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# RNAi Suppression of Hormone Receptor HR3 Blocks Larval Molting and Metamorphosis in the Cigarette Beetle, *Lasioderma serricorne*

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**Abstract:** Hormone receptor 3 (HR3), an early-late gene of the 20-hydroxyecdysone (20E) signaling pathway, plays a critical role in insect metamorphosis and development. In this study, we identified and characterized an *HR3* gene (*LsHR3)* from the cigarette beetle, *Lasioderma serricorne*. The open reading frame of *LsHR3* is 1581 bp encoding a 527 amino acid protein that contains a conserved DNA binding domain and a ligand binding domain. *LsHR3* was mainly expressed in the fourth-instar larvae, prepupae, and pupae and showed high expression in the fat body. The expression of *LsHR3* was induced by 20E, while it was significantly suppressed by silencing of six 20E synthesis and signaling pathway genes. RNA interference (RNAi)-aided knockdown of *LsHR3* in the fourth-instar larvae disrupted the larval–pupal molting and caused 100% mortality. The 20E titer of *LsHR3*-depletion larvae was decreased, and expressions of five 20E synthesis genes were dramatically decreased. Silencing *LsHR3* reduced chitin content and downregulated the expression of genes involved in chitin synthesis and degradation. Hematoxylin and eosin staining of abdominal cuticle showed that no apolysis occurred after silencing *LsHR3*. These results suggest that *LsHR3*-mediated 20E signaling is involved in the regulation of chitin metabolism during the molting process of *L. serricorne*, and targeting this gene by RNAi has potential in controlling this pest.

**Keywords:** cigarette beetle; hormone receptor; molt; 20-hydroxyecdysone; chitin metabolism; RNA interference



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## 1. Introduction

The cigarette beetle, *Lasioderma serricorne* (Fabricius) (Coleoptera: Anobiidae), is distributed worldwide and feeds on stored grains, dry tobacco leaves, traditional Chinese herbal medicine, and herbarium specimens [1]. At present, chemical pesticides are still used as the first choice to control this stored pest *L. serricorne* because of their high efficiency and low cost. However, after long-term application of chemical pesticides, especially fumigation using phosphine or pyrethroid, *L. serricorne* has developed insecticide resistance [2,3]. For example, mutations (T929I and F1534S) on the sodium channel reduced the susceptibility of *L. serricorne* to many kinds of pyrethroid [4]. Therefore, it is necessary to develop alternative control methods for commonly used pesticides.

RNA interference (RNAi) has shown great potential to be developed as a molecular pest control method. It is possible to realize specific gene knockdown with manufactured double-stranded RNA (dsRNA) [5]. Since this technology was proved to work in insects, it has been considered for use as a new approach for pest control [6]. The rapid advances of transcriptome and genome analysis of insects provide a wide range of potential target genes for the application of RNAi [7]. RNAi-mediated knockdown of *coat protein complex* gene (*COPI*) in *Henosepilachna vigintioctopunctata* led to high mortality in both larvae and adults, and the mortality and silencing efficiency of the target gene showed a significant positive relationship [8]. However, suppression of certain genes may not result in the

direct death of pests. In *Nezara viridula*, RNAi experiments showed that the lethal effect of different genes showed diversity, and some of them were almost ineffective [9]. Ideal RNAi target genes should result in high specificity and mortality to pests, while having less effect on non-target organisms, especially mammals [10]. Since the developmental process of insects is quite different from mammals, key genes involved in the unique pathway of hormones related to development are promising for RNAi-target screening.

Molting and metamorphosis are important processes in insect development. When insects enter the next developmental stage, the first step is apolysis and shedding of the old cuticle, and then insects will secrete a new cuticle [11]. This process mainly depends on the regulation of the ecdysone signaling pathway, which is a complex multihormone system with 20-hydroxyecdysone (20E) as the key hormone mediating various physiological and behavioral including molting [12]. The ecdysone receptor works as a switch in the ecdysone pathway, which can be activated by 20E, and trigger downstream reactions by binding to the ecdysone response element of directly responsive genes [13]. Hormone receptor 3 (HR3) is an important early-late gene in the ecdysone signal transduction of insects, and its expression is regulated by the titer of 20E [14]. In *Blattella germanica*, the expression of *HR3* coincides with the peaks of ecdysteroids and could be induced by 20E, and silencing of the *HR3* gene prevents the nymphs from molting [15]. Similar results are also observed in other arthropods, such as *Leptinotarsa decemlineata* [16], *Panonychus citri* [17], and *Aedes aegypti* [18]. However, the specific function of *HR3* in *L. serricorne* remains unknown.

