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Article

# RNA Interference-Mediated Suppression of Ecdysone Signaling Inhibits Choriogenesis in Two Coleoptera Species

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**Abstract:** During choriogenesis in insects, chorion (eggshell) is formed by surrounding follicular epithelial cells in ovarioles. However, the regulatory endocrine factor(s) activating choriogenesis and the chemical components on eggshell deserve further exploration. In two representative Coleopterans, a Coccinellid *Henosepilachna vigintioctopunctata* and a Chrysomelid *Leptinotarsa decemlineata*, genes encoding the 20-hydroxyecdysone (20E) receptor heterodimer, ecdysone receptor (EcR) and ultraspiracle (USP), and two chitin biosynthesis enzymes UDP-N-acetylglucosamine pyrophosphorylase (UAP) and chitin synthase (ChS1), were highly expressed in ovaries of the young females. RNA interference (RNAi)-aided knockdown of either *HvEcR* or *Hvusp* in *H. vigintioctopunctata* inhibited oviposition, suppressed the expression of *HvChS1*, and lessened the positive signal of Calcofluor staining on the chorions, which suggests the reduction of a chitin-like substance (CLS) deposited on eggshells. Similarly, RNAi of *LdEcR* or *Ldusp* in *L. decemlineata* constrained oviposition, decreased the expression of *LdUAP1* and *LdChS1*, and declined CLS contents in the resultant ovaries. Knockdown of *LdUAP1* or *LdChS1* caused similar defective phenotypes, i.e., reduced oviposition and CLS contents in the *L. decemlineata* ovaries. These results, for the first time, indicate that 20E signaling activates choriogenesis in two Coleopteran species. Moreover, our findings propose the deposition of a CLS on the chorions.

**Keywords:** *Leptinotarsa decemlineata*; *Henosepilachna vigintioctopunctata*; choriogenesis; ecdysone signal; chitin-like substance; eggshell

## 1. Introduction

The development of oocytes (oogenesis) in insect ovarioles consists of previtellogenesis, vitellogenesis and choriogenesis stages. Among the three successive physiological processes, choriogenesis is the final developmental period towards oocyte maturation when the chorion (eggshell) is formed by the surrounding follicular epithelial cells [1]. Up to now, however, the regulatory endocrine factor(s) activating choriogenesis and the chemical components on eggshell have not been well explored.

Endocrine control of oogenesis has widely been reported in insects [2–4]. Nevertheless, all these publications are focused on the stimulation of previtellogenesis and vitellogenesis. Three classical hormones, ecdysteroids (20-hydroxyecdysone, 20E, the most active form), juvenile hormone (JH) and insulin-like peptides (ILP), have been confirmed as gonadotropins [2,5]. In *Tribolium castaneum*, for instance, application of JH III induces *vitellogenin* (*Vg*) mRNA in the previtellogenic females, whereas suppressing JH signal impairs *Vg* gene expression and *Vg* accumulation [5]. Similarly, RNAi studies

show that 20E receptor heterodimer, ecdysone receptor and ultraspiracle genes (*EcR* and *usp*), and 20E downstream genes (*E75*, *E93*, *HR3*, *HR4* and  $\beta$ *FTZ-F1*) are required for vitellogenesis and oocyte maturation [6,7]. Moreover, bursicon neuropeptide (downstream 20E component) also triggers *Vg* expression, possibly through activation of JH and ILP pathways [8]. Up to now, whether the same endocrine pathways are involved in the government of choriogenesis remains unclear. In fact, some researchers believe that insect choriogenesis is autonomously initiated when an oocyte has been developed to a certain vitellogenetic phase [9].

As for chemicals on insect eggshells, the most important component is proteins [1]. In the *Drosophila melanogaster* eggshells, for instance, more than 30 protein components are assembled [10,11]. Similarly, in the chorions of the superfamily Bombycoidea insects, more than 100 different polypeptides are accumulated in a stepwise fashion [1,12]. Intriguingly, a chitin-like substance (CLS) accumulates in oocytes during oogenesis, it is also present in ovaries, newly laid and dark eggs in *Aedes aegypti* [13]. Whether the CLS is an eggshell structural component during oogenesis needs to be established in insects other than *A. aegypti* [13].

