]. If the life span of sterile males is shortened, the frequency with which they are released and the number of sterile males (quantity) released should be increased to ensure that there are enough of them to compete effectively with wild males. In this study, we released sterile males twice, with 1250 insects each time, due to the life span of sterile males being significantly shortened owing to their being irradiated with 366 Gy X-ray irradiation. We also found a significant reduction in the mating competitiveness of sterile males. The competitiveness of insects comes from their phenotypes, which are determined by their conditions and surrounding environmental conditions. The physiological environments experienced by insects in the laboratory and the field are completely different, and environmental differences, such as feeding conditions, light stimulation, temperature, food composition, or pH, can directly or indirectly affect the phenotypes of insects [40]. This may lead to a further decline in the mating competitiveness of male insects in the field compared with those kept indoors.

Many experiments and projects have proved the feasibility of using SIT technology to control and eliminate pests [40–42]. Moreover, the economic benefits of a SIT program can far exceed the cost of suppression or eradication, which has been confirmed in many cases [43]. In the sterile insect release (SIR) program carried out in Canada in 1992, the population density of the codling moth was suppressed by releasing sterile moths [40]. Carpenter and Cross (1993) found that the increase in the number of wild *Helicoverpa zea* was significantly delayed after the release of irradiated male adults by the marker-recovery method in the field, indicating that releasing sterile males is an effective way to control wild populations [41]. However, our results showed that there was an insignificant decrease in the fruit infestation rate between the released sterile males and the control groups. The reason for this result may be that the number of sterile male adults released is insufficient to suppress the number of wild populations in the field. It may also be caused by the decrease in male flying ability and mating competitiveness [42]. We need further study to improve the quality of irradiated males and understand the mechanism of how this dose of X-ray causes *C. pomonella* infertility.

The phenomenon of inherited sterility and its connection with radiation-induced chromosome aberrations has been known since 1935 [44]. Ionizing energy breaks chemical bonds within DNA and other molecules, thereby disrupting normal cellular function in the insect. It is well known that the response of insects to irradiation varies with the species and life stage, as well as the absorbed dose received by the insect [45]. Generally, insects in Lepidoptera are radioresistant due to the holokinetic structure of their chromosomes [46]. Therefore, higher radiation doses are usually required to cause sterility. However, the competitiveness of sterile insects irradiated with high doses in the field is weak [47]. Although irradiation may break the chromosomal structure, fragments and functional genes are not completely lost due to the presence of the filament plates of lepidopteran insects, and these can be passed on to the next generation through germ cells [46–49]. Thus, research on the radiation sterility of lepidopteran insects has mainly focused on the sub-sterilization dose because such a dose is more advantageous than using complete sterility to control pests, as the F1 generation of irradiated males usually bequeath sterility genes to their offspring [50–52]. The release test of the sterile codling moth in British Columbia in 1970 showed that the percentage of apples damaged at harvest after the release of sub-sterile moths irradiated with 25 krad was better than that observed after the release of completely sterile moths irradiated with 40 krad, because they had higher mating competitiveness [53]. Therefore, Lepidoptera do not need to be irradiated with a dose producing 100% sterility [46]. Therefore, releasing adults that are not completely

sterile can not only reduce the number of pests in their generation but can also reduce the number of their offspring. A lower irradiation dose can not only reduce the cost of the procedure but can also better realize the continuous control of the number of pests. When the density of the wild population is low, for example, in the overwintering area, genetic sterility technology can be used. Thus, further research is needed to find an incomplete dose more suitable for *C. pomonella*, and a balance between the appropriate sterility and the mating competitiveness of the male is critical to obtain a better control efficacy.

In addition to choosing the appropriate irradiation dose, the release ratio of sterile males is also critical [54]. A proper release ratio can reduce the amount of released sterile male insects, thus saving cost and also making the released sterile male insects more fully utilized [50,54]. Jiang [50] found that the sterility rate of offspring reached 74% when the ratio of irradiated males to non-irradiated males was 12:1 in the mating competitiveness experiment of *Spodoptera frugiperda*. When releasing sterile insects in the field, it may be necessary to have an appropriate release ratio to better control pests. The appropriate proportion for field release may be larger than that measured in the laboratory. For example, in the field experiment of *Anastrepha fraterculus,* the control effect is ideal when the ratio of sterile male insects to wild adult insects reaches 50:1. When the release ratio of sterile males to wild males is 100:1, the sterility rate reaches 70% [54,55]. At present, we are exploring how to determine the proportion of sterile male insects released according to the number of wild pests. A previous study has shown that the recapture rate of *C. pomonella* released on the ground was much lower than that of *C. pomonella* released in the crown, which indicated that different release methods might directly affect the diffusion effect of the sterile moth and that the method of release in high places was more effective. The study also found that the negative impact of releasing the sterile moth in spring would be greater than that of releasing it in autumn [56]. Continuous release for many years also plays an important role in the prevention and control of *C. pomonella*. Following the plan, initiated in August 1992, of producing and releasing the sterile codling moth in Osoyus, Okanagan Canyon, the number of fruit trees damaged by the codling moth was successfully reduced in 1997, which showed that releasing sterile moths was an effective method and could successfully reduce the fruit infestation rate in orchards [57,58]. In the current study, the release of sterile male insects has no significant effect on the fruit infestation rate, which may be associated with the loss of mating competitiveness in irradiated males. Adding active ingredients, such as probiotic microorganisms, into their diets is a potential measure to improve the mating competitiveness of sterile males [59].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects14070615/s1. Table S1: Variance analysis of factors influencing the lifespan of male adults of *C. pomonella* (between-subjects effects); Table S2: Variance analysis of factors influencing the fruit infestation rate of Nanguo pear caused by *C. pomonella* (between-subjects effects).

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Conflicts of Interest: Author Huiyuan Zhao was employed by the Hebi Jiaduoke Industry and Trade Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A

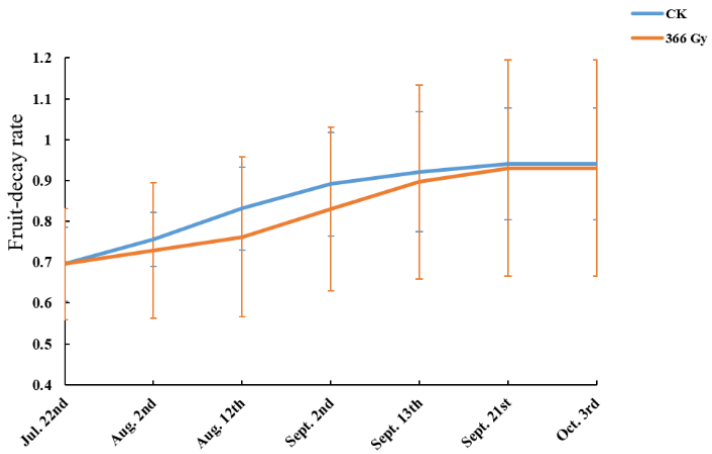

Figure A1. The effect of pilot release of sterile male adults of *C. pomonella* on the fruit infestation rate of Nanguo pear. Male pupae from 1 day before emergence (8-day-old) were irradiated with 366 Gy X-ray, and the emerged adults were released. The fruit infestation rate of orchards with releasing of sterile males was recorded every 10 days post-release to evaluate whether there was a control effect. The results are shown as the mean ± SD. Error bars represent the standard errors calculated from five replicates. An independent sample *t*-test was used to analyze the data ($p = 0.586$).

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Identification and Biocontrol Potential of Entomopathogenic Nematodes and Their Endosymbiotic Bacteria in Apple Orchards against the Codling Moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae)

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Simple Summary: The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is a key pest in apple production. Controlling *C. pomonella* infestations with insecticides can be challenging, as it requires excessive insecticide use during the growing season. A survey of entomopathogenic nematodes (EPNs) and their endosymbionts (ESs) in apple production orchards of Kayseri Province, one of the main apple production areas of Türkiye, was conducted to develop an alternative control strategy to chemicals. Both EPNs and ESs have been studied for their potential control of *C. pomonella* larvae. The results demonstrate that EPNs and their ESs can reduce larval infestations of *C. pomonella*.

Abstract: The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is one of the major pests in pome fruit production worldwide. Heavy treatment of the larvae of *C. pomonella* with insecticides triggered the development of resistance to many groups of insecticides. In addition, the increasing concern about the adverse effects of synthetic insecticides on human health and the environment has led to the development of sustainable and eco-friendly control practices for *C. pomonella*. The entomopathogenic nematodes (EPNs) (*Steinernema* and *Heterorhabditis* spp.) and their endosymbionts (*Xenorhabdus* and *Photorhabdus* spp.) represent a newly emerging approach to controlling a wide range of insect pests. In the present study, field surveys were conducted in apple orchards to isolate and identify EPNs and their endosymbionts and evaluate their insecticidal efficacy on the larvae of *C. pomonella*. EPNs were isolated from 12 of 100 soil samples (12%). Seven samples were identified as *Steinernema feltiae* (Filipjev, 1934) (Rhabditida: Steinernematidae), whereas five samples were assigned to *Heterorhabditis bacteriophora* (Poinar, 1976) (Rhabditida: Heterorhabditidae). The pathogenicity of the EPN species/isolates was screened on the last instar larvae of *G. mellonella*. The two most pathogenic isolates from each EPN species were tested against fifth instar larvae of *C. pomonella* under controlled conditions. The maximum mortality (100%) was achieved by all EPN species/isolates at a concentration of 100 IJs/larva 96 h after treatment. The endosymbionts of selected *H. bacteriophora* and *S. feltiae* species were identified as *Photorhabdus luminescens* subsp. *kayaii* and *Xenorhabdus bovienii*, respectively. The mortality rates ranged between 25 and 62% when the fifth larval instar larvae of *C. pomonella* were exposed to the treatment of cell-free supernatants of symbiotic bacteria. In essence, the present survey indicated that EPNs and their symbiotic bacteria have good potential for biological control of *C. pomonella*.

Keywords: sustainable control; biological control; apple; beneficial nematodes; entomopathogenic bacteria

1. Introduction

Apple (*Malus* spp.) is one of the most economically important fruit crops produced widely in temperate regions of the world. Due to climate and field conditions, Türkiye is one of the leading countries in apple cultivation, with an annual production of 4.3 million tons [1]. Pest and disease management practices heavily influence the profitability of apple cultivation in orchards [2]. Of the numerous pests that infest and damage apple orchards, the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is one of the major pests of apple orchards worldwide because the larvae directly feed on fruits. The adults of *C. pomonella* lay their eggs on the surface of apple fruits after emerging in late spring. The hatching larvae begin feeding by boring into fruits and forming galleries towards the seed chamber of the fruit [3]. Because the internal feeding damage of larvae renders fruits unmarketable, the control of larvae is of great importance for the management of *C. pomonella*. In the absence of appropriate control measures, apple orchards experience serious yield losses, rendering more than 50% of fruits unmarketable [4].

Among the main strategies used in Türkiye to combat codling moths are synthetic insecticides. Insecticides are normally applied more frequently than once a week to prevent neonate larvae from tunneling into fruits following the emergence of adults during the growing season. As a consequence of the intensive use of insecticides, *C. pomonella* has developed resistance to almost all classes of insecticides, leaving their residues on apple fruits that exceed maximum residue levels (MRLs) [5–10]. In addition to dependence on insecticides, these MRLs have led to several environmental and health problems [11]. Therefore, the assessment and development of biologically based alternatives to control *C. pomonella* have been one of the focuses of agricultural research in the last decades [12,13]. Of alternative control strategies for *C. pomonella*, entomopathogenic nematodes (EPNs) (*Steinernema* spp. and *Heterorhabditis* spp.) have attracted great attention due to their biocontrol potential and environmental safety [14]. Entomopathogenic nematodes can reduce the number of insecticide applications and the production costs while providing environmentally safe control. Infective juveniles (IJs) of EPNs are a non-feeding and free-living stage that is able to seek out a potential host in cryptic habitats. Once IJs encounter a host, they penetrate its body using body openings and thin cuticles [15]. The IJs initiate the infection process by releasing their mutualistic bacteria (*Xenorhabdus* and *Photorhabdus* spp.) into insect hemolymph. The symbiotic bacteria multiply rapidly and serve as a nutrition source for the IJs along with host tissues. The host generally dies from septicemia within 48 h after infection due to the toxin complexes and killer proteins produced by the endosymbionts of EPNs [16]. After a few generations, large numbers of IJs abandon the resource-depleted host cadaver to search for a new host [17].

Although EPNs are soil-originated organisms, their ability to find and infect insect pests in cryptic habitats such as crevices in wood and litter at the base of trees where the fifth instar larvae of *C. pomonella* choose to pupate makes them a perfect candidate for the biological management of orchards [18]. Earlier laboratory and field studies showed that first and fifth instar *C. pomonella* larvae were susceptible to EPNs [19–22]. However, to our knowledge, there is no study assessing the effectiveness of EPNs recovered from apple orchards on the larvae of *C. pomonella*. The virulence of EPNs varies greatly among species/isolates depending on the host-finding behaviors, adaptation and penetration capabilities, host specialization, and symbiotic bacteria [23,24]. Therefore, the isolation of native EPN species from orchards and the determination of their virulence on the target host are of crucial importance for the success of EPNs applications. *Photorhabdus* and *Xenorhabdus* spp., the endosymbionts of *Heterorhabditis* and *Steinernema* spp., are Gram-negative bacteria that play a key role in the pathogenicity of EPNs by releasing a wide range of seconder metabolites into host hemolymph, which is lethal to host insects [16]. Numerous studies have revealed that toxin complexes of *Photorhabdus* and *Xenorhabdus* spp. bacteria were highly lethal to tested insects and offered an alternative in the control of many economically important pests [25–28]. However, to date, no study has been conducted to evaluate the cell-free supernatants of *Photorhabdus* and *Xenorhabdus* spp. of bacteria, which

contain insect-killer proteins, proteases, lipases, and toxic compounds, to insects [29]. Thus, the aim of this study was to examine the diversity and the distribution of EPNs in apple-growing areas in Kayseri Province and evaluate their effectiveness and their endosymbiotic bacteria on fifth larval instars of *C. pomonella* under controlled conditions.

2. Materials and Methods

2.1. Soil Sampling

Surveys were conducted in apple orchards between 2021 and 2022 from major apple-growing areas of Kayseri. Soil samples were taken randomly from crown projection areas of apple trees at each sampling site using a hand shovel following rainy days in the spring months (April, May, and June). For each orchard, 5–12 soil samples that consisted of 250 g soil subsamples were collected according to the number of trees in each orchard. All subsamples were pooled together, and 2 kg of a mixture of subsamples in plastic bags was brought to the Entomology Laboratory of Erciyes University in a cool box (12–15 °C). A total of 100 soil samples were taken (Table 1).

Table 1. Soil samples collected from apple-growing areas of Kayseri Province.

Province	EPN-Positive Samples	EPN-Negative Samples	Total	Recovery Rate (%)
Develi	4	26	30	13.3
Yahyalı	3	27	30	10.0
Yeşilhisar	5	35	40	14.2
Total	12	88	100	12

2.2. Isolation of Entomopathogenic Nematodes

Entomopathogenic nematodes were isolated from soil samples using an insect-baiting technique [30,31]. *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) larvae were reared under laboratory conditions (25 ± 2 °C and 60% RH) and the last instar larvae were used as the insect bait [32]. After sieving thoroughly using 1 cm mesh, subsamples of 250 g of soil were put into a clean plastic container (Ø12 cm and 10 cm height) with 10 larvae, and containers were covered with perforated lids. Then, containers were incubated under controlled conditions for 10 days (25 ± 2 °C and 60% RH). The dead and live larvae were checked at 2-day intervals during the incubation period. The dead larvae showing typical signs of nematode infection (soft body and creamy beige or red pigmentation) were placed individually in modified White traps for the emergence of IJs [33]. After washing with sterile water several times, the emerged IJs were applied to eight *G. mellonella* larvae in Petri dishes to verify Koch's postulates for pathogenicity, and dead larvae were re-transferred to modified White traps to obtain pure IJs populations [34]. The newly emerged IJs were rinsed with sterile water and stored horizontally at 11 °C in flasks (250 mL).

2.3. Identification of Nematodes

The morphological and/or morphometric identification of EPN species was carried out using infective juveniles as suggested by [35,36]. The stock cultures of IJs from each population were applied to ten last instar *G. mellonella* larvae (2000 IJs/mL) in a Petri dish (100 × 15 mm) lined with two filter papers and incubated under controlled conditions (25 ± 2 °C and 60% RH). Dead larvae were transferred to the modified White traps and twenty IJs specimens from each EPN population were used for morphometric measurements. Prior to measurements, the IJs were killed by heat (60 °C) in Ringer's solution and placed in TAF (triethanolamine-formalin fixative) [34,37]. Then, the specimens were mounted on microscope slides in a drop of pure glycerin and covered with a coverslip. Observations were made using an MC100 spot microscope equipped with digital image software (Axioskop; Zeiss, Oberkochen, Germany) (Table 2).

Table 2. Morphometric measurements of infective juveniles (IJs) of some of the isolates of entomopathogenic nematode species recovered from survey studies (μM).

Isolates	*L	*MBW	*EP	*ES	*TL	*a	*b	*c	*%D	*%E
Steinernema feltiae, Mean ± SE (Min–max) (n = 20)										
A67	861 ± 54 (805–896)	33 ± 2.6 (27–35)	59 ± 3.8 (54–65)	145 ± 4.1 (133–152)	81 ± 2.9 (75–83)	27 ± 3.3 (23–32)	7.5 ± 1.6 (5.9–7.1)	12.5 ± 2.2 (9.4–13.1)	41 ± 2.3 (37–44)	74 ± 3.2 (69–77)
A93	925 ± 61 (855–971)	31 ± 1.9 (27–33)	60 ± 4.1 (58–67)	126 ± 7.3 (119–142)	85 ± 4.5 (77–89)	29 ± 3.1 (26–33)	6.9 ± 0.9 (5.8–7.4)	11.1 ± 1.5 (9.5–11.4)	42.3 ± 2.9 (39–45)	74 ± 3.9 (70–78)
Nguyen and Hunt (2007)	879 ± 49 (766–928)	29 ± 1.9 (26–32)	63 ± 2.3 (59–67)	136 ± 3.5 (130–143)	86 ± 2.6 (81–89)	30 ± 1.9 (27–34)	6.4 ± 0.3 (5.8–6.8)	10 ± 0.5 (9.4–11)	-	-
Heterorhabditis bacteriophora, Mean ± SE (Min–max) (n = 20)										
A8	611 ± 8.1 (601–620)	21 ± 1.9 (18–24)	115 ± 8.2 (101–127)	145 ± 9.1 (132–155)	87.5 ± 3.7 (82–93)	24.1 ± 1.9 (21–27)	4.9 ± 0.9 (3.9–5.1)	6.9 ± 0.8 (6.6–7.8)	81 ± 3.1 (75–85)	104 ± 4.1 (99–112)
A20	597 ± 8.5 (588–609)	22 ± 2.5 (18–24)	105 ± 7.3 (96–117)	132 ± 4.9 (126–138)	90.7 ± 4.5 (83–96)	23.3 ± 2.7 (20–27)	4.5 ± 0.6 (4.1–4.8)	7.1 ± 0.9 (6.2–8.2)	82 ± 3.5 (77–86)	119 ± 6.5 (112–128)
Nguyen and Hunt (2007)	588 (512–671)	23 (18–31)	103 (87–110)	125 (100–139)	98 (83–112)	25 (17–30)	4.5 (4.0–5.1)	6.2 (5.5–7.0)	84 (76–92)	112 (103–130)

* Total body length (L), body length/body width (a), body length/esophageal length (b), body length/tail length (c), %E:[(EP/TL) × 100], %D:[(EP/ES) × 100], excretory pore (EP), pharynx (ES), maximum body width (MBW), tail length (TL), n: number of specimens examined.

Molecular characterization of EPN isolates was performed based on sequencing of the internal transcribed spacer (ITS) of ribosomal DNA. The isolation of DNA was performed on 8–10 newly emerged, live IJs from each EPN population following the method described by [38]. The ITS region was amplified by a total of 50 μL PCR reaction mixture consisting of 2 μL DNA, 22 μL ddH2O, 25 μL of Dream Taq PCR Master Mix (Thermo Fischer Scientific, Waltham, MA, USA), 0.5 μM of F194 (5′-CGT AAC AAG GTA GCT GTA G-3′), and 5368r (5′-TCC TCC GCT AAA TGA TAT G-3′) primers [39,40]. Thermo-cycling was performed in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) and programmed for initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, extension at 72 °C for 1 min, and the final extension at 72 °C for 10 min. The resulting products were purified and sequenced in both directions using the primers referred to above (Macrogen Inc., Seoul, Korea). The obtained sequences were aligned using the MegAlign module of DNASTAR software, version 7.1.0 (DNASTAR Inc., Madison, Wisconsin, USA) and compared to the sequences deposited in the GenBank. Accession numbers submitted to the GenBank database were given in Table 3.

Table 3. Entomopathogenic nematode isolates found, with the indication of the locality and internal transcribed spacer (ITS) sequence information.

Code	Location	District	Species	Accession Number
A6	38°19′50 K 35°22′52 D	Develi	*Steinernema feltiae*	OM401705
A8	38°21′02 K 35°23′11 D	Develi	*Heterorhabditis bacteriophora*	OM401700
A9	38°24′18 K 35°26′57 D	Develi	*Heterorhabditis bacteriophora*	OM401701
A20	38°24′18 K 35°26′45 D	Develi	*Heterorhabditis bacteriophora*	OM401702
A52	38°20′12 K 35°27′29 D	Yahyalı	*Heterorhabditis bacteriophora*	OM401704
A53	38°19′86 K 35°22′86 D	Yahyalı	*Steinernema feltiae*	OM401706
A54	38°42′58 K 35°31′89 D	Yahyalı	*Steinernema feltiae*	OM401707
A67	38°09′33 K 35°22′01 D	Yeşilhisar	*Steinernema feltiae*	OM401709
A93	38°24′18 K 35°26′57 D	Yeşilhisar	*Heterorhabditis bacteriophora*	OM401703
A94	38°21′84 K 35°05′64 D	Yeşilhisar	*Steinernema feltiae*	OM401708
A97	38°09′52 K 35°21′56 D	Yeşilhisar	*Steinernema feltiae*	OM401710
A100	38°20′53 K 35°05′59 D	Yeşilhisar	*Steinernema feltiae*	OM401711

2.4. Pathogenicity Screening on Galleria mellonella Larvae

The pathogenicity of obtained EPNs was assessed on last larval instar of *G. mellonella* in Petri dishes under laboratory conditions (25 ± 1 °C, 65 ± 5% RH, and 16L: 8D h photoperiod).

The IJs of EPN isolates were applied at the concentration of 100 IJs/Petri dish. Based on the mortality rates 72 h after treatment, the most efficient two isolates of each EPN species were selected to further evaluate their pathogenicity against the larvae of *C. pomonella*.

2.5. Cydia pomonella

Different larval instars of *C. pomonella* were collected from infested apple fruits in large numbers from apple orchards in Kayseri Province during the summer of 2021. The larval instars were reared on apple fruits in cages (60 × 60 × 60 cm) under controlled conditions (25 ± 1 °C, 65 ± 5% RH, and 16L: 8D h photoperiod). The emerging adults from each population were collected using a suction trap and sent to an entomologist for identification (Prof. Halil KÜTÜK, Abant Baysal University). The 5th instar larvae feeding within fruits were gathered and grouped together. The healthy larvae were included in the bioassays.

2.6. Isolation and Identification of Symbiotic Bacteria

The bacterium was isolated using approximately 500 freshly emerged IJs of each isolate. After being surface-sterilized in a sterile Ringer's solution comprising NaClO (10% *w*/*v*) for 10 min, the IJs were washed several times with sterile Ringer's solution and crushed in 1 mL of sterile phosphate-buffered saline. Then, 10 µL of the suspension was streaked onto NBTA medium [16]. After an incubation period of 48 h at 28 °C, primary variants were re-streaked onto the NBTA plates to obtain pure bacterial colonies. Then, each purified bacterial colony was harvested and subjected to DNA extraction using the GeneMATRIX Tissue and Bacterial DNA Purification Kit (EURx) according to the manufacturer's instructions. The 16S ribosomal RNA (rRNA) gene was amplified and sequenced according to primers suggested by [41,42]. The PCR conditions were set as an initial denaturation at 94 °C for 2 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were checked by electrophoresis to verify amplification products and sequenced by Macrogen, Inc. (South Korea). The nucleotide sequences of the isolates/strains were checked and manually corrected. The arrangement of nucleotides in ambiguous positions was corrected by comparisons of the sequences generated from both the forward and reverse primers using MEGA X software [43]. The novel sequences were compared with the sequences registered in GenBank based on nucleotide similarity and deposited in GenBank with Accessions Nos. OP642365 (*X. bovienii* A94 strain), OP630600 (*X. bovienii* A54 strain), OP630601 (*P. luminescens* subsp. *kayaii* A8 strain), and OP642366 (*P. luminescens* subsp. *kayaii* A9 strain).

2.7. Preparation of Cell-Free Bacterial Supernatants

Erlenmeyer flasks (250 mL) containing 100 mL Luria–Bertani (LB) broth (Merck, Darmstadt, Germany) were inoculated with a loopful of the pure bacterial colonies of each isolate and incubated on a rotary incubator at 150 rpm for 144 h (28 °C, 20% RH in the dark) [16,44]. The broth suspensions of bacteria cultures were then poured into 50 mL Falcon tubes. The cell-free supernatants of the solutions were separated by centrifuging the bacterial suspension at 20,000 rpm for 15 min at 4 °C and filtering twice through a 0.22 µM Millipore filter (Sigma–Aldrich). The leakage of bacterial cells was checked by streaking the final cell-free supernatant solutions onto NBTA agar.

2.8. Virulence Tests on Cydia pomonella Larvae

Obtained EPN isolates were tested for their pathogenicity against the last instar larvae of *C. pomonella* in Petri dishes lined with two filter papers. The IJs suspended in 1 mL of tap water from each isolate were inoculated into Petri dishes at concentrations of 10, 25, 50, and 100 IJs/Petri dish, and 10 larvae were added to each Petri dish. A button-sized apple (Approximately 1 cm^2) was provided for the larvae as food. Then, Petri dishes were incubated under controlled conditions (25 °C, 60% RH, and 16:8 h of L/D) after

sealing with a parafilm. The larval mortality was recorded daily for three days, and dead larvae were dissected under a stereo microscope to check the nematode infection. Control groups were treated with tap water. Each treatment consisted of four replicates and 10 larvae were used for each replicate. The bioassays were conducted twice under the same conditions. Only one-week-old IJs were included in the pathogenicity bioassays. Based on the mortality rates obtained on the 3rd day after treatment (DAT), the symbiotic bacteria of the most pathogenic two isolates of EPN species were selected for further evaluation of their effectiveness against 5th larval instar larvae of *C. pomonella*.

2.9. Evaluation of Pathogenicity of Symbiotic Bacteria

The cell-free supernatant solutions were evaluated against ten 5th instar larvae of *C. pomonella* in contact treatments under controlled conditions. A 1 mL aqueous suspension of cell-free supernatants was applied directly onto each larva with the help of a mini spray bottle (50 mL) (Ø 0.5 mm nozzle) and larvae were placed into Petri dishes (Ø 9 cm) individually containing two filter papers and a button-sized apple. The Petri dishes were covered and sealed with parafilm. The Petri dishes were kept at 25 °C, 60% RH, 14 h light, and 10 h darkness for three days and larval mortality was checked daily. In control treatments, larvae were treated with nutrient broth only and the rest of the procedure was repeated. Each treatment consisted of four replicates and 10 larvae were used for each replicate. The bioassays were conducted twice under the same conditions. Only two-week-old cell-free supernatants were included in the bioassays, and they were stored at 9 °C until the bioassays were performed.

2.10. Statistical Analysis

No mortality occurred in control treatments and mortality data were subjected to normality tests and were arcsine transformed. All statistical analyses were carried out using IBM SPSS Statistics, version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Significant differences between treatments were determined by factorial repeated measures ANOVA using a general linear model. The mean differences were grouped using Tukey's multiple range tests ($p \leq 0.05$).

3. Results

3.1. Survey and Identification of EPNs

Out of the 100 soil samples examined for the presence of EPNs, 12 samples were positive (Table 1). Recovery rates of EPNs in the sampled districts ranged between 10.0 and 14.2%. The highest occurrence rate of EPNs (14.2%) was obtained from the Yeşilhisar district, which is the biggest apple cultivation area in Kayseri. Two EPN species were obtained from all districts. However, *Heterorhabditis bacteriophora* (Poinar, 1976) (Rhabditida: Heterorhabditidae) was more prevalent in the Develi district, whereas *Steinernema feltiae* (Filipjev, 1934) (Rhabditida: Steinernematidae) was more common in Yeşilhisar (Figure 1).

Figure 1. Map of Kayseri Province showing districts where entomopathogenic nematodes were found.

Using the taxonomic keys from [36], seven isolates were classified as *S. feltiae*, whereas five isolates belonged to *H. bacteriophora*. The morphometric measurements of IJs of *S. feltiae* and *H. bacteriophora* indicated a remarkable similarity to those described by [35,36] in terms of the most morphometric characteristics (Table 2). The morphological identification of EPNs was also confirmed by the nucleotide sequences of the ITS region of isolates. The DNA fragments of the ITS region were between 770 and 820 base pairs long. The ITS sequences of obtained isolates showed a 99% nucleotide similarity with those of reference isolates deposited in GenBank (Table 3).

3.2. Pathogenicity Screening on Galleria mellonella Larvae

The results showed that all isolates induced significant mortality of G. mellonella larvae (24 h: Df: 11, F-value: 43.111, and p = 0.000; 48 h: Df: 11, F-value: 38.291, and p = 0.000; 72 h: Df: 11, F-value: 6.652, and p = 0.000). The mortality rates ranged between 60 and 100% 72 h after treatment (Table 4). The most efficient isolates of *S. feltiae* and *H. bacteriophora* 72 h after treatment were *S. feltiae* A54 and *H. bacteriophora* A9, and these caused 100 and 96% mortality, respectively.

Table 4. Mortality rates (%) of isolated entomopathogenic nematodes on the last instar larvae of *Galleria mellonella* at concentrations of 100 infective juveniles/Petri dish (100 IJs/Petri) under laboratory conditions (25 °C, 60% RH).

EPNs *	24 h	48 h	72 h
	(Mean ± S. Error)		
S. feltiae A6	23.3 ± 6.6 BC [a]	43.3 ± 3.3 BC	60.0 ± 5.7 A
H. bacteriophora A8	16.6 ± 3.3 ABC	56.6 ± 6.6 CD	86.6 ± 8.8 ABC
H. bacteriophora A9	33.3 ± 3.3 C	66.6 ± 3.3 D	96.6 ± 3.3 BC
H. bacteriophora A20	13.3 ± 3.3 AB	43.3 ± 3.3 BC	86.6 ± 3.3 ABC
S. feltiae A52	16.6 ± 3.3 ABC	43.3 ± 3.3 BC	83.3 ± 3.3 ABC
S. feltiae A53	6.6 ± 3.3 AB	26.6 ± 3.3 AB	63.3 ± 6.6 A
S. feltiae A54	70.0 ± 5.7 D	96.6 ± 3.3 E	100.0 ± 0.0 C
S. feltiae A67	0.0 ± 0.0 A	23.3 ± 3.3 A	60.0 ± 10.0 A
H. bacteriophora A93	0.0 ± 0.0A	26.6 ± 3.3 AB	70.0 ± 5.7 AB
S. feltiae A94	60.0 ± 0.0 D	73.3 ± 3.3 D	86.6 ± 3.3 ABC
S. feltiae A97	13.3 ± 3.3 AB	43.3 ± 3.3 BC	86.6 ± 3.3 ABC
S. feltiae A100	16.6 ± 3.3 ABC	43.3 ± 3.3 BC	83.3 ± 3.3 ABC

* EPNs: Entomopathogenic nematodes. [a] Different capital letters show statistically significant differences among entomopathogenic nematode species/strains for each exposure time level.

3.3. Susceptibility of Cydia pomonella Larvae to Selected Isolates

Statistical analysis revealed that the susceptibility of *C. pomonella* larvae was affected by primary factors and their associated interactions, except for the interaction of nematodes–concentrations (N*C) and nematodes–concentrations–exposure time (N*C*t) (Table 5). Mortality rates tended to increase as exposure time and IJs concentrations increased. The larvae of *C. pomonella* were susceptible to all selected EPN species/isolates and mortalities over 50% were obtained even at the lowest concentration (10 IJs/larva). Maximum mortality (100%) was achieved by only *H. bacteriophora* isolates (H. b. A8 and H. b. A9) at a concentration of 50 IJs/larva. At the lowest exposure time (24 h), *H. bacteriophora* A8 was the only isolate that caused 100% mortality at the concentration of 100 IJs/larva. However, all EPN species/isolates induced 100% mortality 96 h after treatment (Table 6).

Table 5. Repeated measures analysis of variance parameters for the main effects and associated interactions for mortality rates of the 5th instar larvae of *Cyida pomonella* in Petri dish bioassay.

Sources *	df	F-Value	*p*-Value
Nematodes (N)	3	4.605	0.007
Concentrations (C)	3	15.478	0.000
N*C	9	0.767	0.647
Error [1]	48		
Exposure time (t)	3	371.153	0.000
C*t	9	5.984	0.000
N*t	9	3.987	0.000
N*C*t	27	1.071	0.382
Error [2]	144		

Error [1] was used for comparing the mean levels of non-repeating factors when the interaction of the non-repeating factor with the repeated factor was not important. Error [2] was used for comparing the mean levels of the repeated factor. * Tukey ($p \leq 0.05$).

Table 6. The efficacy of the selected EPN species/isolates on the 5th instar larvae of *C. pomonella* (25 °C, 60% RH).

Concentrations	Exposure Time * (h)	Mortality Rates (%)			
		***S. f.* A94**	***S. f.* A54**	***H. b.* A8**	***H. b.* A9**
10 IJs/larva	24	15.0 ± 9.5 A [a] a [b]	10.0 ± 5.7 Aa	15.0 ± 5.0 Aa	10.0 ± 5.7 Aa
	48	35.0 ± 15.0 Ba	35.0 ± 5.0 Ba	30.0 ± 5.7 Ba	30.0 ± 5.7 Ba
	72	45.0 ± 5.0 Ba	40.0 ± 5.7 Ba	50.0 ± 5.7 Ca	40.0 ± 8.1 Ba
	96	65.0 ± 12.5 Ca	50 ± 5.7 Ba	60.0 ± 8.1 Ca	50.0 ± 5.7 Ba
25 IJs/larva	24	50.0 ± 12.9 Ab	35.0 ± 5.0 Aa	55.0 ± 9.5 Ab	50.0 ± 5.7 Ab
	48	60.0 ± 14.1 Aa	55.0 ± 9.5 Ba	75.0 ± 9.5 Bb	65.0 ± 9.5 Ba
	72	85.0 ± 9.5 Bb	60.0 ± 5.7 Ba	80.0 ± 8.1 Bb	75.0 ± 9.5 Bb
	96	90.0 ± 10.0 Bb	70.0 ± 5.7 Ba	85.0 ± 9.5 Bb	85.0 ± 9.5 Bb
50 IJs/larva	24	70.0 ± 10.0 Aa	70.0 ± 5.7 Aa	90.0 ± 10.0 Ab	90.0 ± 10.0 Ab
	48	80.0 ± 8.1 Aa	80.0 ± 8.1 Aa	95.0 ± 5.0 Ab	100.0 ± 0.0 Ab
	72	90.0 ± 5.7 ABa	85.0 ± 9.5 Aa	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
	96	95.0 ± 5.0 Ba	90.0 ± 5.7 Aa	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
100 IJs/larva	24	80.0 ± 8.1 Aa	95.0 ± 5.0 Ab	100.0 ± 0.0 Ab	95.0 ± 5.0 Ab
	48	90.0 ± 5.7 Aba	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
	72	95.0 ± 5.0 Aba	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa
	96	100.0 ± 0.0 Ba	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa

* *S. f.* A94: *Steinernema feltiae* A94 isolate; *S. f.* A54: *Steinernema feltiae* A54 isolate; *H. b.* A8: *Heterorhabditis bacteriophora* A8 isolate; *H. b.* A9: *H. bacteriophora* A9 isolate. [a] Different capital letters show statistically significant differences among entomopathogenic nematode species/strains for each exposure time level. [b] Different lowercase letters show statistically significant differences among the exposure time levels for each entomopathogenic nematode species ($p < 0.05$ Tukey).

3.4. Identification and Evaluation of the Symbiotic Bacteria of Selected Isolates

The bacterial associates of A94 and A54 isolates of *S. feltiae* and A8 and A9 isolates of *H. bacteriophora* were classified as X. bovienii and P. luminescens subsp. kayaii, respectively. Statistical analysis demonstrated that the cell-free supernatant of tested symbiotic bacteria species/strains and exposure time had a significant influence on the mortality rates of *C. pomonella* (Table 7). *Xenorhabdus bovienii* strains generally induced greater mortality at all exposure times compared with P. luminescens subsp. *kayaii* strains. *Xenorhabdus bovienii* strains were able to cause mortalities over 50% only 72 h after treatment. The highest mortality (62%) was achieved by the X. bovienii A94 strain (Table 8).

Table 7. Repeated measures analysis of variance parameters for the main effects and associated interactions for mortality rates of the larvae of *Cydia pomonella* in contact efficacy bioassay of cell-fee supernatants of symbiotic bacteria.

Sources *	df	F-Value	*p*-Value
Supernatant (S)	3	30.727	0.000
Error [1]	12		
Exposure Time (t)	2	144.907	0.000
S*t	6	6.860	0.000
Error [2]	24		

Error [1] was used for comparing the mean levels of non-repeating factors when the interaction of the non-repeating factor with the repeated factor was not important. Error [2] was used for com-paring the mean levels of the repeated factor. * Tukey ($p \leq 0.05$).

Table 8. The mortality rates (%) of the 5th instar larvae of *Cydia pomonella* in contact efficacy bioassay of cell-fee supernatants of symbiotic bacteria of the selected entomopathogenic species/isolates.

Symbiotic Bacteria	Hours (h) after Treatment		
	24 h	48 h	72 h
X. b. A54	25.0 ± 2.5 A [a] a [b]	40.0 ± 2.5 Ab	55.0 ± 5.0 Bc
X. b. A94	12.5 ± 3.1 Ba	50.0 ± 4.1 Ab	62.5 ± 4.1 Bb
P. l. A8	10.0 ± 3.8 Ba	30.0 ± 2.5 Ab	40.0 ± 2.5 Ab
P. l. A9	5.0 ± 2.5 Ba	15.0 ± 3.8 Bab	25.0 ± 3.8 Ab

X. b. A54 and X. b. A94: *Xenorhabdus bovienii*, P. l. A8 and P. l. A9: *Photorhabdus luminescens* subsp. *kayaii*. [a] Different capital letters show statistically significant differences among symbiotic bacteria species/strains for each exposure time level. [b] Different lowercase letters show statistically significant differences among the exposure time levels for each entomopathogenic nematode species ($p < 0.05$, Tukey).

4. Discussion

In the present study, the natural occurrence and distribution of EPNs in apple orchards of Kayseri Province, Türkiye, were determined in order to evaluate the biocontrol potential of EPNs and their bacterial associations against the larvae of *C. pomonella*. Two EPN species were isolated from twelve EPN populations with a recovery rate of 12%. Although the recovery rate of EPNs varied greatly in earlier surveys of EPNs in the world (0.2% to 70%) [45–47], the recovery rate found (12%) in this study was quite similar to those countries in the Mediterranean region such as in Italy (13.8%) [48], in Cameroon (10%) [49], and in Egypt (9.5%) [50]. However, lower and higher prevalence of EPNs were also reported in different countries [51,52]. The difference in recovery rates of EPNs may be associated with different surveying times and methods, and monoculture crop production. Kary et al. [52] reported that sampling times had a significant effect on the recovery of EPNs and the highest recovery frequency was generally obtained in spring (April and May). *Galleria mellonella* is the most used insect in the isolation and pathogenicity testing studies of EPNs due to its susceptibility to EPNs and convenience for in vitro mass culture. However, EPNs differ in host and habitat preference, and this may be another factor behind the varying recovery rates of EPNs [46,52–54].

Although the survey area in this study was limited to apple orchards, seven *S. feltiae* and five *H. bacteriophora* populations were obtained. These results agree with most EPN surveys conducted in Türkiye with similar climatic conditions [55,56]. *Steinernema feltiae* and *H. bacteriophora* are the most frequently detected EPN species in Türkiye and other parts of the world, respectively [57–60]. However, some studies reported a higher occurrence of *H. bacteriophora* than *S. feltiae* from neighboring regions with similar climatic and geographic conditions in Türkiye [61,62]. Although both EPN species have been found in different climatic zones, *S. feltiae* was more dominant in cold and continental climate conditions, whereas *H. bacteriophora* was generally isolated in higher frequencies from tropical and subtropical areas [51,63,64]. The diversity and distribution of EPNs are also related to the presence of suitable insect hosts as well as climatic and environmental conditions. This might be another factor affecting the distribution of EPN species in varying frequencies on Earth [65].

The morphometric measurements of the IJs of *S. feltiae* and *H. bactriophora* isolates showed close similarity with the findings of Nguyen and Hunt [36] and Yüksel and Canhilal [45]. However, they also differed in several morphometric features such as body and tail length, and maximum body width reported by Stock et al. [59] and Laznik et al. [60]. The differences in these measurements might be due to habitat and intraspecific variations among the geographical isolates of EPN species as reported in earlier studies [66–69]. Therefore, in addition to morphometric measurements, the isolates were further confirmed by using molecular methods based on the nucleotide sequences of the ITS region.

Pathogenicity screening of high numbers of candidate biological control agents is a crucial step to revealing the potential of EPN species/isolates and also helps save resources and time before initiating field evaluations [69]. In the present study, preliminary tests were performed on the last instar larvae of *G. mellonella*, and variable mortalities were obtained after 72 h of exposure. Differences in larval mortality among the same EPN and symbiotic bacteria species can be explained by the secondary metabolites that are released by the symbiotic bacteria. Hasan et al. [70] tested the toxicity of different *X. nematophila* on *Spodoptera exigua* (Hübner) (Noctuidae: Lepidoptera) and *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae). They reported a variation in associated secondary metabolites' pathogenic and immunosuppressive activities, which is in line with our study. In addition, a great variation in chemical components among the six bacterial strains of *X. nematophila* was reported by Hasan et al. [70]. Different efficacies of the same EPN and symbiotic bacteria species might be due to variations in chemical components and toxin complexes released by symbiotic bacteria. The most virulent EPN species/isolates on *G. mellonella* were further tested on the last instar larvae of *C. pomonella*. The results revealed that all selected EPN species/isolates were highly pathogenic to *C. pomonella* larvae. However, at concentrations of 50 IJs/larvae, *H. bacteriophora* isolates were more virulent than *S. feltiae* isolates and induced maximum mortality (100%) 72 h after treatment. Contrary to *Steinernema* species, *Heterorhabditis* species possess an additional dorsal tooth that facilitates penetration into a host's body [71]. In addition, the IJs of *Heterorhabditis* species have a smaller body size than *Steinernema* species and this might have enabled them to more easily penetrate the host's body using natural openings [72]. Lacey and Unruh [73] tested the susceptibility of *C. pomonella* larvae against *S. carpocapsae*, *S. riobrave*, and *H. bacteriophora* at 50 and 100 IJs/cm^2 and reported that *H. bacteriophora* was more efficient than *S. riobrave*. However, *S. carpocapsae* performed better than *H. bacteriophora*. In another study conducted by Yağci et al. [22], *S. carpocapsae* and *S. feltiae* exhibited superior virulence to *H. bacteriophora* against the first larvae of *C. pomonella*, which is contradictory to the findings of this study. In a previous study evaluating the field performance of different EPN species against *C. pomonella* larvae, *S. feltiae* and *S. carpocasae* caused similar mortalities at concentrations of 50 IJs/mL [12]. The differences in the larval mortalities may be explained by the variation in the experimental setups such as IJs concentrations, application environment, and tested larval instars as well as different environmental conditions. The virulence of EPN species/isolates varies greatly depending on the host-seeking behaviors, adaptation capabilities, host specificity, and symbiotic association of IJs [23,59,65]. Among these factors,

symbiotic bacteria of EPNs play a crucial role in the pathogenicity process by releasing a wide range of secondary metabolites into host hemolymph [29]. Therefore, in the present study, the cell-free supernatants of bacterial symbionts of the most pathogenic EPN species were also evaluated on *C. pomonella* larvae. To our knowledge, this is the first report on the insecticidal effect of the cell-free supernatants of *Xenorhabdus* and *Photorhabdus* bacteria species on *C. pomonella* larvae.

Based on the identity of the 16S rRNA sequence and BLAST analysis, the symbiotic bacteria of selected *S. feltiae* and *H. bacteriophora* isolates were identified as *Xenorhabdus bovienii* and *Photorhabdus luminescens* subsp. *kayaii*, respectively. The results revealed that the cell-free supernatants of *X. bovienii* and *P. luminescens* subsp. *kayaii* strains were lethal to the fifth instar larvae of *C. pomonella* in the contact efficacy bioassay. However, *X. bovienii* strains exhibited superior mortality against *C. pomonella* larvae causing mortalities over 50% 72 h after treatment. The insecticidal effect of *Xenorhabdus* and *Photorhabdus* bacteria species has been shown against different insect pests in earlier studies and varying degrees of pathogenicity have been reported. Mahar et al. [74] reported that the cell-free supernatant application of *X. nematophila* resulted in 95% mortality in the larvae of *Spodoptera exigua* (Hübner) (Noctuidae: Lepidoptera). In another study, the larvae of *Earias vittella* (F.) (Lepidoptera: Noctuidae) were exposed to the cell-free supernatants of *X. nematophila* and *P. luminescent*, and the mortality rates varied between 65 and 70% 72 h after treatment [75]. Vicente-Díez et al. [76], in their study, tested the efficacy of bacterial secretions of *X. bovienii* on the third larval instar of *Lobesia botrana* (Den. & Schiff.) (Lepidoptera: Tortricidae) and reported 50% mortality 3 days after treatment which is in line with our study. In contrast to the aforementioned studies, Shawer et al. [77] reported lower larval mortality (24%) in the larvae of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) after treatment with *P. luminescens* supernatant. The variation in the mortality rates can be explained by the different toxin complexes produced by *Xenorhabdus* and *Photorhabdus* species/strains as well as differences in the experimental design and larval instars of tested insects [78]. Toxin complexes and secondary metabolites play a major role in the suppression of the host immune system as well as toxicity to the host intestine [76–78]. Earlier studies reported that bacterial toxin complexes such as Tca, Tcb, and Tcc toxins produced by *P. luminescens* were more toxic to the tested insect when digested orally [25–27,78–81]. However, in the present study, cell-free supernatants were sprayed directly onto *C. pomonella* larvae, and this may have restrained the effectiveness of *P. luminescens* subsp. *kayaii* strains. Similar to our findings, the direct application of cell-free supernatants proved to be lethal against *Earias vittella* (F.) (Lepidoptera: Noctuidae) [75], *Aphis gossypii* (Glov.) (Hemiptera: Aphididae) [82], *Macrosiphum rosae* (L.) (Hemiptera: Aphididae) [83], and *Philaenus spumarius* (L.) (Hemiptera: Cercopidae) [76]. The results of the aforementioned studies indicated that secondary metabolites of different *Xenorhabdus* and *Photohabdus* species were able to penetrate through the cuticle and cause remarkable mortalities against tested insects with a varying degree of pathogenicity [44,75,76,83]. Chitinase protein secreted by *Xenorhabdus* and *Photorhabdus* bacteria plays a key role in the virulence of symbiotic bacteria by both degrading the cuticles of insects and accelerating the binding process of toxins to target sites [84,85]. The results suggest that toxic metabolites including chitinase protein are present in the cell-free supernatant of symbiotic bacteria and are responsible for mortality. In addition, Hasan et al. [70] reported that the chemical composition of cell-free supernatants produced by different bacterial strains exhibited great variation. This might be another factor causing varying mortalities in *C. pomonella* larvae by different strains of the same symbiotic bacteria.

5. Conclusions

Surveyed apple orchards yielded twelve EPN isolates belonging to *S. feltiae* and *H. bacteriophora* species. The two most pathogenic isolates of EPN species on *G. mellonella* larvae were further tested for their biocontrol potential on the fifth larval instar of *C. pomonella*. Selected EPN isolates achieved mortalities over 80% at 50 IJs/larva 48 h after treatment.

Bacterial associates of selected EPN isolates were identified as *X. bovienii* and *P. luminscens* subsp. *kayaii*. Direct exposure to the cell-free supernatants of *X. bovienii* and *P. luminscens* subsp. *kayaii* strains were lethal to the fifth larval instar of *C. pomonella*, and the highest mortalities were obtained from *X. bovienii* strains 72 h after application. The results revealed that selected EPN species/isolates and their enteric bacteria have potential in the control of *C. pomonella*. The results also indicate that the cell-free supernatants of *X. bovienii* and *P. luminescens* subsp. *kayaii* strains can be utilized separately from their nematodes against *C. pomonella* larvae. However, further studies are required to reveal the field performance of tested EPN species/isolates and their endosymbiotic bacteria.

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Life Table Parameters of the Tomato Leaf Miner *Tuta absoluta* (Lepidoptera: Gelechiidae) on Five Tomato Cultivars in China

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Simple Summary: As a newly invasive pest in China, the moth *Tuta absoluta* has spread extremely quickly, and now causes serious harm to the Chinese tomato industry. Understanding the resistance of *T. absoluta* to different commercially processed tomato cultivars can improve the integrated management strategy using resistant plants. In this study, we found four processed tomato cultivars (Th9, Th1902, Heinz1015, and Dimen2272) that have higher resistance to *T. absoluta* than fresh tomato (Dafen). Among the four processed tomato cultivars, Th9 was the most suitable cultivar, as *T. absoluta* showed the higher *r* value on Th9 than on the other three processed tomato cultivars.

Abstract: Tomato is the most preferred host plant for *Tuta absoluta*, a newly emerged devastating invasive pest in China. However, no study has evaluated the damage risk of *T. absoluta* on processed tomato worldwide. In the current study, the life table parameters of *T. absoluta* were systematically investigated on five tomato cultivars (one fresh tomato cultivar, four processed tomato cultivars) to determine their susceptibility to *T. absoluta* infestation. *T. absoluta* had a better population growth ability on the fresh tomato, "Dafen", showing shorter duration of the preadult stage, higher lifetime fecundity, and a higher intrinsic rate of increase compared to four processed tomato cultivars. Meanwhile, the life table parameters of *T. absoluta* among different processed tomato cultivars also showed significant differences. Th9 was the most susceptible to *T. absoluta* attack, while Th1902, Heinz1015, and Dimen2272 were the least suitable ones for its development and reproduction. In summary, these tomato cultivars are the most recommended for commercial tomato production to reduce the damage caused by *T. absoluta* and improve the integrated pest management strategy.

Keywords: life table; duration of preadult stage; lifetime fecundity; adult longevity

1. Introduction

The South American tomato leafminer *Tuta absoluta* (Meyrick) is native to Peru, western South America [1]. After invading Spain in 2006, it spread rapidly throughout Afro-Eurasia and has since become a major threat to the world's tomato production [2–5]. *Tuta absoluta* has been considered among the most devastating pests of both open field and greenhouse tomatoes worldwide [6], which can attack all the aboveground plant parts and all the developmental stages (seedling and mature) of the tomato plant, resulting in considerable

reduction in yield and quality of the fruits [1]. Its larvae mine and feed on leaves, stems, petioles, and even bore into the tomato fruits when the population density is high [4,5]. Once it has invaded a new country, *T. absoluta* can not only damage the tomato plants and fruits, directly resulting in economic costs, but it can also indirectly affect the international trade of tomato as the quarantine restrictions are established by importing countries [3,5].

Host range is constrained by the behavioral and physiological traits of the insect [7]. Since 2006, many researchers had paid attention to the biological performance and oviposition performance of *T. absoluta* on different plants (different *solanum* plants or different tomato cultivars) across the world, such as in South America [8–11], Europe [12–15], Asia [16–22], and Africa [23–25]. The general result was that tomato is the most preferred host plant for *T. absoluta* oviposition and is suitable for larval development [4]. However, some *solanum* plants or tomato cultivars can reduce the developmental capacity of the pest without requiring any technical skill on the part of the farmer, so it is a potential pathway to reduce chemical control of *T. absoluta* [4,6]. Resistance may be the result of the plant's phytohormonal system, triggered when the plant is attacked by herbivores [26,27]. Meanwhile, the use of resistant or tolerant cultivars could be part of an integrated management strategy [28].

China is the largest tomato production region in the world. In 2021, China's tomato planting area reached 1.11 million hectares, producing 67.63 million tons, accounting for 36% of the world's total tomato production [29]. *Tuta absoluta* was first found in the Ili country of China in 2017 [30] and, after that, its population quickly expanded and was established in more than 10 provinces in China [31,32]. So far, based on the field investigation, *T. absoluta* could damage four cultured crops (tomato, eggplant, potato, and Chinese lantern) and two wild plants (black nightshade and Dutch eggplant) in China [31]. As a result, it has seriously threatened tomato production in China. Meanwhile, China is the world's largest exporter of processed tomatoes, accounting for 23%, according to statistics from the World Processed Tomato Council in 2022. At present, the most widely planted processed tomato varieties in China are Heinz1015 (Heinzseed division of Kraft Heinz foods company, Stockton, Canada), Dimen2272 (Del Monte Foods company, Walnut Creek, CA, USA), Th9, and Th1902 (Cofco Tunhe Seed Company, Changji, China). However, there has been no study to assess the risk of *T. absoluta* damage to processed tomatoes in the world.

We hypothesized that different tomato cultivars have different genetic backgrounds and could show different resistant levels to *T. absoluta*. In the current study, the main commercial fresh and processed tomato cultivars available in China were collected, and the life table parameters of *T. absoluta* on different tomato cultivars were systematically investigated using the age-stage, two-sex life table method to determine their level of susceptibility to *T. absoluta* infestation.

2. Materials and Methods

2.1. Insects

The original population of *T. absoluta* was collected at Yangrui Organic Agriculture Base, Hongta District, Yuxi City, Yunnan Province, in December 2018. The insect larvae were reared on *Lycopersicon esculentum* ("Dafen" Tomato, Shandong Shouhe seed Industry Co. Ltd., Jinan, China) and kept in an artificial growth chamber (26 ± 1 °C, a photoperiod of 14 L:10 D, and relative humidity (RH) of 65 ± 10%) in the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (IPPCAAS), Beijing.

2.2. Tomato Cultivars

This study selected four main processed tomatoes grown in Xinjiang as the host plants for the experiment, and used a type of fresh tomato, "Dafen", raised with the laboratory *T. absoluta* population as a control. The processed tomato cultivars are "Th1902", "Th9", "Dimen2272", and "Heinz1015". All healthy plants were cultivated and provided in the greenhouse of IPPCAAS. These four cultivars of tomato were grown in a potting mix (a

mixture of nutrient soil and vermiculite = 2:1) in a plastic square basin (10 × 10 × 10 cm). When the effective true leaves of all host plants grew to 5–7 leaves, they were used for the experiments. None of the tomato plants used in the experiment had been exposed to chemical pesticides.