Most of the damage is caused by the larvae of *L. serricorne*, which tunnel in the leaves of tobacco or feed down into the flour in ground food, while the adults rarely feed [19]. Thus, to prevent economic loss from *L. serricorne*, the larvae were considered the priority target stage. In this study, we intend to reveal the function of *HR3* during the larval molting of *L. serricorne*. A gene encoding HR3 of *L. serricorne* was cloned and its molecular characteristics were characterized. RNAi proved its lethal effect on molting and metamorphosis of *L. serricorne*, and network analysis showed that the lethal mechanism was related to abnormal chitin metabolism in larval–pupal transition.

## 2. Materials and Methods

### *2.1. Insects Culture*

The stock colony of *L. serricorne* was initially collected from Guiyang Tobacco Company of Guizhou Province in 2014 and reared on the dried roots of *Angelica sinensis* as described previously [20]. The beetles were cultured in a dark artificial climate box at 28 °C with a relative humidity of 40%.

### *2.2. Molecular Cloning and Sequence Analysis*

Total RNA of *L. serricorne* larvae was extracted by TransZol reagent (TransGen, Beijing, China), and the first-strand cDNA was synthesized by the TransScript Synthesis Supermix (TransGen). The open reading frame (ORF) sequence of *LsHR3* was obtained from *L. serricorne* transcriptome database (SRR13065789) and verified by reverse transcription polymerase chain reaction (PCR) using gene-specific primers (Table S1). Sequence similarities were identified using the basic local alignment search tool at the National Center for Biotechnology Information website (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 28 June 2022). Molecular weight and isoelectric point (pI) were predicted by the ExPASy Proteomics Server (http://web.expasy.org/, accessed on 28 June 2022). Conserved domains were determined by the Simple Modular Architecture Research Tool (http://smart.embl.de/, accessed on 28 June 2022). The amino acid sequences of the *HR3* genes were aligned with Clustal X [21]. A phylogenetic tree was constructed by MEGA7 software (MEGA, PA, USA) using the neighbor-joining method with 1000 bootstrap replicates [22].

### 2.3. Spatiotemporal Expression Analysis

Whole bodies of *L. serricorne* at various developmental stages (fourth-instar larvae, prepupa, pupae, and adults) were collected daily. Seven tissues (brain, epidermis, fat body, Malpighian tubules, foregut, midgut, and hindgut) were dissected from the fourth-instar larvae. Each sample included 30–50 individuals, and three replications were performed. Quantitative real-time PCR (qPCR) was used to detect the expression profiles of *LsHR3*. The qPCR was performed on the CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA) with the TransStart Top Green qPCR SuperMix (TransGen Biotech). The reaction was performed at 94 °C for 3 min, followed by 40 cycles of 94 °C for 5 s and 60 °C for 30 s. A melting curve analysis was used to evaluate the qPCR specificity. *L. serricorne* elongation factor 1-alpha (*EF1α*) and 18S ribosomal RNA (*18S*) were selected as reference genes, and the geometric means of their expression levels were used for normalization [23]. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression levels of target genes [24].

### 2.4. Expression Profiles of LsHR3 in Response to 20E

The 20E (Sigma-Aldrich, St Louis, MO, USA) treatment was performed according to our previous study [23]. Each day-2 fourth-instar larva was injected with 120 nL of 20E solution (120 ng/larva) using a Nanoliter 2010 injector (World Precision Instruments, Sarasota, FL, USA), and the controls were treated with an equal volume of 0.1% ethanol. For expression analysis of *LsHR3*, thirty individuals were randomly collected from each group at 3, 6, 12, and 24 h post-injection. To confirm whether *LsHR3* was responsive to 20E synthesis and signal transduction, we performed RNAi experiments using double-stranded RNAs (dsRNAs) targeting five Halloween genes (*LsCYP302a1*, *LsCYP306a1*, *LsCYP307a1*, *LsCYP314a1*, and *LsCYP315a1*), ecdysone-receptor gene (*LsEcR*), and ultraspiracle gene (*LsUSP*). The dsRNAs were synthesized in vitro by TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific, Wilmington, DE, USA). The green fluorescent protein (*GFP*) served as a negative control. Approximately 200 ng dsRNAs of each ecdysone-related gene were injected into day-2 fourth-instar larvae. After 3 days, insect samples were collected to analyze the RNAi efficiency for the corresponding genes and the expression of *LsHR3*.