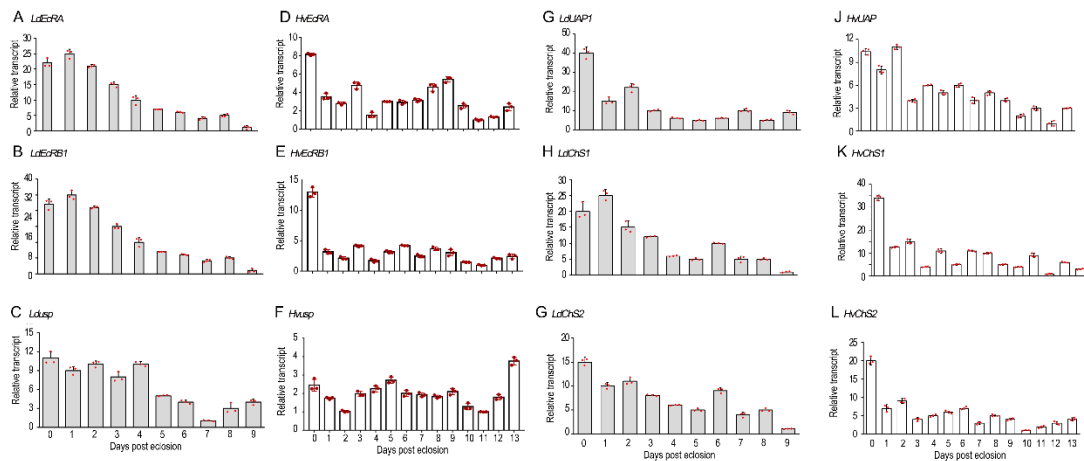
Chitin biosynthesis has well been investigated in insects. UDP-N-acetylglucosamine pyrophosphorylase (UAP) and chitin synthases (ChS) catalyze the final two consecutive biochemical steps [14–17]. In most insects, ChSs have been segregated into two groups, namely, ChS1 and ChS2. ChS1 is exclusively distributed in the ectodermally-derived epidermal cells, it is responsible for chitin generation in epidermis, trachea, salivary gland, foregut and hindgut cells. ChS2 is specifically located in midgut cells and is dedicated to chitin formation in the peritrophic matrix (PM) [14–17]. In *Leptinotarsa decemlineata* larvae, UAPs are also encoded by UAP1 and UAP2 genes. UAP1 and UAP2 specifically catalyze chitin production in ectodermally-derived cells and midgut PM. Moreover, chitin synthesis is tightly controlled by 20E during larval development, through binding to EcR/USP heterodimer in *L. decemlineata* [17,18]. Providing that chitin biosynthesis is strictly coordinated within the cycle of insect molts [19,20], the biochemical process should also be governed by 20E cascade during metamorphosis in other insect species.

A Chrysomelid *L. decemlineata* and a Coccinellid *Henosepilachna vigintioctopunctata* are Coleopteran potato defoliators. Our previous results reveal that vitellogenesis occurs in the 5- to 7-day-old females, while the choriogenesis takes place in the 8- and 9-day-old adults. Copulation and oviposition happen before and at the age of 8 days old in *H. vigintioctopunctata* [21]. RNA interference (RNAi) of either *EcR* or *usp* inhibits oocyte development, dramatically represses the transcription of *Vg* in fat bodies and of *VgR* in ovaries in the two Coleopterans [22]. In the current paper, we performed the same RNAi experiments in the newly-emerged adult females. We intended to test two hypotheses. Firstly, 20E cascade mediates choriogenesis. Secondly, a CLS is deposited on oocyte chorion. Our findings supported the two assumptions.

## 2. Results

### 2.1. The Temporal Expression of Related Genes

Using qRT-PCR, the temporal expression patterns of two 20E receptor genes (*EcR* and *usp*) and three chitin biosynthesis genes (UAP, ChS1 and ChS2) were respectively measured in the 0- to 9-, and 0- to 13-day-old ovaries in *L. decemlineata* and *H. vigintioctopunctata* (Figure 1). All 5 genes in *L. decemlineata* (*LdEcRA*, *LdEcRB1*, *Ldusp*, *LdUAP1*, *LdChS1*, *LdChS2*) and 4 genes in *H. vigintioctopunctata* (*HvEcRA*, *HvEcRB1*, *HvUAP*, *HvChS1*, *HvChS2*) were highly expressed in the young adults (at the ages of 0 to 5 days old), whereas *Hvusp* was constantly transcribed in the 0- to 13-day-old *H. vigintioctopunctata* ovaries (Figure 1).