2.3. Life Table Study

Five tomato cultivars ("Dafen", "Th1902", "Th9", "Dimen2272", and "Heinz1015") were used as the host plant for *T. absoluta* and placed in a net cage (40 × 40 × 60 cm, each plant must have 5–7 or more real leaves). Between 20:00 and 22:00, 50 pairs of *T. absoluta* adults (preferably the newly emerged individual) were placed in each cage, allowing the female to lay eggs. After 4 h, *T. absoluta* were removed. The next day at 8:00, 100 eggs were randomly selected for each treatment. All eggs were put on fresh tomato leaves (one egg per leaf) and maintained in a plastic petri-dish (9 cm); then, they were incubated in an artificial climate chamber as previously described until incubation. The hatching status of the eggs and the survival of individual *T. absoluta* was observed daily at 20:00 under a dissecting microscope (Zeiss, Oberkochen, Germany). During the observations mentioned above, the humidity of the leaves was maintained, and the leaves were timely replaced to ensure an adequate food supply for the larvae. After pupation, the sex of the pupae was determined, and the pupal duration was recorded. After eclosion, the newly eclosed male and female adults were paired in a 1:1 ratio in a specially designed cylindrical plastic device (20 cm height, 10 cm diameter) and placed in a transparent plastic bowl filled with water at the bottom to moisturize the plants below. The base of the tomato plant branch with 3–5 leaves excised from the clean tomato plant was wrapped with cotton and placed in the plastic bowl filled with water, then the foliage portion was enclosed in the cylindrical plastic device described by Zhang et al. [32], in which newly eclosed *T. absoluta* adults were released. We provided each branch with fresh tomato leaves for oviposition and recorded the eclosion rate, the number of eggs, and developmental duration of adult *T. absoluta* until all individuals died. During this experiment, the replication of the treatments of *T. absoluta* on different host plants was 20. The life table studies of all cultivars were conducted under identical conditions and during the same time period.

2.4. Life Table Analysis

The life table raw data of *T. absoluta* were analyzed according to the age-stage, two-sex life table theory and the method described by Chi with the computer program TWOSEX-MSChart [33]. The age-stage life expectancy (e_{xj}), reproductive value (v_{xj}), and the intrinsic rate of increase (r) were calculated according to Chi and Liu [34].

The survival rate (s_{xj}) (x = age and j = stage) is defined as the probability that a newly laid egg will survive to age x and stage j. The age-specific survival rate (l_x) was calculated as follows:

$$l_x = \sum_{j=1}^{m} s_{xj}$$

where m is the number of stages. The age-stage specific fecundity (f_{xj}) and age-specific fecundity (m_x) were calculated as follows:

$$m_x = \frac{\sum_{j=1}^{m} s_{xj} f_{xj}}{\sum_{j=1}^{m} s_{xj}}$$

The net reproductive rate (R_0) is defined as the total number of offspring that an individual can produce during its lifetime and was calculated as follows:

$$R_0 = \sum_{x=0}^{\infty} l_x m_x$$

The intrinsic rate of increase (r) was calculated using the Lotka–Euler equation with age indexed from 0, and was calculated as follows:

$$\sum_{x=0}^{\infty} e^{-r(x+1)} l_x m_x = 1$$

The finite rate (λ) was calculated as follows:

$$\lambda = e^r$$

The mean generation time (T) represents the period that a population is required to increase to R_0-fold of its size as time approaches infinity and the population settles down to a stable age-stage distribution. The mean generation time was calculated as follows:

$$T = \frac{\ln R_0}{r}$$

2.5. Population Projection

To predict the population growth rate and the age-stage structure of 10 *T. absoluta* eggs over the next 60 days, the TIMING-MSChart program [35] was utilized. This prediction was based on multiple data inputs, including the hatching rate, survival rate, fecundity, and duration of each stage [34,36].

2.6. Statistical Analysis

The life history raw data of all *T. absoluta* individuals were analyzed based on the age-stage, two-sex life table using the TWOSEX-MSChart program [33]. The development duration and reproductive (oviposition and fecundity) parameters between different host plants were compared by using one-way ANOVA, complemented with the Tukey's HSD method in SPSS statistics 26.0 version. Due to the non-normal distribution of the offspring sex ratio, the Kruskal–Wallis test was used for analysis. Moreover, according to Akca et al. [37], we used 100,000 re-samplings to obtain stable estimates of standard errors. A paired bootstrap test was used to detect statistical differences on r, λ, R_0, *GRR*, and Doubling time of *T. absoluta* on different host plants. These procedures were embedded in the TWOSEX-MSChart program.

3. Results

3.1. Developmental Duration, Fecundity, and Offspring Sex Ratio of T. absoluta on the Five Tomato Cultivars

Tuta absoluta was able to complete its life-cycles when reared on different tomato cultivars. Tomato cultivar significantly affected the durations of the egg stage ($F_{4,225} = 62.549$, $p < 0.05$), larva stage ($F_{4,225} = 225.203$, $p < 0.05$), pupa stage ($F_{4,225} = 10.375$, $p < 0.05$), and preadult stage ($F_{4,225} = 82.511$, $p < 0.05$) of *T. absoluta*. For the egg stage, the longest duration was generated on Th9, and the shortest on Th1902. The durations of the larval stage and preadult stage of *T. absoluta* feeding on four cultivars of processed tomato were significantly longer than those feeding on Dafen. The longest durations of the larval stage and preadult stage of *T. absoluta* were generated on Th1902, respective up to 12.84 days and 22.74 days. The shortest durations of two stages of *T. absoluta* were generated on Dafen, up to 8.98 days and 19.14 days. Of the pupal stage, the longest pupal duration was seen on Heinz1015, and the shortest on Dimen2272.

The tomato cultivar also significantly affected the adult longevity (male: $F_{4,111} = 8.036$, $p < 0.05$; female: $F_{4,113} = 8.655$, $p < 0.05$), lifetime fecundity ($F_{4,113} = 44.039$, $p < 0.05$), and oviposition period ($F_{4,113} = 15.239$, $p < 0.05$), but did not change the offspring sex ratios ($H_{4,114} = 3.962$, $p = 0.411$). The shortest longevities of male and female adults were generated on Dimen2272, up to 9.83 days and 10.33 days, respectively. However, the longest longevities of male and female adults were seen on Dafen and Th1902 (Table 1). Simi-

larly, the highest lifetime fecundity of *T. absoluta* was generated by Dafen (up to 172 eggs), while the lowest was seen on Dimen2272 (up to 44.88 eggs). The oviposition period of *T. absoluta* feeding on Th9, Th1902, and Heinz1015 was, respectively, 10.32 ± 0.74 days, 9.74 ± 0.87 days, and 8.10 ± 0.81 days, which were significantly longer than that of *T. absoluta* feeding on Dafen. In addition, *T. absoluta* reared on different processed tomato cultivars exhibited a difference in its fecundity. The total numbers of eggs per female significantly varied from 131.32 eggs on Th9, 81.74 eggs on Th1902, 73.95 eggs on Heinz1015, and 45.13 eggs on Dimen2272 (Table 1).

Table 1. Durations of different developmental stages, lifetime fecundity, and offspring sex ratio (mean ± SE) of *Tuta absoluta* on five tomato cultivars.

Parameters	Dafen	Th1902	Heinz1015	Th9	Dimen2272
Duration of Egg stage	3.71 ± 0.06 c	3.03 ± 0.12 d	4.5 ± 0.16 a	4.86 ± 0.05 a	4 ± 0 c
Duration of Larva stage	8.98 ± 0.1 e	12.84 ± 0.13 a	10.35 ± 0.12 c	9.48 ± 0.08 d	11.21 ± 0.06 b
Duration of Pupa stage	6.45 ± 0.1 bc	6.87 ± 0.15 ab	7.25 ± 0.142 a	6.5 ± 0.11 bc	6.29 ± 0.08 c
Duration of preadult stage	19.14 ± 0.11 d	22.74 ± 0.20 a	22.10 ± 0.21 b	20.84 ± 0.17 c	21.50 ± 0.11 b
Adult male longevity	17.04 ± 0.86 a	15.74 ± 1.75 a	13.20 ± 1.02 ab	14.73 ± 0.72 a	9.83 ± 0.72 b
Adult female longevity	10.45 ± 0.56 c	16.26 ± 1.38 a	14.30 ± 0.98 ab	11.45 ± 0.70 bc	10.33 ± 0.80 c
Average oviposition period	5.00 ± 0.25 c	9.74 ± 0.87 a	8.10 ± 0.81 ab	10.32 ± 0.74 a	5.75 ± 0.51 bc
Lifetime Fecundity(eggs/female)	172.17 ± 7.50 a	80.89 ± 6.41 c	74.05 ± 9.24 cd	132.5 ± 11.22 b	44.88 ± 4.89 d
Offspring sex ratio (female/male)	1.07 ± 0.06 a	0.92 ± 0.05 a	1.01 ± 0.07 a	0.98 ± 0.04 a	1.10 ± 0.10 a

One-way ANOVA: Tukey's HSD. This was used to detect the differences between different hosts. Significant differences between different treatments of the same parameter are indicated by a, b, c, d, and e ($p < 0.05$).

3.2. Life Table Analysis

Based on the age-stage specific survival rate (S_{xj}) curves, the survival rate of *T. absoluta*, which feeds on five tomato cultivars from the egg to the pupa stage, maintained a high level, as seen in Figure 1. The lifespan of both male and female adults of *T. absoluta* feeding on Th1902 is longer than that of adults of *T. absoluta* feeding on other cultivars of tomato (Figure 1).

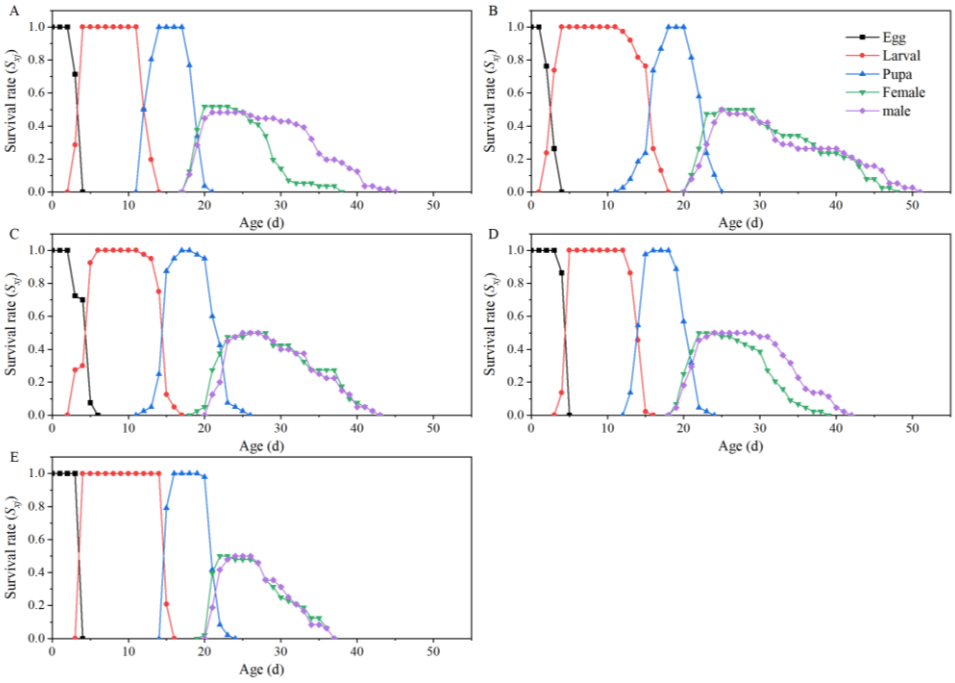

Figure 1. Survival rates at different developmental stages of *Tuta absoluta* feeding on different tomato cultivars. (**A**–**E**) The survival rates of *T. absoluta* after feeding on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272", respectively.

Moreover, there was an initial increase followed by a decrease in the female fecundity and age-specific reproduction of *T. absoluta* on all five tomato cultivars. The age-specific fecundity (m_x) attained the reproductive high point at day 20, 23, 23, 22, and 21 on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272", respectively. The highest m_x value recorded on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272" was 23.5, 5.4, 5.2, 8.7, and 6, respectively. On "Dafen", "Th9", and "Dimen2272", the m_x curve was demonstrated to have only one peak. However, on "Th1902" and "Heinz1015", more than one peak was observed in the m_x curve, suggesting a difference in the oviposition period of the individuals. The female fecundity curves for *T. absoluta* on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272" began on the 17th, 20th, 18th, 18th, and 19th days, respectively. The female fecundity parameters (f_x) of *T. absoluta* feeding on "Th9" were higher compared to the other three processed tomato cultivars. However, the f_x curve of *T. absoluta* feeding on four processed tomato cultivars was lower than that of *T. absoluta* feeding on "Dafen" (Figure 2).

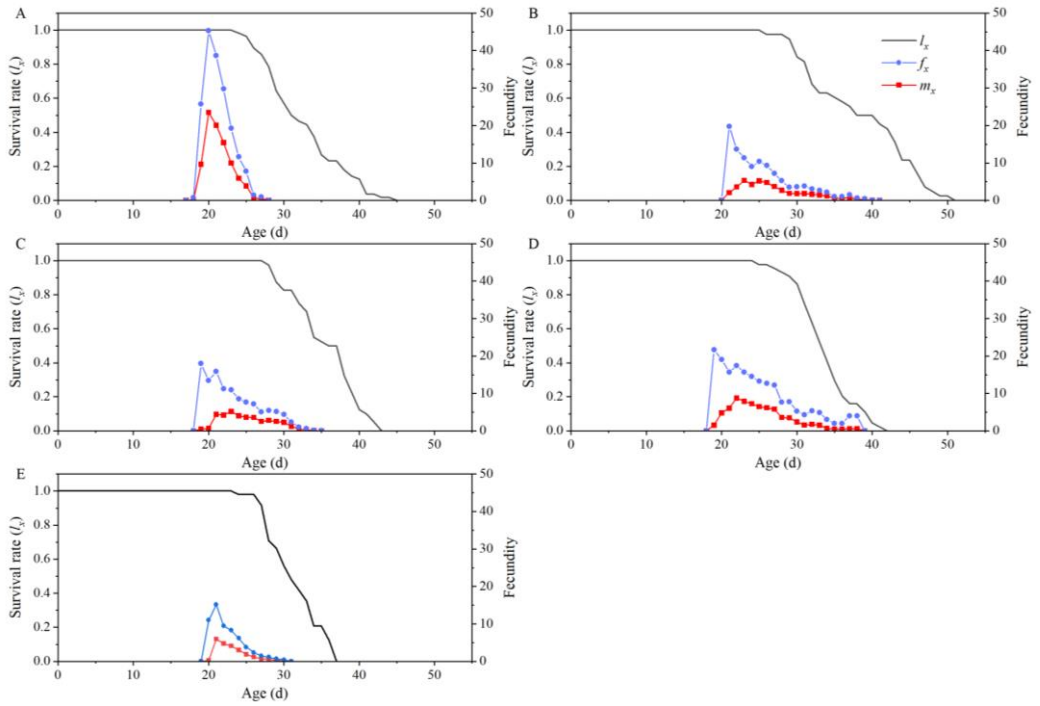

Figure 2. Age-specific survival rates, age-specific fecundity, and female fecundity of *Tuta absoluta* feeding on different tomato cultivars. (**A**–**E**) The reproduction rates and survival rates of *T. absoluta* feeding on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272", respectively. l_x: Population survival rate; m_x: population fecundity; f_x: female fecundity.

The age-stage-specific reproductive values (v_{xj}) of *T. absoluta* increased significantly when adults emerged (18 days on "Dafen", 21 days on "Th1902", 19 days on "Heinz1015", 19 days on "Th9", and 20 days on "Dimen2272"). The reproductive values of *T. absoluta* feeding on "Th9" were significantly higher compared to the other three processed tomato cultivars. Furthermore, as the lifespan of adults increases, reproductive values of *T. absoluta* feeding on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272" gradually decreases to 0 on the 28th, 41st, 35th, 39th, and 31st days, respectively (Figure 3).

The age-stage life expectancy (e_{xj}) refers to the amount of time an individual at ages x and stages j will continue to survive. Therefore, lifespan gradually decreases with age x until it reaches 0. The results revealed that the life expectancy at age 0 (e_{01}) for *T. absoluta* feeding on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272" was 32.77, 38.74, 35.85, 33.93, and 31.58 days, respectively (Figure 4).

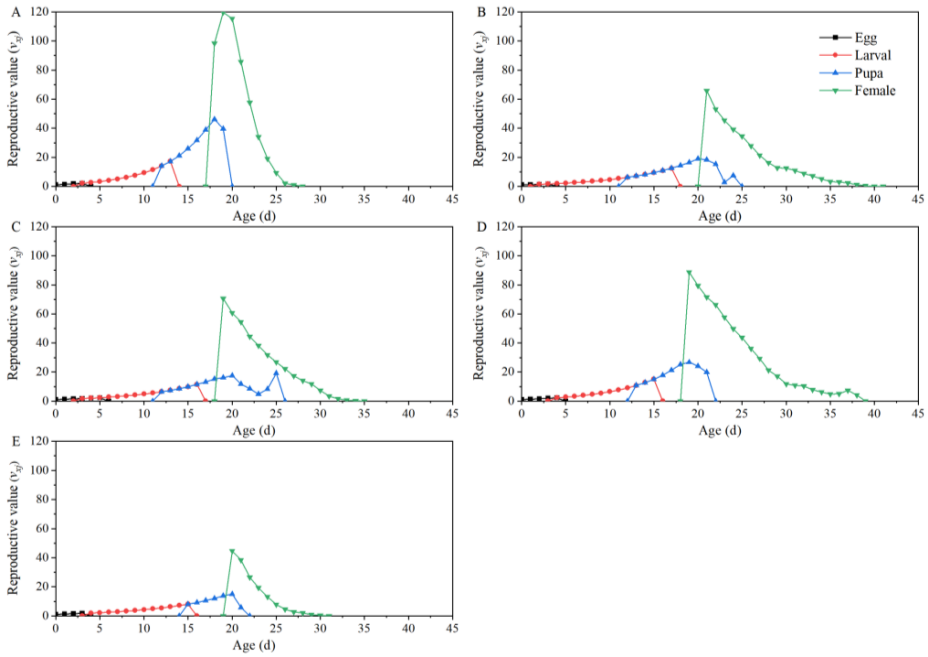

Figure 3. The reproductive value at each development stage of *Tuta absoluta* feeding on different tomato cultivars. (**A**–**E**) The life expectancy values for *T. absoluta* after feeding on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272", respectively.

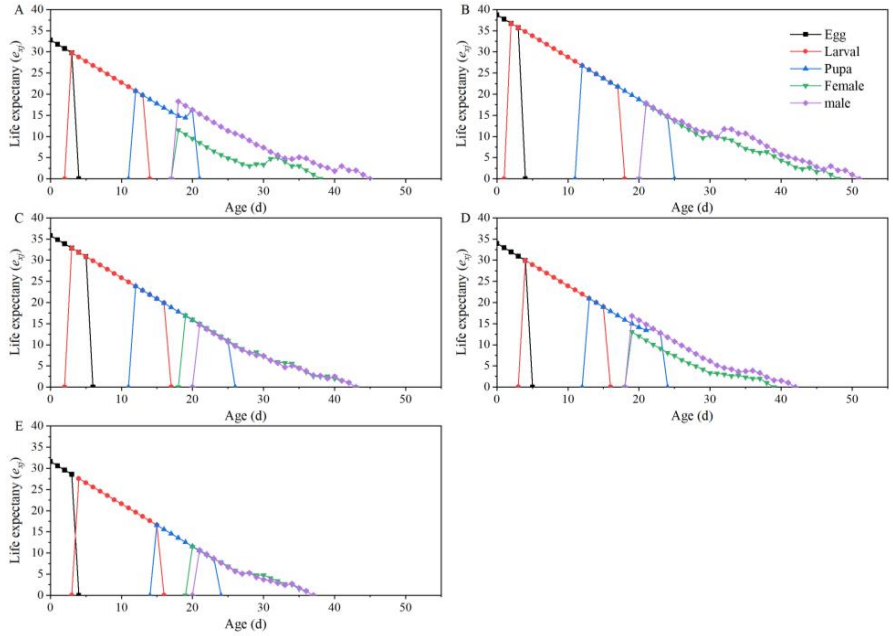

Figure 4. The life expectancy values at each development stage of *Tuta absoluta* feeding on different tomato cultivars. (**A**–**E**) The life expectancy values for *T. absoluta* after feeding on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272", respectively.

The mean of net reproductive rate (R_0), gross reproduction rate (*GRR*), intrinsic rate of increase (r), and finite rate of increase (λ) of the *T. absoluta* population feeding on "Th9" were found to be 66.24 ± 11.43, 71.43 ± 12.99, 0.1690 ± 0.0073, and 1.1531 ± 0.0095, respectively. These values were higher than those observed for *T. absoluta* feeding on the other three processed tomato cultivars. However, these values of *T. absoluta* feeding on four processed tomato cultivars were lower than those of *T. absoluta* feeding on "Dafen" (paired bootstrap test, $p < 0.05$). The mean generation time of the *T. absoluta* population feeding on

"Dimen2272" was 23.74 ± 0.17, which was the lowest among the four processed tomato cultivars (paired bootstrap test, $p < 0.05$). Additionally, the population doubling time of *T. absoluta* feeding on "Th9" was 4.09 days, which was the lowest among all four processed tomato cultivars. However, the population doubling time of *T. absoluta* feeding on "Dafen" was only 3.41 days (Table 2).

Table 2. The population parameters (mean ± SE) of *Tuta absoluta* on five tomato cultivars.

Parameters	Dafen	Th1902	Heinz1015	Th9	Dimen2272
Net reproductive rate (R_0)	89.16 ± 12.10 a	40.45 ± 7.24 bc	37.03 ± 7.40 cd	66.25 ± 11.43 ab	22.44 ± 4.02 d
Gross reproduction rate (*GRR*)	89.54 ± 12.17 a	44.10 ± 8.16 bc	38.25 ± 7.65 cd	71.43 ± 12.99 ab	22.98 ± 4.16 d
Intrinsic rate of increase (r, per day)	0.2034 ± 0.0063 a	0.1404 ± 0.0073 c	0.1432 ± 0.0082 c	0.1696 ± 0.0073 b	0.1310 ± 0.0076 c
Finite rate of increase (λ, per day)	1.2256 ± 0.0077 a	1.1507 ± 0.0084 c	1.1539 ± 0.0095 c	1.1848 ± 0.0086 b	1.1400 ± 0.0086 c
Mean generation time (days)	22.07 ± 0.17 d	26.36 ± 0.44 a	25.22 ± 0.34 b	24.73 ± 0.26 b	23.74 ± 0.17 c
Doubling time (days)	3.41 ± 0.11 c	4.94 ± 0.27 a	4.84 ± 0.29 a	4.09 ± 0.18 b	5.29 ± 0.32 a

One-way ANOVA: Tukey's HSD. This was used to detect the differences between different hosts. Significant differences between different treatments of the same parameter are indicated by a, b, c and d ($p < 0.05$).

3.3. Prediction of the Population Growth of T. absoluta on the Five Tomato Cultivars

The population growth dynamics of *T. absoluta* feeding on five tomato cultivars were observed over a period of 60 days using 10 eggs from each *T. absoluta* population. The results showed that the selected 10 eggs of *T. absoluta* feeding on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272" could produce 90110.99, 14427.61, 13521.36, 42563.78, and 5059.66 offspring on the 60th day, respectively. The population of *T. absoluta* feeding on Th9 exhibited the fastest growth among all four processed tomato cultivars. The numbers of eggs, larvae, pupae, female adults, and male adults of *T. absoluta* feeding on Th9 on the 60th day were 6008.09, 29653.96, 6610.71, 100.08, and 190.94, respectively. After 60 days, the predicted population of *T. absoluta* feeding on Th9 was 2.95, 3.15, and 8.41 times higher compared to *T. absoluta* feeding on "Th1902", "Heinz1015", and "Dimen2272", respectively (Figure 5).

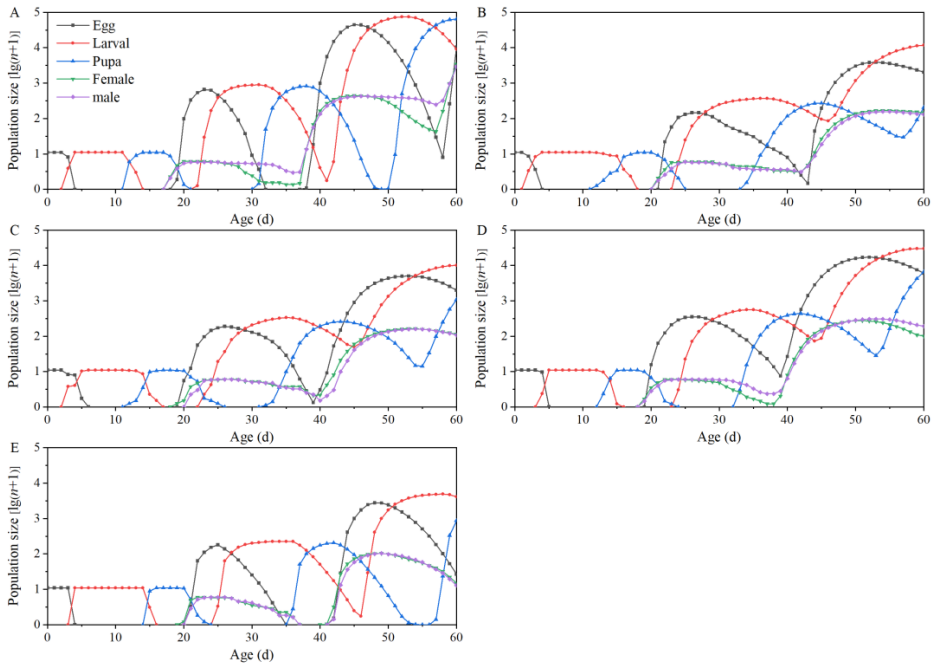

Figure 5. Population projection of *Tuta absoluta* feeding on five tomato cultivars by using the life tables of the original cohort with an initial population of 10 eggs. (**A**–**E**) The simulation of the population growth of *T. absoluta* after feeding on "Dafen", "Th1902", "Heinz1015", "Th9", and "Dimen2272", respectively.

4. Discussion

This is the first study investigating biological performance using a life table of the tomato pinworm on processed tomato plant species and fresh tomato plants. Our results indicated that *T. absoluta* can successfully complete its life-cycles on five tomato cultivars. Different tomato cultivars significantly affected the different developmental stages of *T. absoluta*. The shortest duration of the preadult stage generated on Dafen (up to 19 days), and the longest was seen on Th1902 (more than 22.74 days). These results were consistent with previous research. Rostami et al. [17] indicated that the durations of the preadult stage of *T. absoluta* generated significant differences among three tomato cultivars (Falkato, Grandella, and Isabella). Ghaderi et al. [18] found that tomato cultivars significantly affected the development of the preadult stage of *T. absoluta* compared with seven tomato cultivars in Iran. The longest duration of the preadult stage was found to be Early Urbana Y (up to 26.42 days), and the shortest was Cal JN3 (about 20.83 days). Meanwhile, Krechemer and Foerster [10] also found similar results by comparing the life history traits among six different tomato cultivars in Brazil in a quite low environmental temperature (20 ± 2 °C). In general, a host plant that provides the conditions for a faster development time will allow more generations of that pest to occur in the field. This means that this host plant could be the dominant plant for this pest, which is conducive to a rapid increase in the pest population. That is to say, it is more susceptible to damage from this pest. On the other hand, if a longer duration of the preadult stage was generated, it could increase the exposure to natural enemies and affect female individuals by forcing immature individuals to consume food resources of lower nutritional quality [38,39].

In the current study, *T. absoluta* produced the highest lifetime fecundity (up to 172 eggs) and the shortest female adult longevity (about 10.45 days) on Dafen, and showed the lowest lifetime fecundity on Dimen2272 (about 44.88 eggs) and the longest female adult longevity on Th1902 (up to 16.26 days). Similarly, these results also confirmed that Dafen, as a typical fresh tomato cultivar, is a more susceptible host plant than the rest of the four processed tomato cultivars. However, the lifetime fecundity of *T. absoluta* on Dafen was quite lower than the results in Younes et al. [25] and Heidari et al. [21]. By comparing with the detailed materials and methods of this research, we suggested that the reason for this difference could be that honey solution was absent in our experiment. It is well known that supplemental honey solution can not only increase the lifetime fecundity, but also prolong the adult longevity [40–42]. Moreover, among the four processed tomato cultivars, *T. absoluta* showed the highest lifetime fecundity on Th9 (up to 132 eggs), and the lowest on Dimen2272 (less than 45 eggs). It seems that the four processed tomato cultivars in our study showed quite different levels of resistances to *T. absoluta*.

To assess host plant resistance levels to insects and insects suitedness to host plants, the life table parameters, especially the intrinsic rate of natural increase (r), are the most important usable parameters. r is strongly influenced by the duration of time from birth to the start of reproduction and, to a lesser extent, by survival and reproduction rates [43,44]. Hence, it is a very useful index for evaluating the damage risk of a pest on different host plants. In this study, we found that the r value of *T. absoluta* was significantly affected by different tomato cultivars, and varied from 0.2034 d^{-1} on Dafen to 0.1310 d^{-1} on Dimen2272. Since *T. absoluta* had the shortest duration of the preadult stage, and the highest lifetime fecundity on Dafen, it is reasonable that the higher r value was generated on this cultivar. Conversely, the r values of *T. absoluta* on four processed tomato cultivars were significantly lower than on Dafen, which means that these processed tomato cultivars are unsuitable hosts and have higher resistance to *T. absoluta* compared to fresh tomato. Among the four processed tomato cultivars, Th9 was the most suitable cultivar as *T. absoluta* showed a higher r value on Th9 than on the other three processed tomato cultivars. This result was also reconfirmed by the expected population results, of which the predicted population of *T. absoluta* fed on Th9 after 60 days was 2.95, 3.15, and 8.41 times higher than that of *T. absoluta* fed on "Th1902", "Heinz1015", and "Dimen2272", respectively.

Furthermore, the *r* values on Dafen (0.2034 d^{-1}) and Th9 (0.1696 d^{-1}) were significantly higher than some previous research. Ghaderi et al. [18] found that the *r* value of *T. absoluta* significantly changed among seven tomato cultivars in Iran, ranged from 0.1052 d^{-1} on tomato (Early Urbana Y) to 0.1522 d^{-1} on tomato (Cal JN3). Heidari et al. [21] indicated the *r* values of *T. absoluta* varied from 0.111 d^{-1} on tomato (Atrak) to 0.147 d^{-1} on tomato (King ston). Similar results were also found for six tomato cultivars in Brazil [10]. By conducting in-depth comparative analysis of these references, it was found that the main reason could be that *T. absoluta* was supplemented with honey water in these studies, which led to a significant increase in its adult longevity, as the tomato plants and experimental temperature in this research are similar. Insects have typically been able to acquire nutrients from randomly distributed non-host food resources such as floral and extra-floral nectar, homopteran honeydew, pollen, and leaf trichome exudates in the field [40,45,46]. Strictly speaking, however, these are random events with low probability, not inevitable events [47,48]. Therefore, it may not be very scientific to continuously provide honey solution to *T. absoluta* in indoor experiments, and may even cause the results of the experiment to deviate from the real results to some extent.

5. Conclusions

In conclusion, we systematically assessed the hazard risk of *T. absoluta* to five commercial tomato cultivars in China using the age-stage, two-sex life table method, including four processed tomato cultivars, for the first time. *Tuta absoluta* had better population growth ability on the fresh tomato, "Dafen", showing shorter duration of the preadult stage, greater lifetime fecundity, and a higher intrinsic rate of increase compared to four processed tomato cultivars. Meanwhile, the life table parameters of *T. absoluta* among different processed tomato cultivars also showed significant differences. Th9 was the most susceptible to *T. absoluta* attack, while Th1902, Heinz1015, and Dimen2272 were the least suitable ones for its development and reproduction. Therefore, these tomato cultivars are the most recommended for commercial tomato production to reduce the damage caused by the tomato leafminer.

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Genome-Wide Identification and Expression Profiling of Candidate Sex Pheromone Biosynthesis Genes in the Fall Armyworm (*Spodoptera frugiperda*)

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Simple Summary: The fall armyworm (FAW), *Spodoptera frugiperda*, is a serious worldwide agricultural pest, threatening food security and crop production. Sex pheromone lures are commonly used in population monitoring and biological control of FAWs. Although the sex pheromone components of the FAW have been successfully identified, there are no reports on the molecular mechanism of FAW sex pheromone biosynthesis. In this study, we identified a total of 99 genes related to the biosynthesis of sex pheromones from the *S. frugiperda* genome, which belonged to 11 families of genes. Based on gene expression patterns and phylogenetic analysis, several genes had PG-biased expression, indicating that they may play an important role in sex pheromone biosynthesis. These results could lay a solid foundation for understanding the molecular mechanisms of *S. frugiperda* sex pheromone biosynthesis and provide new targets for developing novel pest control methods based on disrupting sexual communication.

Abstract: *Spodoptera frugiperda* is an agricultural pest causing substantial damage and losses to commercial crops. Sex pheromones are critical for successful mating in Lepidoptera and have been used for monitoring and control of many pest species. The sex pheromone of *S. frugiperda* is known, but the genes involved in its biosynthesis have not been identified. We systematically studied 99 candidate sex pheromone genes in the genome of *S. frugiperda* including 1 acetyl-CoA carboxylase (ACC), 11 fatty acid synthases (FASs), 17 desaturases (DESs), 4 fatty acid transport proteins (FATPs), 29 fatty acyl-CoA reductases (FARs), 17 acetyl-CoA acetyltransferases (ACTs), 5 acyl-CoA dehydrogenase (ACDs), 3 enoyl-CoA hydratases (ECHs), 3 hydroxyacyl-CoA dehydrogenases (HCDs), 6 ethyl-CoA thiolases (KCTs), and 3 acyl-CoA-binding proteins (ACBPs). Based on the comparative transcriptome results, we found 22 candidate sex pheromone biosynthesis genes predominately expressed in pheromone glands (PGs) than abdomens without PGs including *SfruFAS4*, *SfruFATP3*, *SfruACD5*, *SfruKCT3*, *SfruDES2*, *SfruDES5*, *SfruDES11*, *SfruDES13*, *SfruFAR1*, *SfruFAR2*, *SfruFAR3*, *SfruFAR6*, *SfruFAR7*, *SfruFAR8*, *SfruFAR9*, *SfruFAR10*, *SfruFAR11*, *SfruFAR14*, *SfruFAR16*, *SfruFAR29*, *SfruACT6*, and *SfruACT10*. A combination of phylogenetic and tissue-specific transcriptomic analyses indicated that *SfruDES5*, *SfruDES11*, *SfruFAR2*, *SfruFAR3*, and *SfruFAR9* may be key genes involved in the sex pheromone synthesis of *S. frugiperda*. Our results could provide a theoretical basis for understanding the molecular mechanisms of sex pheromone biosynthesis in *S. frugiperda*, and also provide new targets for developing novel pest control methods based on disrupting sexual communication.

Keywords: *Spodoptera frugiperda*; genome; sex pheromone gland; biosynthesis pathway

1. Introduction

Female Lepidoptera (moths) release sex pheromones to attract males for mating [1,2]. Most moth sex pheromones consist of two or more compounds combined in precise ratios with species specificity [3,4]. Based on the difference of their chemical structures and biosynthetic features, sex pheromones are classified into type I and type II [5]. Most known sex pheromones are type I, which are usually synthesized in female sex pheromone glands (PGs) situated in the intersegmental membrane between the eighth and ninth abdominal segments [5,6]. These pheromone components are mainly C10–C18 straight-chain compounds, containing 0–4 double bonds in different positions. The carbon chain ends have alcohol, ester, or aldehyde functional groups [7,8]. During type I sex pheromone biosynthesis, fatty acid intermediates such as palmitic acid or stearic acid are used as precursors. The double bond is generated by the desaturation system, and a short-chain reaction is carried out by a special β-oxidase system [9,10]. Oxidase, fatty acyl-CoA reductase, and acyl transferase catalyze the formation of functional groups such as esters, aldehydes, and alcohols to form a mixture of sex pheromones with specific component ratios and amounts. Acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), fatty acid transport protein (FATP), acyl-CoA dehydrogenase (ACD), 3-ketoacyl-CoA thiolase (KCT), hydroxyacyl-CoA dehydrogenase (HCD), enoyl-CoA hydratase (ECH), desaturase (DES), fatty acyl-CoA reductase (FAR), acetyl-CoA acetyltransferase (ACT), acyl-CoA-binding protein (ACBP) are the key enzymes in sex pheromone biosynthesis of moths [8,11,12].

The fall armyworm (FAW), *Spodoptera frugiperda*, native to tropical and subtropical America, is an agricultural pest. It has a wide plant host range, strong migratory ability, high reproductive capacity, and invaded sub-Saharan Africa, Asia, and other regions [13–15]. In China, the FAW was first found in Yunnan in January 2019. It then spread rapidly to many other food-producing areas in China and threatened food security and crop production [16,17]. Sex pheromone lures are usually used for population monitoring and biological control of FAW [18–22]. The sex pheromone components of FAW usually contain several acetates, including (Z)-9-Tetradeceny1 acetate (Z9-14:OAc) as a major component, (Z)-7-Dodecenyl acetate (Z7-12:OAc), (Z)-9-dodecenyl acetate (Z9-12:OAc), (Z)-11-hexadecell acetate (Z11-16:OAc), and trans-7-Dodecen acetate-1-yl acetate (E7-12:OAc); these acetate esters are all type-I sex pheromones [23–29]. Although the sex pheromone components of FAW have been successfully identified, there are no reports on the molecular mechanism of FAW sex pheromone biosynthesis.

In this study, we identified candidate FAW sex pheromone biosynthesis genes using previously published genome data. Then, we sequenced the transcriptome of PGs and ABs (abdomen without PGs) to analyze the expression of these candidate sex pheromone biosynthesis genes. Quantitative RT-PCR (qRT-PCR) was conducted to validate the transcriptome results. Based on the gene identifications, phylogenetic, and tissue-specific expression analyses, several genes were identified as potentially involved in FAW sex pheromone biosynthesis. Our results could provide potential targets for developing environmentally friendly control methods.

2. Materials and Methods

2.1. Insect Rearing and Tissue Collection

The original population of *S. frugiperda* was collected from a maize field of Dehong Dai and Jingpo Autonomous Prefecture, Yunnan Province, China. Larvae were reared with maize leaves in a growth chamber at (25 ± 1) °C, 55% relative humidity, with a 16:8 h (L:D) photoperiod. Adults were fed with 10% honey water. For the transcriptome sequencing and tissue expression study, 20–25 PGs (with the ovipositor) and 10–15 abdomens (without the PGs) were collected from 3 d old virgin female adults at 6–7 h into the scotophase [24,25]. The FAW shows particularly high mating activity at this time. Three biological replicates were conducted.

2.2. RNA Isolation, cDNA Library Construction, Illumina Sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. The quality and concentration of RNA samples were checked using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the RNA integrity was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Davis, CA, USA).

Oligo (dT) magnetic beads were used to purify mRNA from total RNA, and the mRNA was randomly interrupted in the NEB fragmentation buffer. Fragmented mRNA were used as templates to synthesize the first strand of cDNA using a random hexamer, followed by synthesis of the second strand, cDNA, by adding RNaseH, dNTPs, and DNA polymerase I. After end-repair, poly-A tailing, and ligation of adapters, the cDNA was purified by an AMPure XP system (Beckman Coulter, Beverly, MA, USA), and PCR amplification was performed. The constructed library was quality-tested with an Agilent 2100 Bioanalyzer (Agilent Technologies, Davis, CA, USA).

An Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) was used for sequencing. The raw reads were processed to remove low-quality reads, poly-N reads, and adapter reads to obtain the clean reads. Q20, Q30, and GC content were used to assess the sequencing quality. HISAT2 v.2.0.4 software was used to assemble and compare clean data and obtain annotations in the reference genome of *S. frugiperda* (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/011/064/685/GCF_011064685.1_ZJU_Sfru_1.0/, (accessed on 24 July 2020)). The RNA sequence data was uploaded to the NCBI platform (BioProject Accession: PRJNA885519). Fragments per kilobase of exon per million mapped reads (FPKM) were used to evaluate the expression level.

2.3. Identification and Analysis of Sex Pheromone Biosynthesis-Related Genes

The *S. frugiperda* genome database was obtained from NCBI (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/011/064/685/GCF_011064685.1_ZJU_Sfru_1.0/, (accessed on 24 July 2020)). The amino acid sequences of sex pheromone biosynthesis genes (ACCs, FASs, FATPs, ACDs, ECHs, HCDs, KCTs, DESs, FARs, ACTs, ACBPs) in *Spodoptera exigua*, *Spodoptera litura*, *Helicoverpa armigera*, *Helicoverpa assulta*, *Helicoverpa zea*, *Agrotis segetum*, *Agrotis ipsilon*, *Heliothis virescens* were used as query sequences (Table S1) for local BLASTP search (E-value cutoff of $<1 \times 10^{-5}$ and identity >30%) against the *S. frugiperda* genome database [8,30–38]. The incomplete sequences and duplicated genes were removed to obtain initial genes. All of these potential sex pheromone biosynthesis genes were then verified by BLASTP in NCBI with the E-value $< 1 \times 10^{-5}$ and identity >30%. The location of these genes was generated based on the genome annotation of *S. frugiperda* (BioProject Accession: PRJNA590312) using TBtools software (v1.0987671).

2.4. cDNA Synthesis and Full-Length cDNA Cloning

The cDNA was synthesized using the PrimeScriptTMRT reagent Kit with gDNA Eraser (Perfect Real Time) (TAKARA, Toyoko, Japan). Four differentially expressed genes (*SfurDES2*, *SfurDES5*, *SfurFAR2*, *SfurFAR3*) were randomly selected to amplify the full-length ORF sequence of these genes by using TransStart FastPfu Fly PCR Supermix (TransGen Biotech, Beijing, China). PCR conditions were: 5 min at 94 °C, followed by 40 cycles of 94 °C for 20 s, 20 s at 52 °C, and 45 s at 72 °C, followed by incubation at 72 °C for 10 min, carried out in a Bio-Rad thermocycler (Bio-Rad DNA Engine Peltier Thermal Cycler, Bio-Rad, Hercules, CA, USA). The primers were listed in Table S2, designed by Primer 5.0 software. The products were gel-purified and ligated into a pEASY-blunt vector (TransGen Biotech, Beijing, China). The ligation products were transformed into Trans T1 competent cells (TransGen Biotech, Beijing, China). All sequencing was performed by Tsingke Biotechnology Co., Ltd. (Beijing, China).

2.5. Phylogenetic Analysis

Phylogenetic trees were performed for SfruDESs and SfruFARs with their corresponding homologous genes from *S. exigua*, *S. litura*, *Sesamia inferens*, *A. pernyi*, *Ostrinia nubilalis*, and *H. assulta*, as reported previously [8,30,39]. The DES dataset included 17 sequences from *S. frugiperda*, and 41 from the other six species (12 from *S. litura*, 10 from *S. exigua*, 6 from *S. inferens*, 6 from *A. pernyi*, 3 from *O. nubilalis*, and 4 from *H. assulta*). The FAR dataset included 29 sequences from *S. frugiperda*, and 48 from six other insects (13 from *S. litura*, 13 from *S. exigua*, 3 from *S. inferens*, 11 from *A. pernyi*, 7 from *O. nubilalis*, and 1 from *H. assulta*) (Table S3). Amino acid sequences were aligned by ClatalW, and phylogenetic trees were constructed by MEGA X using the neighbor-joining method with position correction of distances and 1000 bootstrap replications. The final phylogenetic tree is displayed in the form of a circular tree diagram, and the color of each branch is labeled using FigTree v1.3.1.

2.6. Quantitative RT-PCR and Data Analysis

Four differentially expressed genes (*SfurDES2*, *SfurDES5*, *SfurFAR2*, *SfurFAR3*) were verified by qRT-PCR. Primers for qRT-PCR were designed by NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/ (accessed on 24 July 2020)). The 10-fold dilution series of cDNA from the PGs of *S. frugiperda* was used for a standard curve. The corresponding qRT-PCR efficiencies (E) were counted using the equation: $E = (10\,[-1/\text{slope}] - 1) \times 100$ [40] (Table S4). EF1α and RPS18 were selected as internal reference genes [41]. The qRT-PCR was performed on the ABI PRISM 7500 qRT-PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex TaqTM II (TaKaRa, Toyoko, Japan) under the following conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. Relative quantification was performed using the $2^{-\Delta\Delta CT}$ method [42].

3. Results

3.1. Identification and Localization of the Candidate Sex Pheromone Biosynthesis Genes in the S. frugiperda Genome

We identified 99 genes encoding pheromone biosynthesis from 11 gene families, including 1 ACC, 11 FASs, 4 FATPs, 5 ACDs, 3 ECHs, 3 HCDs, 6 KCTs, 17 DESs, 29 FARs, 17 ACTs, and 3 ACBPs (Table 1). To clarify the position of sex pheromone biosynthesis genes in chromosomes, chromosome location analysis of these genes was carried out. The results showed that except for two ACTs that were unplaced, the rest of the candidate sex pheromone biosynthesis genes were distributed across 23 chromosomes (Figure 1). ACC was located on chromosome 29. All of the KCT and HCD genes were distributed on only one chromosome (KCTs: chromosome 8 and HCDs: chromosome 16) (Figure 1). Four FATPs were found on chromosomes 18 (*SfruFATP1*, *SfruFATP2*, and *SfruFATP4*) and 24 (*SfruFATP3*) (Figure 1). Both of ECHs and ACBPs were located on three chromosomes (ECHs: chromosomes 6, 16, and 24; ACBPs: chromosomes 8, 9, and 28) (Figure 1). Seven FASs were clustered together on chromosome 20, followed by chromosomes 10 (*SfruFAS2* and *SfruFAS3*), 7 (*SfruFAS1*), and 17 (*SfruFAS4*) (Figure 1). Five ACDs were independently distributed on five chromosomes (1: *SfruACD4*, 2: *SfruACD5*, 6: *SfruACD2*, 20: *SfruACD1*, and 22: *SfruACD22*) (Figure 1). Thirty FARs were also located on five chromosomes (1, 7, 10, 29, and 30) (Figure 1). DESs and ACTs were widely distributed on more than five chromosomes (DESs: chromosomes 2, 7, 11, 12, 13, 22, and 29; ACTs: chromosomes 1, 2, 4, 6, 10, 15, 17, 21, 22 and 26) (Figure 1).

Table 1. Genes related to sex pheromone biosynthesis in *Spodoptera frugiperda*.