### 2.5. Functional Analysis of LsHR3 by RNAi

To explore the function of *LsHR3* in *L. serricorne* development, 200 ng of dsRNAs for *LsHR3* or *GFP* were injected into the day-2 fourth-instar larvae. All the dsRNA-treated insects were reared under the above conditions. To test RNAi efficiency, relative expression levels of *LsHR3* were measured at 3 and 5 days after dsRNA injection using qPCR as described above. A stereomicroscope (Keyence Corporation, Osaka, Japan) was used to photograph the abnormal phenotype of tested insects, and the survival rate was also recorded. To investigate the effects of *LsHR3* RNAi on ecdysone synthesis, the 20E titer was determined using the Insect Ecdysone Enzyme-linked immunosorbent assay (ELISA) Kit (Shanghai Meilian Biotechnology Co., Ltd., Shanghai, China). The 20E titer was measured by the SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA) 3 days after dsRNA injection. After *LsHR3* was knocked down, the expression levels of *LsCYP302a1*, *LsCYP306a1*, *LsCYP307a1*, *LsCYP314a1*, and *LsCYP315a1* were detected by qPCR at 3 days after the corresponding dsRNA injection. Forty insects were treated as one replication, and three replications were performed.

### 2.6. Knockdown of LsHR3 on Chitin Metabolism and Cuticle Formation

To explore the effect of *LsHR3* RNAi on chitin metabolism, samples were collected from larvae injected with ds*LsHR3* and ds*GFP* for 3 days. The chitin content was assessed by measuring glucosamine from the chitin using chitinase hydrolysis according to the previously described method [25]. Relative expression levels of four chitin synthesis genes, including trehalase (*LsTRE1* and *LsTRE2*), UDP-N-acetylglucosamine pyrophosphorylase (*LsUAP*), and chitin synthase 1 (*LsCHS1*), and six chitin-degrading genes, including *β-N*-acetylglucosaminidase (*LsNAG1* and *LsNAG2*), chitin deacetylase 1 (*LsCDA1*), and chitinase

(*LsCHT5*, *LsCHT7*, and *LsCHT10*), were determined by qPCR. To further investigate the effects of *LsHR3* RNAi on cuticle development, a hematoxylin and eosin (H&E) staining experiment was performed. The abdominal cuticle was dissected from the larvae after injection with ds*LsHR3* and ds*GFP* for 3 days. The insect samples were fixed in 4% paraformaldehyde solution at 4 °C for 12 h, then they were embedded in OCT and trimmed by HM525 frozen microtome (Thermo Scientific). The frozen sections (5 μm) of the abdominal cuticle were prepared and stained with H&E. The slides were viewed and imaged using a LSM 900 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

### *2.7. Statistical Analysis*

Data were analyzed using SPSS 20.0 software (IBM Corp, Chicago, IL, USA). Survival rates were analyzed using the Kaplan–Merier method. The spatiotemporal expression of *LsHR3* was analyzed using a one-way analysis of variance followed by a least significant difference test. The other data were analyzed statistically using Student's *t*-test.

## 3. Results

### *3.1. Identification and Characterization of LsHR3*

The full-length ORF of *LsHR3* (GenBank accession number ON933951) was 1581 bp, which encoded 527 amino acids. The molecular weight and pI of LsHR3 protein are 58.07 kD and 5.77. Domain analysis revealed that LsHR3 contained a DNA-binding domain (DBD, amino acids 61-130) and ligand-binding domain (LBD, amino acids 404-503) (Figure 1). Multiple sequence alignment showed that LsHR3 was highly similar to the HR3 of *Anoplophora glabripennis* (XP_018562864.1) and *Dendroctonus ponderosae* (XP_019758192.1) with identities of 80.2% and 70.9%. Phylogenetic analysis of HR3 from various insect species showed that LsHR3 has a close relationship with that of Coleoptera (Figure 1).