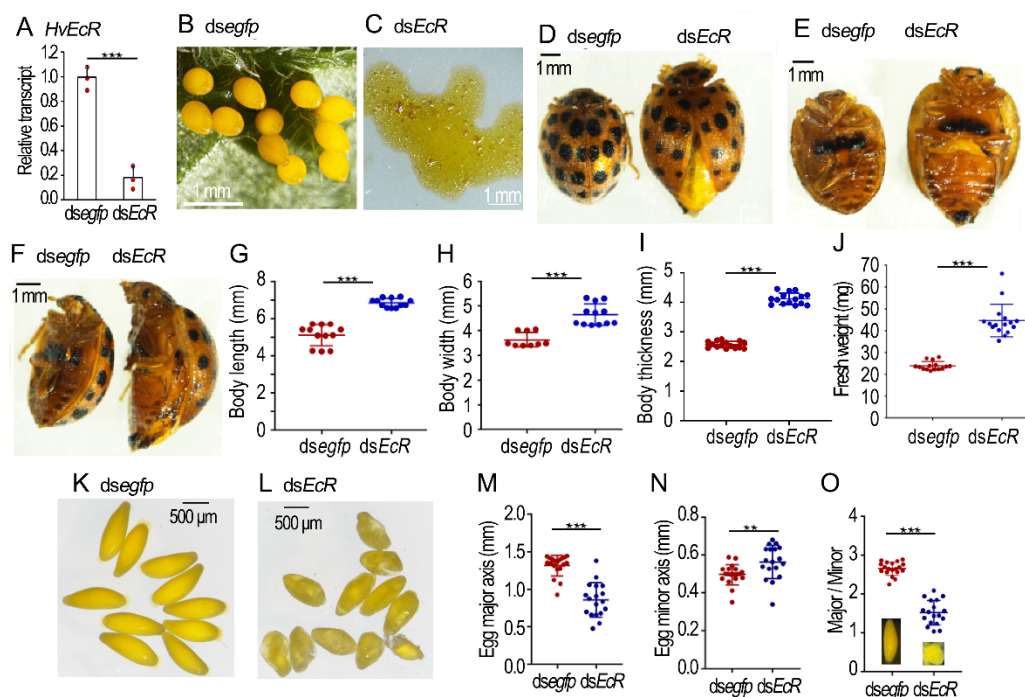


**Figure 1.** The temporal expression patterns of selected genes in the ovaries of two Coleoptera potato defoliators. The cDNA templates were derived from the ovaries of 0- to 9-day-old *L. decemlineata*, and 0- to 13-day-old *H. vigintioctopunctata* female adults respectively. The transcript levels of EcRA, EcRB1, usp, UAP, ChS1, and ChS2 were determined. For each sample, 3 independent pools of 5-10 individuals were measured in technical triplicate using real-time quantitative PCR (red dots). The bars represent  $2^{-\Delta\Delta CT}$  value ( $\pm$ SD) normalized to the geometrical mean of house-keeping gene expression. The lowest transcript levels at a specific developmental time point were set as 1.

## 2.2. Knockdown of *HvEcR* Prevents Oviposition in *H. vigintioctopunctata*

Three days after *dsHvEcR* injection, the *HvEcR* level in the ovary was significantly reduced (Figure 2A). For the *dsegfp*-injected beetles, no female laid eggs until 8 days after adult emergence. They then continuously oviposited. In contrast, the *dsHvEcR*-injected females did not lay any eggs until 20 days post adult emergence. They then laid only turbid liquid (Figure 2B versus 2C).

Consistent with failure of oviposition, the 30-day-old *HvEcR* RNAi females possessed larger body sizes and heavier fresh weights than the 30-day-old controls (Figure 2D-2J). The mature eggs in the 30-day-old control ovaries were ellipse-shaped (Figure 2K). The average major axis and minor axis were 1.30 mm and 0.50 mm respectively. The ratio of major/minor axis was 2.7 (Figure 2M-2O). The eggs in the 30-day-old *HvEcR* RNAi ovaries were misshapen, nearly globular (Figure 2L); the mean major axis, minor axis and ratio of major/minor axis were 0.85 mm, 0.57 mm and 1.5 respectively (Figure 2M-2O).



**Figure 2.** Knockdown of HvEcR in the 0-day-old *Henosepilachna vigintioctopunctata* female adults. The 0-day-old female adults were injected with 0.1  $\mu$ L dseGfp or dsHvEcR (400 ng). The treated beetles were fed on fresh potato foliage. Three days after treatment, transcript level of HvEcR was determined (n=3, A). Relative transcript is the ratio of relative copy number in treated individuals to dseGfp-treated controls, which is set as 1. The body sizes and fresh weights were recorded in the 30-day-old females (n=9-12, G-J). The eggs in the 30-day-old females were measured (n=15, M-O). The averages and SD ranges are drawn. Different stars indicate significant difference at p value < 0.05 (\*), 0.01 (\*\*), or 0.001 (\*\*\*) using t test. The oviposition was observed 0 to 30 days post eclosion (B, C). The body sizes (D-F) and egg shapes (K, L) of 30-day-old females were shown.