Gene Name	Gene ID	ORF (aa)	Gene_chr	Gene Start	Gene End	Best BlastX Match				
						Gene Name	Acc.no.	Species	E Value	Identity (%)
Acetyl-CoA carboxylase (ACC)										
SfruACC1	LOC118280410	2385	NC_049738.1	2,772,652	2,888,149	acetyl-CoA carboxylase	XP_022824105.1	*Spodoptera litura*	0.0	99.20
Fatty acid synthase (FAS)										
SfruFAS1	LOC118265122	1503	NC_049716.1	13,860,534	13,873,617	fatty acid synthase-like	XP_035448777.1	*Spodoptera frugiperda*	0.0	100
SfruFAS2	LOC118267601	2337	NC_049719.1	7,708,838	7,745,487	fatty acid synthase-like	XP_035437568.1	*Spodoptera frugiperda*	0.0	100
SfruFAS3	LOC118267741	2328	NC_049719.1	7,644,074	7,683,559	fatty acid synthase-like	XP_035437791.1	*Spodoptera frugiperda*	0.0	100
SfruFAS4	LOC118272899	1286	NC_049726.1	13,387,368	13,397,181	fatty acid synthase	AGR49310.1	*Agrotis ipsilon*	0.0	85.25
SfruFAS5	LOC118274970	2384	NC_049729.1	88,602	113,004	fatty acid synthase-like	XP_035448683.1	*Spodoptera frugiperda*	0.0	100
SfruFAS6	LOC118275042	1580	NC_049729.1	14,669,977	14,692,392	fatty acid synthase-like	XP_035448779.1	*Spodoptera frugiperda*	0.0	100
SfruFAS7	LOC118274634	2422	NC_049729.1	3,425,920	3,481,831	fatty acid synthase-like	XP_035448162.1	*Spodoptera frugiperda*	0.0	100
SfruFAS8	LOC118274778	2348	NC_049729.1	3,486,227	3,504,960	fatty acid synthase-like	XP_035448370.1	*Spodoptera frugiperda*	0.0	100
SfruFAS9	LOC118274999	2276	NC_049729.1	5,659,108	5,676,788	fatty acid synthase-like	XP_035448720.1	*Spodoptera frugiperda*	0.0	100
SfruFAS10	LOC118274601	2321	NC_049729.1	117,349	133,649	fatty acid synthase-like	XP_047033130.1	*Helicoverpa zea*	0.0	68.91
SfruFAS11	LOC118274969	294	NC_049729.1	84,898	87,981	fatty acid synthase-like	XP_035448682.1	*Spodoptera frugiperda*	0.0	100
Fatty acid transport protein (FATP)										
SfruFATP1	LOC118273519	700	NC_049727.1	6,041,998	6,085,508	fatty acid transport protein	ARD71229.1	*Spodoptera exigua*	0.0	96.43
SfruFATP2	LOC118273025	651	NC_049727.1	6,086,086	6,101,822	fatty acid transport protein	ARD71230.1	*Spodoptera exigua*	0.0	98.00
SfruFATP3	LOC118277430	661	NC_049733.1	5,268,282	5,285,638	fatty acid transport protein	ARD71231.1	*Spodoptera exigua*	0.0	96.37
SfruFATP4	LOC118273026	643	NC_049727.1	6,103,562	6,131,706	fatty acid transport protein	ARD71232.1	*Spodoptera exigua*	0.0	98.29

Table 1. *Cont.*

Gene Name	Gene ID	ORF (aa)	Gene_chr	Gene Start	Gene End	Best BlastX Match				
						Gene Name	Acc.no.	Species	E Value	Identity (%)
Acyl-CoA dehydrogenase (ACD) (β-oxidation enzyme)										
SfruACD1	LOC118274515	408	NC_049729.1	14,890,759	14,893,193	short-chain specific acyl-CoA dehydrogenase, mitochondrial	XP_022831790.1	*Spodoptera litura*	0.0	99.02
SfruACD2	LOC118264809	410	NC_049715.1	14,866,408	14,875,265	acyl-CoA dehydrogenase	QZC92122.1	*Dioryctria abietella*	0.0	72.68
SfruACD3	LOC118276297	418	NC_049731.1	13,012,363	13,027,495	short/branched-chain specific acyl-CoA dehydrogenase, mitochondrial	XP_022827910.1	*Spodoptera litura*	0.0	99.28
SfruACD4	LOC118265626	422	NC_049710.1	13,581,976	13,599,130	putative medium-chain specific acyl-CoA dehydrogenase	AID66670.1	*Agrotis segetum*	0.0	96.45
SfruACD5	LOC118278275	626	NC_049711.1	15,605,655	15,609,993	very long-chain specific acyl-CoA dehydrogenase, mitochondrial	XP_047020249.1	*Helicoverpa zea*	0.0	89.97
Enoyl-CoA hydratase (ECH) (β-oxidation enzyme)										
SfruECH1	LOC118271967	278	NC_049725.1	8,995,073	8,998,854	enoyl-CoA hydratase domain-containing protein 3, mitochondrial-like	XP_035444142.1	*Spodoptera frugiperda*	0.0	100
SfruECH2	LOC118277211	343	NC_049733.1	10,231,633	10,237,455	putative enoyl-CoA hydratase	AID66686.1	*Agrotis segetum*	0.0	77.42
SfruECH3	LOC118264501	298	NC_049715.1	2,819,343	2,822,884	enoyl-CoA hydratase domain-containing protein 2, mitochondrial-like	XP_047024252.1	*Helicoverpa zea*	5×10^{-180}	89.23
Hydroxyacyl-coenzyme A (HCD) (β-oxidation enzyme)										
SfruHCD1	LOC118272082	313	NC_049725.1	1,232,063	1,234,251	hydroxyacyl-coenzyme A dehydrogenase, mitochondrial-like	XP_035451823.1	*Spodoptera frugiperda*	0.0	100

Table 1. *Cont.*

Gene Name	Gene ID	ORF (aa)	Gene_chr	Gene Start	Gene End	Best BlastX Match				
						Gene Name	Acc.no.	Species	E Value	Identity (%)
SfruHCD2	LOC118272083	307	NC_049725.1	1,235,137	1,239,192	putative 3-hydroxyacyl-CoA dehydrogenase	AID66695.1	*Agrotis segetum*	0.0	94.14
SfruHCD3	LOC118272085	80	NC_049725.1	1,234,695	1,242,746	hydroxyacyl-coenzyme A dehydrogenase, mitochondrial-like	XP_022823313.1	*Spodoptera litura*	3×10^{-49}	100
3-ketoacyl-CoA thiolase (KCT) (β-oxidation enzyme)										
SfruKCT1	LOC118266029	396	NC_049717.1	10,904,839	10,911,237	3-ketoacyl-CoA thiolase, mitochondrial-like	XP_035434809.1	*Spodoptera frugiperda*	0.0	100
SfruKCT2	LOC118266244	400	NC_049717.1	12,544,565	12,548,651	3-ketoacyl-CoA thiolase, mitochondrial-like	XP_035435538.1	*Spodoptera frugiperda*	0.0	100
SfruKCT3	LOC118266444	398	NC_049717.1	12,548,954	12,551,900	3-ketoacyl-CoA thiolase, mitochondrial-like	XP_035435800.1	*Spodoptera frugiperda*	0.0	100
SfruKCT4	LOC118266245	394	NC_049717.1	12,538,512	12,544,434	3-ketoacyl-CoA thiolase, mitochondrial-like	XP_035435539.1	*Spodoptera frugiperda*	0.0	100
SfruKCT5	LOC118266304	396	NC_049717.1	12,535,796	12,538,103	3-ketoacyl-CoA thiolase, mitochondrial-like	XP_035435600.1	*Spodoptera frugiperda*	0.0	100
SfruKCT6	LOC118265763	396	NC_049717.1	12,527,729	12,534,164	3-ketoacyl-CoA thiolase, mitochondrial-like	XP_035434809.1	*Spodoptera frugiperda*	0.0	100
Desaturase (DES)										
SfruDES1	LOC118269923	379	NC_049722.1	4,551,252	4,554,527	acyl-CoA desaturase 1-like	XP_035441201.1	*Spodoptera frugiperda*	0.0	100
SfruDES2	LOC118264925	375	NC_049716.1	12,167,231	12,217,589	desaturase	AAQ74260.1	*Spodoptera littoralis*	0.0	96.54
SfruDES3	LOC118268438	444	NC_049720.1	13,813,813	13,831,130	desaturase	ARD71179.1	*Spodoptera exigua*	0.0	91.22
SfruDES4	LOC118274250	336	NC_049711.1	11,647,117	11,651,688	desaturase	ARD71180.1	*Spodoptera exigua*	0.0	98.21
SfruDES5	LOC118276125	339	NC_049731.1	8,780,609	8,784,814	delta 11 desaturase	AGH12217.1	*Spodoptera litura*	0.0	91.15
SfruDES6	LOC118268131	322	NC_049720.1	12,847,464	12,851,906	acyl-CoA Delta(11) desaturase-like	XP_035438325.1	*Spodoptera frugiperda*	0.0	100

Table 1. *Cont.*

Gene Name	Gene ID	ORF (aa)	Gene_chr	Gene Start	Gene End	Best BlastX Match				
						Gene Name	Acc.no.	Species	E Value	Identity (%)
SfruDES7	LOC118264926	371	NC_049716.1	12,252,078	12,286,714	stearoyl-CoA desaturase 5-like	XP_035433487.1	*Spodoptera frugiperda*	0.0	100
SfruDES8	LOC118280447	321	NC_049738.1	9,365,125	9,401,887	putative desaturase des8	ALJ30231.1	*Spodoptera litura*	0.0	97.51
SfruDES9	LOC118264929	354	NC_049716.1	12,362,286	12,378,777	desaturase	ARD71183.1	*Spodoptera exigua*	0.0	98.02
SfruDES10	LOC118268107	390	NC_049720.1	14,173,733	14,179,282	desaturase	ARD71184.1	*Spodoptera exigua*	2×10^{-151}	95.43
SfruDES11	LOC118264952	377	NC_049716.1	10,539,212	10,545,018	acyl-CoA Delta(11) desaturase-like	XP_035433515.1	*Spodoptera frugiperda*	0.0	100
SfruDES12	LOC118264931	358	NC_049716.1	12,104,709	12,122,940	desaturase	ARD71185.1	*Spodoptera exigua*	0.0	92.12
SfruDES13	LOC118264927	369	NC_049716.1	12,146,558	12,164,738	acyl-CoA Delta(11) desaturase-like	XP_035433488.1	*Spodoptera frugiperda*	0.0	100
SfruDES14	LOC118264933	340	NC_049716.1	12,346,231	12,349,171	acyl-CoA Delta(11) desaturase-like	XP_035433497.1	*Spodoptera frugiperda*	0.0	100
SfruDES15	LOC118268104	453	NC_049720.1	14,186,781	14,189,958	Desaturase	KOB71313.1	*Operophtera brumata*	0.0	64.52
SfruDES16	LOC118269215	360	NC_049721.1	3,116,248	3,129,873	acyl-CoA Delta(11) desaturase-like	XP_035440106.1	*Spodoptera frugiperda*	0.0	100
SfruDES17	LOC118269922	396	NC_049722.1	4,554,971	4,557,910	acyl-CoA Delta(11) desaturase-like	XP_035441200.1	*Spodoptera frugiperda*	0.0	100
Fatty acyl reductase (FAR)										
SfruFAR1	LOC118280370	535	NC_049738.1	7,174,208	7,214,859	fatty acyl reductase	ARD71186.1	*Spodoptera exigua*	0.0	96.07
SfruFAR2	LOC118265382	462	NC_049716.1	5,347,090	5,355,248	fatty acyl reductase	ARD71187.1	*Spodoptera exigua*	0.0	82.93
SfruFAR3	LOC118265592	454	NC_049716.1	1,589,115	1,598,988	putative fatty acyl reductase FAR3	ALJ30237.1	*Spodoptera litura*	0.0	93.83
SfruFAR4	LOC118280550	498	NC_049738.1	7,051,936	7,079,859	fatty acyl-CoA reductase 13	AKD01774.1	*Helicoverpa armigera*	0.0	88.76
SfruFAR5	LOC118280458	525	NC_049738.1	7,234,148	7,242,711	putative fatty acyl reductase FAR5	ALJ30239.1	*Spodoptera litura*	0.0	94.26
SfruFAR6	LOC118280491	520	NC_049738.1	1,004,955	1,024,481	fatty acyl reductase	ARD71191.1	*Spodoptera exigua*	0.0	85.38

Table 1. *Cont.*

Gene Name	Gene ID	ORF (aa)	Gene_chr	Gene Start	Gene End	Best BlastX Match				
						Gene Name	Acc.no.	Species	E Value	Identity (%)
SfruFAR7	LOC118263342	520	NC_049710.1	14,027,024	14,057,897	fatty acyl reductase	ARD71192.1	*Spodoptera exigua*	0.0	89.62
SfruFAR8	LOC118280282	526	NC_049738.1	7,122,361	7,154,556	fatty acyl-CoA reductase 1-like	XP_035456141.1	*Spodoptera frugiperda*	0.0	100
SfruFAR9	LOC118265401	490	NC_049716.1	1,634,217	1,641,393	fatty acyl-CoA reductase 1-like	XP_035434130.1	*Spodoptera frugiperda*	0.0	100
SfruFAR10	LOC118280227	624	NC_049738.1	10,266,635	10,318,251	putative fatty acyl reductase FAR10	ALJ30244.1	*Spodoptera litura*	0.0	95.83
SfruFAR11	LOC118280439	510	NC_049738.1	8,122,362	8,131,120	fatty acyl-CoA reductase 2	AKD01763.1	*Helicoverpa armigera*	0.0	56.24
SfruFAR12	LOC118280219	523	NC_049738.1	5,814,548	5,844,700	fatty acyl-CoA reductase 1-like	XP_047038603.1	*Helicoverpa zea*	0.0	68.84
SfruFAR13	LOC118280549	520	NC_049738.1	7,017,686	7,037,406	putative fatty acyl-CoA reductase CG5065	XP_035456531.1	*Spodoptera frugiperda*	0.0	100
SfruFAR14	LOC118280222	528	NC_049738.1	5,956,001	6,001,728	fatty acyl-CoA reductase 8	QLI61998.1	*Streltzoviella insularis*	0.0	86.39
SfruFAR15	LOC118280297	512	NC_049738.1	5,092,043	5,105,984	fatty acyl reductase 15	ATJ44470.1	*Helicoverpa armigera*	0.0	78.52
SfruFAR16	LOC118280293	531	NC_049738.1	7,103,707	7,121,330	fatty acyl reductase 16	ATJ44527.1	*Helicoverpa assulta*	0.0	89.29
SfruFAR17	LOC118265535	450	NC_049716.1	8,947,008	8,959,114	putative fatty acyl-CoA reductase CG5065	XP_035434352.1	*Spodoptera frugiperda*	0.0	100
SfruFAR18	LOC118280263	511	NC_049738.1	3,127,028	3,152,947	fatty acyl reductase 12	ATJ44526.1	*Helicoverpa assulta*	0.0	75.15
SfruFAR19	LOC118280403	513	NC_049738.1	5,124,444	5,137,543	fatty acyl-CoA reductase wat-like	XP_035456297.1	*Spodoptera frugiperda*	0.0	100
SfruFAR20	LOC118280192	523	NC_049738.1	7,287,590	7,295,954	fatty acyl-CoA reductase wat-like	XP_035455970.1	*Spodoptera frugiperda*	0.0	100
SfruFAR21	LOC118280547	542	NC_049738.1	6,979,095	6,990,649	fatty acyl reductase 13	ATJ44468.1	*Helicoverpa armigera*	0.0	66.73
SfruFAR22	LOC118280723	537	NC_049739.1	1,366,062	1,372,472	fatty acyl-CoA reductase wat-like	XP_035456945.1	*Spodoptera frugiperda*	0.0	100
SfruFAR23	LOC118267621	535	NC_049719.1	7,599,750	7,608,971	fatty acyl-CoA reductase wat-like	XP_035437588.1	*Spodoptera frugiperda*	0.0	100
SfruFAR24	LOC118267780	538	NC_049719.1	7,588,680	7,597,048	fatty acyl-CoA reductase 1-like	XP_035438040.1	*Spodoptera frugiperda*	0.0	69.60
SfruFAR25	LOC118267895	548	NC_049719.1	7,574,852	7,582,604	fatty acyl-CoA reductase 1-like	XP_035438040.1	*Spodoptera frugiperda*	0.0	100

Table 1. *Cont.*

Gene Name	Gene ID	ORF (aa)	Gene_chr	Gene Start	Gene End	Best BlastX Match				
						Gene Name	Acc.no.	Species	E Value	Identity (%)
SfruFAR26	LOC118280456	538	NC_049738.1	7,251,597	7,260,794	fatty acyl-CoA reductase wat-like	XP_035456388.1	*Spodoptera frugiperda*	0.0	100
SfruFAR27	LOC118280298	530	NC_049738.1	7,271,670	7,283,596	fatty acyl-CoA reductase wat-like	XP_035456154.1	*Spodoptera frugiperda*	0.0	100
SfruFAR28	LOC118280321	539	NC_049738.1	3,362,799	3,380,634	fatty acyl reductase 5	ATJ44463.1	*Helicoverpa armigera*	0.0	88.27
SfruFAR29	LOC118280434	538	NC_049738.1	8,055,508	8,085,920	fatty acyl reductase	AID66647.1	*Agrotis segetum*	0.0	80.76
Acetyltransferase (ACT)										
SfruACT1	LOC118272936	252	NC_049726.1	7,310,246	7,316,764	N-alpha-acetyltransferase 60-like	XP_035445570.1	*Spodoptera frugiperda*	0.0	100
SfruACT2	LOC118267998	176	NC_049719.1	4,426,976	4,431,010	probable N-acetyltransferase san	XP_022826510.1	*Spodoptera litura*	1×10^{-127}	100
SfruACT3	LOC118275415	199	NC_049730.1	1,741,133	1,742,303	N-alpha-acetyltransferase 80-like	XP_035449252.1	*Spodoptera frugiperda*	8×10^{-143}	100
SfruACT4	LOC118281885	107	NW_023337168.1	95,711	96,749	N-alpha-acetyltransferase 38-B	XP_022826548.1	*Spodoptera litura*	8×10^{-66}	91.35
SfruACT5	LOC118272777	710	NC_049711.1	13,544,018	13,554,256	N-alpha-acetyltransferase 35, NatC auxiliary subunit	XP_028155763.1	*Ostrinia furnacalis*	0.0	91.85
SfruACT6	LOC118278521	469	NC_049735.1	8,174,301	8,181,092	putative acetyltransferase ACT9	ALJ30256.1	*Spodoptera litura*	6×10^{-178}	99.25
SfruACT7	LOC118281877	294	NW_023337168.1	41,788	43,450	N-alpha-acetyltransferase 30-like	XP_035458539.1	*Spodoptera frugiperda*	0.0	100
SfruACT8	LOC118267522	173	NC_049719.1	1,242,030	1,243,082	N-alpha-acetyltransferase 20	XP_022826734.1	*Spodoptera litura*	9×10^{-127}	100
SfruACT9	LOC118272523	180	NC_049726.1	6,776,065	6,778,854	N-alpha-acetyltransferase 10	XP_022830428.1	*Spodoptera litura*	6×10^{-132}	99.44
SfruACT10	LOC118276179	396	NC_049731.1	3,555,474	3,572,627	acetyltransferase	ARD71213.1	*Spodoptera exigua*	0.0	98.49
SfruACT11	LOC118276445	512	NC_049731.1	3,602,442	3,605,363	fatty alcohol acetyltransferase	AIN34682.1	*Agrotis segetum*	0.0	88.85
SfruACT12	LOC118278776	479	NC_049735.1	4,751,837	4,791,014	acetyltransferase	ARD71206.1	*Spodoptera exigua*	0.0	99.79
SfruACT13	LOC118275943	245	NC_049730.1	5,635,234	5,651,704	N-alpha-acetyltransferase 40-like	XP_035449986.1	*Spodoptera frugiperda*	0.0	100

Table 1. *Cont.*

Gene Name	Gene ID	ORF (aa)	Gene_chr	Gene Start	Gene End	Best BlastX Match				
						Gene Name	Acc.no.	Species	E Value	Identity (%)
SfruACT14	LOC118267183	480	NC_049710.1	16,346,539	16,357,150	acetyltransferase 18	ATJ44585.1	*Helicoverpa assulta*	0.0	77.90
SfruACT15	LOC118264603	505	NC_049715.1	8,586,407	8,600,294	acetyltransferase	ARD71205.1	Spodoptera exigua	0.0	90.69
SfruACT16	LOC118262813	195	NC_049713.1	14,325,493	14,326,654	N-acetyltransferase 9-like	XP_035430320.1	*Spodoptera frugiperda*	1×10^{-194}	100
SfruACT17	LOC118271269	869	NC_049724.1	4,989,091	5,073,434	N-alpha-acetyltransferase 15	XP_035442996.1	*Spodoptera frugiperda*	0.0	99.88
Acyl-CoA-binding protein (ACBP)										
SfruACBP1	LOC118267206	85	NC_049718.1	4,519,313	4,523,840	putative acyl-CoA-binding protein	XP_021185372.1	*Helicoverpa armigera*	2×10^{-52}	94.12
SfruACBP2	LOC118266031	470	NC_049717.1	10,902,558	10,904,528	acyl-CoA-binding domain-containing protein 6-like	XP_035434810.1	*Spodoptera frugiperda*	0.0	100
SfruACBP3	LOC118279722	265	NC_049737.1	6,551,474	6,584,040	acyl-CoA-binding domain-containing protein 5-like isoform X4	XP_035455327.1	*Spodoptera frugiperda*	0.0	100

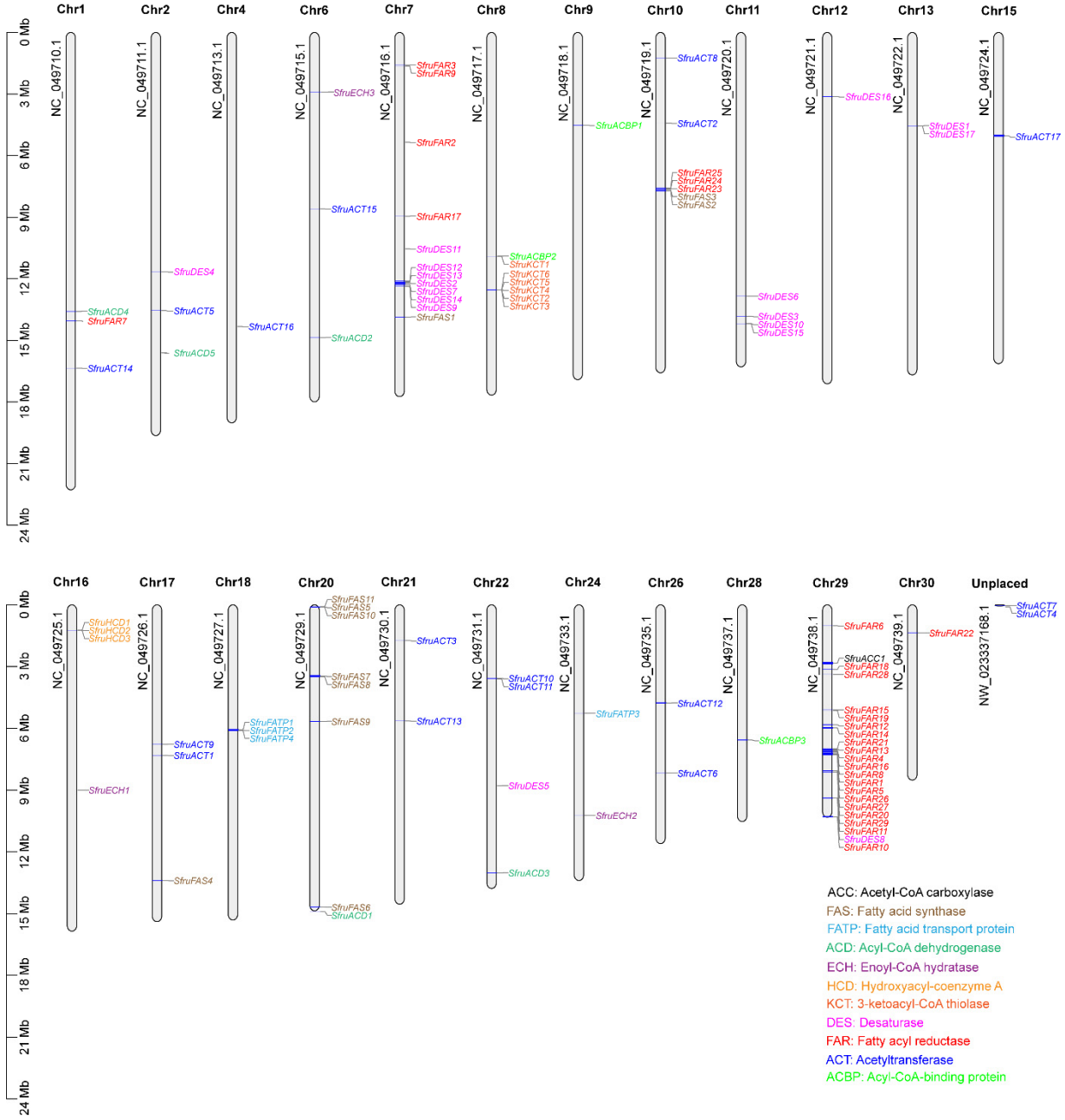

Figure 1. Localization of the candidate sex pheromone biosynthesis genes in the *S. frugiperda* genome. According to the annotation file of *S. frugiperda*, we determined the sex pheromone biosynthesis gene locations in the genome.

3.2. Phylogenetic Analyses of DESs and FARs

To assign putative functions, two phylogenetic trees of DESs and FARs were constructed using protein sequences from *S. frugiperda*, *S. exigua*, *S. litura*, *S. inferens*, *A. pernyi*, *O. nubilalis*, and *H. assulta*. The DESs phylogenetic trees showed that all three identified SfruDESs from the *S. frugiperda* genome were clustered in three different clades of Lepidoptera desaturases: Δ11 desaturase (*SfruDES5*), Δ9 desaturase (18 C > 16 C) (*SfruDES9*), and Δ9 desaturase (16 C > 18 C) (*SfruDES11*) (Figure 2). In the FARs phylogenetic tree, four SfruFARs (*SfruFAR2*, *SfruFAR3*, *SfruFAR9*, and *SfruFAR17*) were clustered within the Lepidoptera pgFAR group, which is a clade with functionally investigated FARs (Figure 3).

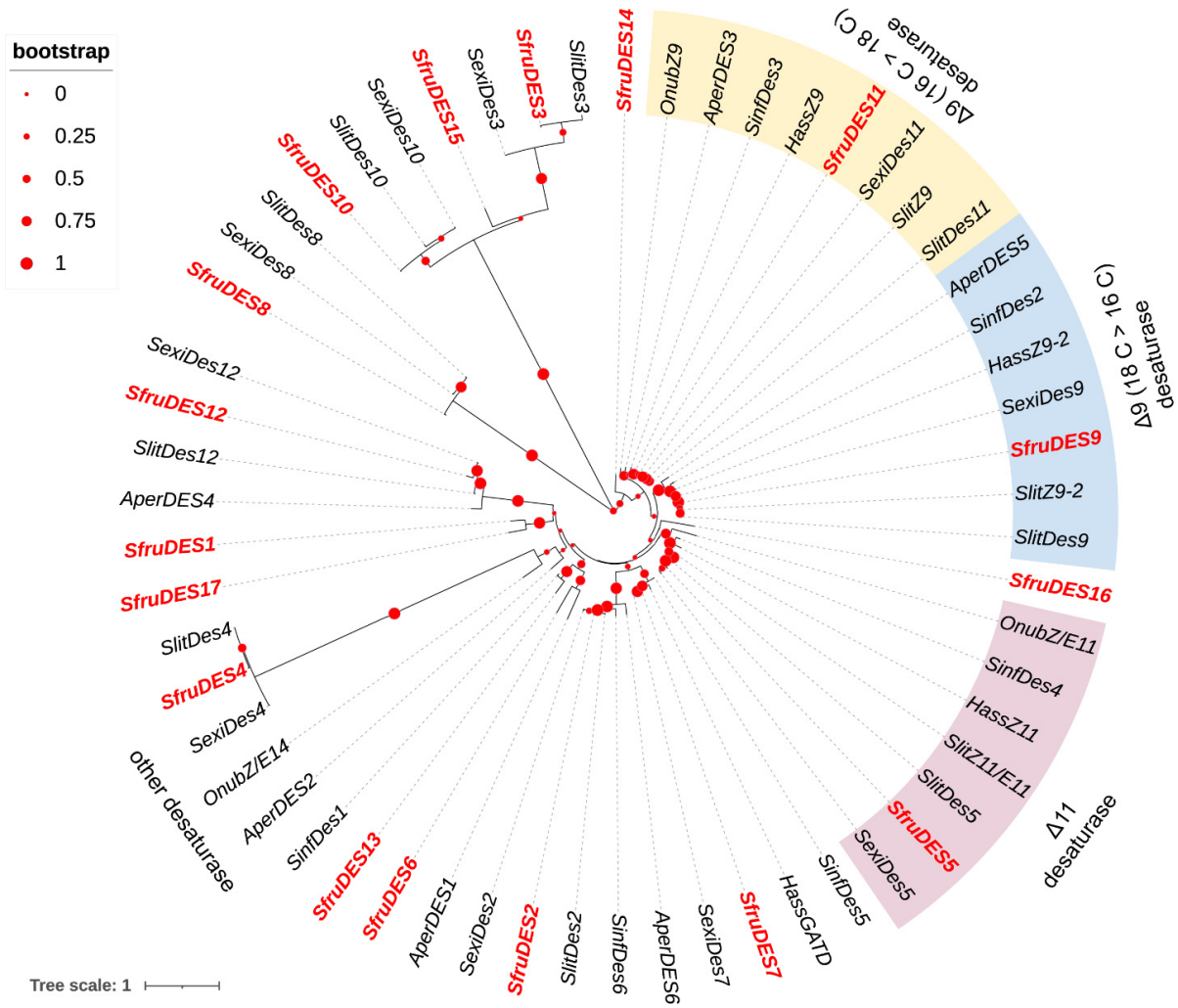

Figure 2. Phylogenetic tree of insect desaturase (DES). The *S. frugiperda* translated genes are shown in red. Sfru: *S. frugiperda*, Slit: *S. litura*, Sexi: *S. exigua*, Sinf: *S. inferens*, Aper: *A. pernyi*, Onub: *O. nubilalis*, Hass: *H. assulta*.

3.3. Expression Profile of Sex Pheromone Biosynthesis Genes

To further screen the candidate sex pheromone biosynthesis genes in *S. frugiperda*, we performed a transcriptome analysis for the genes expressed in female PGs and ABs (Table S5). Based on the transcriptome results (Table S6), we analyzed the expression patterns of all of the candidate sex pheromone biosynthesis genes (Figure 4). None of the genes in ACC, ECH, HCD, and ACBP were significantly higher expressed in the PGs (Figure 4). Only one gene in FAS (*SfruFAS4*), FATP (*SfruFATP3*), ACD (*SfruACD5*), and KCT (*SfruKCT3*) had significantly higher expression levels in pheromone glands (PGs) than abdomens (ABs) (Figure 4). Five DESs (*SfruDES2*, *SfruDES5*, *SfruDES11*, and *SfruDES13*), twelve FARs (*SfruFAR1*, *SfruFAR2*, *SfruFAR3*, *SfruFAR6*, *SfruFAR7*, *SfruFAR8*, *SfruFAR9*, *SfruFAR10*, *SfruFAR11*, *SfruFAR14*, *SfruFAR16*, and *SfruFAR29*) and two ACTs (*SfruACT6* and *SfruACT10*) were predominately expressed in the PGs of *S. frugiperda* (Figure 4). *SfruFAR3* was specifically highly expressed in PGs (Figure 4I). Among the predominately expressed genes in the PGs, the FPKM of *SfruKCT3*, *SfruFAR8*, *SfruFAR9*, *SfruFAR11*, *SfruFAR16*, *SfruFAR29*, and *SfruACT10* were below 10, while the FPKM of *SfruFAS4*, *SfruACD5*, *SfruDES5*, *SfruDES11*, *SfruFAR3*, *SfruFAR10*, *SfruFAR14*, and *SfruACT6* exceeded 100 (Figure 4). The qRT-PCR validation experiments of *SfruDES2*, *SfruDES5*, *SfruFAR2*, and *SfruFAR3* confirmed that all of these four genes were predominately expressed in the PGs (Figure 5).

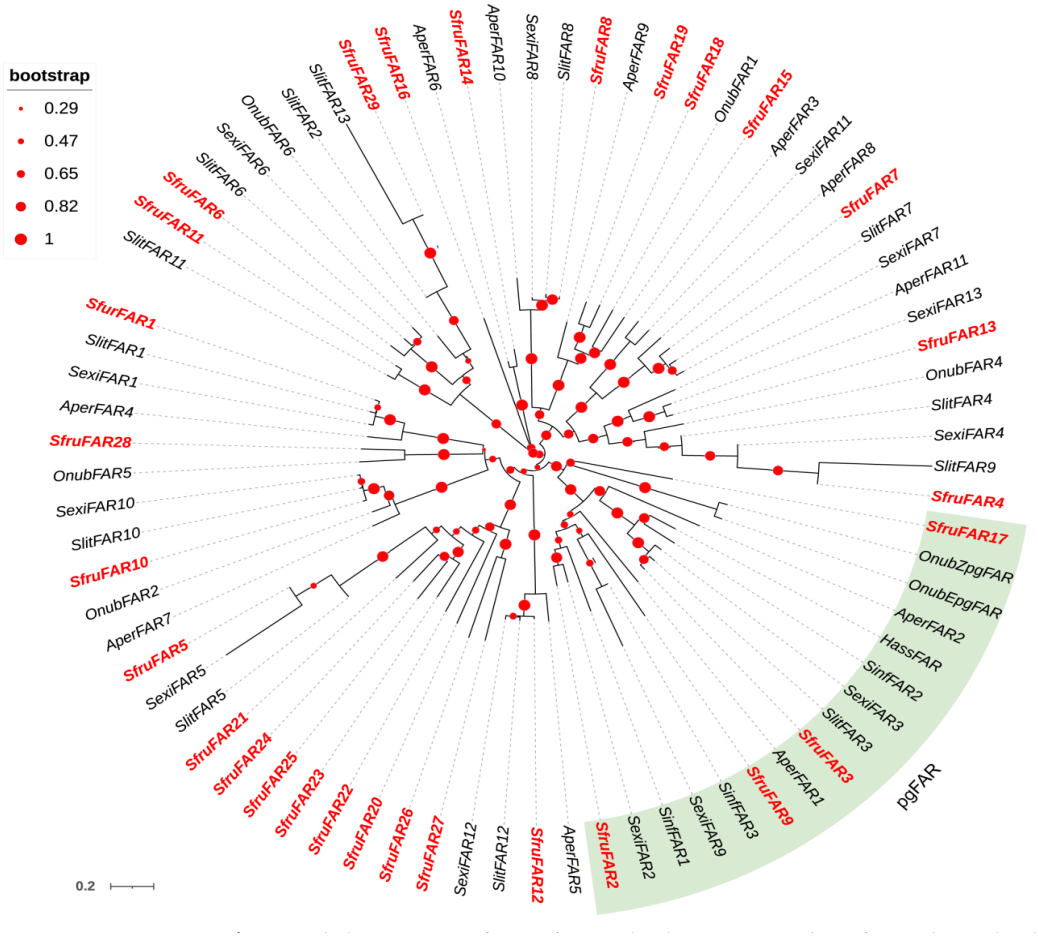

Figure 3. Phylogenetic tree of insect fatty acid reductase (FAR). The *S. frugiperda* translated genes are shown in red. Sfru: *S. frugiperda*, Slit: *S. litura*, Sexi: *S. exigua*, Sinf: *S. inferens*, Aper: *A. pernyi*, Onub: *O. nubilalis*, Hass: *H. assulta*.

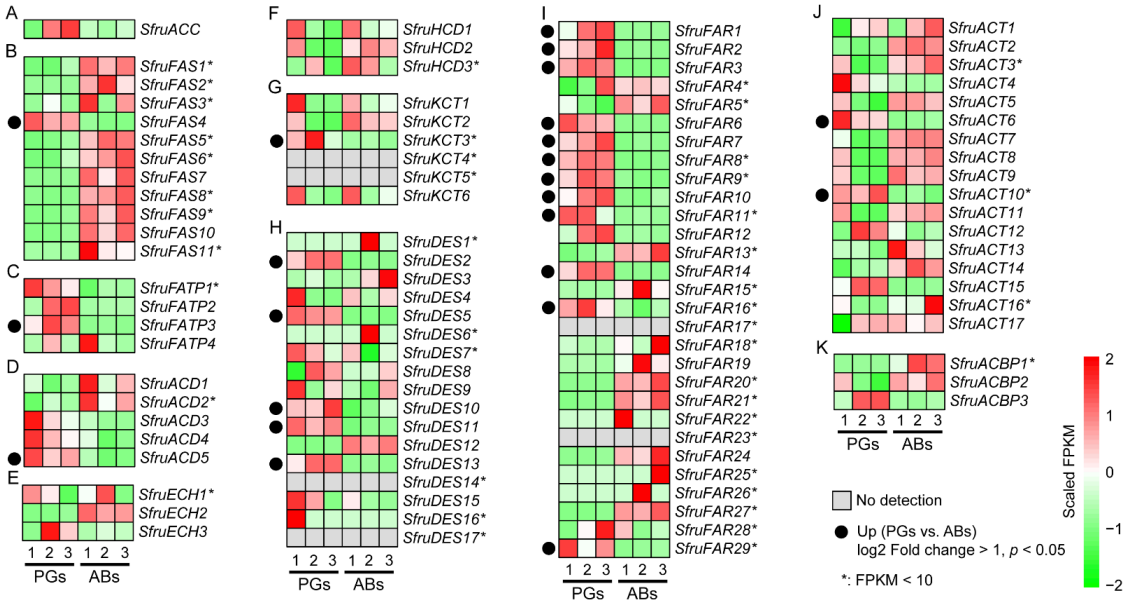

Figure 4. Expression profiles of the candidate sex pheromone biosynthesis genes in *S. frugiperda*. (**A**) ACC; (**B**): FASs; (**C**): FATPs; (**D**): ACDs; (**E**): ECHs; (**F**): HCDs; (**G**): KCTs; (**H**): DESs; (**I**); FARs; (**J**): ACTs; (**K**): ACBPs.

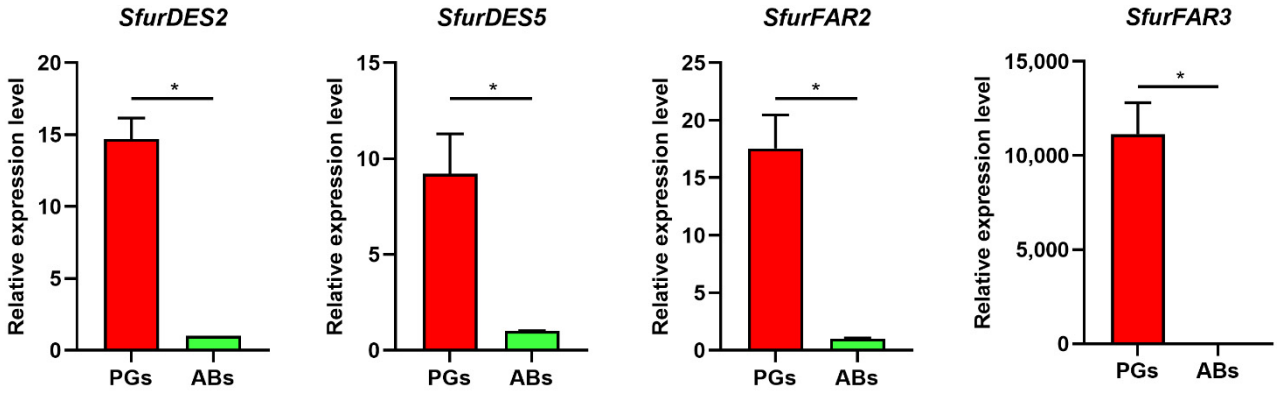

Figure 5. Relative expression levels of four sex pheromone biosynthesis genes. PGs: female pheromone glands, ABs: female abdomens without pheromone glands. The relative expression level (PGs VS ABs) is indicated as mean ± SE (n = 3). The asterisk indicates a significant difference between PGs and ABs ($p < 0.05$, Mann-Whitney U test).

4. Discussion

In moths, the PGs are the most important organ for synthesizing and releasing sex pheromones [35]. Thus, the sex pheromone synthesis genes are usually highly expressed in PGs. In the present study, 99 candidate sex pheromone biosynthesis genes were identified from the genome of *S. frugiperda*. Among them, 22 genes were expressed at significantly higher levels in PGs than in the abdomen, and most of them were key genes involved in the sex pheromone biosynthesis pathway of moths such as DESs and FARs. Consistent with this study, DESs and FARs also showed a trend of high PG expression in *S. litura* and *S. exigua* [8,30].

Sex pheromones released by female moths are composed of a mixture of sex pheromone components in specific ratios that show high species specificity [43,44]. Synthesis of a specific sex pheromone mixture requires the coordination of multiple enzymes, such as ACC and FAS. These two enzyme families mainly work upstream of sex pheromone synthesis and are responsible for the synthesis of fatty acid precursors. Initially, ACC carboxylates acetyl-CoA to malonyl-CoA [45], after which FAS synthesizes malonyl-CoA and NAPDH into fatty acids [46,47]. In this study, one ACC gene and 11 FAS genes were identified from the genome of *S. frugiperda*. Among the 11 FAS genes, *SfurFAS4* had the highest expression in PG, suggesting its important role in fatty acid synthesis. As an evolutionarily conserved membrane-bound protein, FATPs can bind fatty acids and transport them to PG cells via the hemolymph for pheromone biosynthesis [12,48]. Four FATP genes were identified from the genome of *S. frugiperda*, consistent with the number of FATP genes in *S. litura* and *S. exigua* [8,30], and there was a high degree of sequence similarity among these three species. However, only *SfurFATP3* had abundant expression levels in the PGs based on the FPKM values.

There are several sex pheromone components of *S. frugiperda*. Except for Z11-16:OAc, containing 16 carbons, Z9-14:OAc, Z7-12:OAc, Z9-12:OAc, and E7-12:OAc are less than 16-carbon chain length unsaturated fatty acid ester derivatives [24,28]. Therefore, the carbon chain shortening reaction plays an important role in this process, and the pathway to generate sex pheromone precursors with different carbon chain lengths is similar to the local β-oxidation pathway of vertebrate peroxisomes, among which ACD, ECH, HCD, and KCT are four key enzymes in the β-oxidation pathway [9]. A total of five ACD genes, three ECH genes, three HCD genes, and six KCT genes were screened in the FAW genome. *SfurACD5* and *SfurKCT3* had higher expression abundance in PGs than in the abdomen.

DES is a key enzyme in sex pheromone biosynthesis. It removes hydrogen atoms at specific positions and introduces double bonds to form cis-trans isomers [31,49]. DES is classified into Δ5, Δ9 (18 C > 16 C) Δ9 (16 C > 18 C), Δ10, Δ11, Δ12, and Δ14 desaturases according to the position where the double bond is introduced into the catalytic sub-

strate [50–52]. Because several sex pheromone components of *S. frugiperda* have different positions, numbers, and configurations of double bonds, DES is crucial for sex pheromone formation. A total of 17 DESs were identified from the FAW genome. Phylogenetic tree analysis showed that the *SfruDES5* was clustered with the DES5 of *S. litura* and *S. exigua*, and both were clustered in the Δ11 DES branch. Both transcriptome FPKM values and qRT-PCR showed that *SfruDES5* was significantly overexpressed in the PGs of *S. frugiperda*. *SfruDES9* and *SfruDES11* are clustered with the corresponding DES9 and DES11 of *S. litura* and *S. exigua* and belong to the Δ9 (18 C > 16 C) and Δ9 (16 C > 18 C) desaturase groups, respectively, in which *SfruDES11* was specifically expressed in PGs. The Δ9 and Δ11 desaturases are important desaturases in *Spodoptera*. The Δ9 desaturases and Δ11 desaturases can introduce Δ9-double bonds and Δ11-double bonds in precursors [53]. The Z9-14:OAc and Z11-16:OAc are the key components of *S. frugiperda* sex pheromone, and Δ9 and Δ11 desaturases are key enzymes for introducing the Δ9 and Δ11 double bonds into the pheromone. Therefore, *SfurDES5* and *SfurDES11* may participate in the desaturation step from saturated to unsaturated acids during sex pheromone synthesis in *S. frugiperda*.

The precursor substance forms an intermediate product with a specific length and double bond position after the desaturation reaction and chain shortening reaction. It then needs to be catalyzed by reductases to form alcohols. During this process, FAR is responsible for converting unsaturated fatty acids into the corresponding alcohols [11,54]. In our study, a total of 29 FAR genes were identified from the genome; among the 29 FARs of *S. frugiperda*, 12 FARs were specifically highly expressed in the PGs. The phylogenetic tree showed that *SfruFAR3* was clustered with FAR3 of *S. litura* and *S. exigua*, belonging to the PgFAR subfamily of *Spodoptera*, and had a high expression abundance. This indicated that this gene may play an important role in the synthesis of precursor alcohols. ACT can catalyze the formation of esters from alcohols [55,56]. Since there are only esters in the *S. frugiperda* sex pheromone, ACT genes play a key role in sex pheromone biosynthesis. A total of 17 ACT genes were identified in the FAW genome, among which the *SfurACT6* and *SfurACT10* were highly expressed in the PGs. These two genes may play a role in the process of converting alcohol to esters. Furthermore, two ACTs were unplaced in the chromosome, which might be caused by the genome quality. In *Bombyx mori*, ACBP functions as acyl-CoA or cell deposition [12]. We identified three ACBPs from the *S. frugiperda* genome.

According to the sex pheromone components of *S. frugiperda*, we speculated the *S. frugiperda* sex pheromone biosynthesis pathway. Firstly, ACC catalyzes acetyl-CoA to malonyl-CoA, which is followed by FAS to produce the most saturated palmitic acid (16: CoA). The 16: CoA was desaturated by Δ11 desaturase (Z11/E11) to produce Z11-16: CoA/E11-16: CoA. After the desaturase-induced formation of a double bond, specific β-oxidation enzymes shorten the chains to Z9-14: CoA, Z7-12: CoA, or E7-12: CoA. In addition, the 16: CoA was shortened by β-oxidation enzymes to 12: CoA. Then, 12: CoA generated Z9-12: CoA under Δ9 desaturase (Z9). These acyl-CoA precursors were further reduced by FARs to form corresponding fatty alcohols. Finally, pheromone components were produced after the oxidation by ACTs (Figure S1).

5. Conclusions

In conclusion, we identified a total of 99 genes belonging to gene families involved in the biosynthesis of sex pheromones from the *S. frugiperda* genome. Based on gene expression patterns and phylogenetic analysis, several genes highly expressed in the PGs might play an important role in sex pheromone synthesis. The specific functions of these genes in the process of sex pheromone biosynthesis in *S. frugiperda* require further study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13121078/s1. Figure S1: Hypothetical sex pheromone biosynthesis pathways of *S. frugiperda*; Table S1: Query gene sequences; Table S2: Primers used for RT-PCR and qPCR; Table S3: Sequences for phylogenetic tree; Table S4: Primer amplicon characteristics

of 4 genes for qRT-PCR; Table S5: Summary of the transcriptome sequencing data of of *Spodoptera frugiperda*; Table S6: Fragments per kilobase million (FPKM) for the different samples.

Author Contributions: Conceptualization, C.L. and H.Z.; methodology, C.Q.; software, Z.K.; validation, Z.K., B.Z., Y.F. and R.W.; formal analysis, C.Q.; investigation, H.Z.; resources, H.Z.; data curation, C.Q. and F.L.; writing—original draft preparation, C.Q. and Z.K.; writing—review and editing, C.L. and H.Z. visualization, R.W.; supervision, C.L.; project administration, C.Q.; Funding acquisition, C.Q. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The transcriptome data that support the findings of this study are openly available in SRA the Genbank SRA database (BioProject ID: PRJNA885519). Other data presented in this study are available in the article and Supplementary Materials.

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

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Risk Assessment and Area-Wide Crop Rotation to Keep Western Corn Rootworm below Damage Thresholds and Avoid Insecticide Use in European Maize Production

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Simple Summary: *Diabrotica virgifera virgifera* LeConte, the Western corn rootworm (WCR), is a maize-specific pest that has been a serious threat in Europe since the mid-1990s. To properly implement integrated pest management, it is necessary to identify the key factors associated with risks of crop damage from WCR and to evaluate the effectiveness of area-wide strategies based on agronomic measures, such as crop rotation, in reducing those risks. In Italy and Croatia, a survey of agronomic and cultural factors in fields damaged by WCR allowed us to determine that the beetle population size accounts for most of the risk of maize damage from WCR. Crop rotation (without insecticide use), both structural and flexible, was the most effective strategy for keeping WCR populations below the damage threshold. This indicates that WCR management can be carried out in accordance with European Union regulations to limit or avoid insecticide treatments and reduce environmental impacts.

Abstract: The Western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, has been a serious quarantine pest to maize in Europe since the mid-1990s. The integrated pest management of WCR requires an accurate knowledge of the factors that contribute most to risks of crop damage, as well as knowledge of effective area-wide strategies based on agronomic measures, such as crop rotation. In Italy and Croatia, agronomic and cultural factors in fields damaged by WCR were evaluated through a long-term survey. Based on the survey results, high-WCR densities contribute most to risks of damage to maize. Extensive field research in north-eastern Italy compared large areas of continuous maize production with areas under different crop rotation systems (i.e., a structural one with one-time maize planting in a three-year rotation and a flexible one with continuous maize planting interrupted when beetle populations exceed the threshold). The objective was to evaluate the effectiveness of different rotation regimes as possible best practices for WCR management. Captures of beetles in yellow sticky traps, root damage, larval densities, and damage to maize plants (e.g., lodging) were assessed at the center of each area. The results demonstrated the both structural and flexible crop rotation systems were effective strategies for maintaining WCR below damage threshold densities without the need for insecticides.

Keywords: IPM; risk factors; structural crop rotation; flexible crop rotation; damage threshold; area-wide strategies

1. Introduction

The most serious maize pest in North America [1], the Western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte; Coleoptera: Chrysomelidae), is now established in most European maize growing regions [2]. In the last twenty-five years, a significant amount of new knowledge about WCR in Europe has been gained by the scientific community. This knowledge was summarized by Bazok et al. [2] and included information on the monitoring and spread of WCR, on its ecology and damage, and on control methods and management tools, including tools for monitoring and biological control, as well as predictions of further spread and damage. During the last 10 years, WCR has been considered a naturalized pest in many European Union (EU) countries, as it has become a regular part of the entomofauna [2]. There are a number of differences between agricultural systems in the EU and agricultural systems in the USA, where this pest causes the most damage. The approach to WCR control in the EU has, therefore, been adapted to agricultural systems and legislation.

Integrated pest management (IPM) as a cornerstone of sustainable agriculture is strongly advocated by EU policy [3]. IPM aims to improve farmers' practices to achieve higher profits while reducing pesticide use. Successful implementation of IPM will require novel control methods and new strategies that reduce the current reliance on insecticides. The first step in IPM is prevention, i.e., the implementation of a series of agronomic measures such as crop rotation, and where appropriate the use of resistant or tolerant varieties, which create conditions that reduce the risk of pest outbreaks and the need for curative plant protection measures. Crop rotation is the first preventive measure listed in Annex III of Directive 128/2009/EC [3]. Since the most effective strategy against WCR is crop rotation [2–6], its implementation is mandatory under this legislation. Crop rotation can be implemented as a long-term structural measure (e.g., using winter wheat, maize, soybeans) or with flexible modalities. According to the flexible method, to maintain forage production on livestock farms at optimal levels in terms of yield and quality, maize can be grown continuously for two or more years, with other crops planted on a flexible schedule if adult WCR densities increase significantly, and especially if the damage threshold is exceeded. Captures of $\geq$6 beetles/Pherocon AM trap/day over a 6-week period (or even less) as previously recommended in USA has proven to be an effective WCR threshold density in Europe [7,8].

As described many times in the literature, the first records of WCR in Europe were reported from Serbia in the early 1990s [9]; however, WCR is believed to have been introduced into Europe in the early 1980s [10]. After the establishment period, WCR began to cause economic damage to maize production in Croatia and neighboring countries in the 1990s [10,11]. Based on population genetic studies of WCR by Miller et al. [12] and Ciosi et al. [13,14], the population source for the introduction to Serbia was probably Pennsylvania (USA). Based on analysis by Lemic et al. [15] of the genetic variability of WCR populations soon after the introduction in the 1990s and again in the period between 2009 and 2011, control measures (e.g., crop rotation) initiated soon after the introduction did not result in genetic differentiation toward management-adapted populations, as had previously occurred in USA [16]. The results from a range of genetic studies [10–16] suggest that there have been several introductions of WCR into the Veneto region of Italy, where the populations intermingled: in the Veneto region (Venice airport outbreak area), WCR populations had been successfully eradicated [17], but later reinvaded from the east and west, making Veneto an area where WCR populations from different parts of the USA have mixed [18,19]. These included the first reported rotation-resistant strain. Therefore,

understanding the population dynamics of WCR in the Veneto region will be particularly useful as a model for other regions with evolving WCR populations.

The primary objective of the present study is to provide information useful for area-wide management of WCR so that maize can be grown without negatively impacting farmers, in compliance with IPM, and without insecticide use. Our specific objectives were to (i) analyze the risk factors for WCR damage to maize and (ii) evaluate the success of structural and flexible crop rotation as a significant component of best management practices for WCR in Europe.

2. Materials and Methods

Two main extensive and long-term surveys were carried out. These were:

1. A WCR risk assessment survey that included damaged fields in both Italian and Croatian maize cultivation areas (Figure 1) and that considered a large number of potential risk factors;
2. A WCR area-wide management survey in northern Italy where some maize cultivation areas under different rotation approaches (structural or flexible rotation) and pest control practices were monitored to assess the effects of these practices on WCR populations and on the consequent risks of damage.

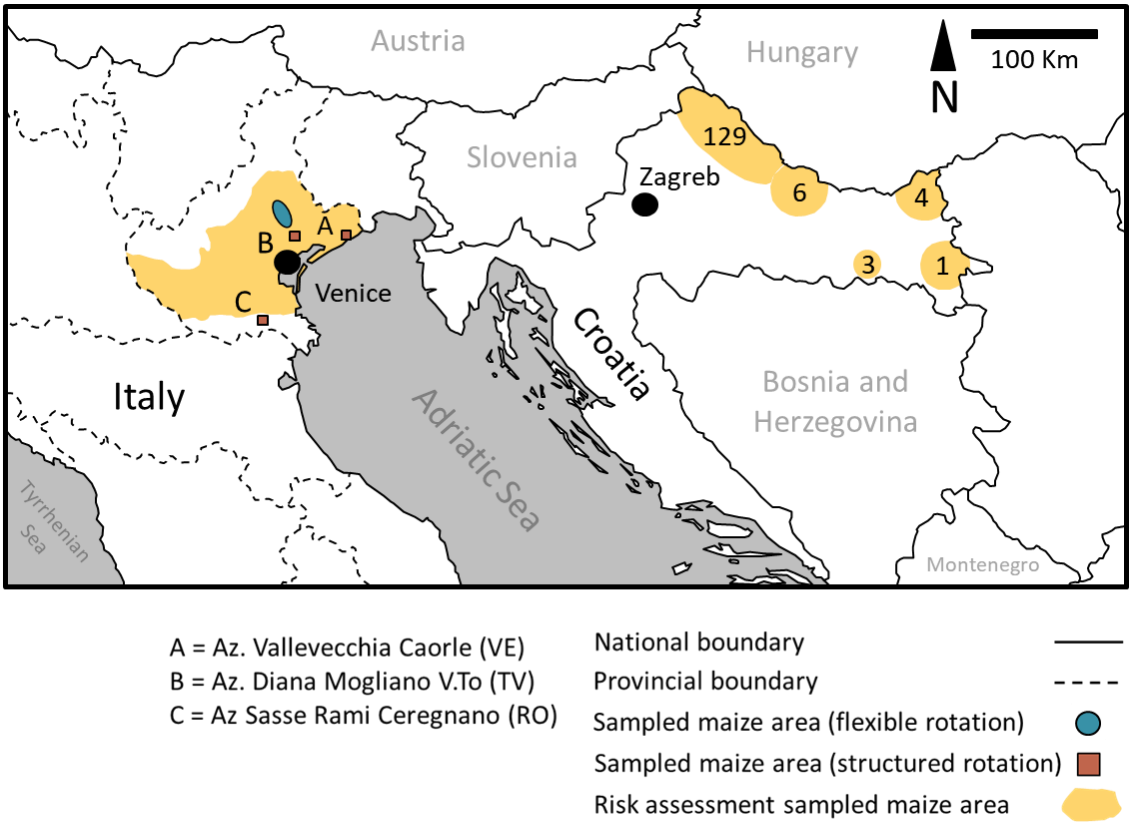

Figure 1. Map showing the regions in Italy and Croatia surveyed for WCR damage between 2003 and 2017. The locations of Venice and Zagreb are indicated by black circles. Orange coloring indicates areas surveyed for risk factors in Italy and Croatia. The numbers associated with surveyed areas in Croatia indicate the numbers of fields that were monitored. In Veneto, Italy, monitored fields were distributed throughout the entire maize cultivation area. Red symbols indicate the areas in Italy where farmers applied structural crop rotation and the blue symbol indicates the area where farmers applied flexible crop rotation. In Croatia, no comparisons of crop rotation regimes were conducted.

2.1. WCR Risk Factors Study in Italy and Croatia

A comprehensive survey of maize fields damaged by WCR was conducted in north-eastern Italy (area covered: 46°06′ N, 12°00′ E and 45°21′ N, 11°38′ E) from 2010 to 2017 and in Croatia (area covered: 46°23′ N, 19°10′ E and 45°15′ N,16°21′ E) from 2003 to 2014 (Figure 1). This produced a large set of infestation and damage data (Table 1). Some additional fields in the vicinity of the damaged fields were additionally surveyed. In Italy, the 1126 ha of maize surveyed from 2010 to 2017 included a total of 602 fields. In Croatia, 143 fields were surveyed. Depending on the year, the number of fields surveyed varied from one to thirty, and the total area surveyed in Croatia was 139.26 ha. The total cultivated area of the farms involved in the study was 2882 ha. The main variables recorded in the extended survey are shown in Table 1.

Table 1. List of the variables included in the database.

Variables	Explanation	Type	Classification [1]	Number of Records		Surveyed Maize-Cultivated Land (ha)
				Croatia	Italy	
Year	Year of data collection	Ordinal	2003–2005	6	0	10
			2006–2010	60	5	62
			2011–2015	77	449	790
			2016–2018	0	148	393
Crop damage	Damage index: percentage of total plants damaged (gooseneck and lodged plants %)	Quantitative	<5%	98	345	864
			≥5%	45	257	391
WCR beetle population level PhAM Y-1	Beetle population level (from pheromone trapping) in the previous year	Qualitative	Low	23	65	137
			Medium	72	83	204
			High	28	110	241
			Very high	20	152	368
			NA *	0	192	305
Soil properties	Texture	Qualitative	F *	0	223	436
			FL **	134	21	216
			FA ***, FLA ****	1	207	269
			FS *****	0	49	117
			NA	8	102	217
Agronomic practices	Rotation type (see Section 2.1.3)	Qualitative	A (100% maize)	21	377	768
			B (70–80% maize)	116	183	416
			C (30–40% maize)	6	42	71
	Sowing date	Qualitative	Early (March)	1	226	414
			Ordinary (April–mid May)	139	240	619
			Late (second half of May)	3	100	143
			Very late (June)	0	36	79
	Hybrid	Qualitative	Dekalb	3	69	140
			KWS	0	37	54
			Pioneer	50	311	595
			BC Institute	84	0	81
			Others	6	67	125
			NA	0	118	260
Treatments	Insecticide treatments against adults in previous year	Qualitative	No	143	444	1022
			Yes	0	147	177
			NA	0	11	56
	Soil insecticide application	Qualitative	No	103	123	354
			In furrow micro-granular insecticide	0	306	510
			Insecticide coating	40	128	329
			Both (granular and coating)	5	27	43
			NA	0	13	19
	Insecticide (active ingredient)	Qualitative	No	0	39	354
			Tefluthrin in furrow	0	22	346
			Teflutrin as seed treatment	0	72	131
			Lambda-cyhalothrin	0	41	
			Others	0	35	

Note: [1]: NA * = not available; F * = loam, clay loam; FL ** = loamy sand, sandy clay loam, silt loam, silty clay loam; FA *** = clay loam; FLA **** = silty clay loam, silty clay, FS ***** = sandy loam.

2.1.1. Soil Properties

Soil texture data for Italy were obtained from the Veneto Region Environmental Protection Agency (ARPAV) database [20]. The soils of each surveyed field were classified based on soil texture, according to the soil characteristics of corresponding soil map units (SMU) (see ARPAV database [20] for more details). Soil texture was determined using the

United States Department of Agriculture (USDA) triangular method [21] based on analysis with a sediment pipette. The soils were loam, clay loam (F), loamy sand, sandy clay loam; silt loam, silty clay loam (FL), clay loam (FA), silty clay loam, silty clay (FLA), and sandy loam (FS) (Table 1). For statistical analyses FA and FLA were grouped together. Soil texture data for Croatia were taken from the database of the interactive soil map of Croatia created for Google Earth [22].

2.1.2. Agronomic Practices

Land management practices were similar at all study sites and included: fertilizer applications at 240–300 kg N/ha; 70,000 to 80,000 seeds/ha; and 75 cm inter-row width in Italy and 70 cm in Croatia. Seeding depth varied from 2.5 to 9 cm (dry seedbed) at the study sites. All sampled fields in Croatia and the majority of sampled fields in Italy were conventionally tilled (i.e., plowing, cultivating, harrowing, and hoeing), while approximately one out of every four fields under structural rotation in Italy were minimally tilled (i.e., one cultivator pass, harrowing, and hoeing). Weed incidence was low and most farmers had applied pre-emergence and post-emergence herbicides.

Rotation Regimes

For the extensive survey, rotation regimes were categorized into three types as follows. Rotation type A: Continuous maize cultivation (for at least four years) even as a second crop after early harvested main crops such as ryegrass; maize accounted for more than 80% of the crops in the rotation. Rotation type B: Maize was the predominant crop ($\geq$40% of crops in rotation, very often 70–80%), often as a permanent crop, but sometimes alternated with different crops in a flexible sequence according to WCR densities, with soybean, winter cereals, and sorghum being the most commonly rotated crops. Rotation type C: Maize was grown once or twice in the period considered and accounted for 30–40% of the crops in the rotation; many other crops were grown as in B, including double crops (e.g., soybean or sorghum after barley or canola) or meadows (e.g., alfalfa, *Festuca* spp., etc.), while continuous maize cultivation was very rare.

Sowing Date

All fields were divided according to sowing date into four categories as early (March), ordinary (April), late (May), and very late.