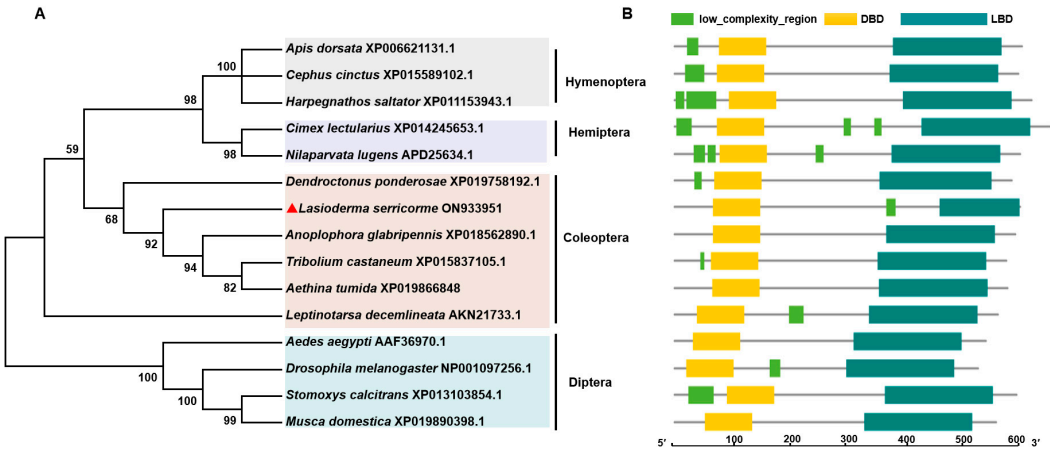


**Figure 1.** Phylogenetic analysis and structure comparison of insect HR3 proteins. (**A**) Phylogenetic tree was constructed with MEGA7 by using neighbor-joining method with 1000 bootstrap replications. LsHR3 is marked with a red triangle. The GenBank accession number of each species is listed in the tree. (**B**) Schematic alignment and comparison of domain architecture of insect HR3 proteins. AA, amino acid; DBD, DNA binding domain; LBD, ligand binding domain.

### *3.2. Spatiotemporal Expression Analysis of LsHR3*

*LsHR3* was constantly expressed in all tested developmental stages. The expression of *LsHR3* was highest in pupae, but high levels also occurred in prepupae and day-3 fourth-instar larvae (Figure 2A). *LsHR3* was expressed in all the dissected tissues of the fourth-instar larvae, with high expression in the fat body, epidermis, foregut, and midgut (Figure 2B).

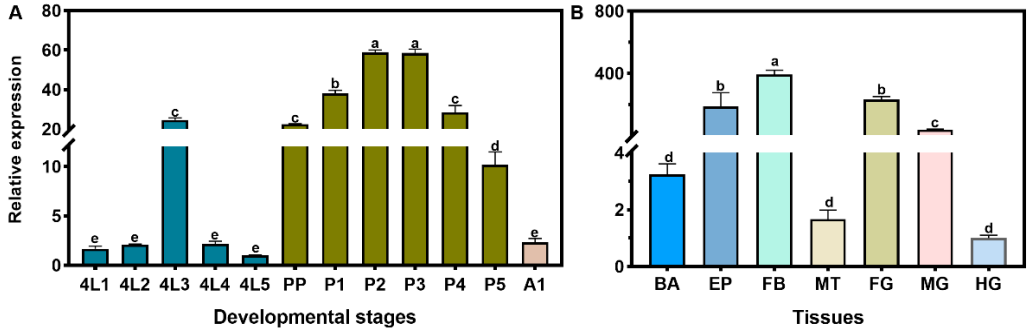


**Figure 2.** Spatiotemporal expression profiles of *LsHR3*. (**A**) Expression level of *LsHR3* in different developmental stages. 4L1, day-1 fourth-instar larvae; PP, prepupa; P1, day-1 pupae; A1, day-1 adults. (**B**) Tissue distribution of *LsHR3* in fourth-instar larvae. BA, brain; EP, epidermis; FB, fat body; MT, Malpighian tubules; FG, foregut; MG, midgut; HG, hindgut. Different letters above each bar represent significant differences based on one-way ANOVA followed by a least significant difference test ($p < 0.05$).