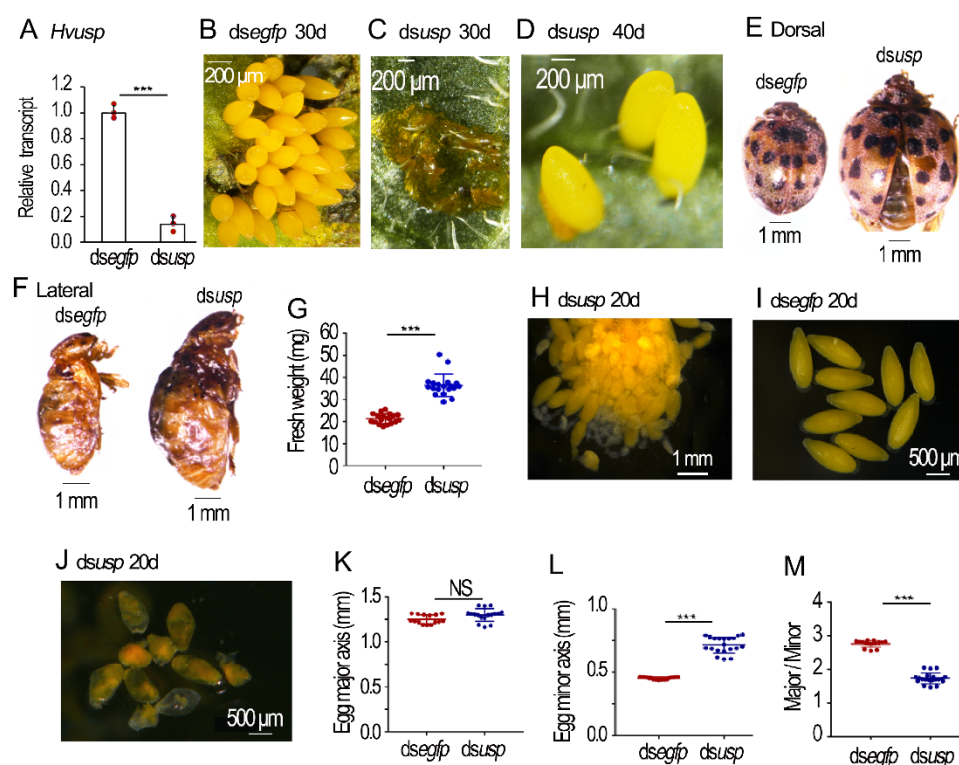
### 2.3. Depletion of Hvusp Affects Oogenesis in *H. vigintioctopunctata*

Injection of dsHvusp into the 0-day-old females successfully knocked down the target gene in the ovary (Figure 3A). Whereas the control females more than 8 days old continuously laid eggs (Figure 3B), the Hvusp RNAi females did not lay any eggs until around 20 days post eclosion. They then laid only turbid liquid (Figure 3C). Interestingly, some Hvusp RNAi females more than 40 days old could lay a few normal eggs daily, along with turbid liquid (Figure 3D).

Similar to the 30-day-old HvEcR RNAi females, the 30-day-old Hvusp RNAi females had larger body sizes and heavier fresh weights than the 30-day-old controls (Figure 3E-3G).

In the 20-day-old Hvusp RNAi ovarioles, there were two kind of mature eggs. Many misshapen eggs were accumulated in the median oviduct and the genital chamber, whereas one or two normally-shaped eggs were sited in the lateral oviduct (Figure 3H).

The mature eggs in the 20-day-old control ovaries were ellipse-shaped (Figure 3I). The average major axis and minor axis were 1.25 mm and 0.45 mm respectively. The ratio of major/minor axis was 2.8 (Figure 3K-3M). Conversely, the eggs in the Hvusp RNAi ovarioles were misshapen (Figure 3J); the mean major axis, minor axis and ratio of major/minor axis were 1.30 mm, 0.70 mm and 1.8 respectively (Figure 3K-3M).