Hybrids

The following commercial maize hybrids were the main ones grown ($\geq$95% of the study plots): in Italy, DKC5276, 5830, 6815, 6718, P1028, P1547, DKC5830 (2015–2019), P1028, P1114, PR32B10, A14, Y43, Kerbanis, LG 33.30, 34.09, and 34.10; in Croatia, BC hybrids (BC Institute) BC 566, 578, 678, 354, 462B, 462, Jumbo 48, 408B, 4982, Pioneer hybrids Colomba, Natalia, Florencia, PR36V52, PR36R10, 33A46-1007, Dekalb hybrids DK571, Occitan, LG 33.30, and 34.09. To our knowledge, none of these hybrids have any varietal resistance or tolerance to WCR.

2.1.3. Insecticide Treatments

Soil insecticide and seed coating treatments are listed in Table 2. The main insecticides used as foliar treatments against the beetles were: lambda-cychalothrin 9.48% (200 mL/ha); alfacypermethrin 15% (0.4 kg/ha); chlorantraniliprole 9.26% + lambda-cychalothrin 4.63% (300 mL/ha).

Table 2. List of soil insecticides used on the fields during the years of monitoring.

Country (Number of Fields)	Product	Active Ingredients	Dose	Type
Croatia (14)	Cruiser®	Thiametoxam	0.63 mg/seed	coating
Croatia (3) Croatia (3)	Gaucho® Macho®	Imidacloprid	1.2 mg/seed	coating
Croatia (18)	Poncho®	Chlothianidin	1 mg/seed	coating
Croatia (1)	Mesurol FS500®	Methiocarb 50%	1.8 L/100 kg of seed	coating
Croatia (6)	Force 1.5G®	Tefluthrin 1.5%	10–12 kg/ha	granules applied in-furrow
Italy (52) Italy (91)	Force ST® Force 0.5 G®	Tefluthrin Tefluthrin	0.5 mg/seed 10–12 kg/ha	coating granules applied in-furrow
Italy (17) Italy (77) Italy (6)	Poncho® Santana® Gaucho®	Clothianidin Clothiadinin 0.7% Imidacloprid	0.5 mg/seed 11 kg/ha 1.2 mg/seed	Coating granules applied in-furrow coating

2.1.4. Damage Assessment

In Italy, at the end of the development period of the WCR larvae, in each monitored field, after an initial general assessment of the homogeneity of the field, two or four (in case of higher variability of crop damage) subplots of four 20 m maize rows were randomly selected. In cases where the fields had obviously different conditions due to cropping density or plant development, the fields were subdivided into relatively homogenous sections; each section was then assessed by randomly selecting at least two subplots and considering the field sections as separate records in the database. In Croatia, four rows of 100 plants were randomly selected in different parts of the fields. In order to reduce the influence of possible 'border effects' [23], only fields that presented visible plant damage exceeding 5% of the plants and distributed throughout the cultivated field were considered for the analysis.

The following parameters were recorded for each subplot (in the case of Italy) or row (in the case of Croatia): (1) the number of normal plants (no symptoms); (2) the number of plants with typical 'gooseneck' symptoms (plants leaning to the ground but not touching it and curving upright); (3) the number of lodged plants (plants touching the ground and not curving to an upright position). Total damage from WCR was calculated as the sum of plants with gooseneck symptoms together with lodged plants. To calculate the proportions damaged, the average number of plants with gooseneck symptoms + lodged plants in each subplot or transect was divided by the average number of total plants (plants with gooseneck symptoms + lodged plants + normal plants). The calculated percentage was entered into the database along with the field characteristics. To ensure that goosenecked and lodged plants were each primarily caused by larval feeding, at least 20 root systems per subplot were excavated and examined for WCR and root damage. For root damage assessments before 2010, the Hills and Peters 1–6 root damage rating scale [24] was used, and after that year the Node Injury Scale 0–3 [25] was used.

2.1.5. WCR Population Level

WCR beetle population assessments were performed as per the description in Section 2.2. In 60% of surveyed fields that had signs of damage, we deployed Pherocon® AM (PhAM) traps (Trece Inc., Adair, OK, USA); in the remaining fields, the mean number of adults from at least three of the surrounding (within 500 m) monitored maize fields was considered (24% of the total number of monitored fields). If properly managed traps were not available, the population size was considered not assessed (NA). This category included 16% of the fields.

2.2. Crop Rotation Study in Italy

Large areas cultivated mainly using maize rotated with other crops were compared against continuously cultivated maize in Veneto, Italy (Figure 1), to assess the effects of two rotation types on beetle and larval densities, as well as on root and plant damage by WCR (see Sections 2.2.1–2.2.3). We assessed the two main crop rotation strategies, structural rotation and flexible rotation, which are used to manage WCR populations in separate comparative studies. The experiment with flexible rotation was initially established as a randomized block design with three replications. However, because of the large areas involved in the study and the differences between the replicated sites in terms of beetle pressures, climatic conditions, and actual rates of rotated maize fields, comparisons are presented separately for each pair of comparative sites (i.e., IR1 and CH1, IR2 and CH2, IR3 and CH3). Fields (1 to 2 ha in size) where maize had been grown previously (continuous maize fields) or with maize fields in rotation were selected in the middle of croplands (about 100 ha in size) (Figure 1). The maize fields were extensively monitored from 2010 to 2020 (area covered: 45°64′ N, 12°96′ E and 45°05′ N, 11°88′ E). Field conditions were generally homogeneous with respect to their main agronomic characteristics.

The field sites are detailed in Sections 2.2.1 and 2.2.2. The rotation types are implemented as outlined below.

Structural crop rotation: A regular rotation regime where maize is sown once out of two or preferably more years (e.g., wheat–maize–soybean or wheat–soybean–maize; maize can represent 33% with three crops in rotation, 25% with four crops, and so on);

Flexible crop rotation: In areas where there is an appreciable incidence of continuous maize cultivation, maize fields are monitored using PhAM traps; when trap captures exceed the damage threshold (6 beetles/trap/day over several weeks), maize cultivation would be interrupted in the next year. With this strategy, it is expected that continuous maize cultivation will be interrupted after 2–3 years.

2.2.1. Structural Crop Rotation

Between 2010 and 2020, observations of WCR densities and crop damage were made at the three sites with structural rotation regimes. The first site was located at the experimental farm in Vallevecchia (Venice Province), an isolated area on the north-eastern Adriatic coast (45.05 N, 11.88 E, 2 m a.s.l.). The soil in Vallevecchia is Gleyic Fluvisol or Endogleyic Fluvic Cambisol [26], with textures ranging from silty-loam to sandy-loam. It has the following average characteristics: sand 34.2%, silt 42.6%, clay 23.2%; pH = 8.3; active carbonate = 3.0%; organic carbon = 1.0%. The climate is sub-humid, with annual rainfall of about 700 mm. Eighty percent of the soils are well drained, with the being remainder poorly drained. The Vallevecchia farm had a cultivated area of about 380 ha and a total area of about 600 ha.

The second site was located at the experimental farm in Diana (Treviso Province, 45°35′ N 12°18′ E, 6 m a.s.l.), which has a cultivated area of 70 ha. The third site was located at the center of 190 ha of cultivated land in Sasse Rami (Rovigo Province 45°30′ N 11°53′ E, 2 m a.s.l.) (Figure 1). Soils at both these sites are characterized by Endogleyan Cambisols [26], mainly silty-loam soils, and are more homogeneous in texture than Vallevecchia. The climate in Diana and Sasse Rami is also sub-humid, with annual rainfall rates of 846 mm and 673 mm, respectively. Temperatures in Vallevecchia, Diana, and Sasse Rami vary from January (minimum average: 0.1 °C, 0.9 °C, and 0.2 °C, respectively) to July (maximum average: 29.6 °C, 29.3 °C, and 30.6 °C, respectively).

A continuous structural crop rotation regime (winter wheat–maize–soybean) was applied at the sites, with some minor variations for experimental needs or because of constraints due to climatic conditions. Maize-after-maize accounted for about 7 to 10% of the cropped area in the first years when some fields were continuously planted with maize (mainly aimed at obtaining as much forage as possible from maize as the main crop). The two cropping regimes were compared for WCR populations as described above.

After a significant increase in the WCR population was detected in 2016 in Vallevecchia, the proportions of continuous maize fields were reduced to 3–4%. At all farms, about half of the fields were conventionally cultivated, i.e., plowed, cultivated, harrowed, and hoed, while the remaining fields were cultivated using conservation farming methods (minimum tillage or no tillage).

The following common agronomic practices were applied in all of the studied fields: fertilizer applications of 240–300 kg N/ha; 70,000 to 80,000 seeds/ha; 75 cm inter-row width; pre-emergence and post-emergence herbicide treatments.

At each site, at least two fields per year were selected for beetle monitoring using traps and root sampling (2016 to 2018 only), while 10 fields were always assessed for symptoms of crop damage using the methods described below.

2.2.2. Flexible Crop Rotation

A two-year experiment was conducted in 2016–2017 in the province of Treviso in an area with intensive maize cultivation in the Veneto region (45°64′ N, 12°96′ E). The experiment compared crop rotation as a crucial IPM tool with conventional management under long-term continuous maize cultivation and using insecticides to control WCR.

Two homogeneous areas (scenarios) of about 100 ha each were selected in three zones with heavy maize cultivation, consisting of one or more farms; the two homogeneous areas in each zone were characterized by:

1. Intensive rotation (agronomic control with intensive rotation) (IR): Maize generally rotated every two years (maize area 66% per year), with no prolonged maize cultivation; monitoring of pests with traps; no use or reduced use of chemical treatments;
2. Chemical approach (chemical control) (CA): Presence of continuous maize plots over many years with adult pest treatments or seed treatments at seeding with insecticides on most or all of the cultivated area.

Based on preliminary surveys conducted during the 2016 and 2017 maize seasons, three pairs (replicates) of regimes (CA and IR) were concretely identified, each at least one kilometer apart. The first pair (1) comprised two main municipalities, Montebelluna (CA1) and Trevignano (IR1); the second pair (2) was located inside the municipality of Paese (CA2 and IR2); the third pair (3) was included in the Quinto di Treviso municipality (CA3 and IR3) [27].

The soils of pairs 1 and 2 were Cutanic Luvisols or Aric Regosols [26] with the following characteristics: stones 24–35%; sand 36–40%, silt 38–34%, clay 26%; pH = 7.6; total carbonate = 0–4%; organic carbon = 1.79–2.56%. The soils of pair 3 were Haplic Cambisols [26], with the following characteristics: sand 55–40%, silt 31–40%, clay 14–20%; pH = 7.6; total carbonate = 1–0%; organic carbon = 1.15–0.89%; as well as Fluvic Cambisols [26], with the following characteristics: stones 6–2%; sand 26–47%, silt 48–27%, clay 26%; pH = 7.6; total carbonate = 2–0%; organic carbon = 1.40–1.66%. The climate is sub-humid, with annual rainfall of about 950 mm. All soil areas are well drained.

The percentages of land under rotational and continuous maize cultivation in the six scenarios are shown in Table 3. In scenarios CA2 and CA3, continuous maize cropping clearly predominated (86.6% and 84.1%, respectively), while in scenario CA1 the proportion of reseeding was just above 60%.

Insecticide use (directed at adult beetles) equaled zero in all IR scenarios and in about 20% of CA scenario plots. Insecticide treatments at seeding (microgranules and coating) were applied to about 80% of the plots in the CA scenarios and to 60% of plots in the IR scenarios. Of these, about 40% were microgranule insecticides, which have the potential to significantly reduce root damage (mainly tefluthrin) [28].

The common agronomic practices used in all fields studied were similar to those described in the Section 2.2.1; the predominant tillage method was conventional tillage based on ploughing. At least three fields were selected in the center of each area for crop monitoring and pest evaluation.

Table 3. Distribution of the rates of cultivated surfaces based on the number of consecutive years of maize cultivation in each rotation scenario under study, province of Treviso.

Scenario	Townland	Surface (ha)	No Maize	Maize							Number of Plots
				1st Year	2nd Year	3rd Year	4th Year	5th Year	6th Year	2nd–6th Year	
IR1	Paese/Trevignano	26.8	30.1	37.7	10.6	6.5	1.5	1.9	11.6	32.1	17
CA1	Montebelluna/Trevignano	30.6	19.8	18.3	1.6	7.2	17.9	1.8	33.2	61.9	54
IR2	Paese	27.3	57.9	16.0	6.9	1.9	0.0	3.5	13.7	26.0	13
CA2	Paese	17.0	0.0	13.4	0.0	6.3	6.8	5.6	67.9	86.6	33
IR3	Quinto TV	15.2	37.9	34.6	23.3	0.0	4.2	0.0	0.0	27.5	30
CA3	Treviso/Quinto/Paese	23.2	9.0	4.5	14.7	8.6	0.0	0.0	60.8	84.1	54

2.2.3. WCR Population Density and Damage Estimation

Within each crop rotation scenario (IR1, CA1, IR2, CA2, IR3, CA3), the following parameters were evaluated: (i) plant density and damage by other soil pests; (ii) number of WCR larvae in root systems (2016 only); (iii) root damage index (Node Injury Scale 0–3 [24]); (iv) catches of adult WCR plants with yellow sticky traps (Pherocon® AM, Trece Inc., Adair, OK, USA), with data from 3 traps per field for up to 6 weeks; (v) crop damage in terms of percentage of "goosenecked" or "lodged" plants, the same as for the root damage index. Data collection linked to each parameter was conducted as outlined below.

Plant stand density and damage from other soil pests were assessed at the 5–8 leaf stage. Two plots of two rows measuring 20 m in length were selected for each of the 5–10 fields monitored in each area (IR1, IR2, IR3, CH1, CH2, CH4); all plants were counted and divided into healthy plants, plants showing signs of damage by wireworms, and plants showing signs of damage by other soil pests (e.g., black cutworm).

WCR Density

To estimate larval WCR densities, five plants for each of the 5–10 (only 1 in IR3) fields monitored in each area (IR1, IR2, IR3, CH1, CH2, CH4) were selected between the second stage and third crop growth stage according to the Davis model [29] at the time of maximum larval presence (late May–early June). Root systems and associated soil samples (intact with the roots) from the middle rows of each plot were randomly sampled with a shovel (approximately 15 × 15 × 15 cm). Samples were placed in funnels (18 cm in diameter) and kept under shelter with an open side allowing winds to enter; no lights were used to accelerate the drying process. Under each funnel, a water-filled vial (2.5 cm in diameter) was attached to collect larvae that moved downward as the soil dried. In order to prevent soil from falling into the vial, a 10 × 10 cm net (0.5 cm bore) was placed just under the sample. The funnels were inspected and larvae collected every two days to determine species and developmental stages. The labelled samples were washed and observed for root damage as described below.

The abundance of WCR adults was estimated using yellow sticky Pherocon® AM (PhAM) or (pair 3) PALs Csalomon® (Csalomon Group, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary) (without pheromones) traps, set out in each of the 3–9 (2 in IR1 2016) monitored fields per area (IR1, IR2, IR3, CH1, CH2, CH4) per year. Three traps were deployed per field and observed according to official monitoring methods for a 6 week (42 day) period (weekly inspection of traps with removal of any attached beetles). The period commenced using predicted pupation times according to the Davis model and based on first captures in PAL pheromone traps (at least 3 per area). Yellow sticky traps attracting beetles by color were placed at least 30 m from the field edge and inside the field. The traps were attached to maize stalks with cable ties at the height of the plant ear, or alternatively on a 1 m high wooden stake in the case of fields with smaller plants. To ensure trap efficacy, the traps were all re-set at least once after the third week or whenever their condition (e.g., drying or wearing of the glue, or clogging with dirt or captured insects) might have affected captures.

Root Damage

Relative levels of root damage were estimated based on root rating or plant lodging. Damage estimates were made after silking.

For root rating, 10 root systems from maize plants from the middle rows of the 4–7 monitored fields per monitored area (IR1, IR2, IR3, CH1, CH2, CH4) were randomly sampled with a shovel. The excavated maize root systems were taken to the farm center for washing; after washing, root damage was assessed using the Node Injury Scale 0–3 [25]. To estimate plant lodging, plants were counted in two subplots of two rows measuring 20 m that were randomly selected in each of the 10–40 monitored fields per area (IR1, IR2, IR3, CH1, CH2, CH4), excluding margins and anomalous areas; damaged plants were recorded and divided into plants with "gooseneck" symptoms and lodged plants (presented as totals in this paper); the percentage of damaged plants ((n° of damaged plants/healthy plants + damaged plants) $\times$ 100) per subplot was calculated and used for statistical analysis.

2.3. Climatic Conditions

Details of climatic variables during the experimental period can be found at the ARPAV site [30] for Italy and through the Croatian Meteorological and Hydrological Service [31] for Croatia.

2.4. Statistical Analysis

2.4.1. WCR Risk Factors Study in Italy and Croatia

To determine the significant predictors of damage risk, the percentages of damaged plants (goosenecked and lodged) at the sampled sites were first dichotomized into $\leq$5% and >5% to eliminate border effects and to include in the analyses only those fields with significant WRC damage. Logistic regression was performed to estimate damage risk based on predictive variables related to adult beetle populations, agronomic practices, soil properties, and treatments. The analysis was conducted using a univariate approach in which each predictive factor was included in the model in a stepwise fashion [32]. Estimated least square means were calculated. Pairwise post-hoc comparisons between levels of factors were Bonferroni adjusted. The relative risk (RR) of damage and 95% confidence intervals (95% CI) were calculated. Significant factors identified in the univariate approach were entered into a multivariate model to test their overall significance while excluding overlap between predictors. This analysis was performed using SAS 9.4 (Institute Inc., Cary, NC, USA).

2.4.2. Crop Rotation Study in Italy

We used parametric and non-parametric analyses to compare WCR densities and damage in maize fields under rotation with those in conventionally managed fields. Normally distributed data (Shapiro–Wilks test) and data that passed the Levene homoscedasticity test were analyzed using univariate ANOVA with two factors (scenario (levels = IR and CH) $\times$ year (levels = 2016 and 2017)) and post-hoc comparisons were conducted using the HSD–Tukey test. Parameters that did not meet the test conditions for ANOVA were analyzed using the non-parametric Kruskal–Wallis test (% damaged plants, nr. larvae/plant) and multiple pairwise comparisons were made using Dunn's procedure. Pearson's correlation was used to assess relations between the percentage of rotation and adults above threshold densities. All analyses were performed using IBM SPSS Statistics version 22.

3. Results

3.1. WCR Risk Factors Study in Italy and Croatia

Results for fields with visible plant damage (gooseneck + lodged plants) greater than 5% are presented in Table 4. In Italy, during the first 3 years of the survey (2010–2012), visible WCR damage was detected exclusively in continuous maize fields (with at least 6 years of previous continuous maize cultivation); this encompassed 37 ha comprising 18 damaged fields. In 2010, outbreaks were limited to the western part of the region (Verona

and Vicenza Provinces); in the two following years, visibly damaged fields were also found in the central part of the region. In 2013, some damaged fields without a long history of continuous maize cultivation (some were in their second or third year under maize) were reported for the first time.

Table 4. Univariate risk analysis establishing the risk of plant damage (gooseneck + lodged plants) exceeding 5%.

Variables	Level	LS Means: % Cases for Total Damage ≥5%	Comparisons	*p*	RR 95%CI
WCR beetle population level PhAM Y-1	Low	8	reference level		
	Medium	23	M vs. L	0.006	2.92 (1.36–6.28)
	High	42	H vs. L	<0.001	5.28 (2.53–11.04)
	Very high	63	VH vs. L	<0.001	7.97 (3.88–16.36)
Soil texture	FS *	26	reference level		
	F **	38	F vs. FS	0.994	
	FA ***	37	FA vs. FS	0.990	
	FL **** + FLA *****	32	FL + FLA vs. FS	0.990	
Rotation type	A (100% maize)	23	C vs. A	0.038	0.57 (0.33–0.97)
	B (70–80% maize)	40	C vs. B	0.017	0.52 (0.31–0.89)
	C (30–40% maize)	44	reference level		
Sowing date	Early (March)	41	early vs. ord.	0.039	1.21 (1.01–1.44)
	Ordinary (April–mid May)	50	reference level		
	Late (second half of May) + Very late (June)	25	very late + late vs. Ordinary	0.002	0.61 (0.45–0.84)
Hybrid variety (producer)	Dekalb	22	De Kalb vs. KWS	0.142	
	KWS	36	reference level		
	Pioneer	40	Pioneer vs. KWS	0.053	
	BC Institute	32	BC Institute vs. KWS	0.259	
	Others	55	Other vs. KWS	0.005	2.53 (1.32–4.84)
Insecticide treatment against adults in previous year	No	40	reference level		
	Yes	43	yes vs. no	0.527	
Soil insecticide application	No	39	reference level		
	In furrow micro-granular insecticide	57	seed vs. No	<0.001	1.45 (1.18–1.79)
	Insecticide coating	33	yes vs. No	0.109	
	Both (granular and coating)	31	yes + seed vs. no	0.400	
Insecticide (active ingredient)	No	39	reference level		
	Tefluthrin in furrow	22	teflutrin in furrow vs. No	0.006	0.56 (0.37–0.85)
	Teflutrin as seed treatment	72	tefluthrin seed treat. vs. No	<0.001	1.82 (1.44–2.30)
	Lambda-cyhalothrin	41	lambda-cyhalothrin vs. No	0.739	
	Other	35	other vs. No	0.325	

FS * = sandy loam, loamy sand, sandy clay loam; F ** = clay loam; FA *** = clay loam; FL **** = silt loam; FLA ***** = silty clay loam, silty clay.

In 2014, a few fields with first-year maize damage were found (Table 5); they occurred exclusively in plots near maize fields with continuous cropping, where continuous maize fields comprised more than 50% of the cultivated area and where high to very high populations of adult WCR occurred (>6 adult/trap/week, mostly >10 adult/trap/week, often more than 20). Damage from WCR was also observed in Croatia in fields under the first year of maize, but significant root damage and lodging were limited to the margins, at up to 20 m from the boundary of the continuous maize fields [23].

Low WCR populations (<2 adult/day) had a low probability of causing maize damage; medium, high, and very high beetle populations (>6 adult/day) increased the risk of damage by 2.92-, 5.28-, and 7.97-fold, respectively, compared to low populations.

Most factors (including soil properties) did not affect the likelihood of maize damage, with the exception of sowing time. Late or very late sowing significantly reduced the risk of damage compared to normal sowing; in contrast, early sowing increased the damage risk by 1.21-fold.

Table 5. Maize fields following no maize crop in the previous year, damaged (>5% of goosenecked or lodged plants) by WCR larvae from 2014 to 2017 in Italy.

Rotation	Fields	Previous Crop	Ha	Adult Population Density (Number of Beetles/Trap Per Day) [1]
C	8	Alfalfa (4) Barley (4)	10.04	Very high *
C	1	Alfalfa	0.14	High **
C	1	Sorghum	1.6	NA ***
B	16	Soybean (2), Winter Wheat (10) Barley (3) Pumpkin (1)	47.46	Very high
B	6	Soybean (4), Winter Wheat (2)	5.77	High
B	8	Soybean (4), Winter Wheat (2) Barley (2)	5.98	NA
Total	40		70.99	

Note: [1]: * Very high beetle population: >10 beetles/trap per year; ** High beetle population: >6 beetles/trap per year; *** NA = not available.

Insecticide treatments against adults showed no effect in reducing the risk of WCR, while rotation C reduced the risk of damage compared to both rotation regimes A and B.

In-furrow insecticide treatments, all considered, did not reduce severe (goosenecklodging) plant damage risk by WCR larval feeding activity; however, whereas Ercole® (lambda-cyhalothrin) treatment caused no risk reduction, in-furrow Force® (tefluthrin) significantly reduced the risk by about 40% (RR = 0.56, 95% CI 0.37–0.85, $p = 0.006$). In contrast, seed treatments, including all active ingredients, caused a slight risk increase. Force® as a seed treatment, considered separately, also caused a slight increase in risk.

Multifactorial Model

Multivariate analysis of factors highlighted some changes in the estimation of risk ratios for the independent contributions by variables included in the final model: high to very high WCR beetle populations continued to be a significant factor ($p < 0.001$), whereas medium beetle populations had a relatively lower impact ($p = 0.03$). Force® (tefluthrin) used as an in-furrow treatment reduced the risk by about 50% (RR = 0.46; 95% CI 0.22–0.93; $p = 0.03$) with respect to no treatment, and late sowing tended to reduce the risk of severe damage by about 40% (RR = 0.56; 95% CI 0.31–0.99; $p = 0.05$) with respect to the ordinary sowing date. No other factors were statistically significant predictors of risk.

3.2. Crop Rotation Study in Italy

3.2.1. Structural Crop Rotation

The first catches with PAL sex pheromone traps were recorded in 2010 (42 specimens, average of two traps placed in continuous maize fields), even though a first occurrence of WCR was expected in 2008 [17]. In 2011, the first catches with PhAM traps (0.5 beetles/trap/day over a 42 day period) were recorded in continuous maize fields. WCR populations increased in continuous maize fields until 2015, when PhAM traps reached 5.8 beetles/trap per day over a 42 day period in some fields. Over the next few years, general reductions in maize cultivation, as well as for continuous maize, probably caused large decreases in populations, which were consistently maintained at negligible levels (SR1, SR2, Figure 2); however, in areas with high proportions of continuous maize, WCR populations remained very high (CM1, CM2, Figure 2), even when PhAM traps had been set in first-year maize fields. Nevertheless, no WCR root or plant damage was observed. The same population patterns were observed in the other two experimental farms (Sasse Rami and Diana), with average beetle population levels never exceeding two individuals/trap/day. Visible WCR damage symptoms were never found at these two farms. Although having a large cultivated area, these two farms were not isolated like Vallevecchia; nevertheless, the WCR populations that colonized these farms in the end of the 2000s [17] showed no significant increase in density over the next 10 years.

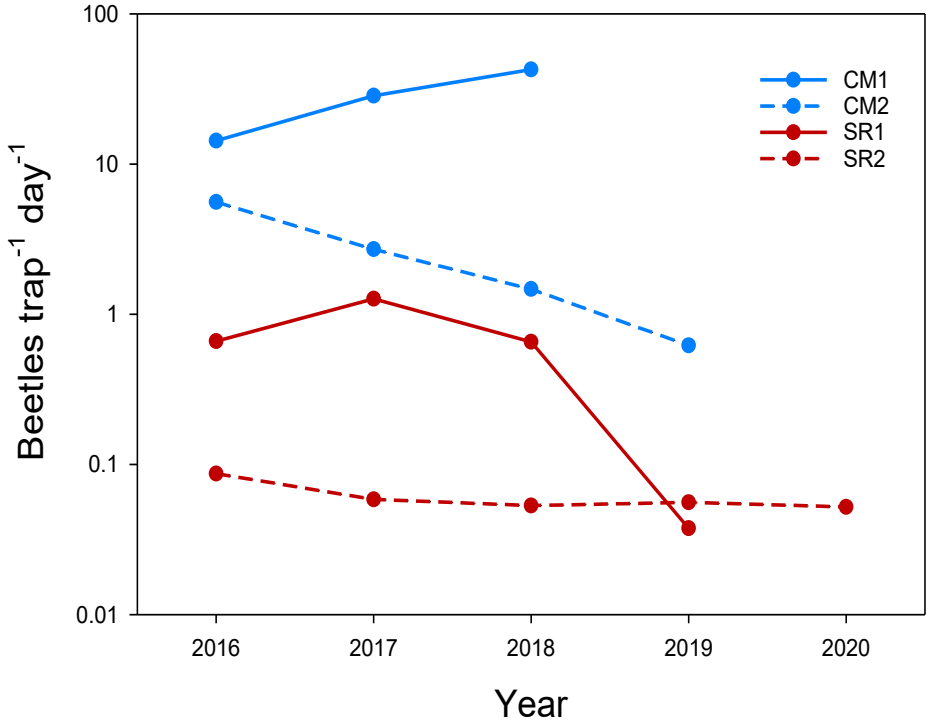

Figure 2. Effects of structural rotation (winter wheat/maize/soybean) on WCR population levels over the years. Numbers of WCR adults/trap/day (total sum at six weeks) in two different scenarios are shown—the first with a high percentage of continuous maize (blue lines and symbols = CM1, CM2) in Treviso province and the second based on structural rotation (red lines and symbols = SR1, SR2) at Vallevecchia pilot farm in Venice province (2016–2020), both in north-eastern Italy. Legend: CM1 = high presence of continuous maize with traps in continuous maize fields (Treviso); CM2 = high presence of continuous maize with traps in rotated maize fields (Treviso); SR1 = extensive structural crop rotation with traps in continuous maize fields (Vallevecchia, Venice); SR2 = extensive structural crop rotation with traps in first-year maize (Vallevecchia, Venice). Numbers are two-point moving averages.

3.2.2. Flexible Crop Rotation

The plant densities were generally good in both survey years; between 85% and 90% of the seeds sown resulted in plants that developed regularly, while infestations by wireworms or other soil pests (such as black cutworms) were negligible in both soil-insecticide-treated and non-treated plots.

The Node Injury Scale 0–3 showed significant differences in the degree of root damage between scenarios, with higher values in CA than in IR, and a statistically significant difference between the two treatments in area 3 (Figure 3A), ($F = 31.39$; $p < 0.001$; $N = 740$), where the maize percentage of cultivated land was the highest.

The percentages of plants with gooseneck symptoms or that were lodged (i.e., WCR damage) were found to be significantly higher in the CA scenarios compared to the IR scenarios (Kruskal–Wallis $K = 30.67$; $p < 0.001$; $N = 260$) (Figure 3A). No damage to maize was observed in year 1.

Cumulative adult catches/trap/day in week 6 were significantly higher in the CA scenario than in the IR scenario in areas 2 and 3 ($F = 12.17$; $p < 0.001$; $N = 201$), where the prevalence of continuous maize was higher (>80% in CA); there was no significant difference in area 1, where there was less difference between the 1CA and 1IR rotational plots (Figure 3B).

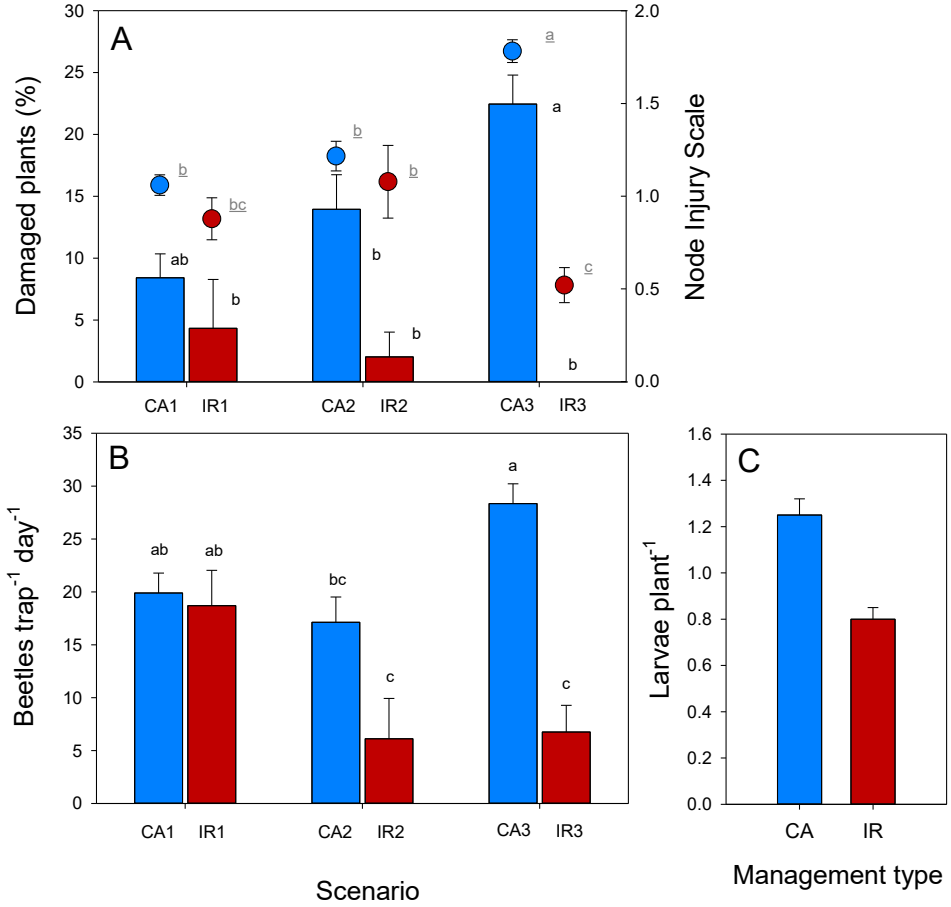

Figure 3. WCR pressure in the surveyed areas with continuous (blue) and rotated (red) maize crops: (**A**) Iowa index scores (Oleson: 0–3) and total (with gooseneck symptoms and lodged) damaged plants (%); (**B**) number of beetles captured by PhAM trap/day (average after six weeks), in the six scenarios under study (three areas hosting the two managements: CA, IR), years 2016–2017; (**C**) number of larvae per plant in the two managements areas: CA = chemical approach; IR = intensive rotation. Standard errors are indicated.

Observations of larval abundance were made only in 2016 and showed significantly higher numbers of larvae in CA scenarios (1.254 larvae/plant) compared to IR scenarios (0.796 larvae/plant) (K = 4.503; p = 0.034; N = 199) (Figure 3C).

The percent of maize area that exceeded the damage threshold and that had an appreciable risk of yield reduction (≥6 beetles/trap/day) and the percent of rotated area were inversely correlated (Figure 4). This confirms the evidence from Szalai et al. [33], who found that the higher percentages of damage occurred in plots with less than 60% rotation; in our case, higher damage rates were found in the 1CA, 2CA, and 3CA scenarios (Figure 4), which had values of 38.1%, 13.4%, and 13.5% of the rotation area, respectively.

The risk of root damage to contiguous corn fields was nearly twice as high in the CA scenario (47%) as in the IR scenario, with the risk of overall crop damage being five times (80%) higher. This illustrates how adult pressure (density) in a given area determines the actual risk of maize damage under the same soil, agronomic, and climatic conditions, as pressure is positively associated with contiguous maize field density, which favors WCR reproduction.

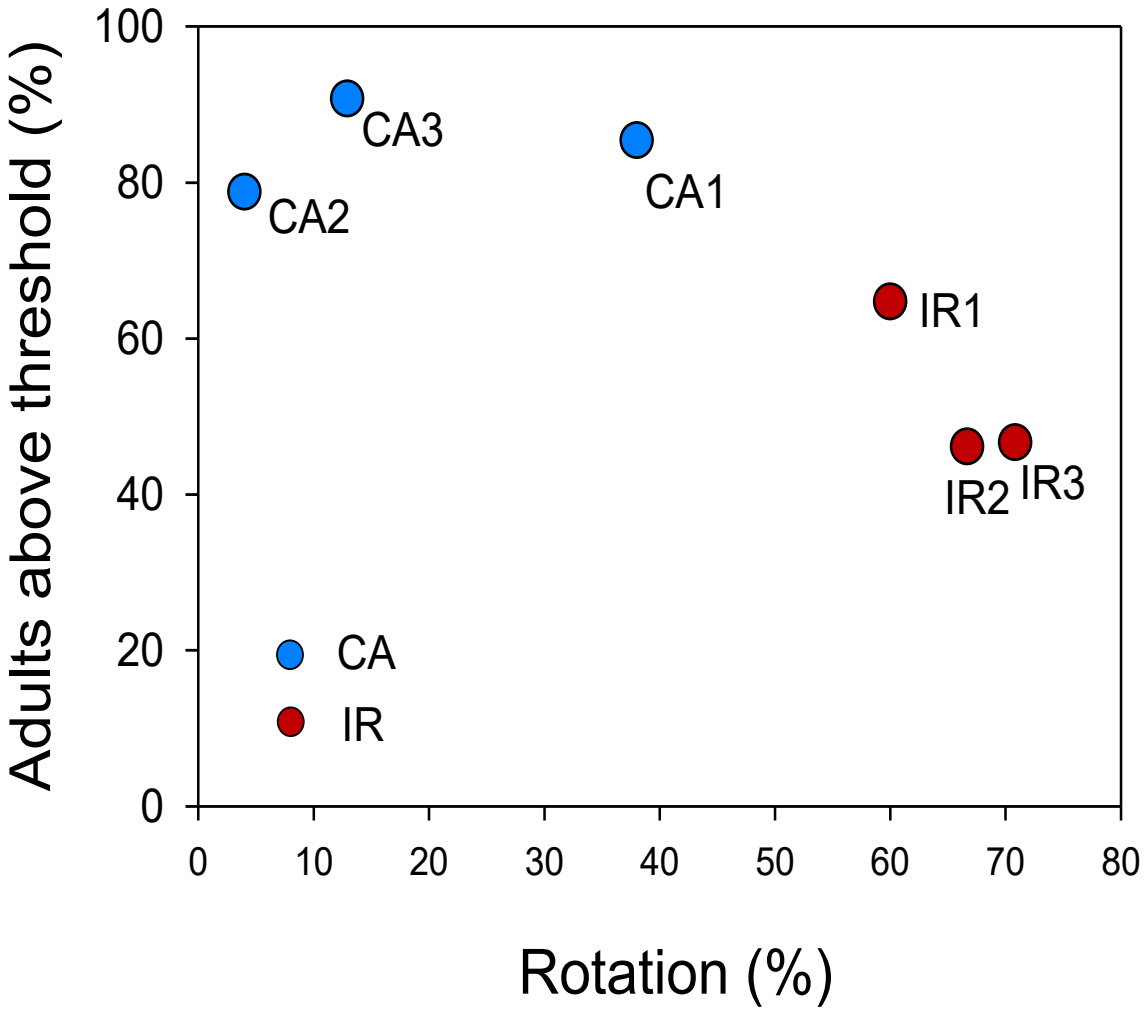

Figure 4. Relation between the percentage of cultivated land in rotation (no continuous maize) and the percentage of land where adult WCR levels exceeded the damage threshold (i.e., >6 beetles/trap/day) accumulated in week 6. CA = chemical approach; IR = intensive rotation. Numbers are scenarios indicated in Figure 3.

4. Discussion

4.1. WCR Risk Factors Study in Italy and Croatia

As shown in Table 4, beetle population densities accounted for most of the risk of maize damage from WCR. Most methods used to assess WCR damage risks use the beetle population density as the main predictor [7,8,34]. Therefore, management actions that reduce WCR population growth rates are essential to reduce damage risks. Very high beetle populations may cause significant damage to maize, even in rotated fields where maize has not had a major presence. In addition to second-year maize fields, first-year maize fields may also be damaged (Table 5), although this is a rare occurrence. It is clear that high WCR populations in cultivated maize promotes the spread of beetles to neighboring maize fields, and occasionally to non-maize fields.

Insecticides (all active ingredients considered) applied as in-furrow applications or as seed coatings did not reduce the risks of severe damage to maize from WCR. Only in-furrow applications of tefluthrin resulted in an estimated 50% risk reduction. In previous studies, this insecticide also showed potential to reduce WCR damage to roots [28,35–38]. Contrary to expectations, univariate analyses indicated that insecticidal seed treatments slightly increased the damage risk. In any case, this factor was weak (not significant after multivariate analysis). The fact that high to very high populations of WCR were found in many more fields with insecticide-treated seeds than in untreated fields, together with

the phytotoxicity of some active ingredients, which may have reduced plant resistance to lodging, might explain these negative impacts of seed treatments. A low efficacy of insecticide seed coatings has also been previously reported by several authors [35,37]. All other factors, including soil texture, maize hybrid, and insecticidal treatments against adults, played no significant role in reducing risks.

4.2. Crop Rotation Study in Italy

Despite the high use of soil and foliar insecticides, larval and beetle densities were significantly higher in the CA (with prevalence of continuous maize and higher insecticide use) scenario than in the IR (high rotation rate and lower insecticide application) scenario. The build-up of WCR beetle populations to densities at which they pose a high risk of damage to maize crops took several years. After a successful eradication program (Venice airport area) that started in 1999 [17], WCR populations in Veneto were negligible until 2005. Afterwards, there was a progressive establishment of populations from the west (Lombardy) and east (Friuli Venezia Giulia), as shown by the use of pheromone traps [39], while no WCR beetles were found in the first eradication focus area in Venice. The spread of WCR from the east and west increased its presence in the region. Population densities continued to increase over several years, with the first visible WCR damage to maize occurring in 2010 in the western part of the region (Verona and Vicenza provinces) [40].

In Veneto, during the first 3 years (2010–2012), visible WCR damage was found exclusively in continuous maize fields (with at least 6 years of previous continuous maize cultivation). In 2013, for the first time, some damaged fields were found where there was no continuous maize cultivation (e.g., some maize fields in the second or third year). Beginning in 2014, when most fields were found to have high beetle densities, many damaged maize fields were observed that did not have a long history of continuous corn planting, including some first-year maize fields (Table 5). This limited number of fields with first-year maize damage (Table 5) were located exclusively in plots near maize fields with continuous cropping, in areas where continuous maize fields comprised more than 50% of the cultivated area, and where high or very high populations of adult WCR occurred. These results are consistent with the "lack of maize fidelity" hypothesis [16,41] as a general mechanism underlying rotational resistance.

The results also support reports by authors from Croatia [23] that indicated damage during the first year of maize cultivation. These authors reported that maize can be damaged in the first year if the field is adjacent to a continuous maize field that had a high adult population in the previous year, and that damage is possible up to 15 m from the border. Kos [42] demonstrated that the type of the previous year's crop had an effect on damage when adjacent to maize fields; fields seeded with sunflower had higher adult populations than fields seeded with soybean, sugar beet, and wheat. However, damage at increasing distances from the edge of the maize field in the first year after sowing the different crops did not differ. In Croatia, economic losses due to the feeding by WCR larvae on maize roots can be expected in first-year fields (up to 10 m from the edge of the "donor" fields) only if WCR beetle populations have reached very high levels (weekly catches in the donor fields at least 70 beetles/PhAM; i.e., 10 beetles/PhAM/day) [42].

The situation observed in Italy in 2014 differed from that in Croatia. The fields in Italy were generally 30–40 m wide and damage was widespread throughout the fields, including in the center and not only at the edge. Some fields were not bordered by maize fields in the year before damage. In contrast to Croatia, where the population was high in only a few continuous maize fields and there were not many continuous maize fields in the study area, in Italy continuous maize fields were prevalent, supplying a large radius of available food and promoting large beetle populations. In subsequent years, probably as a result of a sharp decline in maize fields in the area (from 30 to 50%), no further first-year damaged fields were detected. A dramatic decrease in maize acreage (by 31% on average) was observed throughout the Veneto region [43] (Figure 5), resulting in an overall decrease in the WCR beetle population. Awareness of the damage potential of WCR after visible

damage was observed in some fields and increasing mycotoxin problems (high contents of aflatoxins during drought and in warm summers) led to a reduction in the price of maize grain and a consequent significant reduction in maize acreage.

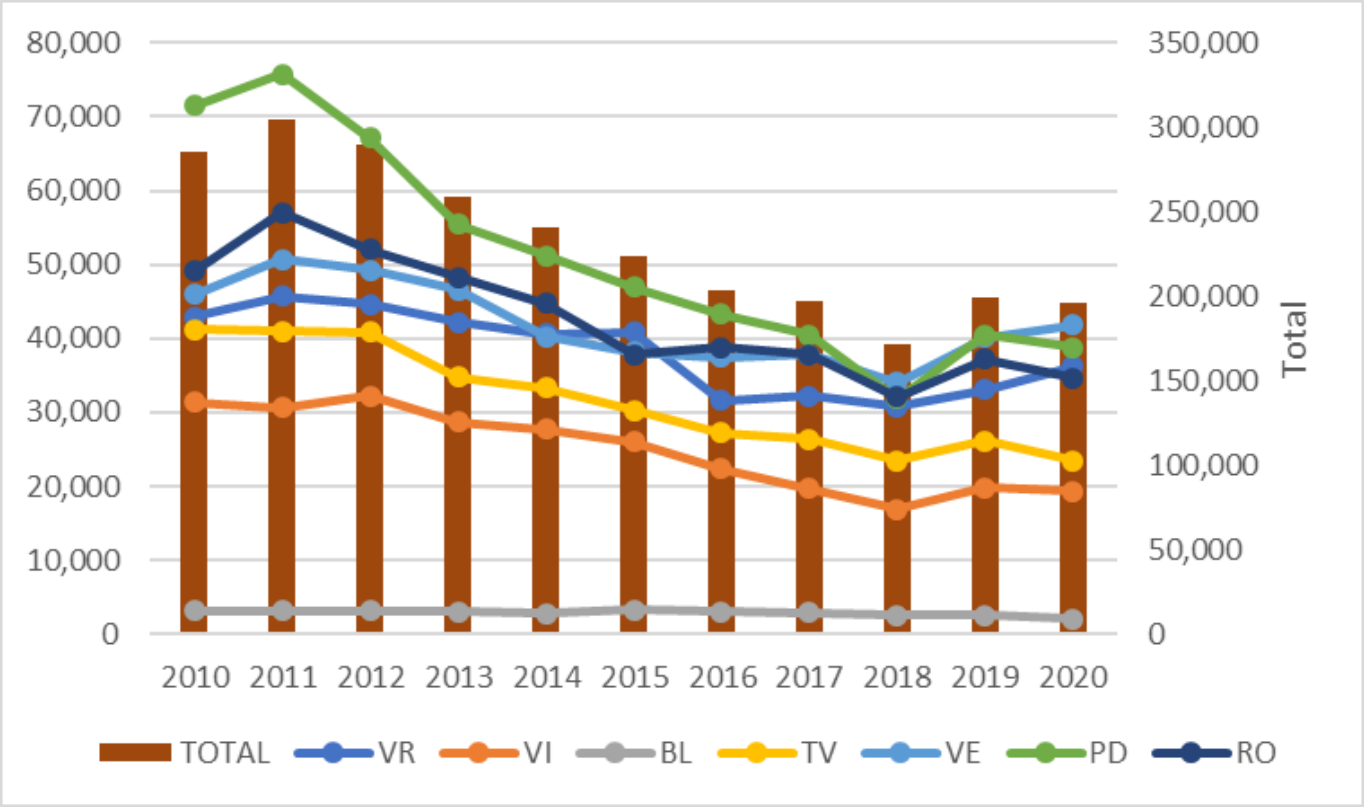

Figure 5. Maize cultivation land (ha) areas in the Veneto provinces from 2010 to 2020 (Source: Veneto Agricoltura 2021 [43]) (VR: Verona; VI: Vicenza; BL: Belluno; TV: Treviso; VE: Venezia; PD: Padova; RO: Rovigo).

The results from Italy may also be indicative of an established rotation-resistant strain that stopped causing severe damage in cornfields due to population declines below the damage threshold. Spencer and Levine 2006 [41] and Knolhoff et al. 2006 [44] described rotation-resistant beetles as more active and better fliers than wild-type beetles. Mikac et al. [45] showed that rotation-adapted beetles have wider wings, while Kadoić Balaško et al. [46] found that rotation-adapted WCR populations have a more stable and elongated wing shape, which means that these individuals can fly longer distances. Elongated wings are more aerodynamic and are considered part of the migratory movement, and could be a useful invasive dispersal strategy for mated females [45]. This behavior is simply an intensification of a solid genetically-based species trait that has been highly expressed wherever the species has invaded new areas from distances greater than 10 km per year. This has been observed in a population that is the result of a "unique" hybridization between WCR populations of different origins (including in the U.S., where rotation-resistant populations occur) and later European populations [12–15,18,47]. Although an increased ability of beetles to digest soybean leaf tissue has been noted in rotation-resistant populations [48], the increased propensity to escape and disperse to any field (lack of fidelity to maize), possibly triggered by the enormous competition at extremely high WCR beetle populations, seems to be the key factor explaining the first-year maize damage observed in north-eastern Italy. Fortunately, this phenomenon is reversible when WCR populations decrease in response to reductions in maize acreage.

In any case, structural and flexible crop rotation regimes both proved to be effective in keeping populations below the damage threshold. This study supports the conclusions

of Szalai et al. [33] that higher damage occurs in plots with less than 60% rotation. Crop rotation has likely fragmented populations and forced them into bottlenecks, resulting in large reductions in population size [49–51].

This study mainly focused on maize fields damaged by WCR and the surrounding fields. Most of the other maize fields in the areas where damaged fields were located did not show visible WCR damage symptoms. At least 100 undamaged fields were found around each damaged field. This ratio confirms data from Agrifondo Mutualistico Veneto and Friuli, which introduced insurance coverage through the Mutual Fund (MF) [52,53]. Applications for compensation for WCR damage represented far less than the 0.5% of the total maize area covered by the innovative insurance instrument.

5. Conclusions

The survey of key agronomic–cultivation factors and beetle population levels in fields damaged by WCR allowed us to determine that beetle population densities accounted for most of the risk of maize damage from WCR. Area-wide studies showed that beetle population levels depend on the number of maize cultivation fields and rotation regimes applied; higher beetle populations occurred in plots with less than 60% rotation.

Insecticide use did not affect WCR populations, while cultural controls based on rotation significantly reduced the damage risk. Structural rotation consistently prevents WCR from damaging maize crops, while flexible rotation may introduce a slightly higher risk of local sporadic damage, as this means that the population is approaching the threshold for control actions.

Maize growers and decision makers must consider practical values (as reported here) for the percentages of maize and continuous maize in terms of acreage as key parameters on which to base effective WCR management.

Moreover, diversified crop rotation strategies that significantly impact pest populations may reduce the risk of pest adaptation. These strategies are also more convenient than costly genetic approaches (WCR GMO resistant hybrids) that can produce pest-resistant strains in a short period of time [4]. The rotational strategies we propose are likely to be valid wherever they are applied, with possible local adaptations.

The results of our study confirm that crop rotation is an effective strategy to keep WCR populations permanently below the damage threshold, thereby preventing the use of pesticides in compliance with current European legislation.

Author Contributions: Conceptualization, L.F. and R.B.; methodology, L.F., B.C., F.C., T.K. and R.B: validation, B.C., R.B., F.C., I.B. and L.F.; formal analysis, B.C., F.C. and L.F.; investigation, R.B., D.L., T.K., F.C., I.B. and L.F.; resources, R.B. and L.F.; data curation, R.B., B.C., D.L., F.C., I.B. and L.F.; writing—original draft preparation, L.F., B.C., F.C., D.L., F.G.H. and R.B.; writing—review and editing, L.F., D.L., RB, B.C., T.K. and F.G.H.; visualization L.F. and F.G.H., supervision, L.F.; project administration, L.F. and R.B.; funding acquisition, L.F. and R.B.; All authors have read and agreed to the published version of the manuscript.

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Effect of Pupal Cold Storage on Reproductive Performance of *Microplitis manilae* (Hymenoptera: Braconidae), a Larval Parasitoid of *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Simple Summary: Parasitoids are one of the most important biological control agents, and there are increasing requirements for long-term breeding. It is critical to figure out the parasitoid biological properties and disclose the effects of cold storage on them to extend their longevity. In this study, we investigated the field parasitism rate and clarified the biological parameters of *Microplitis manilae*, a dominant larval parasitoid of *Spodoptera frugiperda*. Further analysis revealed that the pupal cold storage, including different storage temperatures, storage period and storage time, significantly affected the emergence rate, parasitism rate and longevity of wasp adults, and the optimal storage condition was middle-aged pupae stored at 10 °C for 5–10 d. These results provide a novel insight into the mass-rearing of *M. manilae* and contribute to the biological control using *M. manilae* against *S. frugiperda*.

Abstract: As a major invasive pest in China, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) has caused great damage to crops. Hymenopteran parasitoids, especially the braconid wasps, play crucial roles in depressing pest populations. However, there was little information about the ideal storage of parasitoids to achieve their mass-rearing. Here, we identified a dominant parasitoid of *S. frugiperda*, *Microplitis manilae* (Ashmead) (Hymenoptera: Braconidae), in the Hainan province of China with a field parasitism rate of 5.66–19.10%. The investigation of biological parameters revealed that the parasitism rate of *M. manilae* significantly decreased with an increase in both wasp adult longevity and host age, and the wasp of 1–3 d post eclosion performed best on the first instar of host larvae, showing the highest parasitism rate. We also discovered that the decreased temperature from 30 to 20 °C greatly extended the longevity of wasp adults, and a similar result was observed after feeding on 10% sucrose water compared with sterile water. Then, the effects of different pupal cold storage temperatures (4 and 10 °C), storage period (prepupa, middle-aged pupa, late-aged pupa) and storage time (5, 10 or 20 d) on the emergence rate, parasitism rate, female proportion and longevity of *M. manilae* were investigated. The results demonstrated that the middle-aged wasp pupae stored at 10 °C for 5–10 d possessed a stronger parasitic ability and longer longevity. These findings may promote the flexibility and efficacy of large-scale production of *M. manilae*, thus contributing to its biological field control against *S. frugiperda.*

Keywords: *Spodoptera frugiperda*; parasitoid; *Microplitis manilae*; cold storage; biological control

1. Introduction

As a prominent invasive pest in tropical and subtropical regions, the fall armyworm (FAW), *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) feeds on a broad variety of plants, including at least 353 species belonging to 76 families, such as maize, wheat, rice and

sorghum, which result in huge economic losses to agriculture [1]. Since the beginning of 2019, FAW has rapidly colonized the cornfields of Yunnan Province in southern China, and its invasion in Hainan province was first detected on 30 April 2019. At present, *S. frugiperda* is still primarily controlled by chemical pesticides, albeit the field populations have developed high levels of resistance to various insecticides [2–4], of which the detoxification genes were mainly involved [5]. Biological control is one of the most essential methods in long-term pest management due to its great advantages, such as a persistent effect, low cost, without insecticide resistance and pesticide residues, thus offering considerable ecological, economic and social benefits [6].

Microplitis manilae (Ashmead) (Hymenoptera: Braconidae) is a solitary endoparasitoid parasitizing several lepidopteran pests, of which the species of *Spodoptera* are mainly preferred [7–11]. As an excellent biological control agent, *M. manilae* was first identified in the Philippine Islands [12]. In China, it was found that *M. manilae* showed great potential to parasitize the first to third instar larvae of both *Spodoptera exigua* (Hiibner) and *Spodoptera litura* (Fabricius) in the investigations of 2006 and 2010 [7,13]. Further, several lines of evidence suggested that the dominant parasitic species of both *S. exigua* and *S. litura* was *M. manilae* in Japan [7], Pakistan [8] and Vietnam [9]. Previous research has established that *M. manilae* had a distinct advantage in the interspecific competition after jointly releasing with the egg-larval parasitoid *Chelonus insularis* (Cresson) (Hymenoptera: Braconidae) [14]. Moreover, *M. manilae* parasitization inhibited the host feeding and subsequently led to the growth retardation of host insects [15]. To sum up, *M. manilae* is a promising biological agent for the control of noctuid pests.

The accumulation of sufficient storage methods is necessary for large-scale production and field release of natural enemies [16], and it is of extreme urgency to discover novel methods to extend the shelf life of wasps. Among which cold storage has been commonly used [17], providing us with an optional way to maintain a stable and sufficient wasp population. It has been well established that low temperatures retarded insect development [18], which allows us to synchronously release the wasps according to the pest occurrence regularity. Although there are existing advantages, keeping parasitoids at sub-ambient temperatures adversely affects the fitness of some parasitoids [19,20]. Therefore, to minimize the parasitoid reproductive performance loss after cold storage, it is necessary to investigate the effect of cold storage on the biological parameters of parasitoids, which contributes to establishing technical constraints for their commercial production and application [21–23].