### 3.3. The Response of LsHR3 to 20E

To test whether *LsHR3* is induced by 20E, the fourth-instar larvae were injected with 20E solution. The expression of *LsHR3* was significantly upregulated after 20E treatment compared with the control group with 25.0-, 6.7-, 3.5-, and 3.0-fold increases at 3, 6, 12, and 24 h, respectively (Figure 3A). To further confirm whether *LsHR3* is regulated by 20E signaling, five Halloween genes (*LsCYP302a1*, *LsCYP306a1*, *LsCYP307a1*, *LsCYP314a1*, and *LsCYP315a1*) and two ecdysone receptors (*LsEcR* and *LsUSP*) were individually knocked down using RNAi in the fourth-instar larvae. Among the silencing of the above-mentioned seven genes involved in the 20E signaling pathway, besides *LsCYP302a1*, the rest significantly downregulated the expression of *LsHR3* (Figure 3B).

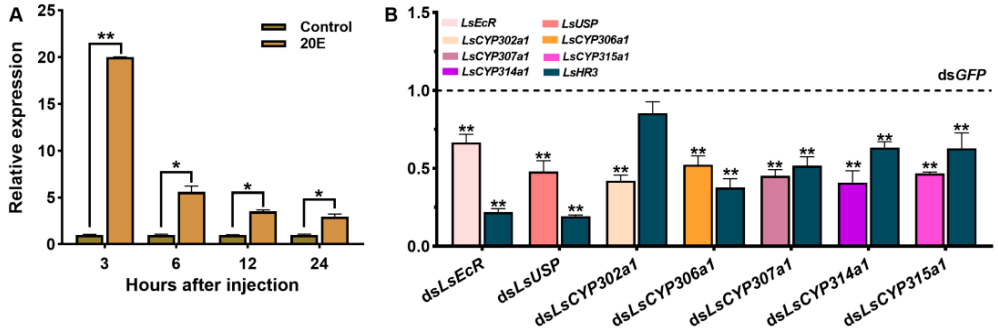


**Figure 3.** Expression profiles of *LsHR3* in response to 20E. (**A**) Effect of 20E on the expression of *LsHR3*. Control: insect injected with distilled water containing 0.1% ethanol; 20E: insect injected with 20E (120 ng/larva). (**B**) Relative expression levels of *LsEcR*, *LsUSP*, *LsCYP302a1*, *LsCYP306a1*, *LsCYP307a1*, *LsCYP314a1*, and *LsCYP315a1* at 3 days after gene-specific dsRNA injection. The gene expression in the ds*GFP* group was set as 1 and indicated by the dotted line. Significant differences between the treatment group and control group were determined using Student's *t*-test (* $p < 0.05$, ** $p < 0.01$).

### 3.4. Knockdown of LsHR3 Disrupts the Larval–Pupal Molting

RNAi was used to evaluate the roles of *LsHR3* in the larval molting process of *L. sericorne*. Compared with the control, the expression level of *LsHR3* was significantly reduced by 43% and 60% at 3 and 5 days after ds*LsHR3* injection, respectively (Figure 4A). In the control group, the larvae could molt normally to pupae 3 days after injection with ds*GFP*. However, 100% of the ds*LsHR3*-injected larvae exhibited abnormal ecdysis and finally died (Figure 4B). Following the injection of ds*LsHR3*, the larvae trapped in their old cuticles remained in the larval stage and failed to complete molting (Figure 4C).