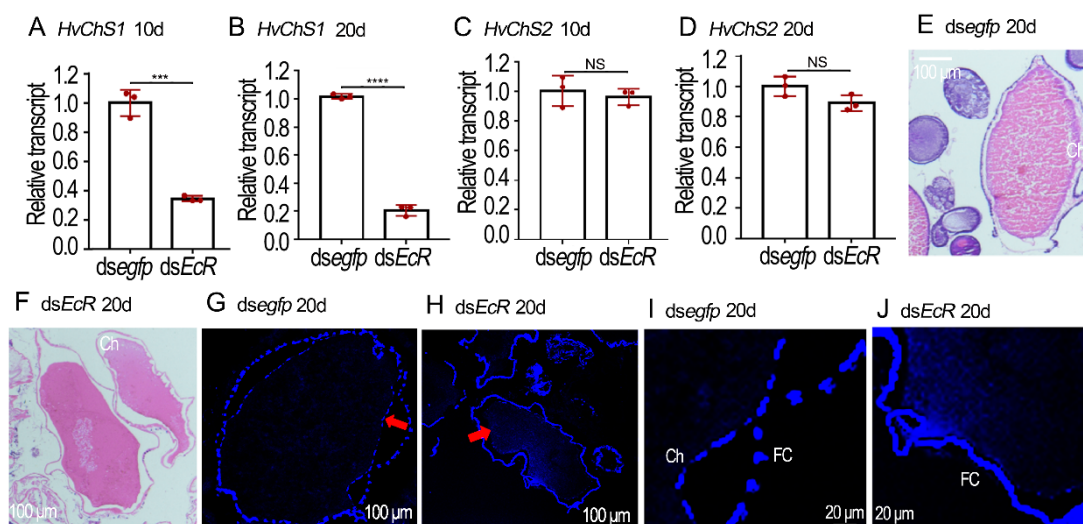
**Figure 3.** Silence of *Hvsp* in the 0-day-old *Henosepilachna vigintioctopunctata* female adults. The 0-day-old female adults were injected with 0.1  $\mu$ L *dsegrp* and *dsHvsp* (400 ng). The treated beetles were fed on fresh potato foliage. Three days after treatment, transcript level of *Hvsp* was determined (A). Relative transcript is the ratio of relative copy number in treated individuals to *dsegrp*-treated controls, which is set as 1. The fresh weights were recorded in the 30-day-old females ( $n=18$ , G). The eggs in the 20-day-old females were measured ( $n=15$ , K-M). The averages and SD ranges are drawn. Different stars indicate significant difference at  $p$  value  $< 0.05$  (\*), 0.01 (\*\*), or 0.001 (\*\*\*) using t test. NS, no significance. The oviposition was observed 0 through 40 days post eclosion (B-D). The body sizes of 30-day-old females (E, F) and egg shapes of 20-day-old females (H-I) were shown.

#### 2.4. RNAi of *HvEcR* Affected CLS Deposition on *H. vigintioctopunctata* Oocytes

Our previous results have documented the identification of two chitin synthase genes (*HvChS1* and *HvChS2*) in *H. vigintioctopunctata* [15]. Here we detected their expression levels in the 10- and 20-day-old ovaries. In contrast to *HvChS2* that is only expressed in the midgut [15], the transcript levels of *HvChS1* were significantly reduced in the *HvEcR* silenced samples, compared with these in the control ovaries (Figures 4A-4D).

HE stained sections of ovaries displayed that the maturing eggs in the control ovarioles had smooth and continuous chorions (Ch) (Figures 4E), whereas the eggshells in the *HvEcR* RNAi developing eggs were irregular and discontinuous (Figures 4F).

The presence of CLS was shown by Calcofluor staining [23]. The fluorescence images of frozen sections displayed that two layers of positive-stained membranes ( $\uparrow$ ) were observed in the control ovarioles, one lining the follicle cells (FC) and the other being deposited to the chorion (Ch) (Figure 4G, 4I). Conversely, only the positive-stained layer along the follicle cells was seen in the *dsHvEcR* treated ovariole samples (Figure 4H, 4J).