There was an abundance of studies that have been implicated in the effects of cold storage on parasitoid biological parameters, including the species of Trcihogrammatidae and Braconidae, such as *Trichogramma evanescens* (Westwood) [24], *Trichogramma chilonis* (Ishii) [25], *Bracon hebetor* (Say) [26] and *Aphidius picipes* (Nees) [27]. Cold storage caused a declined survival rate, survival time and fecundity of wasp adults in *Gonatocerus ashmeadi* (Girault) [28,29], *Anaphes ovijentatus* (Crosby & Leonard) [30], *B. hebetor* [26] and Aphidiine parasitoids [31]. Moreover, low-temperature stress also affected the proportion of fertilized eggs [32], thus leading to the changes in sex ratio [33]. In parallel, numerous studies have demonstrated that host recognition, egg-laying amount and other parasitic behaviors of parasitoids altered after chilling treatment, such as in *A. picipes* [27] and *Anaphes victus* (Huber) [34]. Conversely, it has also been revealed that the emergence rate of wasp adults was unaffected after pupal cold storage in *B. hebetor* [26], *Aphidius matricariae* (Haliday) [35], *Lysiphlebus testaceipes* (Cresson) [36], *Encarsia formosa* (Gahan) [37] and *Muscidifurax raptor* (Girault & Sanders) [38]. Altogether, although much of the research up to date has been conducted, no researchers have been able to draw on any systematic research into the effect of cold storage on biological parameters of *M. manilae*, one of the prevalent parasitoids of noctuid pests.

In this study, we identified a dominant larval parasitoid of *S. frugiperda*, *M. manilae*, in the cornfield of Hainan Province of China in 2020 and investigated the field parasitism rate followed by the determinations of adult longevity and parasitism ability of *M. manilae*. Subsequently, we evaluated the effects of different pupal storage temperatures (4 and

10 °C), storage period (prepupa, middle-aged pupa, late-aged pupa) and storage time (5, 10 and 20 d) on the emergence rate, adult longevity and parasitism ability of *M. manilae*. We speculate that the reproductive performance of *M. manilae* can be improved with the increasing temperature and decreasing time under pupal cold storage, and the middle-aged pupal storage at 10 °C is possibly the most promising strategy for mass rearing. It is hoped that these findings will accelerate the large-scale production of *M. manilae*, thus contributing to its application in the biological control programs against *S. frugiperda*.

2. Materials and Methods

2.1. Insect Rearing

M. manilae and *S. frugiperda* were originally collected from the cornfield located in Yazhou District of Sanya city in Hainan province of China (109°11′ E, 18°22′ N) in 2020. We identified the *M. manilae* by morphological and molecular methods using their *COI* gene (Taxonomy ID: 1427173), which was the first discovery in the Hainan Province of China. A laboratory colony of *S. frugiperda* was established by feeding on an artificial diet made of corn meal, soybean meal and agar (Patent no. 201921652702.2) [39]. *S. frugiperda* were continuously raised in the laboratory for two years (at least 30 generations). Once eclosion, the adults were fed with 10% sucrose water (m/m) and allowed to lay eggs on a piece of gauze. The eggs were collected in a transparent box covered with 200 mesh nets, and the hatched larvae were reared until pupation. For *M. manilae*, we fed the adults with 10% sucrose water post eclosion followed by being fully mated for 24 h. *S. frugiperda* first instar larvae were used for 24 h parasitization to achieve a maximum indoor breeding efficiency with a 1:30 ratio (parasitoid: host). *M. manilae* was continuously bred in the laboratory for at least 20 generations. Both *M. manilae* and *S. frugiperda* were reared under controlled conditions of 25 ±1 °C, 65 ±5% relative humidity (RH) and 16:8 h (L: D). We used the laboratory colonies of *S. frugiperda* and *M. manilae* in subsequent analyses.

2.2. Investigation of Field Parasitism Rate

The larvae of *S. frugiperda* were respectively collected in five cities of Hainan Province, namely Qiongzhong with an average annual temperature of 23.2 °C, 79–88% RH and 2444 mm rainfall, Danzhou with an average annual temperature of 23.3 °C, 77–86% RH and 1815 mm rainfall, Sanya with an average annual temperature of 25.5 °C, 77–87% RH and 1279 mm rainfall, Ledong with an average annual temperature of 25.3 °C, 77–87% RH and 1600 mm rainfall and Dongfang with an average annual temperature of 24.5 °C, 72–81% RH and 1150 mm rainfall, during October and November of 2020, and in May 2021 (www.hainanqx.cn). The collected larvae were kept at 25 °C until the eclosion of either *S. frugiperda* or parasitoids to determine the field parasitism rate of *M. manilae* against *S. frugiperda*. The field parasitism rate (%) was calculated as (number of parasitized *S. frugiperda* larvae/total number of collected larvae) × 100.

2.3. Experimental Set-Up of M. manilae Pupal Cold Storage

The *M. manilae* within 12 h pupariation was maintained at 25 °C for 1, 3 and 5 d to obtain prepupa, middle-aged pupa and late-aged pupa, which were subsequently stored in an artificial climate chamber (YangHui, Ningbo, China) for 5, 10 and 20 d at 4 and 10 °C, respectively. Each treatment included at least 15 cocoons. The adults that emerged from the treated cocoons were considered F1, which were used to examine the effect of pupal cold storage on F1 emergence rate, survival time and parasitism rate, and 26 1st instar larvae of *S. frugiperda* (the maximum parasitized number) were used for 24 h parasitization per F1 female adult after being fully mated. The emerged wasp adults were regarded as F2 generations, followed by the determinations of emergence rate and female proportion in F2. Three repetitive experiments were conducted. *M. manilae* cocoons without pupal cold storage were reared under the optimal conditions of 25 ± 1 °C, 65 ± 5% RH and 16:8h (L: D), and the emerged parasitoids were set as the control.

2.4. Determinations of Parasitoid Emergence Rate and Female Proportion

After pupal cold storage, the number of emerged F1 adults was counted. We calculated the F1 emergence rate (%) as (number of emerged parasitoids/total number of wasp cocoons after pupal cold storage) × 100. The F1 female proportion (%) was calculated as (number of emerged female adults/total number of emerged parasitoids) × 100. Each treatment contains at least 15 cocoons of *M. manilae*. To determine the emergence rate and female proportion of F2, F1 females after being fully mated ($n \geq 15$) were used for subsequent parasitization and these two parameters were calculated as above. Three repetitions were performed.

2.5. Determination of Parasitism Rate

To evaluate the parasitism ability of *M. manilae* at different developmental stages that were raised at optimal conditions without pupal cold storage, we used the female adults of 1, 3, 5, 7 and 9 d post-emergence after being fully mated and the host *S. frugiperda* of different ages, including 1st, 2nd, 3rd and 4th instar larvae, were collected for 24 h after being parasitized at 25 °C. For pupal cold storage treatment, the F1 wasp female adults post 1–3 d eclosion were used for parasitizing the 1st larvae of *S. frugiperda* for 24 h to evaluate the parasitism ability of F1 *M. manila* after pupal cold storage. The parasitism rate (%) was calculated as above. A total of 15–20 replicates were performed in this experiment, and each repetition contained 1 female adult of *M. manila* and 26 1st larvae of *S. frugiperda* (the maximum parasitized number).

2.6. Determination of Parasitoid Longevity

To clarify the effects of different temperatures and foods on the longevity of *M. manilae* adults raised at optimal conditions, at least 15 adults of *M. manilae* without pupal cold storage were collected and respectively raised at 20, 22.5, 25, 27.5 and 30 °C under conditions of 65 ± 5% RH and 16:8 h (L:D) after emergence. All adults were fed with 10% sucrose water or sterile water (control). The number of dead parasitoids was recorded every day. To evaluate the longevity of *M. manilae* F1 adults after pupal cold storage, at least 15 emerged F1 wasps were raised with 10% sucrose water under conditions of 25 °C, 65 ± 5% RH and 16:8 h (L:D). The number of dead parasitoids in F1 was recorded every day. We calculated the average longevity as (total survival time of wasp adults/total number of wasp adults). Three repetitions were performed in this experiment.

2.7. Statistical Analysis

All data were analyzed by SPSS software (SPSS, New York, NY, USA). Before analysis, the percentage data were arcsine square root-transformed to fit a normal distribution. The parasitism rate (response variable) was analyzed using one-way ANOVA in which the host instars and age of parasitoids were set as independent variables. The adult longevity, emergence rate and female proportion of F1 and F2 *M. manilae* adults (response variables) were analyzed using univariate two-way ANOVA (generalized linear model, GLM), in which the storage temperature and storage period of wasp cocoons were set as independent variables. When the means were not normally distributed, a nonparametric Kruskal–Wallis H test was used to determine the F1 parasitism rate after pupal cold storage (response variable) and independent variables were set as above. Statistical significances were marked with different letters (a, b, c, . . .). We plotted all figures using GraphPad Prism 7.0 software (San Diego, CA, USA).

3. Results

3.1. Field Parasitism Rate and Biological Parameters of M. manilae

3.1.1. Field Parasitism Rate Investigation of *M. manilae*

In 2020–2021, the *S. frugiperda* larvae parasitized by *M. manilae* were collected, revealing varied field parasitism rates in different locations (Table 1). In detail, the highest parasitism rate was recorded in Dongfang city, reaching 19.10%, followed by Ledong1 with a 13.74%

parasitism rate in 2020. In comparison, there was a lower field parasitism rate of 5.66% in Ledong2 in 2020. From the results in Table 1, we also observed that the field parasitism rate of *M. manilae* reached 8.09% in Danzhou in 2021. However, *M. manilae* failed to parasitize the *S. frugiperda* population of Sanya.

Table 1. Field parasitism rate of *M. manilae* in different areas of Hainan Province, China.

Collection Time	Location	Coordinate	Number of Collected *S. frugiperda*	Number of Parasitized Larvae	Parasitism Rate (%)
2020.11.10	Dongfang	108°64′ E, 18°84′ N	199	38	19.10
2020.11.03	Ledong1	108°91′ E, N18°47′ N	131	18	13.74
2020.10.23	Qiongzhong	109°90′ E, N19°13′ N	131	16	12.21
2020.11.01	Sanya	109°18′ E, N18°38′ N	238	15	6.30
2020.10.30	Ledong2	108°83′ E, N18°51′ N	53	3	5.66
2021.05.13	Danzhou	109°48′ E, N19°51′ N	235	19	8.09
2021.05.10	Sanya	109°18′ E, N18°38′ N	81	0	0.00
2020.10–2021.05	In total		1068	109	10.21

3.1.2. Effects of Food and Temperature on Adult Longevity of *M. manilae*

Table 2 presented the longevity exposition of *M. manilae*, which revealed that feeding on 10% sucrose water significantly prolonged the wasp longevity from 6.31 to 29.66 d compared with that of sterile water at 20 °C. Closer inspection of the results showed that the longevity of *M. manilae* remarkably shortened with the increase of temperature from 20 to 30 °C, and there was a significant difference ($F = 206.05$; $df = 6, 98$; $p < 0.001$) between the treatment of 20 (29.66 d) and 30 °C (12.26 d). We also found that the survival rate of *M. manilae* decreased significantly as the temperature increased (Figure 1).

Table 2. Longevity of *M. manilae* adults fed different food at different temperatures.

Food	Survival Time (d)					F	*p*
	20 °C	22.5 °C	25 °C	27.5 °C	30 °C		
10% sucrose water	29.66 ± 0.98 a	28.24 ± 0.81 ab	26.51 ± 1.18 b	18.09 ± 0.55 c	12.26 ± 1.04 d	206.05	<0.001
Sterile water	6.31 ± 0.68 e	5.22 ± 0.42 ef	4.75 ± 0.37 ef	4.06 ± 0.12 fg	2.39 ± 0.44 g		

Note: Data are presented as mean ± SE (standard error) ($n \geq 15$). Different lowercase letters indicate significant differences at the 0.05 level according to the Fisher LSD test (two-way ANOVA).

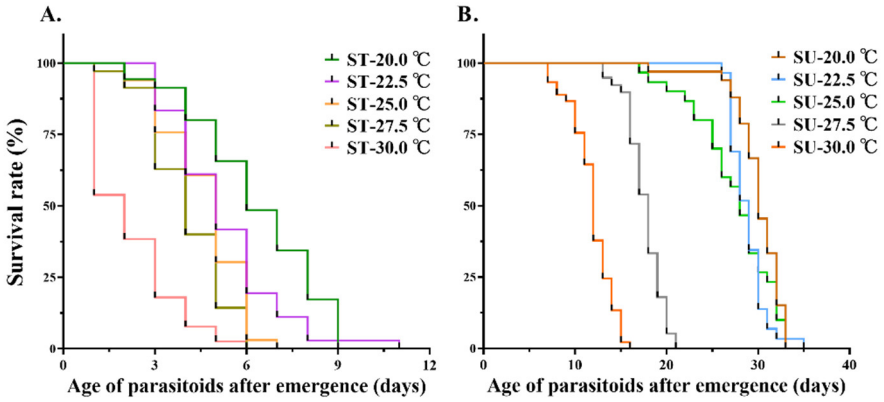

Figure 1. Effects of food on adult longevity of *M. manilae*. (**A**) Survival curves of *M. manilae* adults upon feeding on sterile water at different temperatures. (**B**) Survival curves of *M. manilae* adults upon feeding on 10% sucrose water at different temperatures. SU means 10% sucrose water, and ST represents sterile water ($n \geq 15$).

3.1.3. Determination of Parasitism Rate of *M. manilae*

The next investigation was concerned with the parasitic ability of *M. manilae* on different instars of host *S. frugiperda* larvae at different developmental stages. What stood out in Figure 2A was that the parasitism rate of *M. manilae* was significantly reduced with the increase in the host larval age. *M. manilae* had the greatest potential to parasitize *S. frugiperda* first larvae with a parasitism rate of 82.58% compared to those of 41.86% and 10.86% in second and third larvae, respectively (F = 1090.50; df = 3, 64; $p < 0.001$). Moreover, *M. manilae* failed to parasitize the fourth larvae of host *S. frugiperda*.

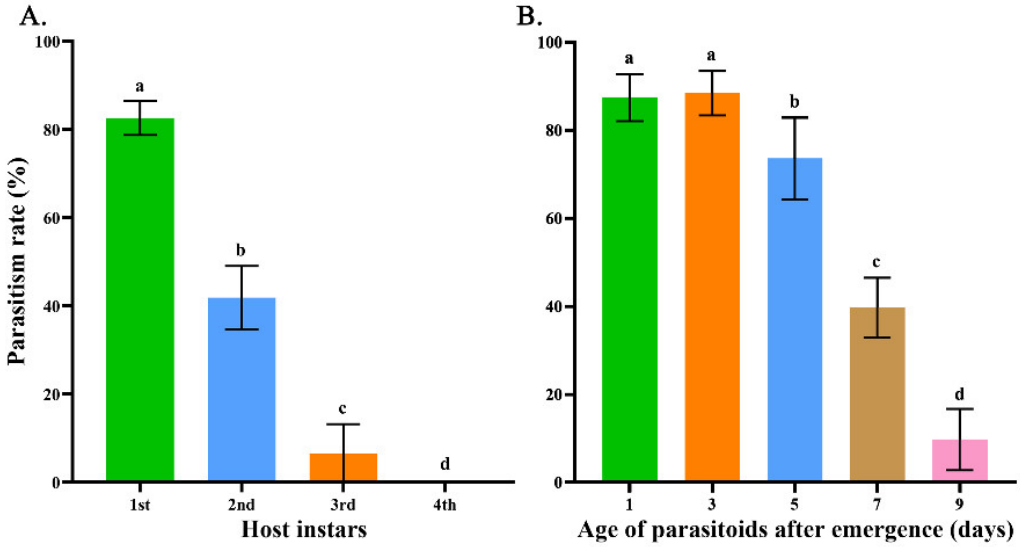

Figure 2. The parasitism rates of *M. manilae* on different instars of *S. frugiperda* larvae at different developmental stages. (**A**) The parasitism rates of *M. manilae* post 1–2 d eclosion on first, second, third and fourth *S. frugiperda* larvae ($n \geq 15$). (**B**) The parasitism rates of *M. manilae* at 1, 3, 5, 7 and 9 d post-eclosion on *S. frugiperda* first larvae ($n \geq 15$). One-way ANOVA between different groups was performed followed by Tukey's multiple comparison tests, and the different letters mean significant differences ($p < 0.05$). Data are presented as mean ± SE.

The following step was to determine the parasitic ability of *M. manilae* at different developmental periods on the first larvae of host *S. frugiperda*. The results, as shown in Figure 2B, indicate that the parasitoid adult after 1–3 d eclosion performed best on *S. frugiperda* larvae with a parasitism rate of 87.44–88.46%. With an increase in the wasp age, there was a significant decline in parasitism rate, merely reaching 9.74% in female wasp adults after 9 d eclosion (F = 374.82; df = 4, 80; $p < 0.001$).

3.2. Effects of Pupal Cold Storage on the Biological Parameters of M. manilae

3.2.1. Determination of *M. manilae* Emergence Rate

From Table 3, we recorded that the emergence rate of *M. manilae* significantly differed upon different treatments of storage temperature, storage time and pupal age. Generally, the emergence rates of parasitoid adults significantly decreased with a temperature reduction from 10 to 4 °C and a prolonged time from 5 to 10–20 d after prepupa, middle-aged and late-aged pupal cold storages. Notably, there was a significantly higher emergence rate in wasp adults upon middle-aged pupal chilling stress compared to those of prepupa and late-aged pupal cold storage schemes at 4 and 10 °C (Table 3). However, the emergence rates of pupal cold storage treatments were significantly lower than that of the control group (F: 108.95–1027.39; df = 6, 15; $p < 0.001$). The most striking result was that 91.88% of wasps successfully emerged after the middle-aged pupal chilling at 10 °C for 5 d, presenting a minimum difference compared with the control group.

Table 3. Emergence rate of *M. manilae* after pupal cold storage treatments.

Storage Time (d)	Storage Temperature (°C)	Emergence Rate (%)			F	*p*
		Prepupa	Middle-Aged Pupa	Late-Aged Pupa		
5	4	65.66 ± 1.01 e	82.32 ± 0.50 c	74.13 ± 1.40 d	108.95	<0.001
	10	76.55 ± 2.11 d	91.88 ± 0.21 b	83.33 ± 0.00 c		
	25 (control)		98.68 ± 0.94 a			
10	4	26.26 ± 4.40 f	63.64 ± 0.00 cd	47.22 ± 2.78 e	138.02	<0.001
	10	58.33 ± 0.00 d	74.88 ± 1.21 b	65.66 ± 1.01 c		
	25 (control)		98.68 ± 0.94 a			
20	4	0 f	27.27 ± 0.00 c	19.39 ± 0.61 e	1027.39	<0.001
	10	0 f	36.56 ± 1.93 b	29.29 ± 2.02 c		
	25 (control)		98.68 ± 0.94 a			

Note: Data are presented as mean ± SE ($n \geq 15$). Different lowercase letters indicate significant differences at the 0.05 level according to the Fisher LSD test (two-way ANOVA).

3.2.2. Determination of *M. manilae* Parasitism Rate

Based on the above results, we recorded relatively low emergence rates after 20 d cold storage, which was excluded from the subsequent analysis. The *M. manilae* adults, after pupal treatments under a lower storage temperature or a prolonged storage time, showed a remarkable decreased parasitism rate on the first instar of host *S. frugiperda* (Table 4). Furthermore, the parasitism rates of wasp adults emerging from middle-aged pupal cold storage was significantly higher than those of prepupa (H = 83.12; $df = 6$; $p < 0.001$) and late-aged pupal treatments (H = 69.64; $df = 6$; $p < 0.001$) under the same storage schedules (Table 4). We further obtained a remarkable outcome that the *M. manilae* adults after middle-aged pupal cold storage at 10 °C for 5 d performed best on host *S. frugiperda*, showing a relatively high parasitism rate of 69.23%.

Table 4. Parasitism rate of *M. manilae* adults after pupal cold storage treatments.

Storage Time (d)	Storage Temperature (°C)	Parasitism Rate (%)			H	*p*
		Prepupa	Middle-Aged Pupa	Late-Aged Pupa		
5	4	23.08(23.08–26.92) f	42.31(38.46–42.31) d	26.92(26.92–26.92) e	83.12	<0.001
	10	50.00(46.15–53.85) c	69.23(65.38–73.08) b	53.85(50.00–57.69) c		
	25 (control)		88.46(84.62–92.31) a			
10	4	7.69(7.69–11.54) d	30.77(26.92–34.62) c	11.54(11.54–11.54) d	69.64	<0.001
	10	34.62(34.62–38.46) c	61.54(57.69–65.38) b	38.46(34.62–42.31) c		
	25 (control)		88.46(84.62–92.31) a			

Note: Data are presented as median (Q1–Q3) ($n \geq 15$). Different lowercase letters indicate significant differences at the 0.05 level according to the Kruskal–Wallis H test (Nonparametric test).

3.2.3. Determination of *M. manilae* Adult Longevity

The most striking result observed in Table 5 was that pupal cold storage had a significant effect on the longevity of *M. manilae* adults compared with the control group. A shorter longevity was shown in *M. manilae* adults after pupal storage at a lower temperature for a longer time, and we also recorded that the longevity of wasp adults after prepupa or late-aged pupal storage for 5 d (F = 34.21; $df = 6, 10$; $p < 0.001$) was significantly shorter than that of middle-aged pupal storage at both 4 and 10 °C. However, no significant differences were shown in adult longevity between late-aged and middle-aged pupal cold storage for 20 d (F = 76.51; $df = 6, 6$; $p < 0.001$) at both 4 and 10 °C. Moreover, the longevity of wasp adults after late-aged pupal chilling was not significantly different from that of prepupae cold storage for 5–10 d (F: 34.21–51.62; $df = 6, 10$; $p < 0.001$) at both 4 and 10 °C. To sum up, the middle-aged pupal cold storage at 10 °C for 5 d had the least effect on *M. manilae* adult longevity, with an average of 21.32 d. Moreover, the survival rates of wasp adults upon

4 or 10 °C pupal storage significantly decreased relative to that of adults raised at 25 °C (26.51 d) (Figure 3A–C).

Table 5. Longevity of *M. manilae* adults after different pupal cold storage treatments.

Storage Time (d)	Storage Temperature (°C)	Survival Time of Adults (d)			F	*p*
		Prepupa	Middle-Aged Pupa	Late-Aged Pupa		
5	4	8.22 ± 1.05 e	13.58 ± 0.11 d	10.54 ± 0.47 de	34.21	<0.001
	10	14.00 ± 0.08 cd	21.32 ± 0.03 b	17.87 ± 1.80 c		
	25 (control)		26.51 ± 1.18 a			
10	4	6.63 ± 0.63 e	10.88 ± 0.43 d	6.39 ± 0.72 e	51.62	<0.001
	10	12.87 ± 0.14 cd	18.78 ± 0.86 b	16.35 ± 0.65 bc		
	25 (control)		26.51 ± 1.18 a			
20	4	-	5.30 ± 0.70 c	3.88 ± 0.63 c	76.51	<0.001
	10	-	13.32 ± 0.12 b	10.63 ± 0.38 b		
	25 (control)		26.51 ± 1.18 a			

Note: Data are presented as mean ± SE ($n \geq 15$). The same lowercase letters indicate no significant differences at the 0.05 level according to the Fisher LSD test (two-way ANOVA).

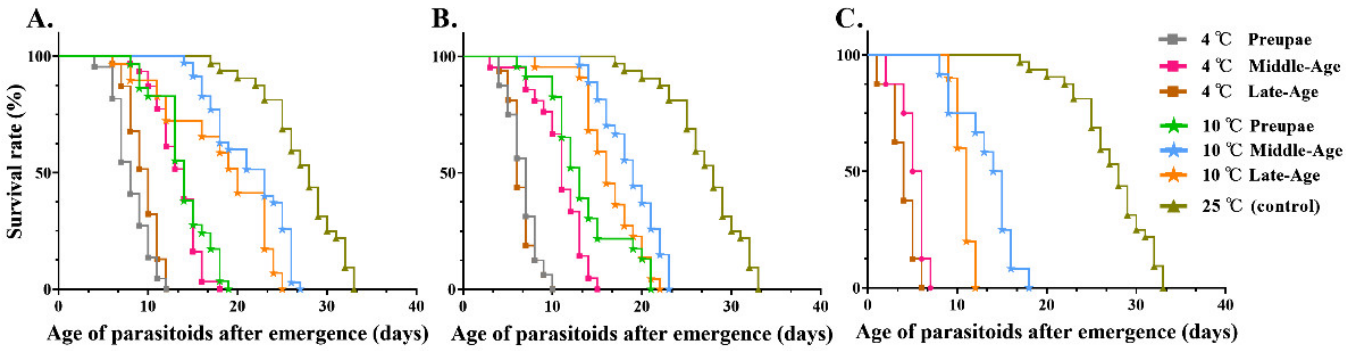

Figure 3. Survival curves of *M. manilae* adults upon different pupal cold storage treatments for different times. (**A**) Survival curves of *M. manilae* adults upon different pupal cold storage for 5 d ($n \geq 15$). (**B**) Survival curves of *M. manilae* adults upon different pupal cold storage for 10 d ($n \geq 15$). (**C**) Survival curves of *M. manilae* adults upon different pupal cold storage for 20 d ($n \geq 15$).

3.2.4. Determination of Biological Parameters in F2 *M. manilae*

In the final part of the survey, we focused on the biological parameters of F2 *M. manilae* upon different cold storage treatments in F1 pupae, mainly involving the emergence rate and female proportion. It was apparent that no significant differences (F: 0.06–0.07; $df = 6, 98$; $p = 0.999$) between different groups were evident in both of the two parameters (Tables 6 and 7). The female proportion of F2 *M. manilae* upon different F1 pupal cold storage treatments was not remarkably different from the control. Whereas further analysis showed that the emergence rate of F2 *M. manilae* ranging from 84.77% to 87.84% significantly decreased (F: 2.88–2.96; $df = 6, 87$; $p < 0.05$) compared to that without pupal chilling (98.68%).

Table 6. Emergence rate of F2 *M. manilae* after different F1 pupal cold storage treatments.

Storage Time (d)	Storage Temperature (°C)	Emergence Rate (%)			F	*p*
		Prepupa	Middle-Aged Pupa	Late-Aged Pupa		
5	4	86.62 ± 1.41 b	87.84 ± 1.70 b	85.47 ± 1.17 b	2.96	0.011
	10	85.69 ± 1.42 b	85.51 ± 1.84 b	86.37 ± 1.89 b		
	25 (control)		98.68 ± 0.94 a			
10	4	86.17 ± 1.51 b	87.74 ± 1.57 b	86.55 ± 1.64 b	2.88	0.013
	10	86.76 ± 1.53 b	84.77 ± 1.81 b	86.63 ± 1.54 b		
	25 (control)		98.68 ± 0.94 a			

Note: Data are presented as mean ± SE ($n \geq 15$). The same lowercase letters indicate no significant differences at the 0.05 level according to the Fisher LSD test (two-way ANOVA).

Table 7. Female proportion of F2 *M. manilae* after different F1 pupal cold storage treatments.

Storage Time (d)	Storage Temperature (°C)	Female Proportion (%)			F	*p*
		Prepupa	Middle-Aged Pupa	Late-Aged Pupa		
5	4	68.12 ± 3.29 a	67.21 ± 2.87 a	67.67 ± 3.26 a	0.06	0.999
	10	67.01 ± 2.97 a	68.42 ± 4.38 a	66.62 ± 3.64 a		
	25 (control)		68.82 ± 1.46 a			
10	4	67.33 ± 4.96 a	68.06 ± 2.51 a	68.93 ± 4.38 a	0.07	0.999
	10	68.59 ± 4.42 a	67.73 ± 3.88 a	66.12 ± 3.75 a		
	25 (control)		68.82 ± 1.46 a			

Note: Data are presented as mean ± SE ($n \geq 15$). The same lowercase letters indicate no significant differences at the 0.05 level according to the Fisher LSD test (two-way ANOVA).

4. Discussion

As is known to all, parasitic wasps are one of the key factors in integrated pest management programs [40]. Therefore, it is critical to utilize local parasitoid resources and develop the most appropriate biological control program considering the various ecological circumstances in different areas of China [41]. The first question in this study sought to determine the field parasitism rate of *M. manilae*, a prevalent larval parasitoid of *S. frugiperda*, in different locations in the Hainan province of China, showing a field parasitism rate of 5.66–19.10%. Prior studies have noted that the primary hosts of *M. manilae* were *S. litura* and *S. exigua* [10,11], and no such investigations about the field parasitism of *M. manilae* on *S. frugiperda* have been conducted. In 2020, we firstly discovered that *M. manilae* successfully parasitized *S. frugiperda* in the Hainan Province of China. Our findings were very encouraging in demonstrating that the *M. manilae* acted as a potential biological control agent to control *S. frugiperda* in China, which greatly expanded the flexibility of biological control strategies using *M. manilae*.

Our next objective of the study was to determine the biological parameters of *M. manilae*. It was found that feeding on 10% sucrose water significantly prolonged the survival time of wasp adults compared to sterile water. Additionally, the longevity of *M. manilae* adults remarkably increased with the decreasing temperature from 30 to 20 °C. These results accord with the previous observations in *Tetrastichus howardi* (Olliff) [42], *Psyttalia incise* (Silvestri), *Microplitis tuberculifer* (Wesmael) [43] and *Anagrus nilaparvatae* (Pang & Wang) [44]. However, the impact of a reduced temperature on adults of *M. manilae* has not been well investigated. Considering that the host insects were optimal vectors for wasp mass-breeding [45,46], the developmental stages of both host larvae and parasitoid adults should be taken into account to achieve maximum reproductive efficiency [47,48]. It was discovered that *M. manilae* showed the highest parasitic potential on the first larvae of *S. frugiperda* and failed to parasitize the fourth instar *S. frugiperda* larvae, which was due to the elevated immune resistance to wasp parasitization in elderly host larvae and the difficulty in penetrating into the host body [49]. In accordance with the present results, the phenomenon extended to the

M. tuberculifer-S. exigua and *Microplitis* sp.-*S. litura* models [50,51]. Simultaneously, we recorded the highest parasitic ability in wasp adults after 1–3 d eclosion, also supporting the previous studies of *M. tuberculife* [52] and *M. mediator* [44]. This part of the study is of great significance in clarifying the biological control potential of *M. manilae* on the control of *S. frugiperda* and provides some theoretical support for the development and breeding of parasitoids.

Cold storage is essential for maintaining a stable and adequate parasitoid population in pest management, thus contributing to its synchronized field release [18]. It has been demonstrated that the cold storage above 0 °C slowed down the metabolic rate of parasitoids and thus delayed their growth and development [17,53]. Although the obvious advantages in improving the efficiency and flexibility of large-scale release programs, the adverse effects on insect quality were worrying after cold storage [19], and a large number of studies have emphasized that the exposure of parasitoids to a chilling condition led to excessive consumption of energy reserves, thus affecting their quality and successful application in the field [20,54]. Accordingly, our next question in this study sought to determine the effects of cold storage on the biological parameters of *M. manilae* compared with the control group reared at optimal temperature conditions. Our results indicated that the emergence rate of F1 *M. manilae* after pupal cold storage was significantly lower than that of the control, showing the fitness cost in F1 caused by pupal chilling. Meanwhile, the decrease in storage temperature from 10 to 4 °C and a prolonged storage time from 5 to 10–20 d led to remarkable declines in the emergence rate of *M. manilae*. This was consistent with previous studies referring to two other species of Braconidae, *P. incisi* and *Diachasmimorpha longicaudata* (Ashmead) [16,55]. Additionally, we found that the emerged adults upon middle-aged pupal chilling showed superior performance in emergence rate compared with those of prepupae and late-aged pupal cold storage under the same storage scheme. Similarly, a study conducted on *P. incisi* showed that prepupae and late pupae were less tolerant to low temperature, showing lower emergence rates than the control group and middle-aged pupal cold treatment group [56]. Furthermore, we found that the prepupae failed to emerge after 20 d cold storage at 4 or 10 °C, which indicated its fatal effect. We proposed that parasitoids need to accumulate energy to strengthen the muscle contraction during the eclosion process [57], and low-temperature exposure for a long time led to the muscle dysfunction [58], possibly accounting for the unsuccessful eclosion of *M. manilae*. Another important finding of this study was that the longevity and survival rate of *M. manilae* adults significantly reduced with the extensions of storage time and the decrease in storage temperature. It was noteworthy that the F1 longevity and survival rate upon middle-aged pupal cold storage was significantly higher than those of prepupal and late-aged pupal treatments under the same storage conditions, which was also evidenced by previous studies [38]. Further, there were remarkable decreases in adult longevity and survival rate after pupal cold storage compared with the control group raised at optimal conditions, further demonstrating the adverse effects of cold chilling on insect physiological activity.

In addition, the reproductive system of parasitoids is extremely vulnerable to cold stress. For instance, the fertility of parasitoid wasps generally declined with the decreased storage temperature and extended storage time [26]. In our present study, the parasitism rate of *M. manilae* adults significantly decreased after pupal cold storage compared with the wasps raised at 25 °C. This result supported the idea that cold chilling led to the disorder of the insect reproductive system. It was also shown that the *M. manilae* adults after middle-aged pupal cold storage performed better than those of prepupal and late-aged pupal cold storage at both 4 and 10 °C, and these results are in good agreement with previous investigations [25,29,59,60]. In some insects, cold exposure also changed their F2 sex ratio [61]. However, no evidence of the differed female proportion was found in F2 *M. manilae* between the cold storage groups, and the control group reared at optimal temperature conditions, which indicated the effects of pupal cold storage on F2 progeny were negligible. This finding provides important cues for the mass-breeding of *M. manilae* after pupal cold storage.

Parasitoids have been widely utilized for the biological control of agricultural pests, and their mass storage is indispensable for subsequent large-scale production and release [53]. Therefore, establishing an optimum storage strategy to increase their breeding efficiency has long been the focus of studies, of which cold storage has been increasingly used [20]. However, minimizing the loss of quantity in parasitoids has become an obstacle to balancing the effects of cold storage and field application efficiency [53]. The present study aimed to clarify the effects of pupal cold storage on the biological parameters of *M. manilae*, a dominant parasitoid of *S. frugiperda* in Hainan Province of China, and provide a reliable basis for optimizing its cold storage scheme. Our results showed that middle-aged pupal storage at 10 °C for 5 d had minimal impact on the adult quality and reproductive performance in comparison with the control group reared at optimal temperature conditions. Taken together, these findings provide significant theoretical support for the commercialization of breeding and large-scale field release of *M. manilae* against noctuid pests.

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Sublethal Dose of β-cypermethrin Impairs the Olfaction of *Bactrocera dorsalis* by Suppressing the Expression of Chemosensory Genes

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Simple Summary: The oriental fruit fly, *Bactrocera dorsalis*, is a pest that causes huge economic losses in the fruit and vegetable industry. β-cypermethrin has been widely used in the orchard to control this major pest. According to a previous study, the oriental fruit fly developed significant resistance against β-cypermethrin in fields, which indicated that the oriental fruit fly has been exposed to sublethal concentrations of β-cypermethrin in the field for a long time. However, the sublethal effect and its mechanism are still unclear. In our present study, after treatment with sublethal concentrations of β-cypermethrin, the olfaction was disrupted significantly and the chemosensory genes were suppressed obviously. Our data demonstrated that the sublethal dose of β-cypermethrin impairs the olfaction of the pest insects by suppressing the expression of chemosensory genes, which provided theoretical guidance for the rational use of pesticides in fields.

Abstract: The oriental fruit fly, *Bactrocera dorsalis*, is one of the most destructive fruit insect pests. β-cypermethrin has been widely used in the orchard to control this major insect. Based on the resistance monitoring in 2011, *B. dorsalis* developed significant resistance against β-cypermethrin in fields. This indicated that the *B. dorsalis* has been exposed to sublethal concentrations of β-cypermethrin in the field for a long time. Thus, it is urgent to understand the sublethal effects of β-cypermethrin on this fly to guide the rational use of an insecticide. According to the olfactory preference assays and electroantennogram (EAG) recording, the *B. dorsalis* after β-cypermethrin exposure (LD_{30} = 10 ng/fly) severely decreased the ability to perceive the tested odorants. Moreover, we then performed quantitative real-time PCR and found the chemosensory genes including odorant receptor co-receptor (*BdorORco*) and ionotropic receptor co-receptors (BdorIRcos) were obviously suppressed. Our results demonstrated that the sublethal dose of β-cypermethrin impairs the olfaction of the pest insects by suppressing the expression of chemosensory genes (*BdorORco* and BdorIRcos), which expanded our knowledge of the sublethal effects of the pesticide on insects.

Keywords: oriental fruit fly; sublethal effect; olfactory genes; pyrethroids

1. Introduction

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), attacks more than 600 fruit and vegetable crops [1,2]. It causes great economic losses through oviposition and larval feeding inside of the host plants [1,3]. Due to its rapid population growth, wide distribution and invasiveness, it has become one of the most destructive pests in tropical and subtropical countries [4,5]. Nowadays, chemical control is still the main method to control *B. dorsalis* and plays a significant role in the management of this fly [6–8]. β-cypermethrin, a pyrethroid, represents one of the major insecticides against *B. dorsalis* [5,7]. It behaves as

a fast-acting neurotoxin in insects and it eventually leads to the death of target insects by prolonging the opening time of sodium ion channels [9,10].

In addition to the direct killing effect of insecticides on insects, a sublethal effect also existed in some individuals with different exposure amounts and times. It has been well documented that exposure to sublethal insecticides may cause multiple effects on the behavior, development and reproduction of insects [11,12]. On one hand, sublethal effects have negative effects on insects. For example, researchers found that a sublethal dose of indoxacarb and β-cypermethrin could significantly inhibit the growth and reproduction of *Rhopalosiphum padi* and *Plutella xylostella* and their offspring [9,13]. Exposure to a sublethal dose of deltamethrin detrimentally affects the reproduction and wing shape of *Chironomus columbiensis* [14]. Furthermore, spinosad exposure was found to impair the mobility of *Adoxophyes honmai* [15]. On the other hand, sublethal effects have "positive" effects on insects. The resurgence led by the hormesis effect of sublethal insecticides on the target insect pests is a great challenge to the rational use of pesticides [16]. For instance, exposure to sublethal concentrations of pesticides was also reported to stimulate the reproduction of several insects such as *Myzus persicae*, *Bemisia tabaci* and *Frankliniella occidentalis* [17–19].

Furthermore, an increasing number of studies have shown that sublethal doses of insecticides can severely damage olfaction, resulting in abnormal olfactory behaviors, especially in non-target organisms [20]. Currently, the sublethal effects of insecticides on non-target pests have become an interesting topic in the pesticide environmental toxicology study [20]. In *Oncorhynchus mykiss*, the sublethal dose of chlorpyrifos impaired olfactory signal transduction resulting in olfaction sensitivity disruption [21]. Moreover, the dose-dependent sublethal concentration of the imidacloprid impaired host finding and sexual communication in *Nasonia vitripennis* [22]. After exposure to a sublethal dose of imidacloprid, the nervous system in the calyces regions responsible for both olfaction and vision was damaged, causing decreased olfaction learning ability in *Apis mellifera* [23].

In 2011, the resistance monitoring of *B. dorsalis* in mainland China showed that 24 out of the 25 field populations developed different resistance against β-cypermethrin ranging from 3.0 folds to 44.0 folds [7]. Due to the rapid development of the resistance, *B. dorsalis* has probably been exposed to sublethal concentrations of β-cypermethrin in fields since 2011. However, the sublethal effect of β-cypermethrin on *B. dorsalis* still remains unknown, especially whether there are any significant effects on its olfaction. Therefore, we conducted the olfactory behavior and electroantennogram (EAG) assays to investigate the disruption of the olfaction in *B. dorsalis* exposed to a sublethal dose of β-cypermethrin. Moreover, to figure out its potential mechanism, we then performed quantitative real-time PCR to determine the expression profiles of chemosensory genes including *BdorORco* and BdorIRcos. The results not only demonstrated that the sublethal dose of β-cypermethrin impairs the olfaction of *B. dorsalis* by suppressing the expression of chemosensory genes (*BdorORco* and BdorIRcos), but also provided insights into the physiological effects of β-cypermethrin on the target insect pest besides its insecticidal effects. Our data will also provide a reference for the scientific use of pesticides in pest management in fields.

2. Materials and Methods

2.1. Insects

The *B. dorsalis* population used in our experiments was originally collected from Hainan province, China, in 2008. All life stages of the oriental fruit flies were kept in a growth chamber at 27.5 ± 1 °C, 75 ± 5% relative humidity, with a 14 h light:10 h dark photoperiod [24]. Adults were fed an aqueous artificial diet of yeast powder, honey, sugar and vitamin C [25]. Male and female adults were separated within 3 days of the eclosion. All the adults were synchronized under the same conditions before experimentation.

2.2. β-Cypermethrin Bioassay

β-cypermethrin (96% purity) was provided by the Institute for Control of Agrochemicals, Sichuan province, China. The topical application method was used in the bioassay [26]. A serial dilution of *β*-cypermethrin in acetone (0, 5 ng/fly, 10 ng/fly, 15 ng/fly, 20 ng/fly, 25 ng/fly) was applied to the pronotum of 6-day-old flies, using a PB600-1 repeating dispenser (Hamilton, Reno, NV, USA). Flies applied with acetone served as a control. Mortality was recorded at 24 h after *β*-cypermethrin treatment. There were three biological replications with 60 flies for each.

2.3. Olfactory Preference Assays

The four choice olfactometer assay was carried out to detect the olfactory preference of treated flies (*β*-cypermethrin, 10 ng/fly) to 1-octen-3-ol (Sigma-Aldrich, St Louis, MO, USA), methyl eugenol (Acros, Morris Plains, NJ, USA) and ethyl acetate (Sigma, St Louis, MO, USA) followed the published method [27] with slight modification. The apparatus consisted of a central chamber connected to four 50 mL cylindrical glass bottles. Two of the glass bottles at the opposite corner were designated for odor stimuli treatments and the other two for controls. Purified air at a rate of 200 mL/min was drawn towards the center of the olfactometer simultaneously from each arm and removed via the vacuum pump at the rate of 1000 mL/min.

1-octen-3-ol was regarded as a female attractant [28], while methyl eugenol was a male-specific attractant [29]. Meanwhile, ethyl acetate was proved to attract both male and female flies [30]. Therefore, only females were used for the assay of 1-octen-3-ol, while only males were used for the assay of methyl eugenol. Meanwhile, both females and males with a sex ratio of 1:1 were employed in the assay of ethyl acetate. Each glass bottle was filled with a quarter piece of filter paper (Newstart, Hangzhou, China) that was impregnated with 100 µL of diluted odorant in the concentration of 1% (*v*/*v*) or control. In detail, the control for 1-octen-3-ol and ethyl acetate is mineral oil (MO, Sigma-Aldrich, St Louis, MO, USA), and the control for methyl eugenol is dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA). Twenty 7-day-old flies (after treatment) were placed in the center of the olfactometer and continuously monitored for 10 min. Each group contained 6 replicates. Flies that stayed at the center of the apparatus longer than 10 min were considered "non-responders". The attraction rate was calculated by dividing the number of flies attracted per treatment by the total number of flies entering the olfactometer. In addition, the *B. dorsalis* were tested at the same time point to guarantee the consistent rhythm.

2.4. Electroantennogram (EAG)

The EAG responses of the *β*-cypermethrin treated (LD_{30} = 10 ng/fly) flies were measured with a standard protocol [31]. Briefly, the whole head of the 7-day-old fly (after treatment) was excised and connected to two glass micropipettes filled with 0.9% NaCl. One glass micropipette was in contact with the distal tip of the antenna as the recording electrode, the other one was inserted into the base of the head, which served as the reference electrode. The response signal was recorded using EAG-2000 software (Syntech, Hilversum, The Netherlands) with an IDAC4 amplifier (Syntech, Hilversum, The Netherlands). A controller generated 100 mL/min of pulse flow to stimulate the fly's antenna.

Three plant volatiles (1-octen-3-ol, methyl eugenol and ethyl acetate) were selected for EAG experiments [28–30]. In detail, only females were used for the assay of 1-octen-3-ol, while only males were used for the assay of methyl eugenol. Meanwhile, both females and males with a sex ratio of 1:1 were employed in the assay of ethyl acetate. As described above, MO or DMSO was used as the solvent and the negative control. Ten microliters of each dilution or control were applied to a paper strip (5 × 1 cm) which served as the stimulus source. Each stimulation lasted 1 s, and stimulus interval was at least 30 s [31]. There were about 10 individuals for the EAG assay of each plant's volatile.

2.5. Quantitative Real-Time PCR

The flies were treated with acetone or β-cypermethrin (LD_{30} = 10 ng/fly) at 6 days old. After 24 h, their heads were dissected and immediately frozen in liquid nitrogen. In detail, the samples of males and females were separately dissected and mixed in equal proportions. Total RNA was extracted from the collected samples with TRIzol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using 1μg of total RNA and PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China).

A standard protocol [32] was applied in this study, and the amplification efficiencies of primers were validated by generating a standard curve based on a double-fold cDNA dilution series. The 10 μL qPCR system consisted of 5 μL of SYBR Supermix (Novoprotein, Shanghai, China), 10 pM of the specific primers of each gene, 250 ng cDNA and nuclease-free water. The qPCR reaction was conducted on a CFX Conncet^{TM} Real-Time System (Bio-Rad, Jurong, Singapore) with the following program: 2 min at 95 °C for pre-denaturation, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A melting curve analysis generated from 60 °C to 95 °C was used to verify the specificity and consistency of the products for each primer pair [33]. *α-tubulin* (GenBank: GU269902) and *ribosomal protein S3* (*rps 3*, GenBank: XM_011212815) were selected as the internal reference genes. All the primers used in this study were designed by Primer Premier 5 (PREMIER Biosoft International, Palo Alto, California, USA) as shown in Table 1. Four biological and two technical replicates were set for each experiment. The relative expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method [34].

Table 1. Primer sequences of chemosensory genes used for quantitative real-time PCR.

Primer	Sequence (5′-3′)	Amplification Efficiency	Product Length (nt)
qPCR-*α-tubulin*-F	CGCATTCATGGTTGATAACG	97.5%	184
qPCR-*α-tubulin*-R	GGGCACCAAGTTAGTCTGGA		
qPCR-*rps 3*-F	TAAGTTGACCGGAGGTTTGG	98.3%	169
qPCR-*rps 3*-R	TGGATCACCAGAGTGGATCA		
qPCR-*ORco*-F	TTGACATCCACCATTATGCTGAC	95.3%	209
qPCR-*ORco*-R	TCCTCGGAGCCATCATACCA		
qPCR-*IR8a*-F	ATTGCGGCGTTGGTGGGTA	97.7%	185
qPCR-*IR8a*-R	GAGACGGCTTTTGGTGCTT		
qPCR-*IR25a*-F	TTGCTCCAGGTAATGCCTCC	96.5%	189
qPCR-*IR25a*-R	TCGTTTTCCCTCCTTCGCAA		
qPCR-*IR76b*-F	CCACTTTGGACGAGGGTGAA	101.5%	194
qPCR-*IR76b*-R	AGGCTTCTGCTCCTTATCGC		
qPCR-*IR93a*-F	AAGTGTAGCGGTCATGGTGG	90.7%	171
qPCR-*IR93a*-R	TGCAAAGACACCTCGCTTCT		

2.6. Statistical Analysis

All experiments were performed with at least three biological replications, and all the data were analyzed with SPSS software version 25.0 for Windows (IBM, Chicago, IL, USA). The concentration-mortality data of β-cypermethrin bioassay were analyzed by probit analysis. The results of olfactory preference assays, EAG recording and chemosensory genes relative expression after β-cypermethrin induction were analyzed with Student's *t*-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3. Results

3.1. Bioassay

We applied β-cypermethrin to the pronotum of cold-sedated *B. dorsalis* in 6-day-olds. The mortality for the tested concentrations was assessed 24 h after the β-cypermethrin application, which ranged from 11.7% to 78.3%. Based on the probit analysis, the dose causing 50% mortality (LD_{50}) within a 24 h observation period was 14.50 ng per fly (Table 2).

According to the calculation, the LD_{30} would be 9.70 ng/fly. Thus, we used this sublethal dose for the subsequent study.

Table 2. Toxicity of *β*-cypermethrin against 6-day-old adults of *B.dorsalis*.

Insecticide	*n*	Slope ± SE	X^2	df	Concentration (95% CI) (ng/fly)	RR *
β-cypermethrin	360	2.85 ± 0.47	1.96	4	LD_{30} = 9.70 (7.91–11.18) LD_{50} = 14.50 (12.76–14.46)	11.7

* Resistance ratios: LD_{50} divided by LD_{50} of susceptible strain.

3.2. Olfactory Preference

The alteration in olfactory preference of the 7-day-old flies treated with *β*-cypermethrin was determined by an olfactory behavior assay using the four-choice olfactometer. Three plant volatiles including 1-octen-3-ol, methyl eugenol and ethyl acetate were used at the concentration of 1% (*v*/*v*). As the results indicated, the *B. dorsalis* exposed to sublethal doses of *β*-cypermethrin significantly disrupted the discernment of the tested odorant (Figure 1). After *β*-cypermethrin treatment, the attraction rate of 1-octen-3-ol to the females decreased by 14.3% (from 55.0% to 40.7%), while the attraction rate of methyl eugenol to the males decreased by 18.5% (from 67.4% to 48.9%). Furthermore, the attraction rate of ethyl acetate to both females and males (with a sex ratio of 1:1) decreased by 14.2% (from 46.6% to 32.4%), specifically. Notably, there was no significant difference between the male and female flies in the ethyl acetate attraction assay.

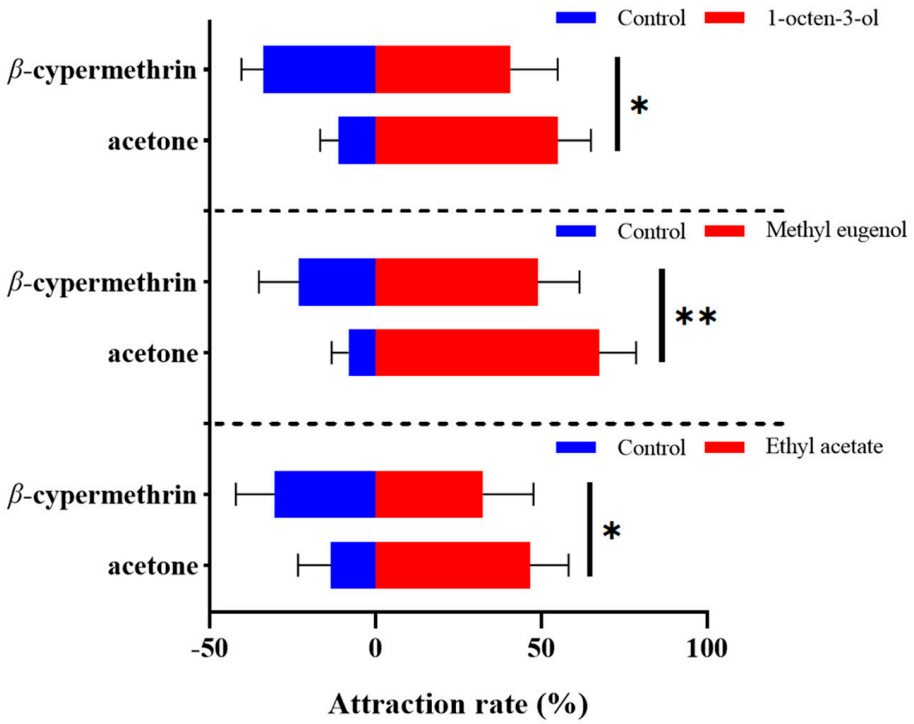

Figure 1. Four-way olfactometer assay of 7-day-old adults treated by *β*-cypermethrin (LD_{30} = 10 ng/fly). We used 1-octen-3-ol, methyl eugenol and ethyl acetate at 1% (*v*/*v*) concentration as the attractants. Females, males and both sexes were employed in the assays of 1-octen-3-ol, methyl eugenol and ethyl acetate, respectively. Data were presented as mean ± SE (*n* = 6). Asterisks represent a significant difference determined by Student's *t*-test (* $p < 0.05$; ** $p < 0.01$).

3.3. EAG Analysis

To study the electrophysiological response of *B. dorsalis* treated with β-cypermethrin, the EAG responses of 7-day-old adult flies to 1% (*v*/*v*) concentration of 1-octen-3-ol, methyl eugenol and ethyl acetate were recorded (Figure 2). The EAG signal ranged from −0.6 to −3.5 mV for 1-octen-3-ol, −0.6 to −3.9 mV for methyl eugenol, and −0.3 to −3.3 mV for ethyl acetate. However, the EAG responses of the flies exposed to β-cypermethrin were significantly weaker than the control for all three odorants. After β-cypermethrin treatment, the EAG response of the females to 1-octen-3-ol was reduced by 26.6%, while the EAG response of the males to methyl eugenol was reduced by 66.7%. In addition, the EAG response of both females and males (with a sex ratio of 1:1) to ethyl acetate was reduced by 63.9%. Notably, there was no significant difference between the male and female flies in the EAG assay of ethyl acetate.