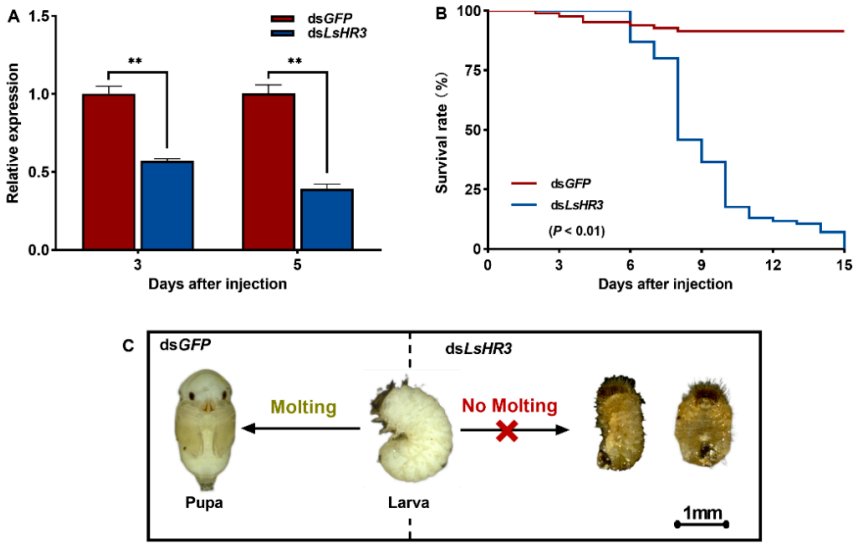


**Figure 4.** Effect of *LsHR3* RNAi on larval-pupal molting in *Lasioderma serricorne*. (**A**) Relative expression levels of *LsHR3* at 3 and 5 days after *LsHR3* or *GFP* dsRNA injection in the fourth-instar larvae. (**B**) Kaplan–Meier survival curves of larvae after *LsHR3* or *GFP* dsRNA injection. (**C**) Representative phenotypes of larvae after *LsHR3* or *GFP* dsRNA injection. Significant differences between the RNAi group and control group were determined using Student's *t*-test (** $p < 0.01$).

### *3.5. Knockdown of LsHR3 Disturbs 20E Synthesis*

The 20E titer was significantly decreased by 35.6% at 3 days after injection with ds*LsHR3* ($p < 0.01$) compared with the ds*GFP* group (Figure 5A). Accordingly, the mRNA levels of five 20E synthesis genes, including *LsCYP302a1*, *LsCYP306a1*, *LsCYP307a1*, *LsCYP314a1*, and *LsCYP315a1* were significantly decreased after knockdown of *LsHR3* (Figure 5B).

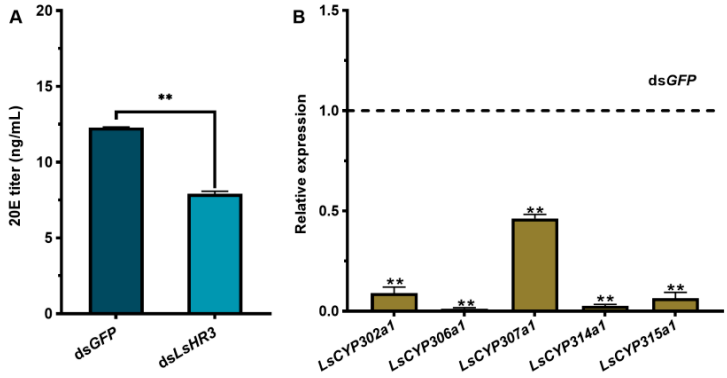


**Figure 5.** Effect of *LsHR3* RNAi on 20E synthesis. (**A**) 20E titer in fourth-instar larvae after *LsHR3* RNAi. (**B**) Relative expression levels of 20E synthesis genes after *LsHR3* RNAi. Significant differences between the RNAi group and control group were determined using Student's *t*-test (** $p < 0.01$).

### *3.6. Knockdown of LsHR3 Inhibits the Chitin Metabolism and Cuticle Formation*

The chitin content of *LsHR3* knockdown individuals was significantly lower than those injected with ds*GFP* (Figure 6A). The mRNA levels of *LsTRE1*, *LsTRE2*, *LsUAP1*, and *LsCHS1* responsible for chitin synthesis were dramatically downregulated by 99.2%, 70.4%, 85.6%, and 37.2%; *LsCHT5*, *LsCHT7*, *LsCHT10*, *LsCDA1*, *LsNAG1*, and *LsNAG2* of key genes responsible for chitin degradation were also significantly downregulated by 44.0%, 50.5%, 98.6%, 63.7%, 94.4%, and 28.4%, compared to those of the ds*GFP*-injected group (Figure 6B). H&E staining results showed that apolysis occurred in the control insects, where the old cuticles digested and successfully separated from the underlying new epidermis; however,

the ds*LsHR3*-treated insects were unable to form the new cuticles and failed to molt without apolysis (Figure 6C).