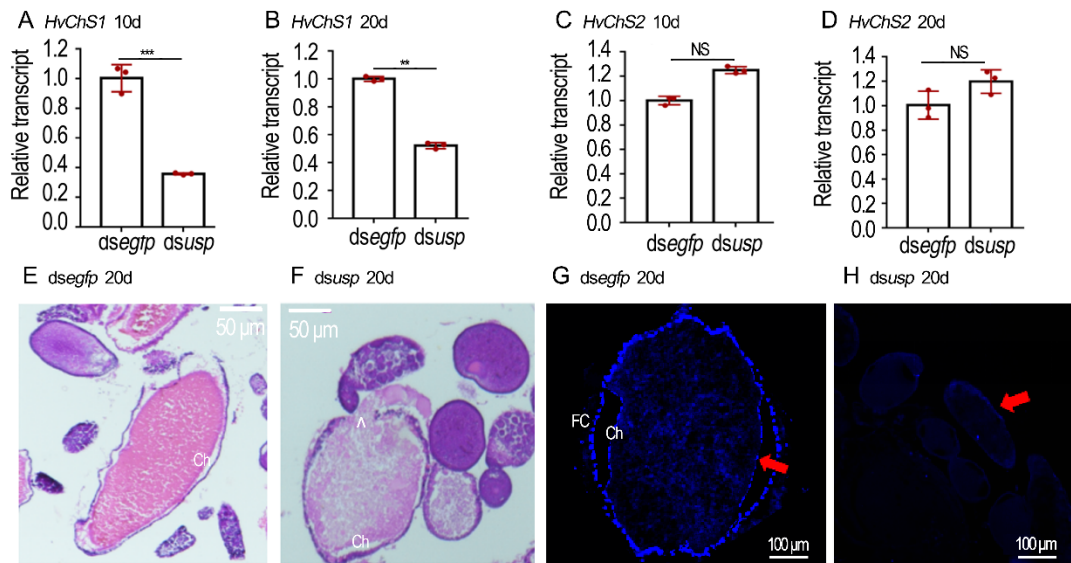
**Figure 4.** RNAi of HvEcR represses choriogenesis in *Henosepilachna vigintioctopunctata*. Ten and twenty days post injection of dsegfp and dsHvEcR, the ovaries of resultant females were dissected. The expression levels of HvChS1 and HvChS2 from 10- and 20-day-old ovaries were determined using the  $2^{-\Delta\Delta CT}$  method (A-D). Relative transcripts are the ratios of relative copy numbers in treated individuals to dsegfp controls, which are set as 1. The columns represent averages with vertical lines indicating SD. Different stars indicate significant difference at p value < 0.05 (\*), 0.01 (\*\*), 0.001 (\*\*\*) or 0.0001 (\*\*\*\*) using t test. NS, no significance. The paraffin sections of ovaries from 20-day-old treated and control female adults were treated with hematoxylin-eosin staining (E and F), or dyed with Calcofluor-White and 10% KOH (G-J). The images were showed. Blue color in G to J (shown with red arrow) marks chitin layer.

### 2.5. Silence of Hvusp Blocked CLS Deposition on *H. vigintioctopunctata* Oocytes

CLS deposition on the Hvusp RNAi *H. vigintioctopunctata* oocytes was also compared (Figure 5). The expression levels of HvChS1 were significantly diminished in the 10- and 20-day-old Hvusp silenced ovaries, compared with these in the control specimens (Figures 5A, 5B). In contrast, the transcript levels of HvChS2 varied little between Hvusp RNAi and control samples (Figures 5C, 5D).

From HE stained sections, it was easily noted that the normal maturing eggs had smooth and continuous chorions (Ch) (Figures 5E). Conversely, the egg shells in the Hvusp RNAi eggs were irregular and discontinuous. Some yolk substances were seeped out (^) (Figures 5F).

The fluorescence images of frozen sections stained with Calcofluor exhibited two layers of positively-stained membranes (↑) in the follicle cells (FC) and chorion (Ch) (Figure 5G). Conversely, the two positively-stained layers were almost disappeared in the dsHvusp treated ovarian specimens (Figure 5H).



**Figure 5.** Depletion of Hvusp blocks choriogenesis in *Henosepilachna vigintioctopunctata*. Ten and twenty days post injection of dsegfp and dsHvusp, the ovaries of resultant females were dissected. The expression levels of HvChS1 and HvChS2 from 10- and 20-day-old ovaries were determined using the  $2^{-\Delta\Delta CT}$  method (A-D). Relative transcripts are the ratios of relative copy numbers in treated individuals to dsegfp controls, which is set as 1. The columns represent averages with vertical lines indicating SD. Different stars indicate significant difference at p value < 0.05 (\*), 0.01 (\*\*) or 0.001 (\*\*\*) using t test. NS, no significance. The paraffin sections of ovaries from 20-day-old treated and control female adults were treated with hematoxylin-eosin staining (E and F), or dyed with Calcofluor-White and 10% KOH (G, H). The images were showed. Blue color in G and H (shown with red arrow) marks chitin layer.