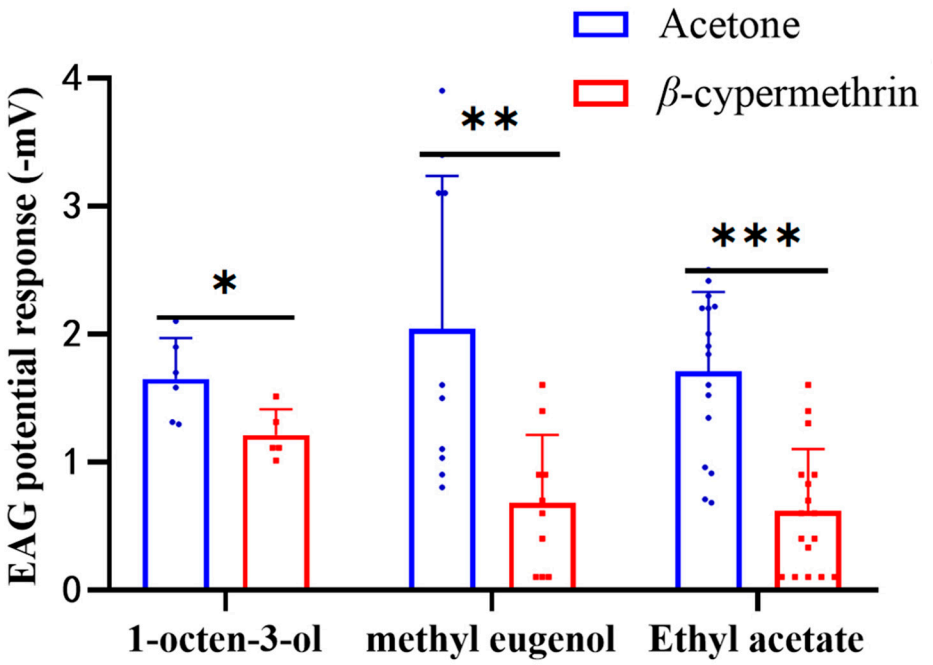

Figure 2. EAG recording of 7-day-old flies treated by β-cypermethrin (LD_{30} = 10 ng/fly). The EAG data was obtained by stimulating flies with three odorants including 1-octen-3-ol, methyl eugenol and ethyl acetate at 1% (*v*/*v*) concentration. Ethyl acetate and 1-octen-3-ol were diluted with MO and methyl eugenol was diluted with DMSO. Females, males and both sexes were employed in the assays of 1-octen-3-ol, methyl eugenol and ethyl acetate, respectively. Data were presented as mean ± SE, and asterisks represent a significant difference with analysis of Student's *t*-test (n = 8–12, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.4. Differential Expression of Olfactory Genes upon β-Cypermethrin Exposure

The relative expressions of chemosensory genes in the 7-day-old adult flies under β-cypermethrin treatment were analyzed by RT-qPCR (Figure 3). The amplification efficiencies of *α-tubulin*, *rps 3*, *BdorORco*, *BdorIR8a*, *BdorIR25a*, *BdorIR76b* and *BdorIR93a* were 97.5%, 98.3%, 95.3%, 97.7%, 96.5%, 101.5% and 90.7%, respectively (Table 1). As the results indicated, a sublethal dose of β-cypermethrin impaired the expression of chemosensory genes in *B. dorsalis*. Compared with the control group, the expression of *BdorIR8a*, *BdorIR93a* and *BdorORco* of β-cypermethrin treated flies significantly decreased by 48.2%, 53.3%, and 62.1%, respectively. However, the relative expression of *IR25a* and *IR76b* after treatment had no significant differences.

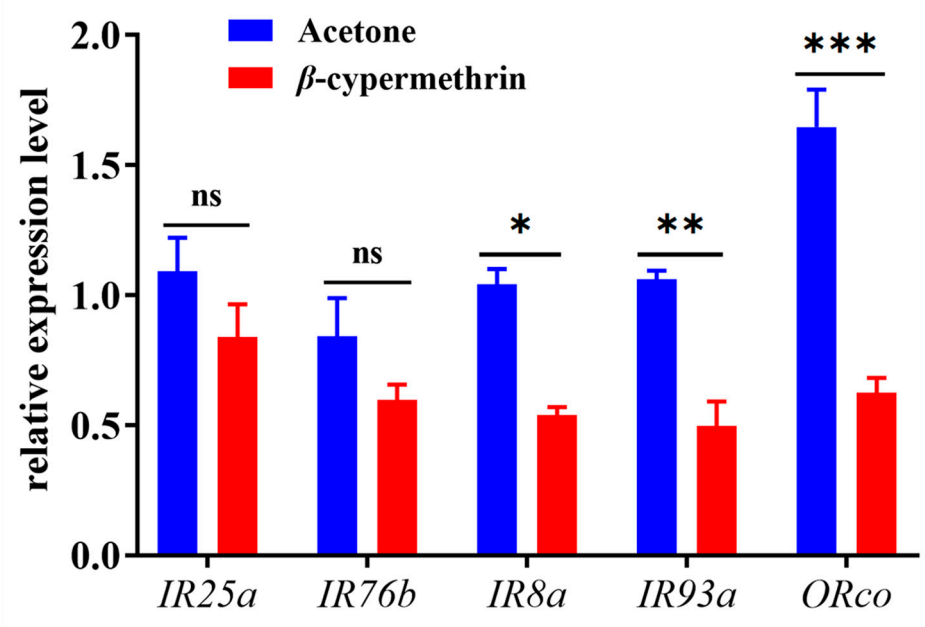

Figure 3. Transcriptional expression profiles of *ORco* and IRcos after β-cypermethrin induction (LD_{30} = 10 ng/fly). Data were presented as mean ± SE, and asterisks represent a significant difference with analysis of Student's *t*-test ($n = 4$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). No significant difference was represented by "ns".

4. Discussion

The oriental fruit fly has probably been exposed to sublethal concentrations of insecticides in fields for a long time, since the resistance monitoring of *B. dorsalis* in mainland China showed that only 1 of the 25 populations stay sensitive to β-cypermethrin [7]. Therefore, it is necessary to investigate the sublethal effects of β-cypermethrin on this fly. Compared with the susceptible strain described in the previous study, the *B. dorsalis* strain in our laboratory possessed a medium resistance with a resistance ratio of 11.7 (based on the LD_{50}) to β-cypermethrin [7]. Thus, we exposed the flies to β-cypermethrin with the dose of LD_{30} (10 ng/fly) in this study.

Sublethal insecticide residues have been shown to cause multiple effects on insects. For example, *Bacillus thuringiensis* exposure was found to reduce the lifespan and reproductive capacity of *Helicoverpa armigera* [35]. In *Cyrtorhinus lividipennis*, researchers found that sublethal concentration of triazophos and deltamethrin disturbed foraging ability, reduced predatory capacity, and decreased pepsin activity [36]. Compared with the susceptible strain, the cyantraniliprole-resistant (19.44 folds) strain of *B. dorsalis* shows a prolonged larval duration, higher pupa weight, and longer timing of sexual maturation [37]. Interestingly, a very low-dose exposure of chlordimeform induced an increased sensitivity to sex pheromone in *Carposia niponensis* [38]. Likewise, exposure to a sublethal dose of β-cypermethrin also improved the abilities of motility and respiration in *Harmonia axyridis* [39]. In the current study, *B. dorsalis* exposed to a sublethal dose of β-cypermethrin showed an altered olfactory behavior. However, we do not know whether development and reproduction were influenced or not. Moreover, we do not know whether the β-cypermethrin residue resulted in hormesis effects on this fly either. Hence, future studies on its developmental biology and ecological fitness are needed to investigate whether the sublethal effects of β-cypermethrin are contributing to the resurgence of *B. dorsalis* in a field or not.

The olfactory system of *B. dorsalis* is essential for finding habitats and mates, foraging, ovipositing, and avoiding predators [40–42]. Olfactory reception in *B. dorsalis* is represented by two main types of molecular receptors, the odorant receptors (ORs) and the ionotropic receptors (IRs) [43]. Recently, studies have shown that sublethal doses of insecticides can

seriously damage insect olfaction, resulting in abnormal olfactory behaviors. Our olfactory preference assays and EAG recording indicated that the *B. dorsalis* after β-cypermethrin exposure (LD_{30} = 10 ng/fly) severely decreased the ability to perceive the tested odorants. We are aware that we tested the females and males separately either for the behavior or the EAG for 1-octen-3-ol and methyl eugenol, due to the characterizations of the tested odorants. However, we concluded that the impaired olfaction by a sublethal dose of β-cypermethrin is not sex-biased, since there was no significant difference between sexes in the assays for ethyl acetate. Moreover, our results are consistent with several previous studies. In *N. vitripennis*, sublethal doses of the imidacloprid damaged the olfaction thereby impairing sexual communication and host finding [22]. In *Spodoptera littoralis*, the sublethal dose of deltamethrin residue reduced the repolarization time to sex pheromone and host–plant odorants and overexpresses some detoxification metabolic enzyme genes [44]. It would be very interesting to further study whether the olfactory alteration contributed to the development of resistance in insects or not.

According to the previous studies, a sublethal dose of chlorpyrifos impaired olfactory signal transduction resulting in olfaction sensitivity disruption in *O. mykiss* [21]. Besides, in *A. mellifera*, the olfaction learning ability decreased after exposure to a sublethal dose of imidacloprid because the density of the synaptic units in the region of the calyces that are responsible for olfactory and visual functions decreased [23]. In *Agrotis ipsilon*, sublethal doses of clothianidin residue decreased behavioral sex pheromone responses by downregulating the sensitivity of antennal lobe output neurons [45]. In the present study, according to the EAG and qPCR data, we found that the impaired olfaction may be due to the significantly reduced expression of chemoreceptor genes closely related to olfaction. This is consistent with the result that a sublethal dose of imidacloprid suppresses the *ORco* and *IR8a-2* in *Aphidius gifuensis* by analyzing transcriptome [46]. As our qPCR results indicated, the relative expression of *ORco*, *IR8a* and *IR93a* after treatment were significantly lower than control while the relative expression of *IR25a* and *IR76b* after treatment had no significant differences. Hence, we speculated that *IR8a* and *IR93a* are more closely associated with olfaction rather than *IR25a* and *IR76b* which have a more widespread expression (undocumented) [47,48]. However, due to the limitation of technical conditions, we do not know whether insecticide residue impairs the sensilla present in various tissues such as antennae and maxillary palp, olfactory signal transduction system, or the nervous system, which is worthy of further study.

In general, our data suggested that it might be difficult for *B. dorsalis* to proliferate and invade under sublethal β-cypermethrin stress. However, when non-target insects other than *B. dorsalis* encounter β-cypermethrin stress in the field, their olfaction is most likely affected. Further study on the ecological and environmental toxicology of sublethal dose of β-cypermethrin on non-target insects will provide answers for the effects on their populations. Therefore, our data not only expanded the knowledge on the sublethal effects of insecticides but also provided theoretical guidance for the rational use of pesticides in fields.

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Involvement of *Laccase2* in Cuticle Sclerotization of the Whitefly, *Bemisia tabaci* Middle East–Asia Minor 1

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Simple Summary: *Bemisia tabaci* Middle East–Asia Minor I (MEAM1) is a widely distributed invasive agricultural pest that causes extensive damage to agricultural, horticultural, and ornamental crops. *Laccase2* (*Lac2*), a phenol oxidase, plays an important role in cuticle tanning of some insects. However, the function of *Lac2* in whitefly remains unclear. In this study, we identified a *BtLac2* gene in MEAM1 that is expressed in all developmental stages, and its expression in the cuticle is especially high. Knockdown of *BtLac2* in nymphs produced thinner and fragile cuticles, which significantly increased the mortality rate and extended the development duration of nymphs, as well as further decreasing the emergence rate of adults. Overall, *BtLac2* plays an important role in cuticle hardening of whitefly, suggesting a potential management strategy using RNAi to knock down *BtLac2* expression.

Abstract: Cuticle sclerotization is critical for insect survival. *Laccase2* (*Lac2*) is a phenol oxidase that plays a key role in cuticle formation and pigmentation in a variety of insects. However, the function of *Lac2* in whitefly, *Bemisia tabaci*, remains unclear. In this study, we identified a *BtLac2* gene in *B. tabaci* MEAM1 and found that *BtLac2* was expressed in all stages. It was highly expressed in the egg stage, followed by nymph and adult. Moreover, the expression of *BtLac2* was higher in the cuticle than in other tissues. Knockdown of *BtLac2* in nymphs produced thinner and fragile cuticles, which significantly increased the mortality rate, extended the development duration of nymphs, and decreased the emergence rate of adults. This result demonstrates that *BtLac2* plays an important role in the cuticle hardening of *B. tabaci* and suggests a potential management strategy using RNAi to knock down *BtLac2* expression.

Keywords: *Bemisia tabaci*; *Laccase2*; cuticle; expression profiling; RNA interference; insect pest control

1. Introduction

Laccase (EC 1. 10. 3. 2) is a dioxygen oxidoreductase that contains four copper atoms in the catalytic center. Laccases are widely distributed in fungi, higher plants, insects, and some bacteria [1–7] and belong to the family of blue multi-copper oxidases (BMCOs) [8–10]. The wide range of substrates catalyzed by laccase contributes to the diversity of laccase function [11]. Previous studies have found that insect laccases mainly include *Laccase1* (*Lac1*) and *Laccase2* (*Lac2*); *Lac1* may play an important role in metal ion metabolism, detoxification of secondary plant compounds, and lignocellulose digestion [12–15], whereas *Lac2* plays an important role in insect cuticle tanning, morphology, and pigmentation [16–19].

Lac2 is a highly conserved multicopper oxidase that has three classic Cu-oxidase domains with a Cu^{2+} in the catalytic center, and it is known that the enzyme is synthesized by the epithelial cells and is secreted to the locations where new epidermis synthesis occurs [17,20,21]. The red flour beetle *Tribolium castaneum* injected with dsRNA for *Lac2*

showed soft cuticles, and the insects were deformed and subsequently died [16]. Similar phenotypes were also observed in other insects, such as *Monochamus alternatus* [17], *Apis mellifera* [18], and three hemipteran stinkbugs, *Riptortus pedestris* (Alydidae), *Nysius plebeius* (Lygaeidae), and *Megacopta punctatissima* (Plataspidae) [19]. In addition, many studies have indicated that *Lac2* also has other biological functions. For example, *Lac2* plays roles in *Vanessa cardui* butterfly wing pigmentation and scale development [22]. *Lac2* knockdown in *Aedes albopictus* resulted in pale and incomplete sclerotization of eggs and caused high mortality [23]. However, information is lacking on the specific role played by *Lac2* in whiteflies, which are important pests and vectors of plant viruses.

In this paper, we investigated the function of *Lac2* in *B. tabaci* cryptic species Middle East–Asia Minor I (MEAM1), also known as the B biotype, which is an invasive agricultural pest that is widely distributed and causes billions of dollars in crop losses worldwide [24–27]. In this study, we cloned the cDNA sequence of *BtLac2* of MEAM1. We characterized the temporal and spatial expression pattern in the developmental stages and body tissues of MEAM1 via real-time quantitative PCR (RT-qPCR). We also investigated the phenotype of MEAM1 after knockdown of *BtLac2* expression and its effect on the emergence and mortality of *B. tabaci*.

2. Materials and Methods

2.1. Whitefly Rearing

The MEAM1 whiteflies were maintained on tomato plants (*Lycopersicon esculentum*, cv. Zhongza 9) at 26 ± 1 °C, under a photoperiod of 16:8 h light/dark and 70 ± 10% RH. The purity of the whitefly colony was monitored by gene sequencing of *mtCOI* every two months.

2.2. Cloning, Sequence Retrieval, and Phylogenetic Analysis

Total RNA was extracted from approximately 200 *B. tabaci* adults using Trizol (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed into cDNA using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Bio Inc., Shiga, Japan). The *BtLac2* nucleotide sequence was found in the MEAM1 *B. tabaci* genome database (http://www.whiteflygenomics.org/cgi-bin/bta/index.cgi, accessed on 6 April 2019), and three pairs of primers were designed based on the putative *BtLac2* sequence to obtain the full open reading frame (ORF). PCR products were purified and subcloned into PMD-19 vector (Takara, Bio Inc., Shiga, Japan) for sequencing. The primers are listed in Table 1.

Table 1. Primers Used in This Study.

Primer Name	Primer Sequence (5′→3′)	Amplicon (bp)	Remarks
LAC2-1	F:ATGAAGGTGAAAATGTCACG R:GTTTCCTTGTAAGATGGGGC	777	fragment
LAC2-2	F:CCCATCTTACAAGGAAACACT R:CAGTTCTTGGAGTAAAGCCTT	795	fragment
LAC2-3	F:AGGCTTTACTCCAAGAACTGC R:TCAATGTAAACTGACCGGG	704	fragment
q-*LAC2*	F:AGTGTCTGCCCAGCTCAACT R:CAATTGCTGCACTCGTTTGT	213	qRT-PCR
q-*SDHA*	F:GCGACTGATTCTTCTCCTGC R:TGGTGCCAACAGATTAGGTGC	141	qRT-PCR
ds*BtLAC2*	F: taatacgactcactatagggGATCGACGACATCCCACCAG R: taatacgactcactatagggATCGAGGGAAACCTTCTGGC	479	RNAi
ds*EGFP*	F: taatacgactcactatagggCACAAGTTCAGCGTGTCCG R: taatacgactcactatagggTGCCGTTCTTCTGCTTGTCG	420	RNAi

DNAMAN was used to translate the full-length sequence of the nucleotide ORF to an amino acid sequence. Transmembrane helices were predicted using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM, accessed on 7 May 2019). The SignalP server (http://www.cbs.dtu.dk/services/SignalP, accessed on 7 May 2019) was used to predict signal peptides. Alignment and phylogenetic analyses were performed using DNAMAN and MEGA 7.0 software, respectively.

2.3. RT-qPCR Analysis of BtLac2 Expression Patterns

RNA was isolated from different developmental stages (eggs, 1st-instar nymph, 2nd-instar nymph, 3rd-instar nymph, 4th-instar nymph, and adults), and different tissues (head, thorax, cuticle, and ovary) of adults. Approximately 200 individuals of *B. tabaci* were used for one biological replicate. Adults that emerged within two days were used in the experiment. Subsequently, RT-qPCR was performed. The RT-qPCR primers (Table 1) were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/, accessed on 7 May 2019) and synthesized by Sangon Biotech (Shanghai, China). Succinate dehydrogenase complex subunit A (SDHA) was selected as a housekeeping gene for the RT-qPCR [28]. The RT-qPCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The reactions were performed on a LightCycler® 96 (Roche group, Basel, Switzerland). Three biological replicates, taken as three independent samples, were performed for each treatment.

2.4. Functional Analysis of the BtLac2 Gene in B. tabaci by RNAi

To explore the function of *BtLac2* in MEAM1, RNAi bioassay for the delivery of dsRNA by nanomaterial was performed. A unique region of the MEAM1 *BtLac2* gene was chosen as a template for double-stranded RNA (dsRNA) synthesis (Data S2). The fragments of the *BtLac2* and enhanced green fluorescent protein (*EGFP*) genes were amplified by reverse transcription PCR (RT-PCR) using specific primers conjugated with 20 bases of the T7 RNA polymerase promoter (Table 1). The PCR products of *BtLac2* (478 bp) and *EGFP* (420 bp) were used as templates for dsRNA synthesis using the Transcript Aid T7 High Yield Transcription Kit (Thermo Scientifific, Vilnius, Lithuania). The dsRNA was ethanoprecipitated, resuspended in nuclease-free water, quantified using a NanoPhotometer N50 (Implen, Munich, Germany), and stored at −20 °C until use. The concentration of the dsRNA solution was 0.5 μg/μL, as the solution contained 1 μg/μL dsRNA and an equal volume of nanomaterial provided by Professor Jie Sheng from China Agricultural University [29,30]. Twenty nanoliters of dsRNA mixture was dropped on the center of the back of a 4th-instar nymph using a micro-injector. The RNAi whiteflies were kept in a climate incubator (26 ± 1 °C, 60–70% RH, and 16L:8D photoperiod).

The RNAi efficiency was determined after 72 h. Thirty nymphs in each of the three replicates were used for total RNA extraction and RT-qPCR to determine the effect of RNAi. The RT-qPCRs were performed in a 20 μL mixture containing 1 μL of cDNA, 10 μL of 2× SYBR Premix Ex Taq II (Takara Biotechnology, Dalian, Liaoning, China), 1 μL of each primer (Table 1), and 7 μL of H_2O. The RT-qPCR program and housekeeping gene used were the same as those described above. The enhanced green fluorescent protein (*EGFP*) gene was used as a control. The same-aged nymphs (13 days from egg stage) were used for RNAi.

Phenotypes were observed and captured 3 days, 8 days, and 10 days after dsRNA treatment using a stereomicroscope. Ten nymphs were used for phenotypic observation, and fifty nymphs for each condition were used for analysis of the emergence rates of RNAi and control; three biological replicates were performed. The numbers of emerged *B. tabaci* in both the control and treatment groups were counted every 24 h for a total of 10 days.

2.5. Cuticle Morphology Analysis

After 72 h of ds*BtLac2*/ds*EGFP* treatment, nymphs were prepared for histological sectioning and HE staining. The samples were fixed overnight in 4% (*w*/*v*) paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. Slides were prepared by soaking twice in xylene for 20 min, twice in 100% alcohol for 5 min, and in 75% (*v*/*v*) alcohol for 5 min. The slides were then rinsed with water. The slides were immersed in hematoxylin solution for 3–5 min and rinsed with water. The sections were differentiated with acid alcohol, rinsed again, stained with ammonia solution, and washed in slowly running tap water. The sections were then stained in eosin using 85% (*v*/*v*) alcohol for 5 min, 95% (*v*/*v*) alcohol for 5 min, and eosin for 5 min. The sections were dehydrated thrice with 100% alcohol for 5 min, twice with xylene for 5 min, and, finally, in colorless hyaloid resin. Digital images of the sections were acquired using an Olympus fluorescence microscope (Olympus Optical, Tokyo, Japan) coupled to cellSens Standard software. The thickness of the dorsal section of the nymphs was measured using cellSens Standard software (Olympus Optical, Tokyo, Japan).

2.6. Data Analysis

Different developmental stage and body tissue data were used to calculate the mean ± SEM and were analyzed by one-way analysis of variance (ANOVA) using SPSS 20.0 (IBM, Armonk, NY, USA). Student's *t*-test was used to determine the significance of differences between the treatment and control groups. The figures were made using SigmaPlot 12.0 (Systat Software Inc., Palo Alto, CA, USA). The genes' relative expression levels from RT-qPCR were calculated by the $2^{-\Delta\Delta Ct}$ method.

3. Results

3.1. Characterization of BtLac2 from B. tabaci

The complete cDNA of *BtLac2* contains an open reading frame (ORF) of 2238 bp encoding a protein of 745 amino acid residues with a predicted molecular weight of 82.53 kDa, and the theoretical isoelectric point (pI) is 6.31. GenBank detection revealed that *BtLac2* has high homology with *Lac2* of other insects and contains three Cu-oxidase domains (Data S1), belonging to the laccase family. By using the biological software signalP, it was predicted that the signal peptide was located at amino acids 1–26, and we speculated that *BtLac2* is a secreted protein. There were 63 basic amino acids (Arg + Lys) and 73 acidic amino acids (Asp + Glu) in the sequence, accounting for 8.4% and 9.7% in the sequence, respectively. The instability coefficient was 36.73, suggesting that *BtLac2* is a stable protein.

The *Lac* family genes of different insects were found through NCBI's BlastP, and a multiple amino acid sequence comparison of *Lac2* between *B. tabaci* cryptic species MEAM1 and other insects was performed to construct a phylogenetic tree of Lac using MEGA 7.0 (Figure 1A). Phylogenetic analysis based on the *Lac2* amino acid sequences showed that *BtLac2* is closely related to *Lac2* of *Nephotettix cincticeps*, *Megacopta punctatissima*, and *Riptortus pedestris*, sharing more than 78% homology. As a character of laccases, the well-conserved cysteine-rich region was found in the predicted *BtLac2* protein when it was aligned with laccase protein sequences from other insect species (Figure 1B). The *Lac2* gene is relatively distant from the *Lac*, *Lac1*, and *Lac4* genes of *B. tabaci*.

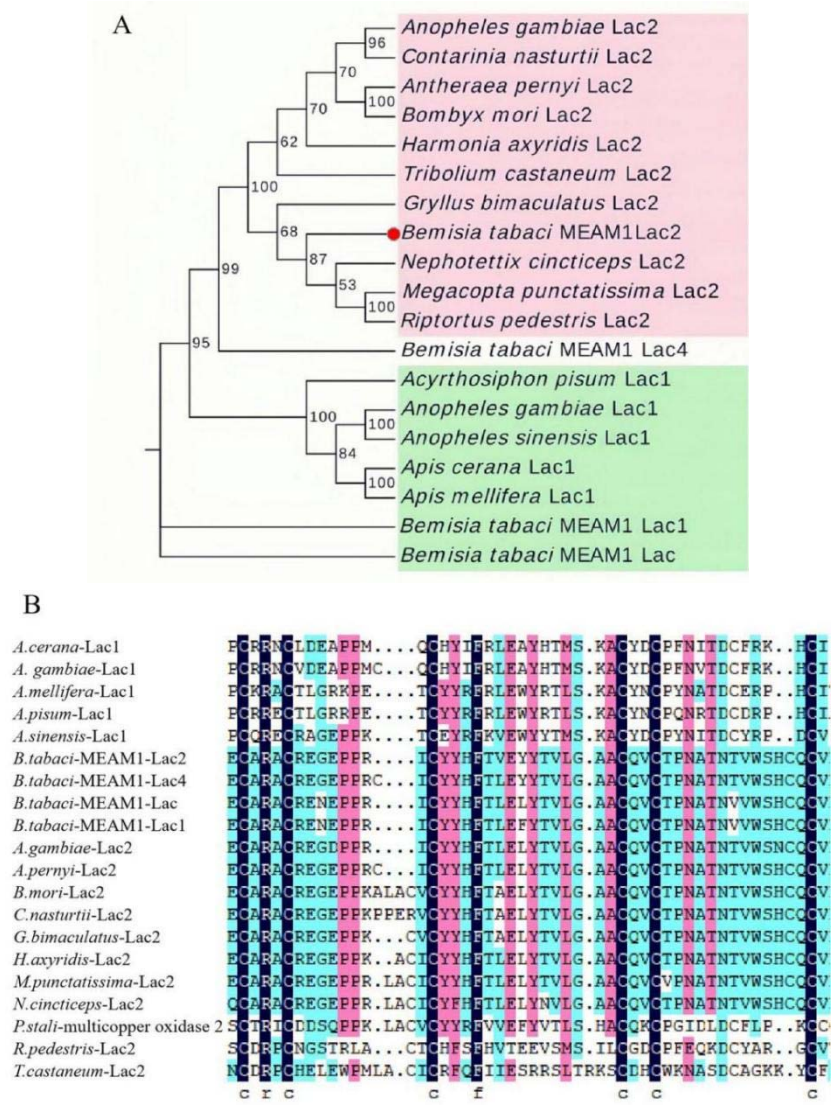

Figure 1. (**A**). Phylogenetic tree of Lac amino acid sequences in *B. tabaci* and other insects. The phylogenetic tree was generated by MEGA 7.0 based on the neighbor-joining method according to amino acid sequences. Accession numbers: *Apis cerana Lac1* XP_016918769.1; *Anopheles gambiae Lac1* AAN17505.1; *Apis mellifera Lac1* XP_001120790.2; *Acyrthosiphon pisum Lac1* XP_029344899.1; *Anopheles sinensis Lac1* KFB43437.1; *Bemisia tabaci* MEAM1 *Lac4* Bta00306; *Bemisia tabaci* MEAM1 Lac Bta11878; *Bemisia tabaci* MEAM1 *Lac1* Bta15284; *Anopheles gambiae Lac2* AAX49501.1; *Antheraea pernyi Lac2* AII19522.1; *Bombyx mori Lac2* BAG70891.1; *Contarinia nasturtii Lac2* XP_031632951.1; *Gryllus bimaculatus Lac2* BAM09185.1; *Harmonia axyridis Lac2* QNH91383.1; *Megacopta punctatissima Lac2* BAJ83488.1; *Nephotettix cincticeps Lac2* BAJ06133.1; *Plautia stali* multicopper oxidase 2 BBM95978.1; *Riptortus pedestris Lac2* BAJ83487.1; *Tribolium castaneum Lac2* AAX84203.2. (**B**). Multiple amino acid sequence alignment of the cysteine-rich consensus region in insect laccase proteins. Numbers on the right are the position of the final amino acid. Six cysteine residues conserved in the *Laccase1* and *Laccase2* proteins are marked by the letter C below the sequences.

3.2. Expression Profiling of BtLac2

To understand *BtLac2* function, the expression levels of the *BtLac2* gene in different tissues of adults and all development stages were detected by RT-qPCR (Figures 2 and 3). The results indicated that *BtLac2* mRNA was expressed consistently in all different developmental stages (eggs, 1st–4th-instar nymphs, and adults). However, the relative expression level of *BtLac2* in eggs was significantly higher than that in adults and nymphs. The expression level of the *BtLac2* gene in eggs was the highest, 130-fold higher than that in adults,

which showed the lowest expression. In addition, the relative expression levels of *BtLac2* in 2nd- and 3rd-instar nymphs were significantly higher than that in adults ($p < 0.05$). The expression of *BtLac2* was detected in the head, thorax, cuticle, and ovary. It is worth noting that *BtLac2* was expressed the most in the cuticle. There was no significant difference between male and female individuals.

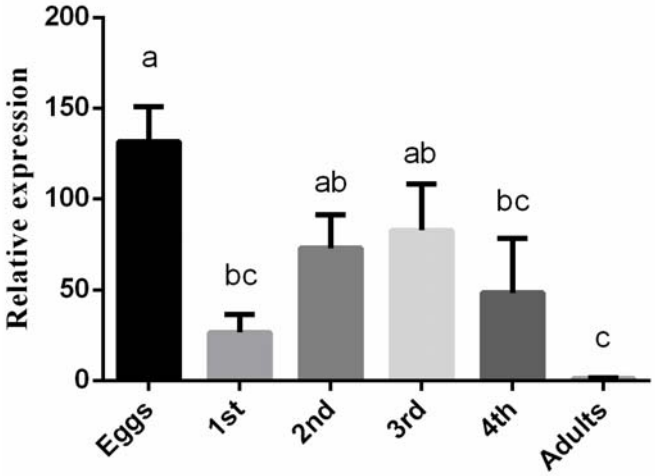

Figure 2. Relative expression levels of *BtLac2* in different developmental stages of *B. tabaci* cryptic species MEAM1. Bars indicate standard errors (n = 200–300, biological replicates = 3). Different letters indicate statistical significance ($p < 0.05$).

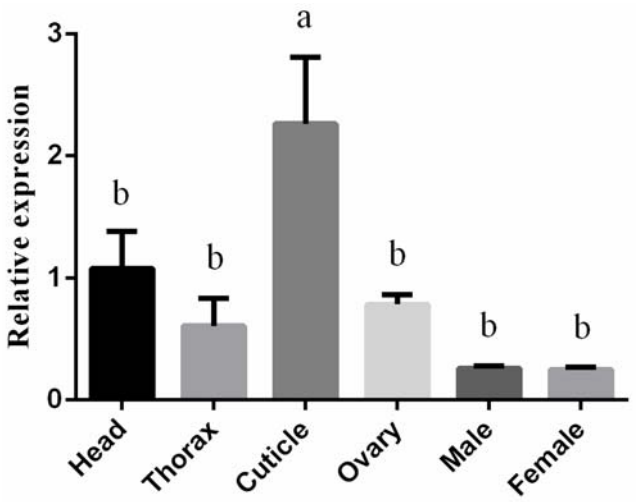

Figure 3. Relative expression levels of *BtLac2* in different tissues of *B. tabaci* cryptic species MEAM1. Bars indicate standard errors (n = 200–300, biological replicates = 3). Different letters indicate statistical significance ($p < 0.05$).

3.3. Low Expression of BtLac2 Results in Nymph Cuticle Sclerotization Defects and Emergence Retardation

The thickness of the dorsal section of the ds*BtLac2*-treated nymphs (Figure 4B) was only about 37% (Figure 4C) of that in the control group (Figure 4A). The expression of the *BtLac2* gene was detected by RT-qPCR at 72 h post RNAi. It was shown that the *Lac2* gene can be successfully silenced by a nanocarrier-based cuticle penetration method, and the expression of the *Lac2* gene in the whitefly was decreased by 58% at 72 h (Figure 4D).

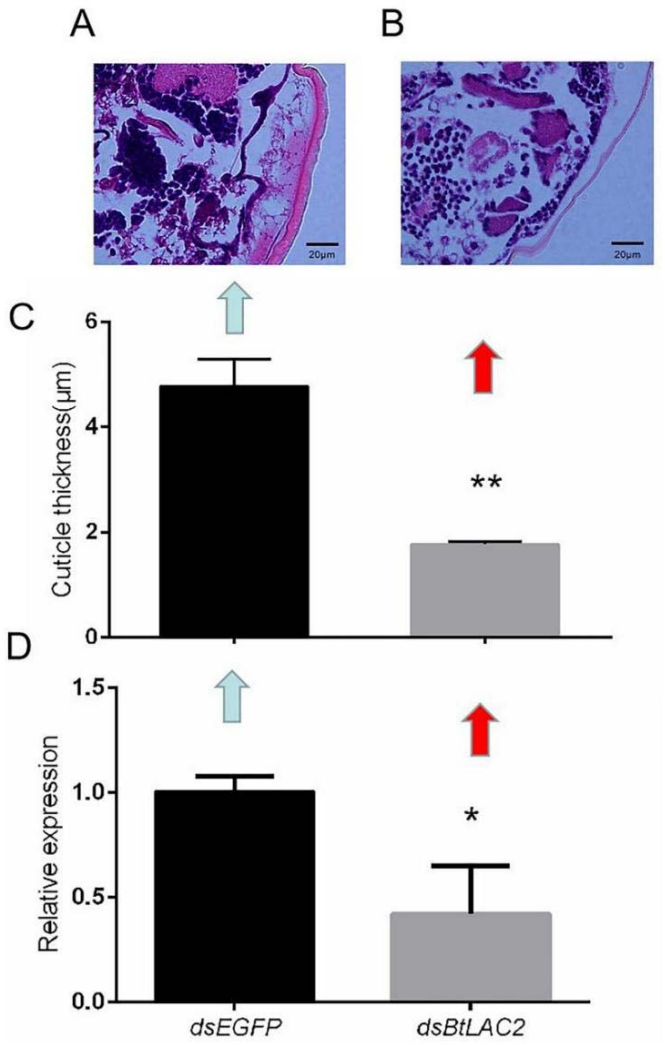

Figure 4. Effects of silencing *BtLac2* on physical characteristics of the *B. tabaci* nymph cuticle. Observation and thickness measurement of the dorsal cuticle of individual nymphs in the ds*EGFP*-treated (**A**) and ds*BtLac2*-treated (**B**) groups. (**C**) The dorsal cuticle thickness of *B. tabaci* 72 h after the RNA interference ($t = 18.329$, ** $p < 0.01$, $n = 10$). (**D**) The relative expression of the *BtLac2* gene 72 h after the RNA interference. Bars indicate standard errors ($t = 3.863$, $n = 50$, biological replicates = 3, * $p < 0.05$). dsRNA targeting enhanced green fluorescent protein (*EGFP*) was used as a negative control.

Meanwhile, compared to the control nymphs, ds*BtLac2*-treated nymphs were very fragile and easy to break (Figure 5(A1)); the nymphs could not develop and emerge as adults normally, and their bodies gradually dried out (Figure 5(C1)). The emergence rate of RNAi-treated nymphs (42 ± 2.49%) was significantly lower than that of control nymphs (78.67 ± 2.37 %) ($p < 0.05$) (Figure 6A), and the mortality rate of ds*BtLac2*-treated nymphs (0.21 ± 0.06) was significantly higher than that of control nymphs (0.58 ± 0.03) ($p < 0.05$) (Figure 6B). In addition, the development duration of the RNAi-treated nymphs was significantly increased ($p < 0.05$), and the time for emergence was nearly two days longer than that of the control nymphs (7.4 d ± 0.1 in control vs. 8.7 d ± 0.1 in treatment) (Figure 6A). Our data indicate that *BtLac2* dysfunction can lead to cuticle defects in the sclerotization processes, suggesting that it is crucial for cuticle tanning. The obvious time lag, low emergence rate, and physical weakness therefore affected the whitefly population.

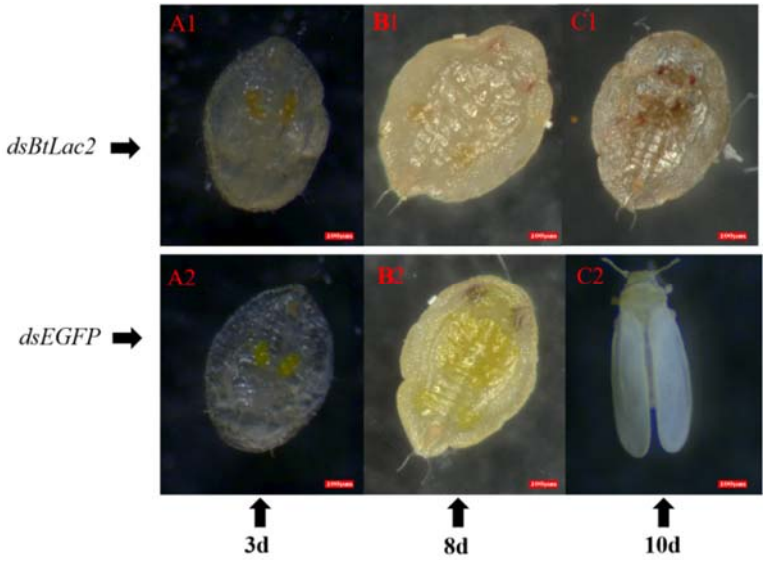

Figure 5. Lethal phenotypes of *B. tabaci* nymphs post RNA interference by *BtLac2*: after 3 days, 8 days, and 10 days. dsRNA targeting enhanced green fluorescent protein (*EGFP*) was used as a negative control.

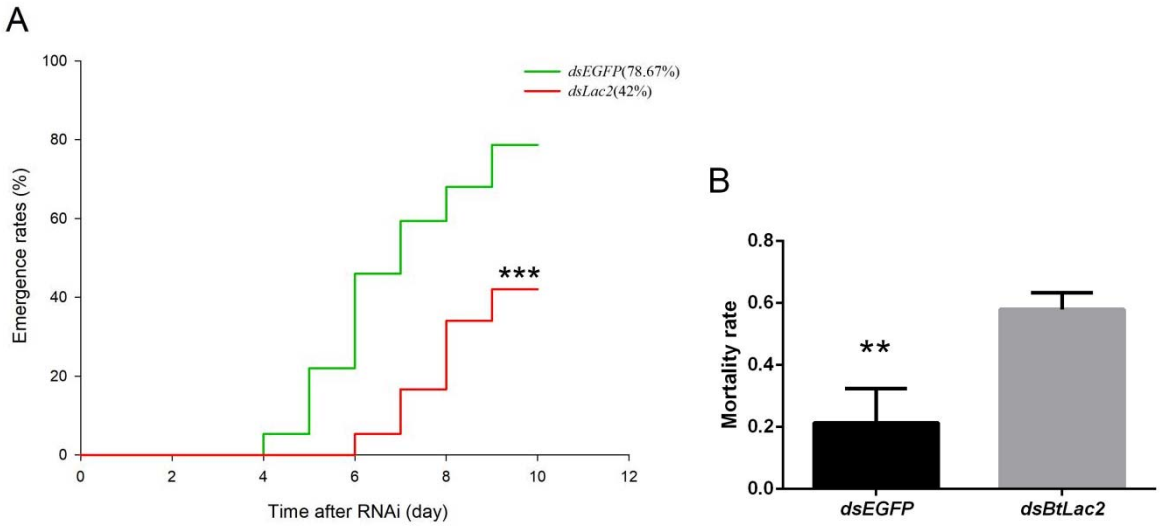

Figure 6. (**A**) The emergence rates of *B. tabaci* in the 10 days after knocking down of *Lac2*. The same-aged nymphs (13 days from egg stage) were used for the RNAi (t = 1.564, n = 50, biological replicates = 3, *** $p < 0.001$). (**B**) Mortality rate of *B. tabaci* nymphs 10 days after RNA interference by *BtLac2*. Bars indicate standard errors (t = 5.197, n = 50, biological replicates = 3, ** $p < 0.01$).

4. Discussion

Prior studies on *Lac2* genes in insects such as *T. castaneum*, *M. alternatus*, *A. mellifera*, *Bombyx mori*, and stinkbugs showed that *Lac2* is mainly involved in the hardening and darkening of the insect epidermis [16–19,30]. However, no studies had yet been performed to investigate the function of *Lac2* in the important agricultural pest *B. tabaci*. This is the first study to explore the important role of the *Lac2* gene in *B. tabaci*.

In this work, we successfully cloned the CDS sequence of the *Lac2* gene from *B. tabaci* cryptic species MEAM1. *BtLac2* showed high similarity with *Lac2* from other insects at the amino acid level. Phylogenetic analysis based on *Lac2* amino acid sequences showed that *BtLac2* is closely related to *Lac2* of *N. cincticeps*, *M. punctatissima*, and *R. pedestris*, sharing more than 78% homology with all three proteins, indicating that *BtLac2* is related closely to laccase from these insect species. A comparison of the *Lac*, *Lac1*, *Lac2*, and *Lac4* genes

of *B. tabaci* in the phylogenetic tree revealed that these genes are distinct from each other, suggesting that different laccases play different functions in insects. An analysis of the structural characteristics of the amino acid sequence showed that *BtLac2* has the typical laccase characteristic of Cu-oxidase domains. Copper ions are the active center of the laccase catalytic reaction and play an important role in the catalytic oxidation process. There are generally four Cu-oxidase domains in laccases from plants and fungi. However, similarly to those of other insects, the laccase of *B. tabaci* contains three Cu-oxidase domains [31,32], indicating that the function of *Lac2* in insects may differ from that in other organisms.

The RT-qPCR results indicated that *BtLac2* mRNA was expressed consistently in all developmental stages of the whitefly, suggesting that *BtLac2* is expressed throughout development and growth and plays a crucial role in biological processes. Higher expression levels of *BtLac2* were found in *B. tabaci* eggs and nymphs compared to adults. *Lac2* has been found in the eggshell [33,34], and pigmentation of the eggshell was blocked when the expression level of *Lac2* was lower [23]. Eggs and nymphs can protect themselves from external adverse factors mainly through passive defenses. A high expression level of *Lac2* in this stage can promote quinone accumulation to enhance, harden, and steady the cuticle [35]. Especially in the nymphal stage, *B. tabaci* must constantly molt and form a new epidermis during growth. The high expression level of *BtLac2* indicates that its function may play an important role in the formation of the epidermis of the whitefly nymphs. The RNAi study showed that silencing of *BtLac2* softened the whitefly nymphal epidermis, and the body gradually shriveled up, indicating that *BtLac2* is involved in the tanning process of the whitefly's epidermis. It is also beneficial for adult cuticle formation to adapt to the environment [16,35]. *Laccase2* plays an important role in the cross-linking of cuticular proteins, a process that is involved in the oxidative conjugation of quinones and quinone methides [36]. Adaptation of the cuticular composition often leads to increased insecticide resistance due to inhibition of insecticide penetration capacity [33]. It has been demonstrated that *Lac2* contributes to multiple-resistance phenotypes [37].

Due to their limited feeding capacity, or maybe because they cannot transmit viruses, the egg and nymphal stages of whiteflies are considered less harmful to plants than the adult stage [38]. So, it is reasonable to disturb the normal development of whiteflies in their egg or nymphal stages to prevent further loss. In this study, a nanocarrier-based RNAi technology was used to silence the *Lac2* gene in the nymphal stage of *B. tabaci*. Nanomaterial could deliver dsRNA into cells, achieving a good silencing effect [27,29,39]. *Bemisia tabaci* with successful silencing of the *Lac2* gene showed thinner cuticles and fragile bodies, and they died due to their weak ability to resist the external environment. In addition, we also found that the nymphs in which the *BtLac2* gene was successfully interfered with took longer for emergence than the control group, and the emergence rate also decreased, indicating that *BtLac2* plays an important role in the development of *B. tabaci*. These results, along with those of previous research, enrich our knowledge about the functions of insect *Laccase2*, so that it may potentially act as a new target for pesticides and pest control in the future.

5. Conclusions

In this study, we cloned the cDNA of *BtLac2* from *B. tabaci* MEAM1. After gene expression analysis, we found that *BtLac2* was expressed in all developmental stages, but the highest expression level was observed in the egg stage, followed by nymphs and adults. Further, Bt*Lac2* was expressed more in the cuticle than in other tissues. Knockdown of *BtLac2* in nymphs produced thinner and fragile cuticles, which further impeded the emergence rate of adults, as well as their fitness. Our data could clarify the structures, phylogeny, expression pattern, and biological functions of *BtLac2* in *B. tabaci*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13050471/s1, Data S1: The protein-coding DNA sequence and amino acid sequence for B. tabaci MEAM1 *Lac2* gene. Note: start codon (ATG); stop codon (TGA);

glycosylation sites are double-underlined; blue areas are signal peptide site; yellow areas represent copper ion domains; Data S2: The sequence of the gene region used for dsRNA syhthesis.

Author Contributions: Conceptualization, C.-H.Y. and T.-X.L.; data curation, Q.Z. and W.-Q.Z.; methodology, C.-H.Y. and Y.S.; validation, H.-H.C.; software, H.-H.C.; investigation, W.-Q.Z.; resources, Y.S.; formal analysis, C.-H.Y.; writing—original draft preparation, C.-H.Y. and Z.L.; writing—review and editing, C.-H.Y., Q.Z., L.G., D.C. and T.-X.L.; supervision, T.-X.L. and D.C.; funding acquisition, C.-H.Y., T.-X.L. and L.G.; project administration, Z.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

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Purification and Functional Characterization of a Soluble Trehalase in *Lissorhoptrus oryzophilus* (Coleoptera: Curculionidae)

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Simple Summary: The rice water weevil, *Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae), is indigenous to the United States and has become a significant invasive agricultural pest in China. In this study, we identified and cloned one trehalase gene (*LoTRE1*) encoding a soluble protein in *L. oryzophilus* and compared the relative expression levels of *LoTRE1* in different tissues. The purified *LoTRE1* protein was obtained using a prokaryotic expression system, and its enzymatic properties were explored. Amino acid sequence homology modeling of *LoTRE1* and molecular docking between the *LoTRE1* protein and substrate trehalose were simulated, which further provided a theoretical basis for revealing the role of *LoTRE1* in the degradation mechanism of trehalose. In addition, the *LoTRE1* double-stranded RNA (dsRNA) was synthesized in vitro, and its RNAi effect in *L. oryzophilus* was detected via feeding. The results suggested that *LoTRE1* played a vital role in *L. oryzophilus* development, which could be useful for providing information for insect pest control in the future.

Abstract: Trehalase is the only enzyme known for the irreversible splitting of trehalose and plays a major role in insect growth and development. In this report, we describe a basic study of the trehalase gene fragment encoding a soluble trehalase from *Lissorhoptrus oryzophilus* (*LoTRE1*). Sequence alignment and phylogenetic analysis suggested that *LoTRE1* was similar to some known insect trehalases and belongs to the Coleoptera trehalase group. Additionally, *LoTRE1* was expressed mainly in the fat body. Purified protein was obtained using heterologous expression of *LoTRE1* in *Escherichia coli*, and the recombinant protein exhibited the ability to decompose trehalose. Enzyme–substrate docking indicated the potential involvement of other residues in the catalytic activity, in addition to Asp 333. Moreover, feeding of adults on *LoTRE1* dsRNA silenced the transcription of *LoTRE1* and thereby reduced the activity of trehalase and increased the trehalose content; it also led to a 12% death rate. This study reveals essential molecular features of trehalase and offers insights into the structural aspects of this enzyme, which might be related to its function. Taken together, the findings demonstrate that *LoTRE1* is indispensable for adults of this pest and provide a new target for the control of *L. oryzophilus*.

Keywords: *Lissorhoptrus oryzophilus*; soluble trehalase; molecular docking; RNAi; prokaryotic expression; homology modeling

1. Introduction

Trehalose is a nonreducing disaccharide that consists of two α-glycosidically linked glucose units. This disaccharide is found in many organisms, such as plants, nematodes, bacteria, insects, and some other invertebrates; however, it does not exist in mammals [1]. Trehalose is mainly considered an important cell protective metabolite in vivo, and cells can

synthesize trehalose in large quantities under stress and degrade it rapidly under normal conditions [2]. Trehalose exhibits multiple physiological effects in various organisms, and it has been shown that trehalose can shield proteins and cellular membranes from inactivation or denaturation caused by a range of stress conditions, including dehydration, desiccation, cold, heat, and oxidation [3–8]. Additionally, trehalose is the major blood sugar in insects, playing an important role as an instant source of energy and in the response to abiotic stresses. Trehalose is specifically synthesized in insect fat bodies and quickly discharged into the hemolymph and other tissues [7,8]. To utilize hemolymph trehalose, insect tissues contain trehalases (EC 3.2.1.28) that catalyze the hydrolysis of one mole of trehalose to two moles of glucose. Thus, trehalase is the enzyme that is required for the uptake or utilization of trehalose in the hemolymph of insects. Two types of trehalase, soluble trehalase (TRE1) and membrane-bound trehalase (TRE2), have been cloned and characterized in several insect species, such as *Acyrthosiphon pisum*, *Bombyx mori*, *Helicoverpa armigera,* and *Harmonia axyridis* [9–12]. Both TRE1 and TRE2 include a signal peptide, two signature motifs (PGGRFREFYYWDSY and QWDYPNAWPP), and one glycine-rich region [13]. Gibson et al. [14] have shown that the catalytic domain of *E. coli* trehalase displays an aspartate (Asp312) and a glutamate (Glu496) residue, which play the role of a general acid and a general base, respectively, similarly to hydrolases from the GH37 family. By site-directed mutagenesis in *Spodoptera frugiperda*, three arginine residues essential to the enzyme activity were identified inside the active site [15]. To date, no three-dimensional (3D) structure is available from experimental data for TREs from plants, animals, or fungi, whereas molecular modeling studies have predicted the 3D structure of insect TREs in *Bombyx mori* [10], *Helicoverpa armigera* [11], *Drosophila melanogaster* [16], *Chironomus riparius* [17], and *Delia antiqua* [18]. There are also individual protein differences among different insects [10,17]. In vivo, soluble trehalase accounts for the majority of the overall trehalase enzyme activity, according to previous studies [19]. The main function of TRE1 is to decompose trehalose in cells. TRE1 is an essential enzyme in insect energy metabolism and the first enzyme in the chitin synthesis pathway in insects [11].

The rice water weevil, *Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae), is the most harmful and widely distributed early-season pest of rice in the USA [20] and causes serious economic issues in wetland rice agriculture, resulting in losses of up to 25% in untreated fields [21]. Parthenogenetic female *Lissorhoptrus oryzophilus* (*L. oryzophilus*) have quickly invaded many rice-growing regions around the world [20,22,23]. In China, *L. oryzophilus* were first discovered in 1998; they have rapidly invaded 78% of the provinces and have become the foremost widespread invasive pest [24]. *L. oryzophilus* have caused serious harm to the rice in the invaded area.

In this paper, we analyzed the sequence, structural characteristics, and expression patterns of *LoTRE1*. We also expressed recombinant *LoTRE1* and studied its physicochemical properties to gain insight into the optimum conditions for its activity, including temperature and pH. Furthermore, we used RNAi to study the function of the *LoTRE1* gene and detected changes in target gene expression, trehalase activity, and trehalose content. The findings of our research are critical for understanding the performance of trehalase at the molecular level, as well as the potential to adapt to future invasions. The characterization of trehalase genes could facilitate the development of novel ways to manage *L. oryzophilus*.

2. Materials and Methods

2.1. Insect Culture and Tissue Collection

L. oryzophilus adults were collected from Changchun, Jilin Province, China, and reared on rice seedlings under a 16 h light/8 h dark photoperiod at 26 ± 1 °C with 80 ± 5% relative humidity. The adults and different tissues (hemolymph, midgut, head, wing, fat body, and leg) of dissected *L. oryzophilus* were promptly immersed in liquid nitrogen and stored at −80 °C until use.

2.2. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from adults and different tissues using RNAiso Plus (Takara, Dalian, China) according to the manufacturer's instructions for gene cloning and spatial expression. The RNA integrity and concentration were checked using agarose gel electrophoresis and spectrophotometry (NanoDrop2000, Wilmington, DE, USA), respectively. Then, approximately 1 μg of total RNA was utilized for the synthesis of first-strand cDNA by using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Dalian, China). The synthesized cDNA was stored at −20 °C until use.

2.3. Identification of the LoTRE1 Gene and Bioinformatics Analysis

The sequence of *LoTRE1* was identified using our unpublished transcriptome data. The amino acid sequence of *LoTRE1* was deduced using DNAMAN software. Open reading frames (ORFs) of genes were predicted using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/, accessed Date: 25 June 2021). Signal peptides and transmembrane domains were predicted using SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/, accessed Date: 25 June 2021) and TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/, accessed Date: 25 June 2021), respectively. The molecular weight and theoretical isoelectric point of the deduced protein were calculated using the ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi/, accessed Date: 25 June 2021) [25]. Multiple amino acid sequence alignments were performed by using DNAMAN software (http://www.lynnon.com/pc/alignm.html, accessed Date: 25 June 2021). The tertiary structures of *LoTRE1* proteins were predicted using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl, accessed Date: 25 June 2021) and SWISS-MODEL (https://swissmodel.expasy.org/, accessed Date: 25 June 2021). PDB files and molecular ligand data were obtained from ZINC (http://zinc15.docking.org/substances/home/, accessed Date: 25 June 2021). The molecular model docking calculations of *LoTRE1* with ligands were performed by using the Autodock 4.2 program. BLASTX best hits were found using the BLASTX program provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed Date: 25 June 2021). Phylogenetic trees were constructed using MEGA 6.0 software with maximum-likelihood phylogenetic analysis. The tree was colored and arranged using iTOL (https://itol.embl.de/upload.cgi, accessed Date: 25 June 2021).

2.4. Quantitative Real-Time PCR

Primer pairs for qPCR were designed using Primer 5 software, as shown in Table 1. GAPDH was used as a reference gene. mRNA expression levels were measured using qPCR using SYBR qPCR SuperMix (TransGen, Beijing, China). Each amplification reaction was carried out in a total volume of 20 μL, with 1 μL of cDNA, 10 μL of green qPCR SuperMix, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 8.2 μL RNase-free water. qPCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) under the following conditions: initial denaturation at 94 °C for 30 s, followed by 40 cycles of denaturation at 94 °C for 5 s, annealing at 55 °C for 15 s, and extension at 72 °C for 10 s. To calculate the relative expression levels, melting curves were evaluated to confirm the single peak and check amplification specificity after qPCR. Standard deviations and means were obtained from the mean of three biological replicates with three corresponding technical replicates. The relative expression value of the *LoTRE1* gene was calculated using the $2^{-\Delta\Delta Ct}$ method [26].

Table 1. Primer information.

Primer Name	Primer Sequence (5′-3′)	Purpose
TRE1	F: CGCGGATCCATGATGAAGAATATTTATGTAACGA R: CCGCTCGAGTCACCCTATAAATCCTGCTGATAAG	Prokaryotic expression
dsRNA-TRE	F: TAATACACTCACTATAGGGGTGGGCTAAGAAGCTCAACG R: TAATACACTCACTATAGGGCCGAATACGATTCCGGTCTA	RNAi
dsRNA-GFP	F: TAATACGACTCACTATAGGGGACGTAAACGGCCACAAGTT R: TAATACGACTCACTATAGGGTGTTCTGCTGGTAGTGGTCG	RNAi
TRE1-q	F: AAAATTACACTTTGGCCCTCTA R: GTCCCAACCGGATTCAGC	RT–qPCR
GADPH	F: ACCACTGTCCACGCAACT R: ACTCTGAAGGCCATACCG	RT–qPCR

The boxes mark the restriction sites and the T7 promoter sequence is underlined.