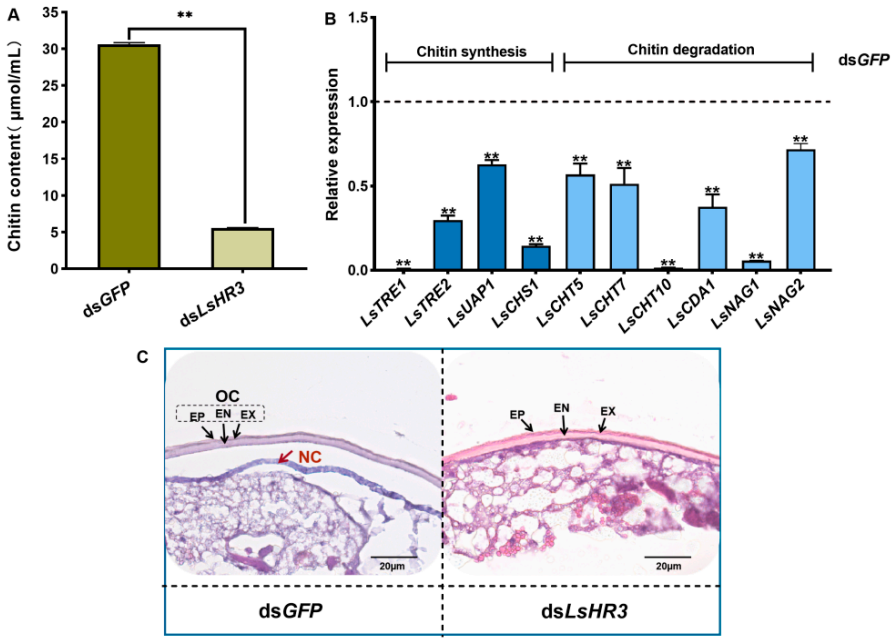


**Figure 6.** Effect of *LsHR3* RNAi on chitin metabolism and cuticle formation. (**A**) Chitin content in *Lasioderma serricorne* after *LsHR3* knockdown. (**B**) Relative expression levels of genes involved in chitin degradation and synthesis after *LsHR3* RNAi. (**C**) Hematoxylin and eosin staining of the abdominal cuticle after *LsHR3* or *GFP* dsRNA injection. EP: Epicuticle, EX: Exocuticle, EN: Endocuticle, OC: Old cuticle, NC: New cuticle. Significant differences between the RNAi group and control group were determined using Student's *t*-test (** $p < 0.01$).

## 4. Discussion

Nuclear receptors function as key control points in the molting process of insects, which can be triggered by specific hormones and then regulate the expression of downstream genes [26]. A highly conserved DNA-binding domain (DBD) and a less-conserved ligand-binding domain (LBD) in the protein sequence are typical characteristics of nuclear receptors. A low-complexity region was also predicted in LsHR3 protein, indicating that it might play functional roles in the modulation of protein–protein interactions [27]. Subfamilies of nuclear-receptor genes can be classified by phylogenetic analysis according to clusters of receptors that share significant sequence conservation between their respective DBDs and LBDs [28]. Our phylogenetic tree showed that LsHR3 was closely related to the HR3 of other coleopteran insects, indicating high conservation of HR3 within orders.

The expression of hormone receptor genes was regulated by 20E in different developmental stages of insects [29,30]. In *Locusta migratoria*, the expression of *LmHR3* reached the peak on the day-6 fifth-instar nymph [31]. In the current study, the expression of *LsHR3* had a specific pattern in day-3 fourth-instar larvae and the pupae. It was consistent with the expression mode of *HR3* in *L. decemlineata*, in which *HR3* was mainly expressed in certain time points of larval and pupal stages [16]. Further, the expression of *LsHR3* could be induced by additional 20E in the fourth-instar larvae. 20E with ecdysone receptor acted as a key to start the network of the molecular pathway [13]. In this case, these nuclear receptors are considered important pest control targets either in the development of traditional chemical pesticides or nucleic acid pesticides with RNAi [32].