### 2.6. Shortage of 20E Signal Prevents CLS Contents in *L. decemlineata* Ovaries

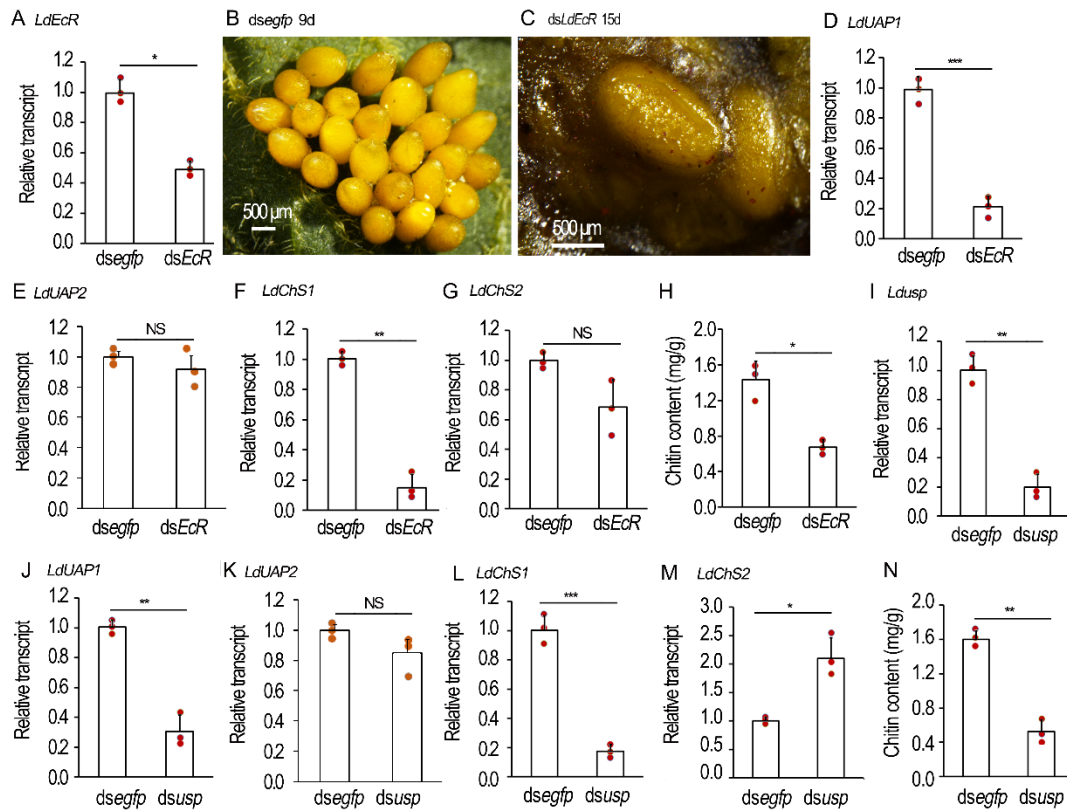
In the newly-eclosed *L. decemlineata* females, we repeated the RNAi experiments and examined the defective phenotypes (Figure 6).

Three days after dsLdEcR or dsLdusp injection, the target genes LdEcR (Figure 6A) and Ldusp (Figure 6I) in the ovaries were respectively silenced.

When the 9-day-old control *L. decemlineata* females normally oviposited (Figure 6B), the 15-day-old LdEcR and Ldusp RNAi females laid turbid liquid, along with a few deformed eggs (Figure 6C). These misshapen eggs had shrunk eggshells and wet surface (Figure 6C).

The expression of LdUAP1, LdUAP2, LdChS1 and LdChS2 in the 10-day-old ovaries was determined. The transcript levels of LdUAP1 and LdChS1, but not LdUAP2 and LdChS2, were significantly lowered in the LdEcR (Figure 6D-6G) or Ldusp (Figure 6J-6M) RNAi ovaries.

The chitin contents in the 10-day-old LdEcR (Figure 6H) or Ldusp (Figure 6N) RNAi ovaries were tested. As expected, the chitin amounts were greatly declined in the treated ovaries when evaluated by N-acetylglucosamine (GlcNAc) concentrations using the chitinase degraded method, compared with control specimens.