2.5. Protein Expression and Purification

The coding region of the *LoTRE1* gene was subcloned into the *BamHI/XhoI* sites of the pET28a (+) vector and then transformed into Rosetta (DE3) *E. coli* competent cells. The colonies were grown on Luria–Bertani culture medium with kanamycin (50 mg/mL). The positive monoclones were cultured in liquid LB medium (supplemented with 50 mg/mL kanamycin) overnight at 37 °C. The culture was diluted at 1:100 in liquid LB and incubated at 37 °C for 3–4 h until the OD600 reached 0.4–0.6. Isopropyl-β-d-thiogalactoside (IPTG) was added at final concentrations of 0.1, 0.4, 0.8, and 1.0 mmol/L, and then the culture was incubated at different temperatures (28 and 37 °C) for 8 h. Centrifugation (5000 rpm, 5 min, 4 °C) was performed to harvest the cells, and the collected cells were suspended in 1× phosphate-buffered saline (PBS). The suspension was sonicated on ice and centrifuged (5000 rpm, 5 min, 4 °C) for a second time. Protein present in the supernatant was purified with a Ni-NTA Gravity Column (Sangon, Shanghai, China). The purified protein was assessed using SDS–PAGE and then quantified via the Bradford assay with BSA as the standard.

2.6. Western Blot Analysis

The purified protein was separated using 10% SDS–PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane (100 V, 1 h). The membrane was treated with 5% blocking protein powder in TBST (1 M Tris-HCl pH 7.5, 500 mM NaCl, and 0.2% Tween 20) for 1 h at room temperature and reacted with the anti-6 × His tag mouse monoclonal antibody (Sangon, Shanghai, China) in TBST for 1.5 h at room temperature. The membrane was washed with TBST 4 times for 5 min each time and then incubated with AP-conjugated goat anti-mouse IgG (Sangon, Shanghai, China) in TBST for 1 h at room temperature. The NBT/BCIP substrate solution (Sangon, Shanghai, China) was utilized to visualize the protein band after the PVDF membrane was washed again.

2.7. Enzyme Activity Assay In Vitro

The 3,5-dinitrosalicylic acid technique was used to indirectly measure trehalase activity [27]. The reaction mixture (1 mL) consisted of 10 μL purified protein, 50 μL trehalose (200 mmol/L), and 940 μL PBS. PBS was prepared for a pH range of 3.0–7.0 The mixture was incubated in each pH buffer at 25 °C for 30 min and subjected to boiling for 5 min. The coagulated protein was removed by centrifugation at 12,000 rpm for 10 min at 4 °C. Trehalase activity was determined by measuring the content of glucose released during incubation. Similarly, for the measurement of trehalase activity at different temperatures, the mixture containing PBS (pH 7.0) and other components was incubated at the 9 individual temperatures, which ranged from 5 to 75 °C. To determine the kinetic parameters (K_m and V_{max}) of trehalase, substrates at different concentrations (1, 2.5, 5, 7.5, and 10 mmol/L) were

added to the reaction mixture and incubated at 50 °C and pH 7.0 for 30 min. The trehalase activity was recorded with a UV-2450 spectrophotometer (Shimadzu, Japan). One unit of enzyme activity (U) was defined as the amount of protein that released 1 μmol of glucose in 1 min. Each experiment was replicated three times.

2.8. dsRNA Synthesis and Feeding

The dsRNA of *LoTRE1* was synthesized using the T7 RioMAX Express RNAi System (Promega, San Luis Obispo, CA, USA) according to the manufacturer's recommendations. Green fluorescent protein (GFP) dsRNA was used as the control. The primers used to synthesize the dsRNA are listed in Table 1.

A total of 270 *L. oryzophilus* adults were divided into three groups, and each group was fed rice leaves with dsGFP, ds*LoTRE1*, and RNase-free water. Approximately 30 individuals were fed per treatment. Each group contained three biological replicates. Edges of approximately 1 cm were cut from both ends of fresh rice leaves, and the leaves were dried for 30 s at 55 °C in a drying oven. The rice leaves were immersed in 10 mL centrifuge tubes containing 500 ng/μL dsRNA for 6 h. The adults of *L. oryzophilus* were transferred to a new 10 mL centrifuge tube, and each tube contained 30 insects. The treated rice leaves were put into the centrifuge tubes containing *L. oryzophilus* adults, and then the centrifuge tubes were sealed with gauze. dsRNA feeding was continued for 12 h, and the leaves were replaced with fresh rice leaves without dsRNA every 24 h. The dead insects were picked out with a brush, and the number of dead insects was recorded. Fresh leaves and dsGFP-treated leaves were used as controls. Total RNA from treated *L. oryzophilus* adults was extracted as a template, and specific primers (Table 1) were used for quantitative RT–qPCR. Each RT–qPCR biological replicate contained three surviving *L. oryzophilus* adults. Three biological replicates and three technical replicates were set for each treatment group.

2.9. Determination of Trehalase Activity and Sugar Content In Vivo

A total of 30 *L. oryzophilus* adults were placed in a 10 mL centrifuge tube and fed with rice leaves treated with dsRNA for 12 h, then transferred to fresh rice leaves without dsRNA. The surviving individuals were picked out with a brush after 48 h. The enzymatic analysis of the activity of trehalase (THL) was performed using a THL kit according to the manufacturer's instructions (Solarbio, Beijing, China), and the trehalose content was determined according to the manufacturer's instructions for the Trehalose Content Kit (Solarbio, Beijing, China). Each experiment was replicated three times.

2.10. Statistical Analysis

All data obtained during this study were expressed as the means ± standard deviations of three replicates and were tested with a one-way ANOVA and *t* tests using SPSS 22.0. *p* values of less than 0.01 indicated significant (**) differences.

3. Results

3.1. Sequence Analysis of LoTRE1

LoTRE1 was identified from our unpublished transcriptome data of *L. oryzophilus*. Through homology searching in our transcriptional sets, the sequence of *LoTRE1* was found to contain an 1839 bp-long open reading frame encoding 612 amino acid residues (approximately 70833 Da and a theoretical isoelectric point (pI) of 5.38). A signal peptide of 25 amino acids and a cleavage site (IAI-YK) between residues 25 and 26 were identified (Figure 1). *LoTRE1* had two signature motifs (PGGRFREFYYWDSY and QWDYPNAWPP) and a highly conserved glycine-rich region (GGGGEY). Four N-glycosylation sites (residues 336, 579, 580, and 581) were identified in the *LoTRE1* sequence (Figure 1). TMHMM predicted that there were no transmembrane domains.

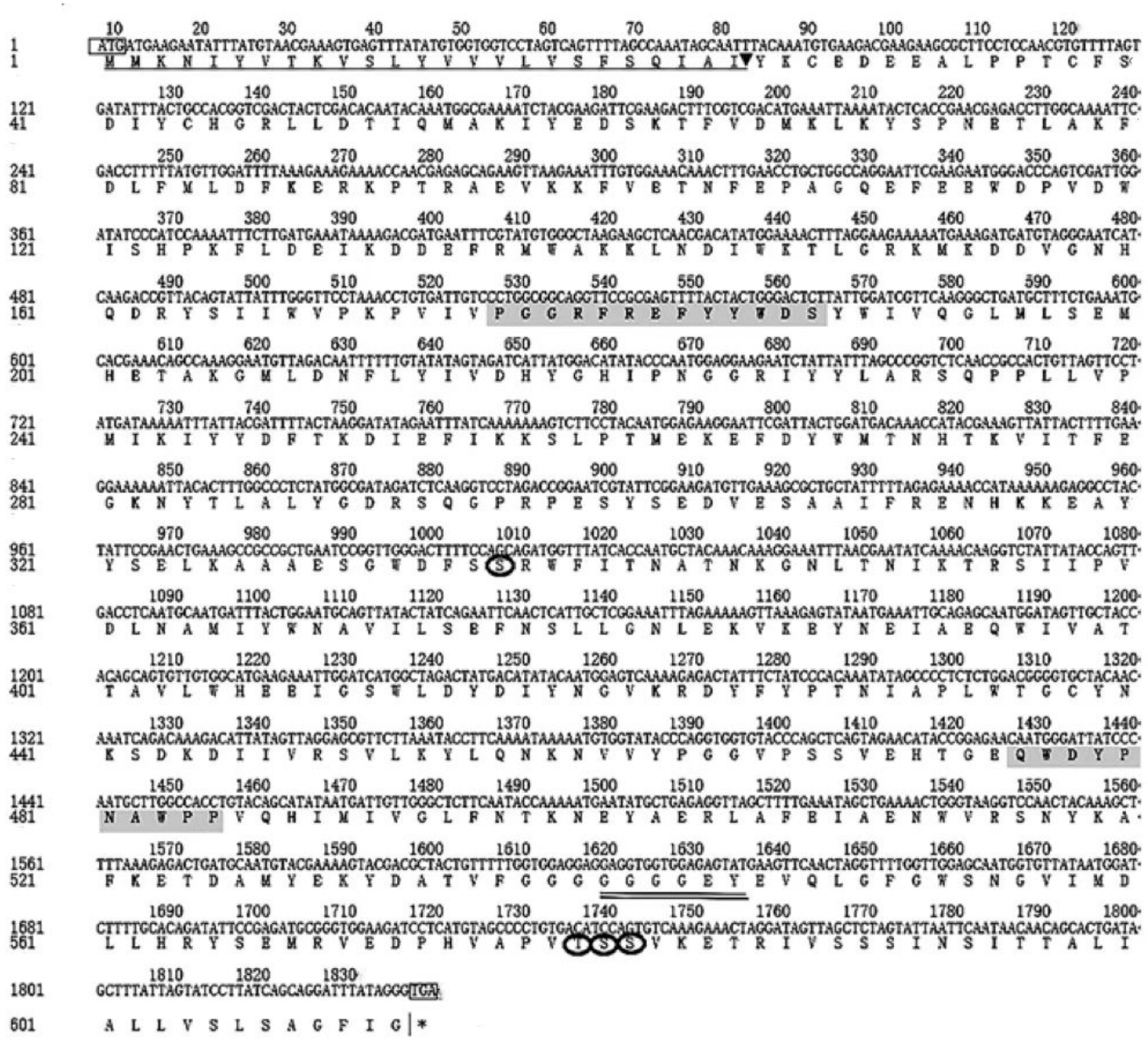

Figure 1. Nucleotide sequence and deduced amino acid sequence of *LoTRE1*. The black box in the figure indicates the start codon (ATG) and stop codon (TGA). Underlined amino acid residues (1–25) and the arrowhead represent the signal peptide and putative cleavage site, respectively. Trehalase signature motifs (amino acid residues 176–189 and 476–485) are shaded. Four potential N-glycosylation sites (amino acid residues 336, 579, 580, and 581) are encircled. The highly conserved glycine-rich region is double underlined.

Multiple sequence alignment was performed to analyze evolutionarily or structurally related positions between *LoTRE1* and its homologues based on the amino acid sequence (Figure 2). *LoTRE1* showed approximately 55–72% sequence identity with other Coleoptera insect trehalases. The greatest sequence identity (72%) was with trehalase (GenBank ID: XP 030756586.1) from *Sitophilus oryzae,* and the lowest sequence identity (55%) was with trehalase (GenBank ID: XP_031336857.1) from *Photinus pyralis.* To investigate the evolutionary relationships of *LoTRE1*, a phylogenetic tree of *LoTRE1* was constructed along with several previously studied TREs from 24 species of Coleoptera, Hemiptera, Blattaria, and Hymenoptera using maximum-likelihood phylogenetic analysis (Figure 3). The results

showed that *LoTRE1* shared the highest homology with the genes from *Sitophilus oryzae* and *Rhynchophorus ferrugineus.*

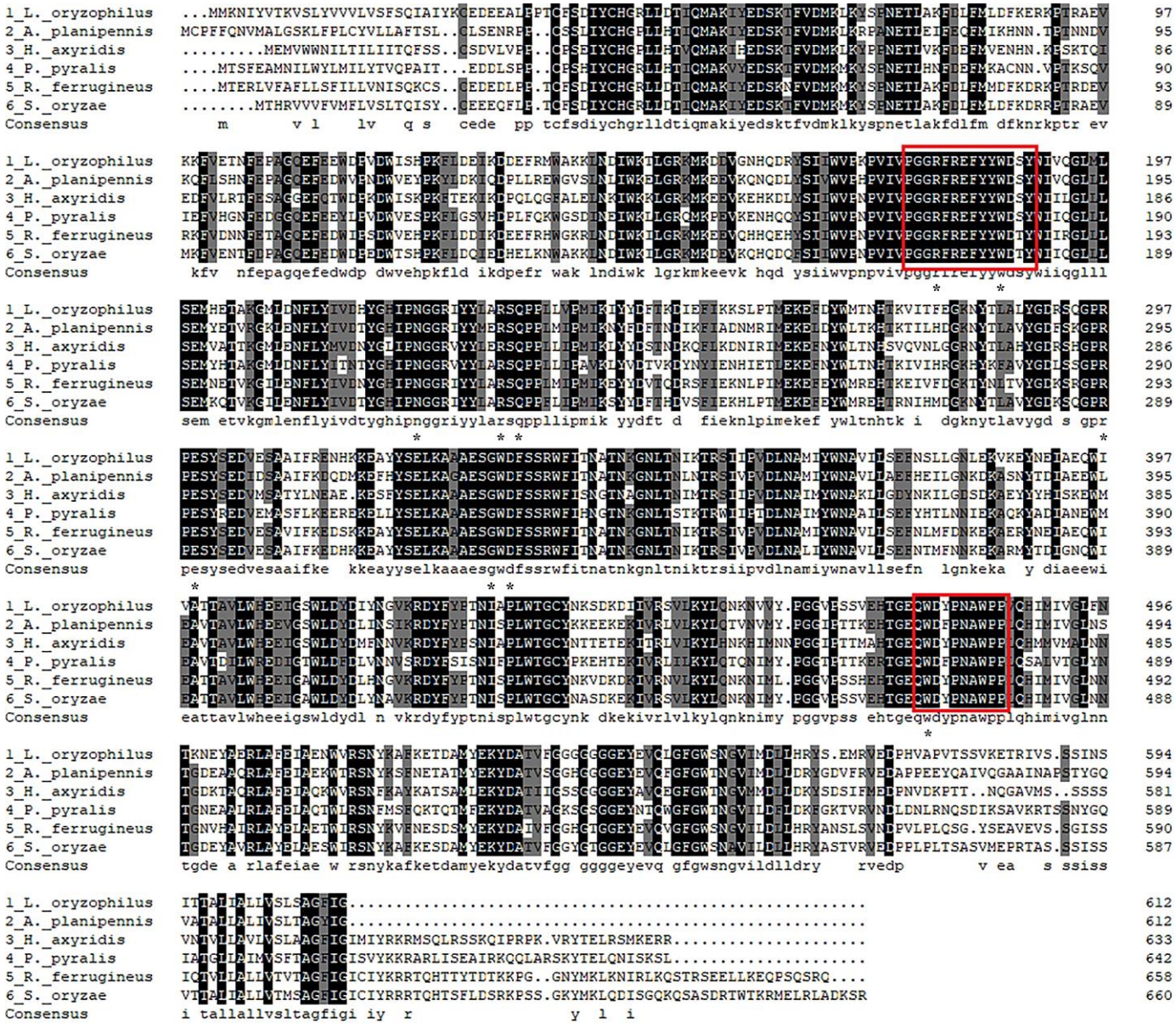

Figure 2. Multiple alignment of soluble trehalase amino acid sequences from Coleoptera insects. The following sequences were utilized in the alignment: *ApTRE* from *Agrilus planipennis* (XP_018322659.1), *HaTRE* from *Harmonia axyridis* (AOT82130.1), *PpTRE* from Photinus pyralis (XP_031336857.1), *RfTRE* from *Rhynchophorus ferrugineus* (KAF7271780.1), and *SoTRE* from *Sitophilus oryzae* (XP 030756586.1). Alignments were performed with ClustalX2. The interaction sites between trehalose and *LoTRE1* are indicated by (*). The two signature motifs based on *Lissorhoptrus oryzophilus* TRE1 are in red boxes in the alignment. Conserved and highly conserved amino acid residues are highlighted in grey and black, respectively.

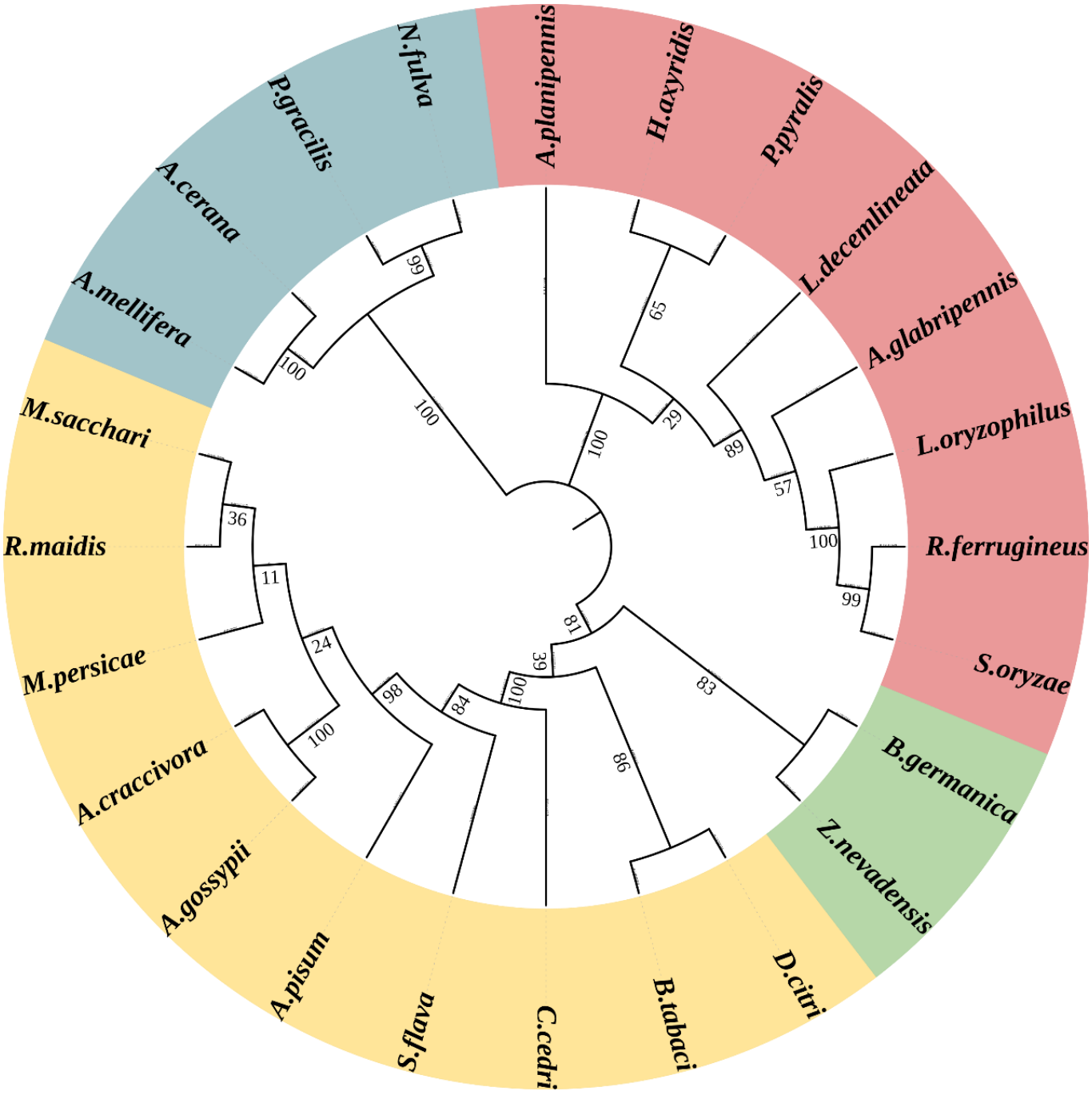

Figure 3. The phylogenetic tree was constructed using the maximum-likelihood method based on amino acid sequence alignment. Full-length amino acid sequences were aligned using the Mega 6 program to generate a phylogenetic tree. A bootstrap analysis was carried out, and the robustness of each cluster was verified in 1000 replications. Red represents Coleoptera, brown represents Hemiptera, green represents Blattaria, and blue represents Hymenoptera. *A. mellifera*, NP_001106141.1; *A. cerana*, XP_016903816.1; *P. gracilis*, XP_020290660.1; *N. fulva*, XP_029165580.1; *A. planipennis*, XP_018322659.1; *H. axyridis*, AOT82130.1; *P. pyralis*, XP_031336857.1; *L. decemlineata*, XP_023020910.1; *A. glabripennis*, XP_018571289.1; *R. ferrugineus*, KAF7271780.1; *S. oryzae*, XP 030756586.1; *B. germanica*, PSN49112.1; *Z. nevadensis*, KDR17472.1; *D. citri*, P_008474901.1; *B. tabaci*, XP_018905428.1; *C. cedri*, VVC30114.1; *S. flava*, *XP_025410170.1*; *A. pisum*, XP_003248025.1; *A. craccivora*, KAF0772952.1; *M. persicae*, XP_022174955.1; *R. maidis*, XP_026821537.1; *M. sacchari*, XP_025190889.1.

3.2. Spatial Expression Patterns of LoTRE1

The expression profiles of *LoTRE1* in numerous tissues were probed using qPCR. *LoTRE1* was shown to be expressed in a variety of tissues, including the head, midgut, hemolymph, wing, leg, and fat body (Figure 4). Remarkably, *LoTRE1* had high expression within the fat body; however, it had the lowest expression in the wing. The relative expression in the fat body was 120 times that in the wing.

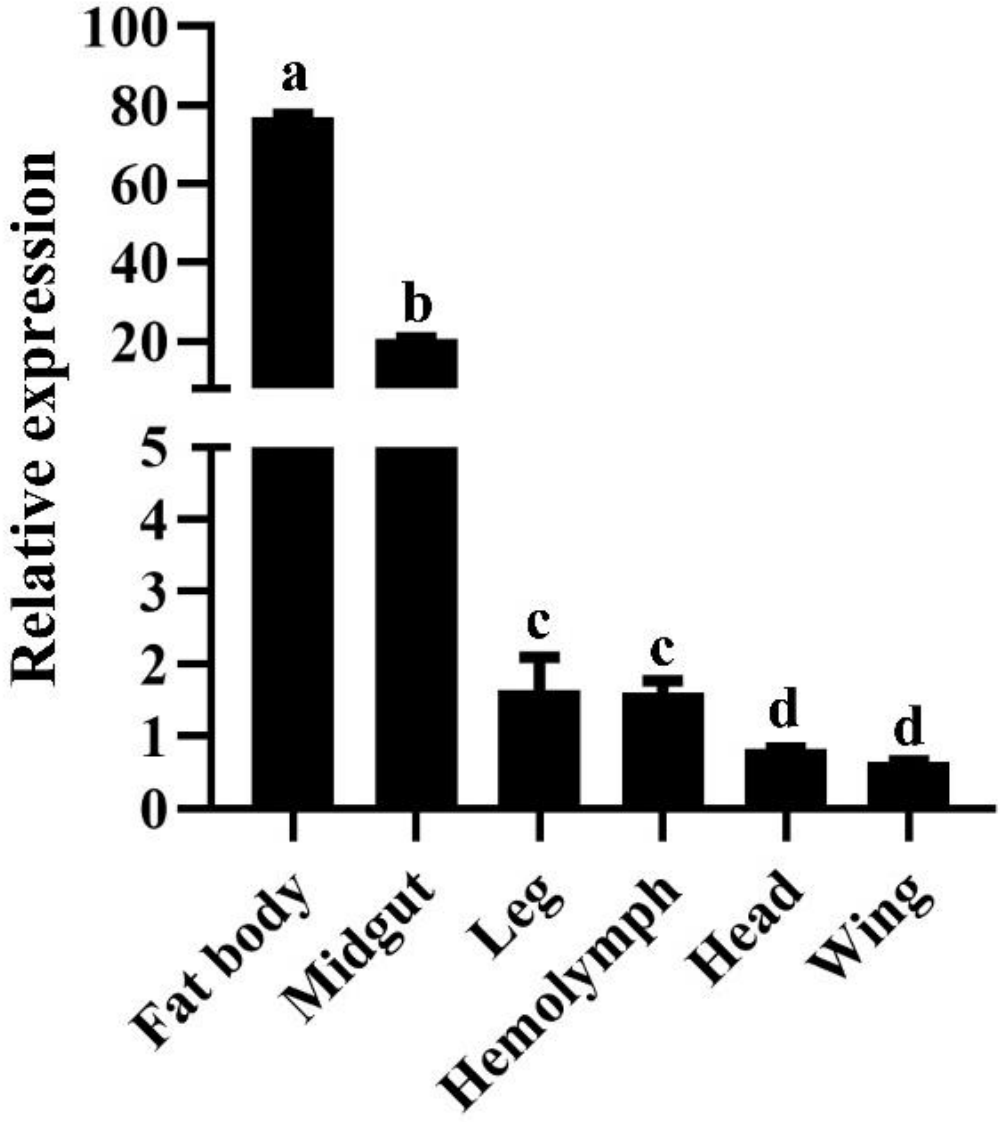

Figure 4. Relative expression levels of *LoTRE1* in different tissues of adult *L. oryzophilus*. All data obtained were expressed as the means ± standard deviations of three replicates and were tested using a one-way ANOVA and *t* tests using SPSS 22.0. The significant changes are indicated by different letters above the bars. ($p < 0.05$).

3.3. Molecular Docking and Key Binding Sites of LoTRE1

The SWISS-MODEL service was used to predict the three-dimensional structure of *LoTRE1* based on the resolved crystal structure of the periplasmic trehalase of *E. coli* (PDB ID 2WYN; Figure 5). A Ramachandran plot and the Procheck server were used to verify the homology model's dependability (Figure 6). A total of 89.7% of amino acid residues were in the most favored regions, 9.9% of amino acid residues were in the additional regions, and 0.2% of amino acid residues were in the disallowed regions. Thus, this was a

high-quality model. The predicted α-toroidal structure of *LoTRE1* was very similar to that of the periplasmic trehalase of *E. coli*, but one β-sheet was absent in *LoTRE1* at position 1.

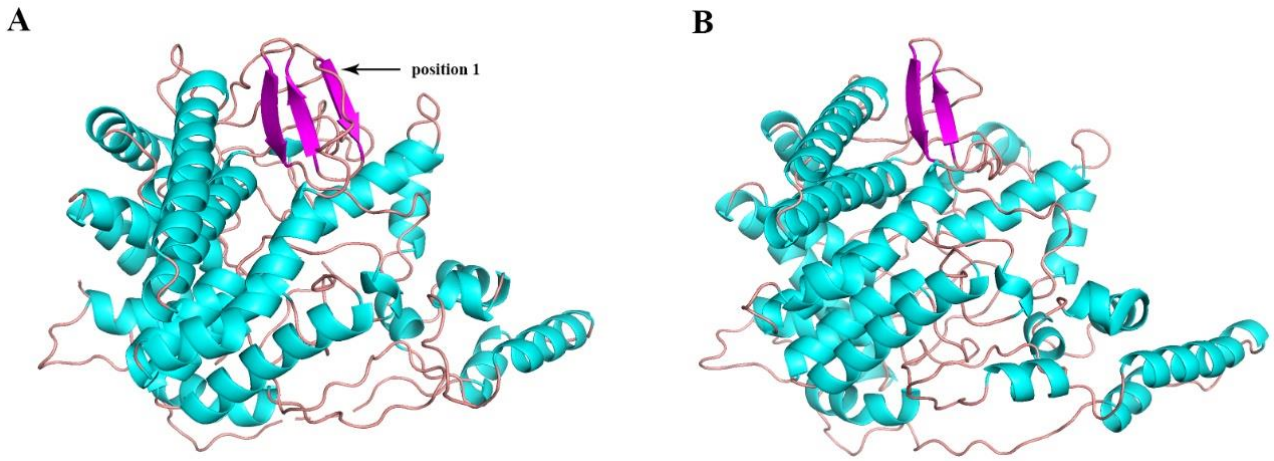

Figure 5. Three-dimensional structure of the *E. coli* periplasmic trehalase (**A**) and the homology model of the *L. oryzophilus* trehalase (**B**). The differences between the structures are highlighted with arrows.

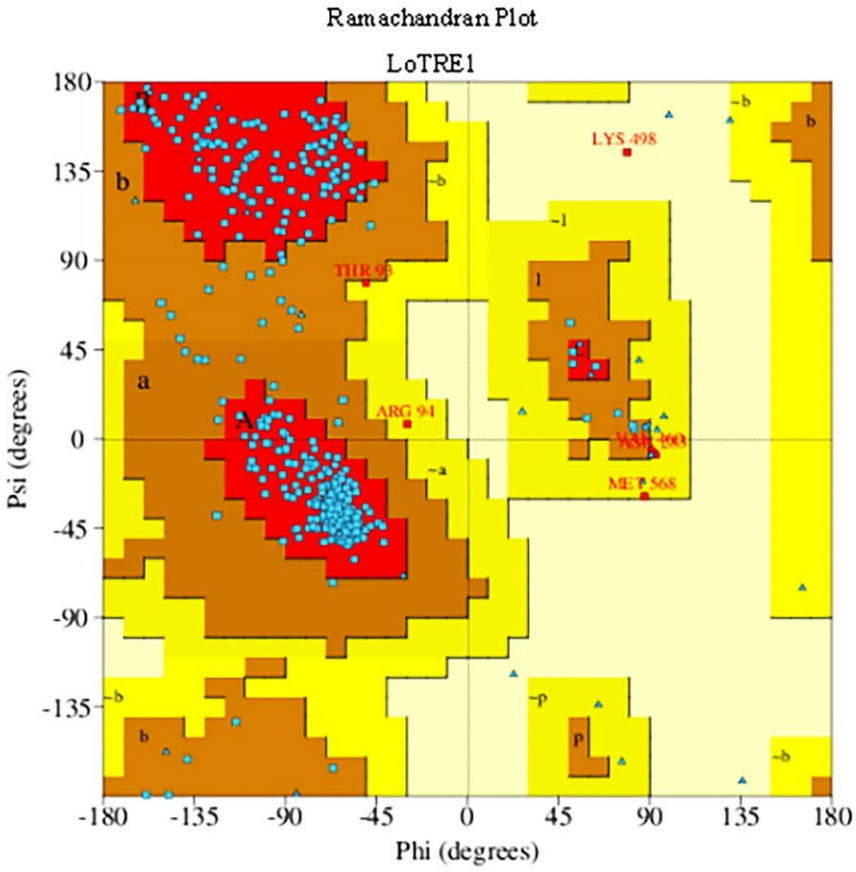

Figure 6. Ramachandran plot of *LoTRE1*.

A molecular docking study was performed to further understand the interaction of trehalose with trehalase and to obtain insight into the binding location of this molecule on trehalase. The 3D protein structure with small molecular compounds was studied using

molecular docking simulations (Figure 7). The results showed that trehalose bound tightly to *LoTRE1* (G = −1.36 kcal/mol). The docking results revealed that the interactions of trehalose with *LoTRE1* were mediated by ARG 179, TRP 186, ASN 223, ARG 232, GLN 234, ARG 297, GLU 299, GLY331, ASP 333, and TRP 477.

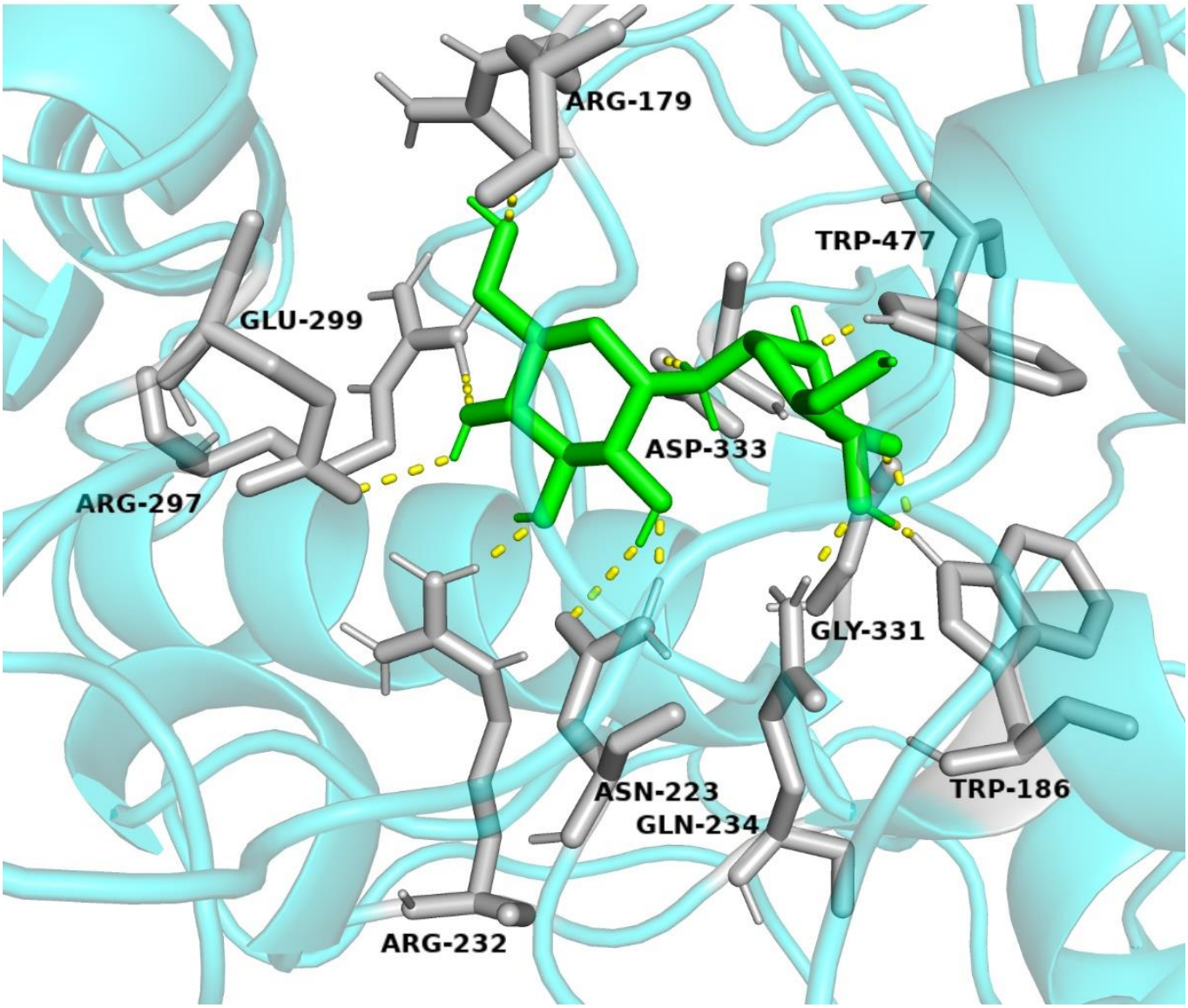

Figure 7. Molecular docking of *LoTRE1*. The ligands and the key residues are shown as stick models. Hydrogen bonds are shown as yellow lines, ligands are shown in green, and key residues are shown in grey. The figure was drawn using PyMOL.

3.4. Protein Expression and Purification

To obtain recombinant *LoTRE1* protein in large quantities, we induced protein production in *E. coli* Rosetta (DE3) cells under different induction conditions (Figure 8A). The molecular weight of the trehalase protein was found to be close to the predicted size in all the tests (70 kDa). Therefore, we chose a final concentration of 0.1 mmol/L IPTG at 28 °C to obtain large quantities of recombinant protein. In addition, the *LoTRE1* recombinant protein was mostly expressed as soluble protein (Figure 8B). The purified *LoTRE1* protein was analyzed using SDS–PAGE (Figure 8C), and one obvious unique band appeared at the predicted size (70 kDa). The expression of the recombinant *LoTRE1* protein

was verified using Western blotting (Figure 8D). The concentration of the purified *LoTRE1* protein was 0.98 mg/mL.

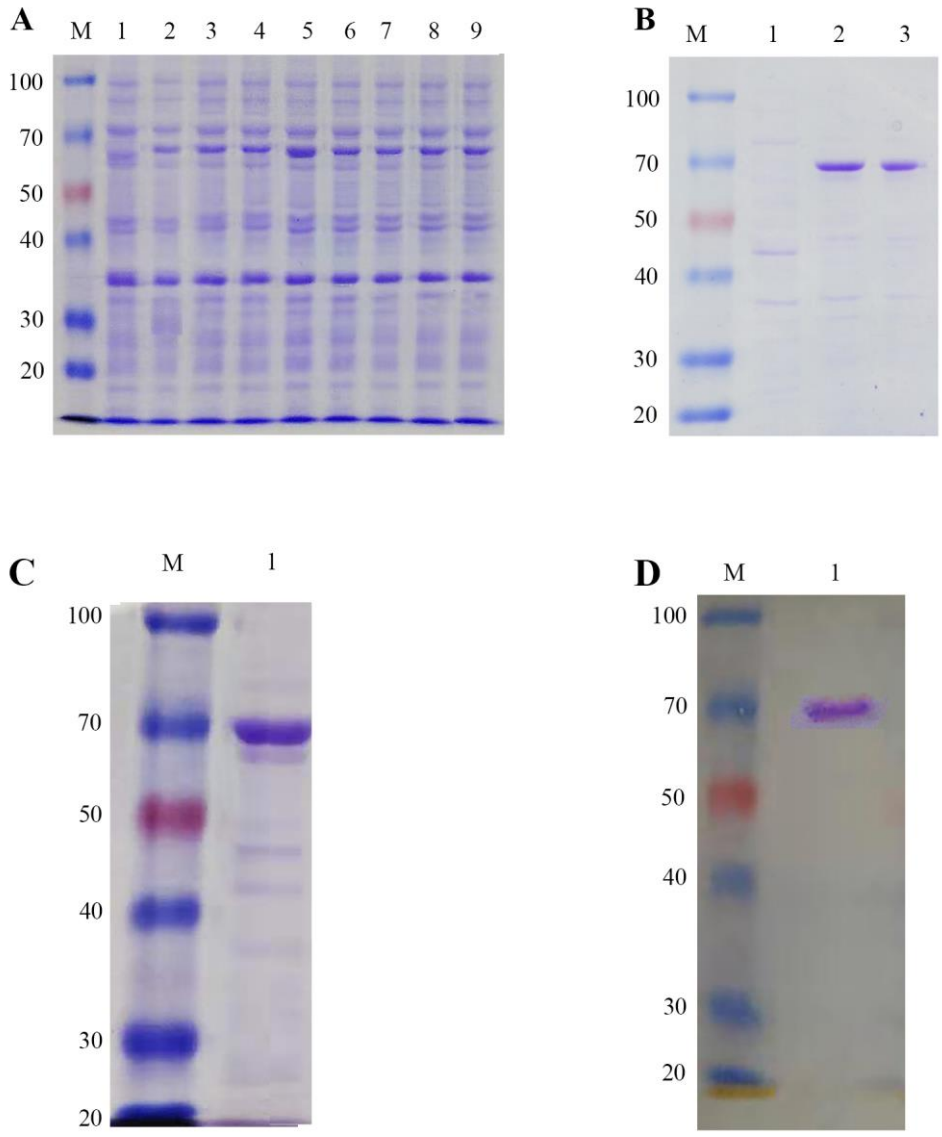

Figure 8. The recombinant protein *LoTRE1* was expressed and purified. (**A**) Expression of recombinant *LoTRE1* protein induced with IPTG under various conditions. Lane M: molecular weight marker. Lane 1: Uninduced cells of *E. coli* Rosetta (DE3). Lanes 2–4: The protein production in the *E. coli* Rosetta (DE3) cells induced with 0.1, 0.4, 0.8, and 1.0 mmol/L IPTG at 28 °C. Lanes 5–9: Protein production in the *E. coli* Rosetta (DE3) cells induced with 0.1, 0.4, 0.8, and 1.0 mmol/L IPTG at 37 °C. (**B**) Determination of the target protein's expression form. Lane M: molecular weight marker. Lane 1: Uninduced cells of *E. coli* Rosetta (DE3). Lane 2: The target protein in the supernatant after sonication and centrifugation. Lane 3: The target protein in the sediments after sonication and centrifugation. (**C**) Purification of the *LoTRE1* recombinant protein. Lane M: Molecular weight marker. Lane 1: Purified target protein. (**D**) Western blot. Lane M: Molecular weight marker. Lane 1: Purified target protein.

3.5. Enzymatic Assays of LoTRE1 In Vitro

The enzyme activity increased with increasing temperature and pH. The optimum activity of *LoTRE1* was found at 50 °C and pH 7.0, beyond which the enzyme activity decreased. No catalytic ability was found at temperatures above 75 °C or below 5 °C, indi-

cating that the catalytic performance of *LoTRE1* had a restricted temperature range. Under the optimal conditions, the specific activity of purified *LoTRE1* was 58.47 ± 1.74 U/mg. The kinetic parameters *km* and *Vmax* of *LoTRE1* were 48.6 mmol/L and 1.108 mmol/(L·min), respectively (Figure 9).

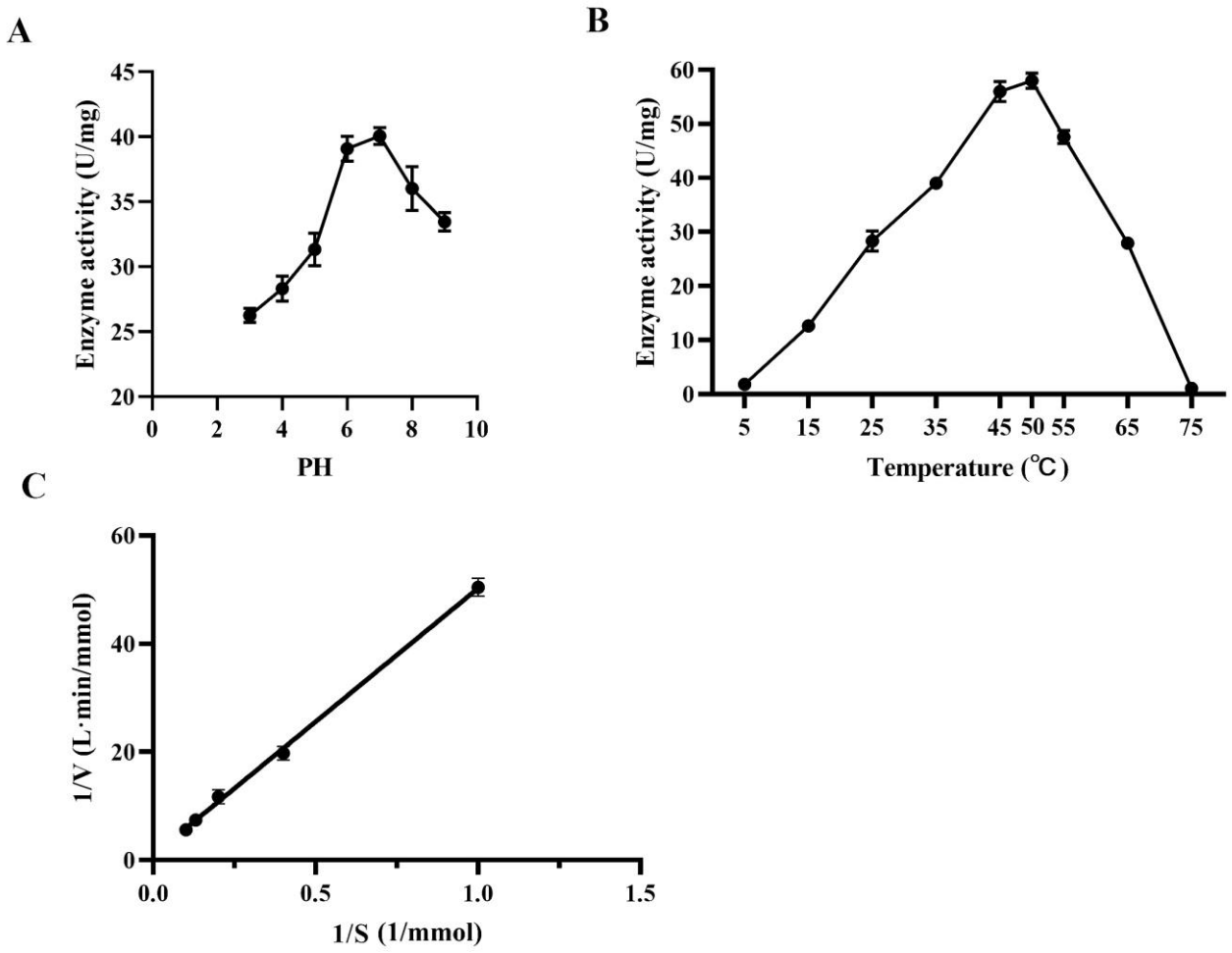

Figure 9. Enzymatic properties of the *LoTRE1* recombinant protein. (**A**) Enzyme activity of trehalose under different pH conditions. (**B**) Enzyme activity of trehalose under different temperature conditions. (**C**) Steady state kinetic equation of *LoTRE1*.

3.6. Silencing of LoTRE1 by RNAi

qPCR was performed to prove the effectiveness of RNAi in *L. oryzophilus*. Feeding dsRNA-*LoTRE1* significantly reduced the expression levels of trehalase in the rice water weevil compared with that in the nontarget control group within 24 and 48 h ($p < 0.01$). The results showed that RNAi reduced the expression levels of *LoTRE1* to 40 % at 24 h and 56 % at 48 h in *L. oryzophilus* (Figure 10A). The mortality rate of *L. oryzophilus* increased significantly ($p < 0.01$) at 24 h (9%) and 48 h (12%) after dsTRE1 feeding (Figure 10B).

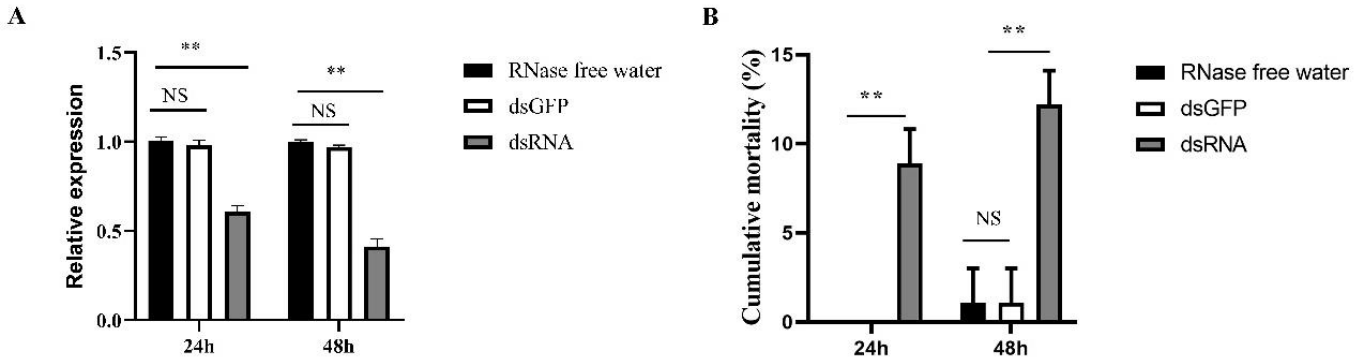

Figure 10. Silencing of *LoTRE1* using RNAi. (**A**) The relative expression levels of the *LoTRE1* gene after silencing for 24 and 48 h. (**B**) The mortality rates of adults after feeding on different treated leaves for 24 and 48 h. All data obtained were expressed as the means ± standard deviations of three replicates and were tested using a one-way ANOVA and *t* tests using SPSS 22.0. No significant differences are indicated by NS. The significant differences are indicated by ** ($p < 0.01$).

3.7. The Effect of LoTRE1 Silencing on Trehalose Metabolism

At 48 h after feeding dsRNA-*LoTRE1*, the trehalase activity of *L. oryzophilus* was 13.14 ± 0.47 U/g, which was 31.6% lower than that of the control group (Figure 11A), and there was a significant difference compared with the control group ($p < 0.01$). The trehalose content of *L. oryzophilus* was 5.73 ± 0.60 mg/g, which was 130% higher than that of the control group (Figure 11B), and there was a significant difference compared with the control group ($p < 0.01$).

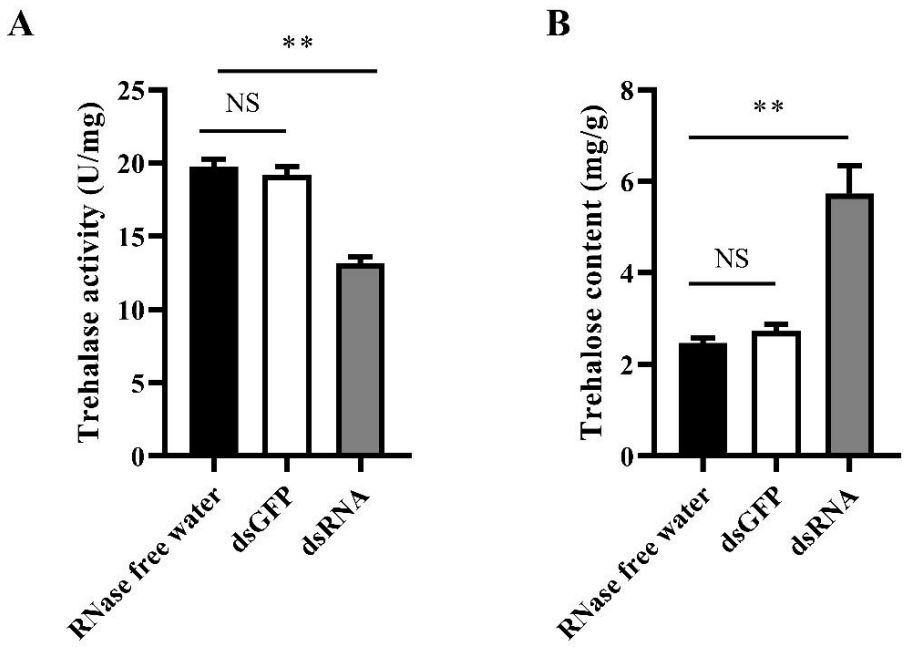

Figure 11. Detection of enzyme activity and trehalase content after RNAi treatment. (**A**) Trehalase activity after RNAi treatment. (**B**) Trehalose content after RNAi treatment. All data obtained were expressed as the means ± standard deviations of three replicates and were tested using a one-way ANOVA and *t* tests using SPSS 22.0. The standard deviation of the computed means based on three biological replicates is represented by the error bars. No significant differences are indicated by NS. The significant differences are indicated by ** ($p < 0.01$).

4. Discussion

Trehalase catalyzes the hydrolysis of trehalose and plays a vital role in insect metabolism. The amount of trehalases has been found to differ among insect species. A total of three trehalase genes were found in *N. lugens*, and three trehalase genes were identified in *Tribolium castaneum* [28,29]. One trehalase gene, *LoTRE1*, was identified from our previous transcriptome database of *L. oryzophilus*, and the analysis of the deduced amino acid sequence demonstrated that *LoTRE1* encodes a soluble trehalase. *LoTRE1* contained some conserved regions, including one signal peptide, two signature motifs, four putative glycosylation sites, and a highly conserved glycine-rich sequence. The findings were consistent with those for trehalase genes found in other Coleoptera species. Amino acid sequence alignment showed that *LoTRE1* shared high identity with some known insect trehalases. The *LoTRE1* sequence shared the highest identity with that of *S. oryzae* (72% identity GenBank ID: XP 030756586.1), followed by those of *R. ferrugineus* (70% identity GenBank ID: KAF7271780.1), *A. planipennis* (65% identity GenBank ID: XP_018322659.1), *H. axyridis* (59% identity GenBank ID: AOT82130.1), and *P. pyralis* (55% identity GenBank ID: XP_031336857.1). The phylogenetic tree revealed that *LoTRE1* shared the highest homology with the genes from *Sitophilus oryzae* and *Rhynchophorus ferrugineus.*

The model of *LoTRE1* shared a general α-toroidal architecture with the template protein. According to the CAZy database, insect trehalases are mostly members of GH family 37 and possess glutamic acid (Glu) as a nucleophile, while their proton donor is aspartic acid (Asp) [16]. We identified Asp 333 and Glu 299 as important catalytic residues in the *LoTRE1* model by comparing it to the *E. coli* periplasmic trehalase. The trehalose molecule occupies a space in the active center pocket of the enzyme that contains potential acid (Asp 333) and base (Glu 299) residues, as well as three conserved Arg residues (R 179, R 232, R 297). Our results with *LoTRE1* were consistent with those reported in other insect trehalases [9,30]. The primary three-dimensional structure of *LoTRE1* was similar to the periplasmic trehalase of *E. coli.* Both structures mainly consisted of α-helixes and were surrounded by α-toroidal structures. This structure was also observed in the trehalase of other insects [9,17]. According to Silva et al., guanidine groups and Arg residues are required for soluble trehalase activity, and chemical alteration of these residues results in enzyme inactivation. This was later confirmed by site-directed alterations in three Arg residues within the enzyme's active site pocket [15].

In some insect species, TRE1 has been purified from the goblet cell cavity, hemolymph, egg homogenates, and midgut [31]. The tissue expression pattern analysis revealed that *LoTRE1* was most highly expressed in the fat body, followed by the midgut. Yu et al. (2021) found that trehalase was most highly expressed in the head and wings of *Diaphorina citr* [32]. Ma et al. (2015) found the highest expression of trehalase in the midgut in *Helicoverpa armigera* [33]. These results indicate that TRE1 could serve completely different functions in several tissues during the development of various insects. The insect fat body functions as an energy storage center. It is also the organization center of the metabolic processes of insects, such as growth, development, metamorphosis, and reproduction. Trehalase could cooperate with the hormones in fat to dynamically control the concentrations of trehalose and glucose in insects [34].

Trehalases can be divided into acidic trehalases and neutral trehalases according to their optimal pH. Inagaki et al. (2001) found that the optimum pH of the trehalase from *Acidobacterium capsulatum* was 2.5 [35]. Lee et al. (2001) revealed that the optimum pH of the trehalase from *Apis mellifera* was 6.7 [36]. In this study, the recombinant *LoTRE1* protein had enzyme activity in the range of pH 3.0~9.0, but the highest activity was observed at pH 7.0, which indicated that the *LoTRE1* protein has the best enzyme activity in neutral environments. Previous studies have shown that the optimum temperature of trehalase is generally high, usually at 40~65 °C [37]. In this experiment, *LoTRE1* had no activity at temperatures below 5 °C, and the enzyme activity increased gradually with increasing temperature. When the system temperature exceeded 75 °C, *LoTRE1* activity was lost. Shukla et al. (2016) revealed that the optimum temperature of the trehalase from *Drosophila*

melanogaster was 55 °C [16], Ai et al. (2018) revealed that the optimum temperature of the trehalase from *Helicoverpa armigera* was 55 °C [11]. Since trehalase remains the only enzyme known for the irreversible splitting of trehalose under physiological conditions, heterologous expression of *LoTRE1* can be used to explore the biochemical characteristics and kinetic properties of the enzyme.