RNAi-based disruption of the normal molting process of arthropods has been proved to be ideal in pest control [33]. For instance, silencing of genes related to ecdysone signal transduction with exogenously dsRNA could interrupt the normal developmental process. In *Tetranychus cinnabarinus*, the knockdown of *EcR* disrupted the metamorphic transition,

and the pest mites failed to develop to adult stages [34]. Ultraspiracle (USP) is a homologue of the vertebrate RXR and dimerizes with EcR by ligand binding. Decreasing expression of *RXR1* and *RXR2* by RNAi in *P. citri* showed the same phenotype as that of silencing *EcR* [35]. Once the dimerization of EcR-USP is activated by 20E, a series of hormone receptors downstream of this pathway will give a response to regulate the expression of genes involved in molting. Silencing of *HR4* in fourth-instar larvae of *L. decemlineata* resulted in high lethality and impaired pupation [36]. *E75* is a dimer partner of *HR3*, and RNAi of *E75* in *Daphnia magna* significantly delayed molting, reduced the number of offspring, and caused developmental abnormalities [37]. In this study, suppression of *LsHR3* expression disrupted the larval–pupal transition and caused 100% mortality. Transgenic algal strains were constructed to express dsRNA of *HR3* to feed *Aedes aegypti*, and all the treated larvae died on the 10th day at the latest [18]. In *L. decemlineata*, silencing of *HR38* caused high larval mortality and the impairment of pupation and adult emergence [30]. These results suggest that genes involved in the ecdysone signaling pathway could be considered an ideal lethal target of RNAi control.

Insect cuticles consist of epicuticle (lipids and proteins) and procuticle (chitin filaments arranged within a protein matrix). Chitin architecture is a key factor that affects the mechanical properties of insect cuticle, and its degradation and synthesis are extremely important for insect molting [11]. The process of chitin metabolism is regulated by the 20E pathway, starting from the titer change of 20E, and then the signal is transmitted by *EcR* and a series of nuclear receptor genes [26]. Molecular network analysis indicated that *LsHR3* is involved in insect molt and metamorphosis via regulation of chitin synthesis and degradation. Genes such as *E74*, *E75*, and *HR3* were induced directly by EcR/USP, and these nuclear receptors could start a cascade transmission to the downstream functional genes [38]. The signal triggered by 20E is transmitted in this way down to the genes related to chitin metabolism and cuticular formation. Studies in *L. migratoria* demonstrated that *HR3* is related to nymph molting by regulating the expression of chitin synthesis and degradation genes [31]. In *Bombyx mori*, certain cuticular protein genes were also regulated by the expression of *HR3*, and *HR3* expression was induced by *EcR* [38]. In the present study, RNAi of *LsHR3* substantially decreased the chitin amounts and the expression levels of ten chitin synthesis and degradation genes, which led to abnormal cuticular formation during the molting process. Thus, the unsuccessful exchange of old and new cuticles should be the direct lethal reason for RNAi-based silencing of *LsHR3*.

*HR3* was located downstream of *EcR*, and its expression could be induced by 20E. Simultaneously, we noticed that decreasing *LsHR3* expression could affect 20E synthesis. This influence might be due to the feedback between *HR3* and *EcR*. In *Helicoverpa armiger*, knockdown of *EcR* by transgenic tomato significantly reduced mRNA levels of *HR3* and other downstream genes involved in the 20E signaling pathway [39]. In *L. decemlineata*, silencing of *EcR* also inhibited the expression of *HR3* and blocked larval–pupal–adult transition [40]. In conclusion, RNAi targeting of *HR3* had a lethal effect on larval–pupal transition of *L. serricorne*, and its mechanism was to disturb chitin metabolism and cuticle formation during development.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12081257/s1. Table S1. Primer sequences used in this study.

**Author Contributions:** Conceptualization, L.-X.M. and W.-J.Y.; methodology, L.-X.M.; validation, L.-X.M. and R.-T.H. Investigation, L.-X.M. and S.-Y.Y.; data curation, S.-Y.Y. and W.-J.Y.; writing—original draft preparation, L.-X.M.; writing—review and editing, W.-J.Y.; funding acquisition, W.-J.Y. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data presented in this study are available in article.

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

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