RNAi is a highly conserved mechanism initiated by sequence-specific double-stranded RNA (dsRNA), leading to target-specific endogenous gene silencing [38]. RNAi has been widely accepted as a powerful tool for gene function research and is relatively well-established in a variety of insects, such as *Bemisia tabacii* [39] and Hemiptera [40]. Previous studies have shown that silencing trehalase in *Leptinotarsa decemlineata* caused larval death [32]. Silencing of trehalase in *Nilaparvata lugens* caused phenotypic deformities [41]. These results suggested that trehalase has a biological function in insect development and survival. In this study, RNAi was performed to determine whether the levels of *LoTRE1* would affect *L. oryzophilus* development and survival. The results indicated that dsRNA feeding-mediated silencing of the *LoTRE1* gene caused not only downregulation of the transcript level of *LoTRE1* but also decreased trehalase activity in treated adults. In contrast, silencing of *LoTRE1* increased the trehalose content and resulted in lethal effects in adults. In agreement with our results, knockdown of the trehalase gene led to mortality in treated *Harmonia axyridis* and *Spodoptera exigua* [12,42]. In addition, silencing HaTRE1 led to significant decreases in the ability of females to attract males and successful mating proportions [43]. Trehalose has been well demonstrated in insect physiology as an energy source for insects, maintaining the glucose level [44]. The energy level and blood glucose content are affected in various cells of insects when the hydrolysis of trehalose to glucose is inhibited, and thus other physiological pathways are affected [42,45]. Trehalase can decompose the important energy storage material and the stress metabolite trehalose in insects. The changes in gene expression and enzyme activity affect the life processes of insects, including molting, metamorphosis, and reproduction [34]. Studies have shown that the TRE gene affects the levels of three sugars by regulating gene expression and enzyme activity [44,46]. These results collectively indicated that the *LoTRE1* gene plays a vital role in *L. oryzophilus* by regulating the trehalose content. Feeding with dsTRE1 can disrupt the metabolism of trehalose in the body. The results lay a foundation for exploring the potential functions and regulatory mechanisms of insect TRE1, which could be useful for providing information for insect pest control in the future.

In conclusion, we identified one soluble trehalase gene from *L. oryzophilus*, analyzed its molecular characteristics, and explored its biochemical characteristics, kinetic properties, and optimum reaction conditions through heterologous expression analysis. The importance of *LoTRE1* was inferred through RNAi. *LoTRE1* is a key gene regulating the expression of trehalase and the trehalose content. However, the particular role of this gene is still unknown, and more research is needed to gain better knowledge of *LoTRE1's* functions. The results of this study provide a good basis for further studies on the regulation of the expression of this gene and provide a potential target for the control of *L. oryzophilus* in the field.

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insects

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Article

Attraction of Egg Parasitoids *Trissolcus mitsukurii* and *Trissolcus japonicus* to the chemical cues of *Halyomorpha halys* and *Nezara viridula*

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Simple Summary: When an alien species reaches a new environment, the natural enemies present in that habitat might fail to regulate its population as they might not be host-adapted. Hence, the best solution might be the use of alien biological control agents that are co-evolved with the exotic pest in question. This is the case of *Halyomorpha halys*, which is native to Asia and has recently invaded Europe and the Americas. *Trissolcus japonicus* and *Trissolcus mitsukurii* are among its main parasitoids. Adventive populations of the latter were found in Northern Italy, suggesting its employment within augmentative biological control. Homologous programs with *T. japonicus* are already ongoing in Italy. This procedure implies releasing the parasitoid to increase its population and spread to new areas invaded by *H. halys*. However, a fundamental aspect that must be investigated is the risk-assessment beforehand, i.e., the systematic process of identifying the hazard associated with such a procedure. In this context, the preference of *T. japonicus* and *T. mitsukurii* between two stinkbugs was evaluated in this study. We found that *T. japonicus* preferred the naturally released traces of *H. halys* while *T. mitsukurii* exhibited a higher preference for the natural and synthetic chemical cues of *N. viridula*.

Abstract: *Trissolcus mitsukurii* and *Trissolcus japonicus* are two Asian egg parasitoids associated with different pentatomids such as *Halyomorpha halys*. Adventive populations of *T. mitsukurii* were found in Northern Italy, suggesting its employment as a biological control agent (BCA) against *H. halys*. Nevertheless, to reduce the latter's population, *T. japonicus* was released in Italy. Releasing an exotic parasitoid requires investigating the interaction between the BCA and the environment to avoid negative impacts on the entomofauna of the new habitat. *Trissolcus mitsukurii* is mainly associated with *Nezara viridula* in its native area. Therefore, we investigated and compared the ability of female *T. mitsukurii* and *T. japonicus* to distinguish between naturally released cues of *H. halys* and *N. viridula.* A single parasitoid was exposed to contact kairomones of both pests to evaluate its modifications in orthokinetic and locomotory behaviour. The behaviour of female *T. mitsukurii* was also tested on synthetic compounds simulating the cues of the two pentatomids. When naturally released cues were used, *T. japonicus* preferred the traces of *H. halys*, while *T. mitsukurii* preferred *N. viridula*'s cues. Moreover, the attraction *of T. mitsukurii* to *N. viridula*'s cues was confirmed with synthetic cues. Additional studies are needed to judge if this parasitoid can be used as a BCA.

Keywords: *Trissolcus mitsukurii*; *Halyomorpha halys*; behavioural trials; biological control; risk assessment

1. Introduction

Trissolcus mitsukurii and *Trissolcus japonicus* Ashmead (Hymenoptera: Scelionidae) are micro-Hymenopteran egg parasitoids. Both are thought to be native to eastern Asia, including Japan and China. *Trissolcus mitsukurii* is mainly associated with *Nezara viridula* (L.) (Hemiptera: Pentatomidae), and for this reason it was allowed to be released in Brazil and Australia for the control of *N. viridula*, a stinkbug pest [1–3]. *Trissolcus mitsukurii* has been reported to attack several pentatomid species, among which it presents a considerable rate of parasitism on eggs of the brown marmorated stink bug (BMSB), *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) [4–7]. It is thought that the range of *T. mitsukurii* could be spreading following the global spread of BMSB [8]. BMSB is an invasive pest native to Eastern Asia that is now widespread in various European regions, including Northern Italy [9,10]. It is a highly polyphagous pest, as it can attack many host plants depending on the season. This results in substantial damages and economic losses, owing to its feeding with its piercing sucking mouthparts [11,12]. *Trissolcus japonicus* (known as the samurai wasp) is also an Asian egg parasitoid of BMSB. In China, it has been able to reach a 90% parasitism rate [7,13]. Both *T. japonicus* and *T. mitsukurii* have been found in multiple regions of Northern Italy and have been shown to have a greater impact in controlling BMSB when compared with other indigenous species of egg parasitoids [14–16]. With the attempt to reduce the population of this harmful pest, *Trissolcus japonicus* has been released strategically in Italy. Given the considerable presence of *T. mitsukurii* individuals in northern Italy [16], this parasitoid could potentially be used as a biological control agent (BCA) in the framework of an integrated pest management (IPM) of BMSB. Handling an exotic parasitoid with the intention of augmenting its adventive populations should of course be carefully planned by studying its interactions with native potential host populations and highlighting its preferences [17]. Therefore, to avoid any negative impact on the native fauna, risk assessment is necessary for the potential use of *T. mitsukurii* as a BCA.

Whenever parasitic Hymenoptera perceive signals of the host presence, a series of behavioural steps are initiated. The successful recognition of a potential host depends on these. Such steps account for host habitat location, host location and host selection [18]. Among the factors that lead to the host selection, infochemicals play the most important role [19]. Infochemicals are chemical cues that concern the interaction between different organisms [20,21]. Perceived by the olfactory system at a long distance, when in the form of volatile substances, and by gustatory receptors at a short distance, when in the form of contact substances, infochemicals can allow the detection of an infested area and the appropriate host stage [18]. Kairomones are a type of infochemical that mediate the relationship between organisms belonging to different species, favoring the perceiver [19,21]. Parasitic wasps are known to benefit from them for foraging purposes, in that they induce a motivated search behaviour when received [18,21]. Moreover, parasitoids can use indirect host-related cues, vicariously associated with the host, in order to get into the vicinity of the suitable host stage [21,22]. The strategy of using cues from an unsuitable host stage to reach the right stage is called infochemical detour [19].

When walking on a surface, insects leave traces such as hydrocarbon compounds that remain as footprints, constituting a signal for other species including egg parasitoids [22]. Footprints appear to derive from the insect cuticle and are lipophilic compounds of low volatility secreted by the tarsal pads when in contact with the plant surface [23–25]. Diglycerides and triglycerides of high molecular weight, along with long-chain alcohols and fatty acids, were found in *N. viridula* footprints [26]. The epicuticular waxes of the plant can retain chemical footprints by virtue of their lipophilic properties, allowing the traces to be detected by egg parasitoids as kairomones [19,25]. In the literature, there are different studies about the use of contact kairomones by scelionid parasitoids [27–29]. When perceiving host footprints, parasitoids can adopt a systematic or random search. The latter is usually used when the cues derive from a host stage indirectly associated with the suitable ones [18,30]. Colazza et al. (2009) [30] reported that platygastrid egg parasitoids, in response to adult pentatomid footprints, increase the host searching behaviour moving

around the contaminated area and return to the chemical cues after losing track of them. Egg parasitoids such as *Trissolcus basalis* and *T. brochymenae* displayed decreased walking speeds when exposed to a substrate contaminated by the adult host [27,28]. The preference for the coevolved host was shown in *Trissolcus* wasps [31], suggesting their capacity to discriminate among different host species with the purpose of choosing the most suitable ones [29,32].

In this framework, the aim of the present work was to assess and compare the discrimination capability of *T. japonicus* and *T. mitsukurii* between the chemical cues of *N. viridula* and *H. halys*. Considering that several previous studies on the searching behaviour of *T. japonicus* proved to have a positive response for *BMSB* footprints, here, we tested its foraging behaviour applying the same approach [33,34]. In the trial with *T. mitsukurii*, we used both chemical cues naturally released by adult female stink bugs, as well as specific ratios of synthetic compounds that simulate the main volatile cues addressed to the two stink bugs. The outcome of this study could contribute to a better understanding of the recognition mechanisms to the different pentatomid hosts by *T. mitsukurii,* allowing us to predict the potential effects of its use as a BCA through augmentation strategies.

2. Materials and Methods

2.1. Parasitoids

Trissolcus mitsukurii and *T. japonicus* were collected in Trento province in summer 2019 [15], and colonies were established in the quarantine facility at Fondazione Edmund Mach (Italy). The parasitoids were reared in plastic vials (50 mL), at 25 ± 1 °C, 60 ± 5% RH and a 16:8 day:night photoperiod. Pure honey droplets were supplied and replaced twice a week. Stored (at −80 °C, up to 6 months) *BMSB* egg masses were exposed to female specimens for progeny production.

2.2. Hosts

The BMSB colony was established from overwintering adults collected in Trento province in October 2019 using live traps [35]. The insects were kept in mesh rearing cages (BugDorm ®, Taichung, Taiwan, 30 × 30 × 30 cm) at 25 ± 1 °C, 60 ± 5% RH and a 16:8 day:night photoperiod, provided with French beans (*Phaseolus vulgaris* L.), tomatoes (*Solanum lycopersicum* L.), carrots (*Daucus carota* L.) and raw peanuts (*Arachis hypogaea* L.). Food and water were replaced twice weekly. The cages were inspected daily to collect fresh egg masses that were used for the rearing of the parasitoids.

Nezara viridula specimens for experiments were collected in Trento province in June 2020 and reared following the same process used for BMSB.

2.3. Foraging Behaviour on Footprints

The foraging behaviour of *T. mitsukurii* and *T. japonicus* on BMSB and *N. viridula* footprints was assessed using cellulose filter papers (6 cm ø, VWR® n.401) contaminated by the cues laid by an adult female of each of the two pentatomid species. The abovementioned filter papers were obtained by forcing a mated female pentatomid to walk on the filter papers inside a plastic Petri dish (6 cm ø) for 30 min. Uncontaminated filter papers were used as control. Filter papers with frass deposits were discarded. The experimental arena consisted of a glass Petri dish (10 cm ø) with the filter paper, placed on a light panel (Medalight LP-1218) to attract the parasitoid to the lower surface and thus to increment the color contrast and to facilitate the behavioural obeservations. The behavioural responses were recorded using a video camera (CANON EOS 80D). Each filter paper was employed for five observed individual parasitoids, i.e., for five replicates. The no-choice trials were performed using a naïve mated individual female (N = 30) of *T. mitsukurii* or *T. japonicus* (3–5 days old) and placed inside the arena. The observations lasted for a maximum of 10 min and were interrupted after one cumulative minute spent outside the filter paper (behaviour that indicates no interest in the filter paper). The recorded videos were analyzed with Ethovision ® XT 9 software. The variables considered for investigating the *T. mitsukurii*

preference towards the two pentatomids were angular velocity mean (deg/s), in-zone cumulative duration (i.e., total residence time on filter paper in seconds), mean velocity (m/h) and distance moved (cm). The trials were carried out in a dark climatic chamber, lit only by the light panel, at 26 ± 1 °C and 70 ± 1% RH from 10.00 to 12.00.

2.4. Foraging Behaviour on Synthetic Compounds

The exposure of *T. mitsukurii* to the synthetic chemical cues of BMSB and *N. viridula* was carried out considering the naturally released compounds of the two pentatomids [33,36], using their main components. These components were tridecane, (*E*)-2-decenal and (*E*)-2-hexenal (Sigma-Aldrich, Sent Luis, MO, USA). According to Malek et al. (2021) [33], the 4:1 ratio of tridecane to (*E*)-2-decenal (1.6: 0.4 nl/mL) has a significant positive effect on the foraging behaviour of *T. japonicus*. In this study, we used the same ratio for *T. mitsukurii* in the case of BMSB. As for *N. viridula,* we used a 3:1 ratio for the two main components of its footprints, tridecane and (*E*)-2-hexenal (1.5: 0.5 nl/mL), as indicated by the study of Aldrich et al. (1978) [36]. Pure compounds were prepared using aliquots containing 0.2 nL of each compound in 100 µL of the solvent dichloromethane (Sigma-Aldrich Chemie, Steinheim, Germany). For the control, 100 µL aliquots of the solvent dichloromethane were deployed. The above-mentioned compounds were applied on sterile filter papers (6 cm ø) and left to dry for 2 min. The experimental design remained the same as in footprint trials.

2.5. Statistical Analysis

The differences among behavioural parameter groups were analyzed using Wilks' Lambda type non-parametric interference for multivariate data [37]. All statistical analyses were performed in R (https://www.R-project.org/, accessed on 16 June 2021) through the application of *nonpartest* and *ssnonpartest* functions in the "npmv" package, which provided post hoc analysis as well [38].

3. Results

3.1. Foraging Behaviour on Footprints

An interested parasitoid, when triggered by chemical cues released by a suitable host, demonstrates higher angular velocity, slower walking velocity, greater residence time and longer covered distances [23,39].

For *T. japonicus*, the statistical analysis highlighted significant differences among groups (Wilks Lambda = 27.304, df = 8, 168, $p < 0.001$) (Figure 1). Analyzing the foraging behaviour of *T. japonicus* females, a preference for the substrate contaminated by the BMSB female compared to *N. viridula* and the control emerged. The time spent on the BMSB-contaminated surfaces was higher than in *N. viridula* and the control, in that order. The same trend of preference occurred for the mean velocity, where the lowest values were associated with BMSB, succeeded by *N. viridula* and the control. The distance moved was the highest for BMSB. Similar to the distance moved, the greater recorded value was for BMSB, followed by *N. viridula* and the control. The values featured in Table 1 represent the probability that a value obtained from one treatment sample is higher than a randomly chosen value of the other treatment samples.

In the trial with *T. mitsukurii*, a significant difference between groups was observed (Wilks' Lambda = 8.490, df = 8, 168, $p < 0.001$) (Figure 2). The tested parasitoids showed a clear preference toward *N. viridula* footprints, in comparison to those released by BMSB and the control. The time spent on filter paper was higher for *N. viridula*, followed by BMSB and control, respectively. Greater values for mean velocity were recorded for the control. As for the time spent on filter paper, higher values were observed for *N. viridula* in the distance moved, followed by BMSB and the control. The same trend was recorded for the angular velocity as well (Table 1).

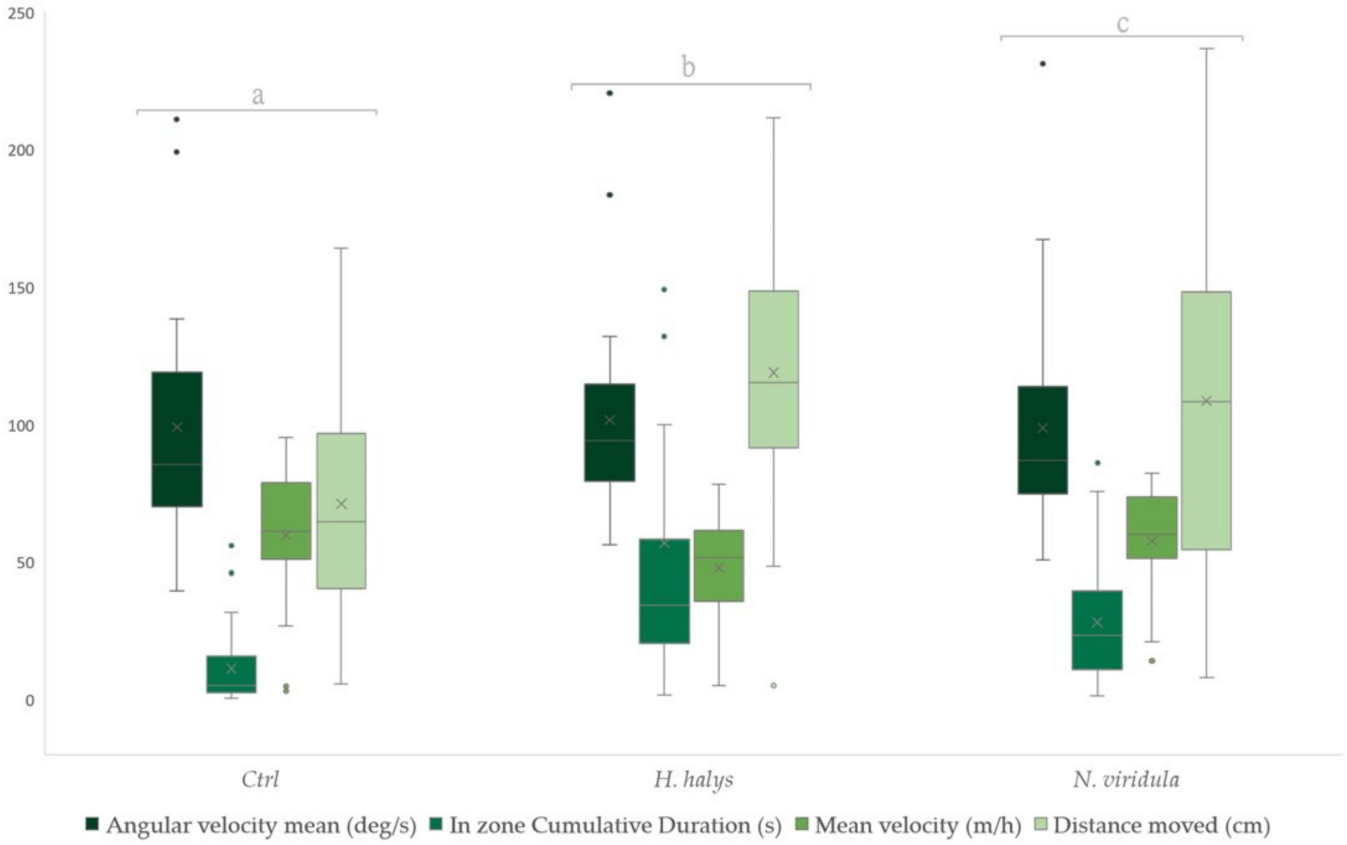

Figure 1. Boxplots of foraging behaviour parameters for females of *T. japonicus* following contact with footprint-contaminated substrate from female adults of *H. halys* and *N. viridula*. Behavioural parameter groups indicated by different letters show significant differences (Wilks' Lambda type non-parametric interference at $p < 0.001$). Boxplots represent the interquartile range, with a horizontal bar as the median. The bottom whisker and the upper whisker represent the minimum and maximum values, respectively. The "X" symbol represents the mean value. The dots show the outliers.

Table 1. Relative effects that quantify the tendencies observed in the data in terms of probabilities for each species within different treatments (i.e., footprints and synthetic compounds). All reported values were calculated using the npmv package in R program [37].

			Parameters			
			Angular Velocity Mean	**In Zone Cumulative Duration**	**Mean Velocity**	**Distance Moved**
Footprints	*T. japonicus*	Control	0.46852	0.30000	0.56778	0.51556
		H. halys	0.53815	0.66815	0.39000	0.73704
		N. viridula	0.49333	0.53185	0.54222	0.24741
	T. mitsukurii	Control	0.34630	0.24352	0.64074	0.27111
		H. halys	0.45815	0.57389	0.47333	0.56407
		N. viridula	0.69556	0.68259	0.38593	0.66481
Synthetic compounds	*T. mitsukurii*	Control	0.45185	0.31833	0.54593	0.42704
		SC *H. halys*	0.55111	0.54481	0.47296	0.51074
		SC *N. viridula*	0.49704	0.63685	0.48111	0.56222

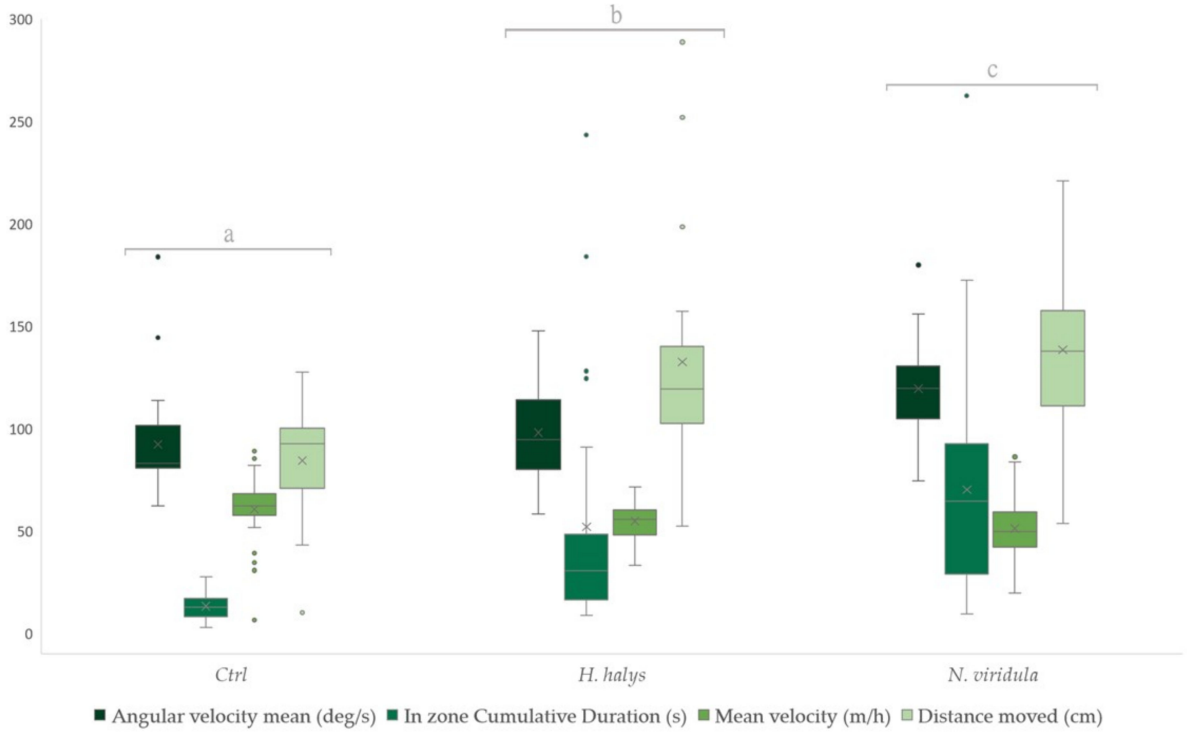

Figure 2. Boxplots of foraging behaviour parameters for females of *T. mitsukurii* following contact with footprint contaminated substrate from female adults of *H. halys* and *N. viridula*. Behavioural parameter groups indicated by different letters show significant differences (Wilks' Lambda type non-parametric interference at $p < 0.001$). Boxplots represent the interquartile range with a horizontal bar as the median. The bottom whisker and the upper whisker represent the minimum and maximum values, respectively. The "X" symbol represents the mean value. The dots show the outliers.

3.2. Foraging Behaviour on Synthetic Compounds

Regarding the trials with the use of synthetic compounds, there was a significant difference between the three treatments (Wilks' Lambda = 3.397, df = 8, 168, $p = 0.001$) (Figure 3). In particular, *T. mitsukurii* female parasitoids preferred *N. viridula* synthetic chemical cues. The data collected revealed no significant preference for the BMSB mixture compared to the other two treatments. Nevertheless, the parasitoids showed low interest for the uncontaminated filter papers. Higher values in terms of time spent on the filter paper and distance moved were observed for *N. viridula* compared to the control and BMSB. The mean velocity was lower for BMSB and *N. viridula,* respectively. Greater values were displayed in the angular velocity for BMSB, *N. viridula* and control, in that order. The values featured in Table 1 represent the probability that a value obtained from one treatment sample is higher than a randomly chosen value of the other treatment samples.

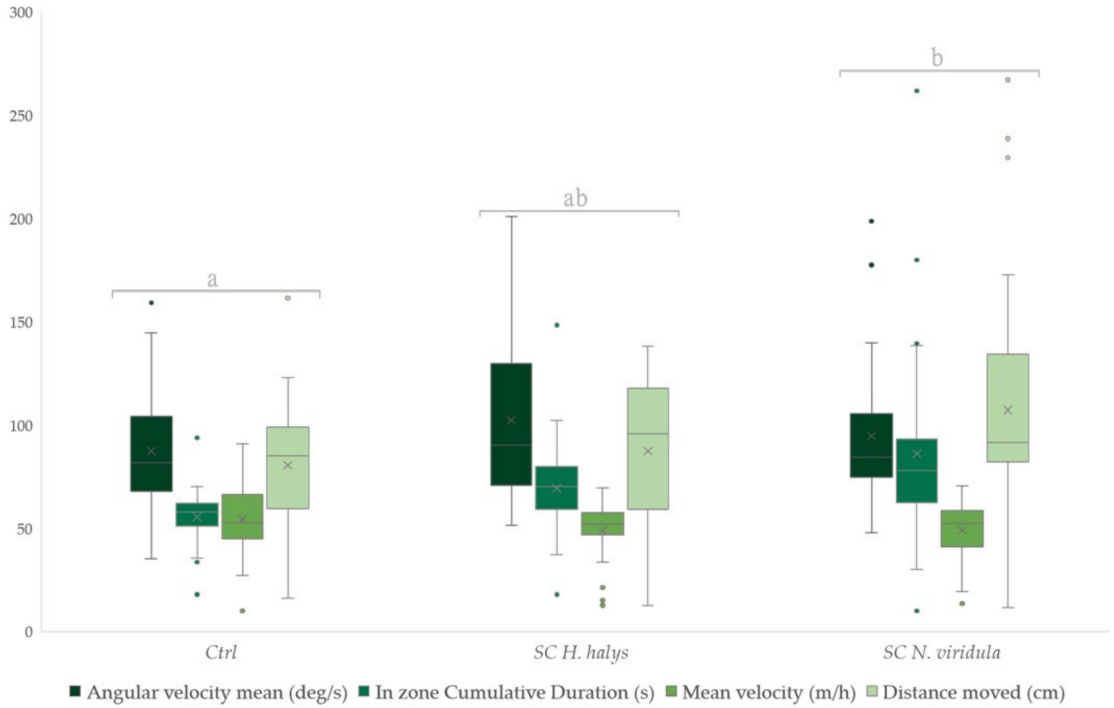

Figure 3. Boxplots of foraging behaviour parameters for females of *T. mitsukurii* following contact with contaminated substrates by synthetic compounds (SC) of *H. halys* and *N. viridula*. Behavioural parameter groups indicated by different letters show significant differences (Wilks' Lambda type non-parametric interference at $p < 0.001$). Boxplots represent the interquartile range with a horizontal bar as the median. The bottom whisker and the upper whisker represent the minimum and maximum values, respectively. The "X" symbol represents the mean value. The dots show the outliers.

4. Discussion

Egg parasitoids have adapted to employ infochemical detours from herbivore adults, with the aim of detecting the suitable host stage [19]. Chemosensors are used to recognize odors produced in minute concentrations by hosts, while the antennae and the brain perform a filter function, reducing the complexity of the stimuli received [40,41]. This elaborate mechanism provides details about the presence of the host and, consequently, parasitism success. Kairomones act as signals, inducing in *Trissolcus* wasps some specific habits consisting of arrestment behaviour characterized by slow-going speed and small distance displacement [28,33]. Studies that concern the interaction between a parasitoid and host trails, involving chemical ecology, should be considered as an essential integration to conventional host no-choice experiments (physiological host range) to understand the actual host range under conditions that mimic as closely as possible natural ones and incorporate part of the environmental complexity. In this regard, this work provides elements about the ecological host range of *T. mitsukurii*, investigating its principal hosts as per the literature.

In the present study, for both parasitoids *T. mitsukurii* and *T. japonicus*, an alteration in their orthokinetic and locomotory behaviour was noticed, which was elicited by chemical traces released by the two pentatomids. The preference towards their respective primary host was demonstrated. Indeed, *T. mitsukurii* manifested a deceleration when in contact with *N. viridula* traces, probably to better perceive the trail marked out by the host. In contrast, *T. japonicus* revealed a preference for BMSB cues, with a higher residence time and lower velocity. The use of a synthetic mixture that mimicked *N. viridula* cues induced an arrestment response in *T. mitsukurii*, exhibiting an inclination in the choice of this pest.

When investigating singular parameters, the angular velocity revealed the most heterogeneous response in different trials. This parameter indicates the change in direction,

meaning the more the parasitoid returns to the trails, the higher its value will be. Various studies demonstrated a discrepancy in the behavioural response connected to this parameter. Indeed, for *T. basalis*, when it comes to angular velocity, no significant difference was found in patches contaminated by different bugs and the control, despite the residence time being significantly higher in patches with *N. viridula* kairomones [29]. *Trissolcus brochymenae* exhibited no difference between chemical traces of different host stages and the control in the angular velocity as well [27]. On the contrary, Boyle et al. (2020) [34] illustrated a greater angular velocity in *T. japonicus* on leaves contaminated by its main host BMSB, even if no difference existed among treatments with the use of soybean leaf as substrate. In this study, the remaining parameters agreed with similar previous tests, showing a certain preference for one species over another. In previous studies, *T. japonicus* experienced a stronger arrestment behaviour to BMSB footprints compared to those of *Podisus maculiventris* (Hemiptera: Pentatomidae), a suboptimal host species, showing higher residence time and covering greater distance in response to BMSB female kairomones [33]. The same pattern occurred in the residence time with the use of a contaminated leaf as a substrate, though no significant differences were observed between the two groups in the linear walking speed [34]. This proves that the contact with the treated surface could affect locomotory paths in different manners, denoting an interest for the attractive substances produced by stink bugs in relation to the control substrate. Conti et al. (2003) [27] noticed the ability of *T. brochymenae* to discriminate among various stages, different sexes and physiological conditions of its host *Murgantia histrionica* (Hemiptera: Pentatomidae). Indeed, the female parasitoids preferred to spend more time on the patches contaminated by gravid or virgin pentatomid females, whereas there was no difference in the residence time between patches with virgin pentatomid male kairomones and the control ones. The host's physiological condition influenced the arrestment behaviour of *T. basalis* when it encountered chemical traces of *N. viridula*, revealing an arrestment response and a reduced speed for pre-ovipositional females [28]. The use of arenas contaminated by the co-evolved *N. viridula* induced *T. basalis* to spend higher residence time compared to the arenas containing the trails of a non-coevolved host [29]. The locomotory path was affected as well, with a lower linear speed not only in substrate contaminated by *N. viridula* but also by the non-coevolved *M. histrionica* and *Eurydema ventralis* (Hemiptera: Pentatomidae) [29]. Furthermore, the preference for the co-evolved host was observed in *T. brochymenae*, highlighted by the highest residence time in areas contaminated by *M. histrionica*. Nevertheless, *Trissolcus simoni* (Hymenoptera: Scelionidae) reacted to the stimuli of different hosts with similar intensity to that of the co-evolved *E. ventralis* [32]. Thus, it seems clear that *Trissolcus* species have a great discriminatory capability that allows them to select the trails released by the hosts' stage correlated to the suitable oviposition substrate. This strategy is related to successful reproduction to ensure the generation of a new progeny. Moreover, this ability enables them to discern between co-evolved and non-coevolved hosts.

In the present study, *T. japonicus* revealed a preference for the principal host, BMSB, which is in accordance with literature [33,34]. In contrast, *T. mitsukurii* reacted differently, demonstrating a preference for *N. viridula* kairomones. This detail could presume the existence of some sort of co-evolutionary or adaptation process. A recent survey on the olfactory response of *T. mitsukurii* confirms this inclination for *N. viridula* [42]. Indeed, the parasitoid manifested an interest in the arm containing a plant contaminated by *N. viridula*, which was previously exposed to individuals for feeding and oviposition [42].

Examples of *N. viridula*/*T. mitsukurii* association are present in the literature [1,3], confirming the parasitoid tendency to perform a motivated searching behaviour when entering in contact with the cues of this phytophagous species. However, a recent study on the physiological host range of *T. mitsukurii* surprisingly highlighted the low suitability of *N. viridula* egg masses, with a reduced parasitoid emergence rate and elevated amounts of dead eggs [43]. The introduction of the parasitoid in a new area could have driven a genetic drift, in response to different biotic factors but also in reaction to the evolutionary trap represented by *N. viridula*, which results in a dead end of *T. mitsukurii* progeny. Moreover,

the development in BMSB eggs positively influences parasitoids' fitness, ensuring survival in a foreign zone.

The specimens of *T. mitsukurii* used for our trials were reared on BMSB eggs. Such a condition could have affected the foraging behaviour, causing the wasp to be less assertive in its choice when exposed to the two stimuli [44]. The response to chemical trails may change with the learning process of the parasitoid. The development of a particular host could influence the preadult learning [45,46]; however, contact kairomones are mainly responsible for inbred responses [19]. The degree of host specificity can affect the learning experience as well [44].

5. Conclusions

In conclusion, *T. mitsukurii* preferred the contact kairomones produced by *N. viridula*, especially when naturally released when walking. Conversely, BMSB cues elicited a modification in the kinetic reaction with a reduced intensity. The aforementioned interest was confirmed in olfactory investigation [42], while discordant results were highlighted in the physiological host range [43]. Additional analyses are needed to prove this preference. The employment of an exotic BCA to control a pest could imply collateral effects on the entomofauna present in the area of its release. Therefore, the incorporation of chemical ecology studies within the risk assessment might help with the comprehension of the natural mechanisms conducted by the BCA.

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insects

MDPI

Communication

Lithium Chloride Shows Effectiveness against the Poultry Red Mite (*Dermanyssus gallinae*)

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Simple Summary: The poultry red mite (*Dermanyssus gallinae*) is the major pest of poultry and a vector for many animal and human pathogens. Control is limited, and alternative substance efficacy may imply resistance and inconsistency problems. As a consequence of this, there is an increasing demand for alternative substances. In this pilot study, we report for the first time that lithium chloride, a currently promising potential miticide for *Varroa* mite (*Parasitiformes*) control in bees, showed effectiveness against poultry red mite in vitro. However, further comprehensive studies are needed to reveal whether lithium compounds can be considered an alternative miticide to control *Dermanyssus gallinae*.

Abstract: The poultry red mite (*Dermanyssus gallinae*) is the main pest of poultry, causing severe problems by being a vector of several animal and human pathogens. The number of miticides is few, and their efficacy in practice implies problems of residues and resistance; therefore, the demand for a new and safe agent is constant. The present publication investigated the effectiveness of lithium chloride under in vitro conditions on poultry red mites. This chemical currently appears to be one of the most promising alternatives to study amongst potential applicants to treat varroosis, a fatal disease of honey bees. In Experiment I, the previously used experimental doses (5.52 M, 2.76 M, 1.38 M) on *Varroa* mites confirmed their in vitro activity on the poultry red mite. Three event times (uncontrolled movement, immobilisation and death) were recorded to base the response to treatment for each concentration. In Experiment II, the LD 50 value was calculated, i.e., the value at which 50% of the mites were killed by the treatment. This Experiment showed that the LD50 of lithium chloride = 0.265 M in the poultry red mite. It is to note that the study remained restricted to in vitro confirmation of lithium chloride's effectiveness on the parasite. Thus, further extensive studies are needed to decide whether it has any relevance in practice against *D. gallinae*, and also to assess potential residue problems that could affect poultry products.

Keywords: lithium chloride; contact effect; poultry red mite

1. Introduction

Poultry red mite (*Dermanyssus gallinae*) has been a severe threat to animal health and welfare for decades, adversely affecting egg production and having significant public health implications. In infected animals, the first clinical symptom is anaemia due to subacute, recurrent mite bites. In extreme cases, *D. gallinae* infestation may be so intense that chicken may die of severe anaemia [1–3]. Mite infestation results in aggressive feather pecking and cannibalistic behaviour increased feed and water consumption, and thus deterioration of the general health status of the animals [4–7].

In addition to its direct impact on blood-sucking parasitism, *D. gallinae* is considered a vector for several avian viral and bacterial pathogens and can be a reservoir for numerous pathogens [5,8–15]. These have recently included Lyme borreliosis and avian influenza A virus [8,15]. Of the human pathogens, *Salmonella enteritidis* should be highlighted as having the highest global mortality rate among human zoonotic diseases [10,16]. The majority of cases are foodborne, and poultry products are the most common source of the disease [11,13]. In addition, *D. gallinae* infection is increasingly responsible for human dermatological lesions, notably gamasoidosis, especially in people living or working near poultry farms [8]. The disease is underdiagnosed [9], with an actual prevalence higher than is generally assumed [17]. Persistence in a fasting state for prolonged periods increases the vector's role in pathogen maintenance [18].

Poultry infestation affects all types of production, from the backyard or organic farms to more intensive, improved cage or shed farming [19]. A recent epidemiological review found that 83% of European farms are infected, and this prevalence reaches 94% in the Netherlands, Germany and Belgium [20]. Mite damage is expected to increase due to recent changes in legislation on poultry production [1,16,17,21–23], climate change [24] and increased miticide resistance. The withdrawal of many miticides from international markets due to safety concerns and the persistent lack of new effective control methods has exacerbated the occurrence of *D. gallinae* in Europe. The need for effective and safe control of mites is therefore ongoing. Currently, there are a very limited number of active substances available for the treatment of mite infestations [19,25], and in addition, they show little or no efficacy against mite eggs, i.e., they do not prevent the recurrence of the pest population. The main active substances belong to the group of carbamates (carbaril, methomyl, propoxur), organophosphates (dichlorphos, phenitrothion, chlorpyrifos, diazinon) and pyrethroids (cihalothrin). The use of some of these (e.g., organophosphates) is already limited due to resistance issues [25,26].

Furthermore, the treatment of *D. gallinae* can pose critical risks to food safety and cause resistance development as a consequence of subtherapeutic doses [27–30]. Alternatively, used silica-based compounds and essential oils carry inconsistent efficacy and pose serious safety risks to the user and animals [5]. Developing new vaccine-based control strategies is promising; however, their efficacy is currently unsatisfactory [31,32]. For the above reasons, there is a continuous demand for new, lower residue risk and nature-identical insecticides.

Also belonging to *Parasitiformes and* order *Mesostigmata*, mite *Varroa destructor* is another important pest in agriculture. Although it has no human implications, it is also considered a viral vector. The resistance to different agents and the hidden lifestyle of reproductive individuals makes it the greatest challenge to apiculture and threatens the stability of pollination. Controlling the pest is challenging, and the need for new active substances is constant. Alongside novel approaches for the treatment of varroosis, lithium salts have been shown to be effective in vitro for eradicating *V. destructor* [33]. In addition to its systemic mechanism of action, a strong contact effect was confirmed to contribute to its high varroacidal efficacy [34]. In situ, it has outperformed oxalic acid, a widely used alternative to synthetic miticides to control varroosis of the honey bee. The *Varroa* mite and the poultry red mite share numerous common features of parasitism and result in similar impacts on resistance and residual-related issues. Lithium chloride might represent a practical, relatively inexpensive and potential alternative in apiculture worth further investigation [35]. In addition, preliminary results on chemical residues suggest that the risk of residues is expected to be low in beekeeping use [35,36]. However, for the moment, lithium is registered as a veterinary product only in Serbia [37].

Although it may be a promising miticidal agent in apiculture, the miticidal effectivity of lithium has not yet been reported on other taxa in the literature. Since problems similar to that of *V. destructor* arise concerning poultry red mite, we aimed to test whether the miticidal effectiveness of lithium may be extended to *D. gallinae*.

2. Materials and Methods

2.1. Mites

The mites were of mixed age, sex, and origin collected in July 2021, from poultry farms (Dabronc N: 47.0333, E: 17.1667, Cserszegtomaj N: 46.80613300, E: 17.23126210 and Miháld N: 46.265999, E: 17.076000 Hungary). Individuals were held at 24 ± 1 °C and 55 ± 5% relative humidity under a photoperiod of 16 h:8 h (light/dark) in a closed container with mesh prior to bioassays. Mites were tested within two days after collection. A total of 732 individuals were tested (Table 1).

Table 1. The number of mites tested (N) in Experiments I. and II.

Licl Concentration	Experiment I. N	Experiment II. N
5.520 M	50	32
2.760 M	50	33
1.380 M	50	29
0.690 M		66
0.345 M		56
0.173 M		111
0.086 M		82
0.043 M		33
0.014 M		22
0.000 (CONTROL)	50	68
TOTAL	200	532

2.2. Experimental Setup

Two experiments were conducted. Experiment I. tested the dynamics of the response of mites immersed in an aqueous lithium chloride solution of the following concentrations: 5.52 M, 2.76 M, and 1.38 M, similar to those applied in an earlier experiment with lithium chloride against the mite parasite of the honey bee [34]. The first recorded event was the onset of tremorous, uncontrolled movements. The second event was recorded when the mite could no longer change position but was still moving in one place, called the immobility state. Subsequently, the time of death was recorded as the third event.

Experiment II. was conducted to determine the concentration (LD50) at which lithium chloride kills half of the tested mites. To establish a concentration-response relationship, the mortality of mites was tested at ten concentrations: 5.520 M, 2.760 M, 1.380 M, 0.690 M, 0.345 M, 0.173 M, 0.086 M, 0.043 M, 0.014 M and 0.000 M (control).

Sample sizes are specified in Table 1.

2.3. Immersion Test

The response of mites to lithium chloride was investigated using the immersion method following Thomas et al. [38] with slight modifications. Each mite was immersed in a 1.5 mL Eppendorf tube containing 1 mL lithium chloride solution. The tubes were flipped up and down ten times for 10 s (one turn per second) in the bioassay. Subsequently, they were then placed on a filter disc (Sartorius, d = 150 mm, Grade: 1292). Dead specimens were discarded, leaving only the viable, undamaged specimens to examine, then placed onto Petri dishes, sealed, and held under laboratory conditions.

The control treatment was also carried out with an aqueous solution, and all treatments were replicated three times.

2.4. Statistical Analysis

In Experiment I., the elapsed time from the immersion was analysed separately for each event. Abbott's formula [39] was applied to compute mortality rates. For the statistical analysis, data were $LOG_{10}(x + 1)$ transformed; then normality testing was carried out by the Jarque-Bera method; all group's data were normally distributed ($p > 0.05$). Statistical

differences were analysed with one-way ANOVA for each event separately. The homogeneity of variances was justified by Levene's test ($p > 0.05$) for the log-transformed values of time to immobility and death but not for time to uncontrolled movement. Therefore, pairwise differences were analysed with Tukey's post hoc test for time to immobility and time to death, with homogeneous variances, and Tamhane's post hoc test for the time to uncontrolled movements, with inhomogeneous variances [40].

A classic logistic growth curve (also known as the Pearl-Reed logistic curve) was fitted to the Abbott corrected mortality rate data for each treatment, of the form $y = K/(1 + b \times e^{-cx})$ with K = 100, and y representing the mortality rate and x the exposure time. Parameters c and b define the position and slope of the fast-growth section of the curve, with $x = \ln b/c$ leading to $y = K/2$. This means that higher c values make the curve steeper, while with higher b values, the 50% value (LD50 stage) is reached later at higher × values.

The statistical tests were computed by SPSS 22.0 software [41].

In experiment II., Hill model (i.e., 4 parameters logistical) [42] was fitted to mortality data of mites by lithium chloride concentrations, and the LD50 value was calculated based on the nett effect of the tested substance (i.e., mortality above the control level) by using the calculator of AAT Bioquest Inc. (Pleasanton, CA, USA) [43].

3. Results

In Experiment I., differences were detected between all concentrations for the mean exposure times to each recorded event (i.e., uncontrolled movement, immobility, death), with higher concentrations leading to shorter mean times (Figure 1 and Table 2).

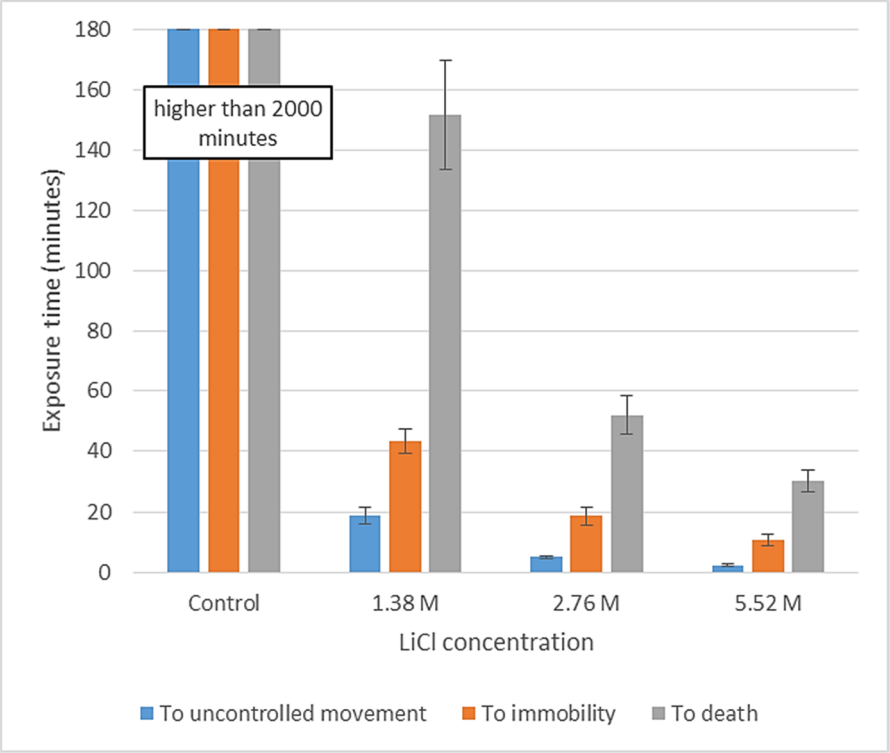

Figure 1. Mean exposure time from immersion to occurrence of an event, with error bars denoting ± SE (standard error) of the mean, by LiCl concentration. For statistics based on $LOG_{10}(x + 1)$ transformed data, see Table 2.

Table 2. Mean ± SE (standard error) for the log-transformed exposure times by concentration and results of the ANOVA statistics.

Licl Concentration	Uncontrolled Movement	Immobility	Death
1.38 M	1.183 ± 0.051 a	1.5775 ± 0.045 a	2.062± 0.061 a
2.76 M	0.750 ± 0.027 b	1.1851 ± 0.043 b	1.602 ± 0.054 b
5.52 M	0.459 ± 0.036 c	0.9275 ± 0.055 c	1.388 ± 0.052 c
ANOVA	F(2,108) = 87.549 $p < 0.05$	F(2,108) = 45.446 $p < 0.05$	F(2,108) = 36.626 $p < 0.05$

Note: (a, b, c) indicate concentrations significantly different from others, according to the Tukey and the Tamhane post-hoc tests. F shows the F-test statistic value to compare with the theoretical F-distribution, high F-values indicating significant differences between the concentrations. *p* values are the probability of erroneously concluding significant differences between the concentrations.

Classic logistic growth curves fitted to the Abbott corrected mortality rate data by treatment concentrations are shown in Figure 2. All R2 values are above 0.94, indicating good fits of the estimated trend lines. With higher doses of treatment, the speed of mortality increases. The exposure times needed to LD50 decrease with higher concentrations relatively fast; when doubling the concentration from 1.38 M to 2.76 M, the exposure time decreases to one-third (from 134 to 45) and then doubling the concentration from 2.76 M to 5.52 M results in nearly halving the exposure time again (from 45 to 25). Table 3 gives the elapsed time elapsed for the three concentrations till the 50% (LT50), and 90% (LT90) death rates, respectively, clearly showing the effectiveness of the higher concentrations.

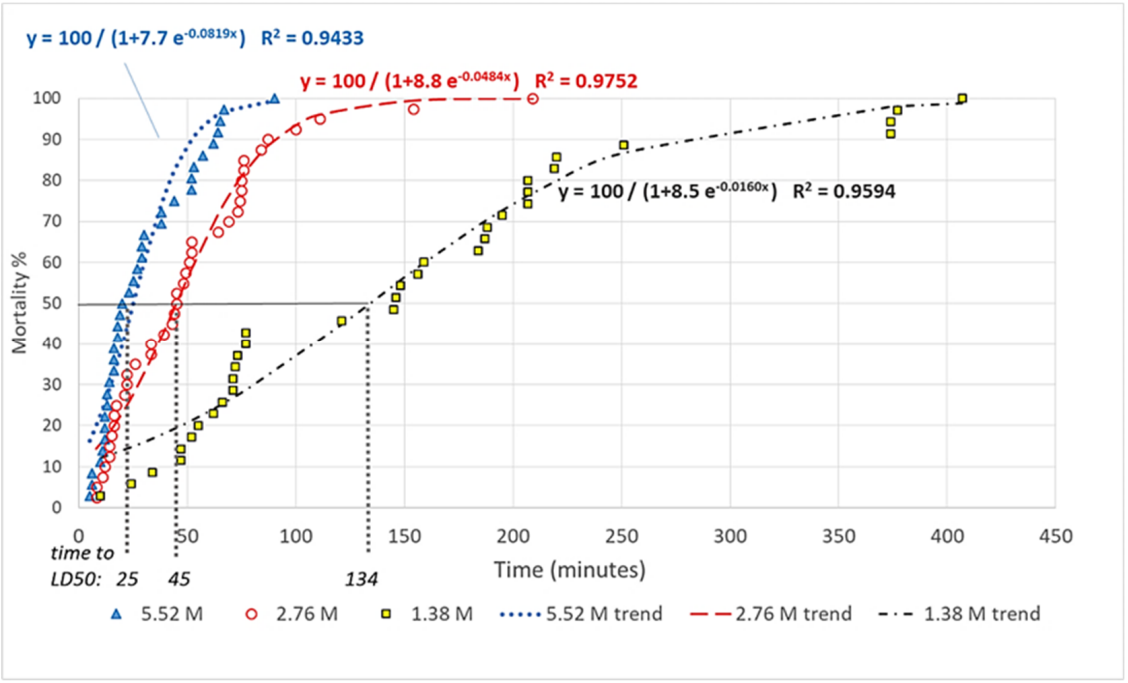

Figure 2. Abbott corrected mortality rates and fitted trends for the three LiCl-concentrations.

Table 3. Time to the death of 50% (LT50) and 90% (LT90) of animals (in minutes).

Licl Concentration	LT50 (Minutes)	LT90 (Minutes)
5.52 M	25	64
2.76 M	45	87
1.38 M	134	312.5

Experiment II. revealed that LD50 = 0.265 M for lithium chloride in poultry red mite (Figure 3).

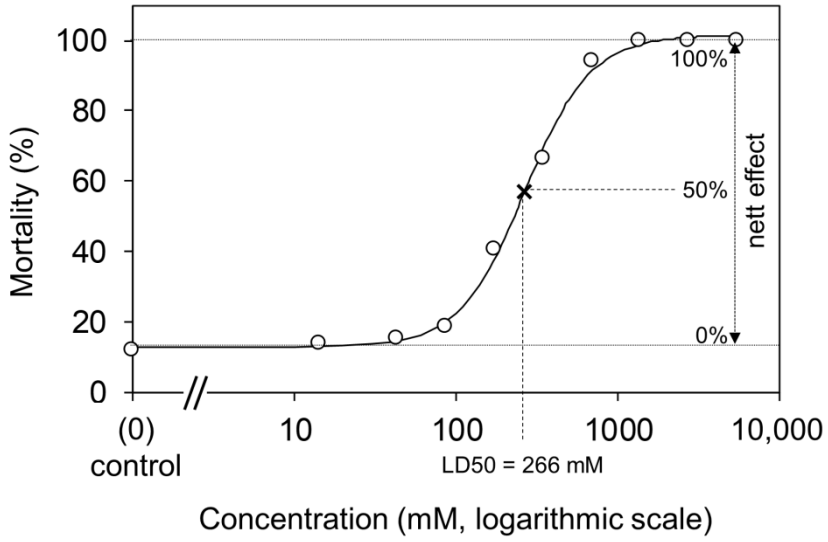

Figure 3. Concentration-response curve and LD50 concentration value for lithium chloride in poultry red mite. Note that LD50 value was calculated based on the net effect of the tested substance (i.e., mortality above the control level).

4. Discussion

In the present study, we have demonstrated that lithium chloride shows effectiveness against poultry red mite in vitro. All the treated groups developed detectable signs of lithium poisoning, and the time courses of intoxication were inversely proportional to concentration for all treatments. These results support recent findings with lithium chloride as an effective potential acaricide in beekeeping [33,34,44,45], indicating that the potential applicability of lithium salts for mite infections in cultured animals might deserve further testing.

The risk of residues, however, is critical for using the microelement as an acaricide. Results so far indicate that the residues of lithium in honey as a consequence of anti-*Varroa* treatment are not alarming [35,36], supported by the fact that many foods may contain lithium as a trace element [46,47]. Foods of animal origin are generally rich in naturally occurring micro elemental lithium, also involving milk, poultry meat and eggs (>7000 μg dry matter) [46–49]. Furthermore, chemical residues can be judged as residues of a trace element, the daily intake of which in the milligram range can prevent progression or delay the onset of chronic diseases and may elicit a positive effect on life expectancy in general, mainly by suppressing the function of the glycogen synthase kinase-3 (GSK3) enzyme [50,51]. Moreover, 1 mg of lithium per day has been proposed as a micronutrient supplement [48], with a recommended daily intake of 1.0 mg lithium/day for 70 kg adult [52]. However, in higher amounts than trace element levels, lithium is a common alternative to treating bipolar disorder in human medicine with side effects on the kidney. It should be noted that the lithium intake, in this case, is several times the range of the microelement (~170 mg Li^+/day).

Present results for the first time indicate an interesting aspect of lithium as having acaricidal effectiveness on the most important mite parasite of poultry, with LD50 effect at 0.265 M concentration. Still, comprehensive research is required to uncover if there would be any effective method of administering lithium in situ, to know whether lithium chloride may have practical relevance. Furthermore, taking that lithium overdose may pose the risk of building chemical residues, and extensive studies on the kinetics of lithium are needed in poultry products in situ.

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