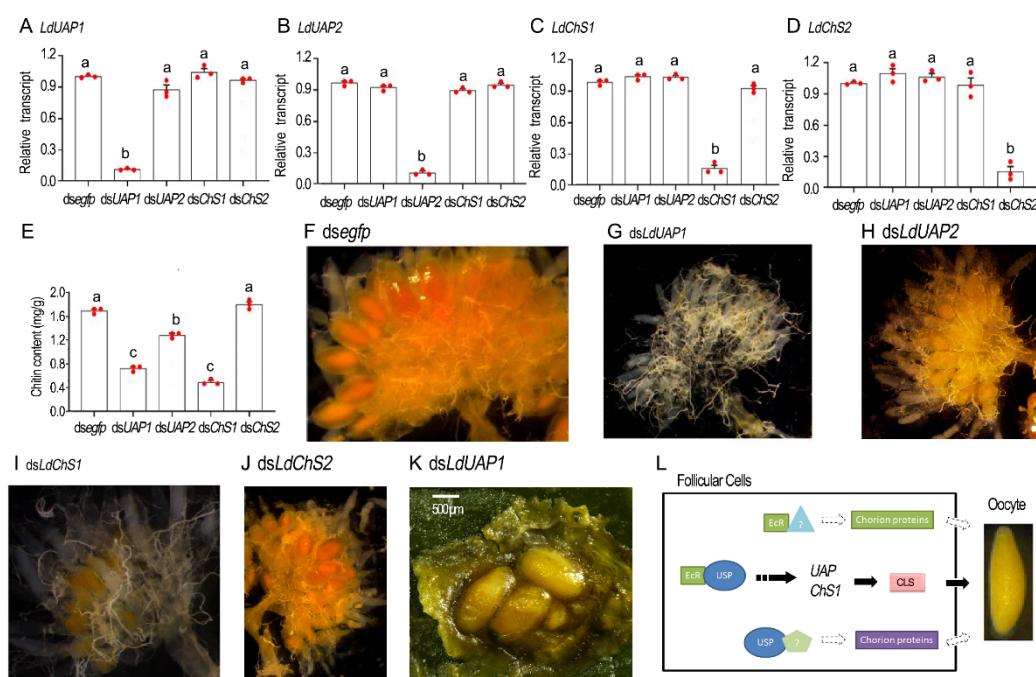


**Figure 6.** RNAi of LdEcR or Ldusp in the 0-day-old *Leptinotarsa decemlineata* female adults. Two biologically independent experiments were carried out using different generations, with two treatments: (1) dsegfp and (2) dsLdEcR or dsLdusp. The treated beetles were fed on fresh potato foliage. Three or ten days days after treatment, transcript levels of LdEcR, LdUAP1, LdUAP2, LdChS1 and LdChS2 (A, D-G), or Ldusp, LdUAP1, LdUAP2, LdChS1 and LdChS2 (I-M) in the ovaries were determined. Relative transcripts are the ratios of relative copy numbers in treated individuals to dsegfp-treated controls, which are set as 1. The chitin contents of the 10-day-old ovaries were measured by N-acetylglucosamine (GlcNAc) concentrations using the chitinase degraded method (H, N). The columns represent averages with vertical lines indicating SD. Different stars indicate significant difference at p value < 0.05 (\*), 0.01 (\*\*), or 0.001 (\*\*\*) using t test. NS, no significance. The oviposition was observed 0 to 15 days post eclosion (B, C).

### 2.7. Chitin Deficiency Causes Similar Defects on *L. decemlineata* Oocytes

To determine whether chitin shortage causes similar defects on oocytes to those in the LdEcR or Ldusp RNAi ovaries, we knocked down LdUAP1, LdUAP2, LdChS1, or LdChS2 by injected corresponding dsRNA into 4-day-old female adults (Figure 7A-7D). Introduction of dsLdUAP1, dsLdUAP2, dsLdChS1, or dsLdChS2 significantly lowered its target mRNA when detected 3 days post treatment, but displayed little influences on non-target transcripts (Figure 7A-7D).

Depletion of LdUAP1 and LdChS1 significantly reduced chitin contents in the ovaries when measured with GlcNAc concentrations using the chitinase degraded method, in contrast to control, and LdUAP2 and LdChS2 RNAi specimens (Figure 7E). Moreover, no mature oocytes were seen in 10-day-old LdUAP1 and LdChS1 RNAi ovaries, in contrast to the control, and LdUAP2 and LdChS2 RNAi samples (Figure 7G, 7I versus 7F, 7H, 7J). Twenty days after treatment, the LdUAP1 and LdChS1 RNAi female adults began to lay turbid liquid, along with a few deformed eggs (Figure 7K).



**Figure 7.** Knockdown of chitin biosynthesis genes in the 4-day-old *Leptinotarsa decemlineata* female adults. The 4-day-old female adults were injected with 0.1  $\mu$ L dseGfp, dsLdUAP1, dsLdUAP2, dsLdChS1 or dsLdChS2 (400 ng). The treated beetles were fed on fresh potato foliage. Three days after treatment, transcript levels of LdUAP1, LdUAP2, LdChS1 and LdChS2 were determined (A-D). Relative transcripts are the ratios of relative copy numbers in treated individuals to dseGfp-treated controls, which are set as 1. The chitin contents of the 10-day-old ovaries were measured by N-acetylglucosamine (GlcNAc) concentrations using the chitinase degraded method (E). The columns represent averages with vertical lines indicating SD. Different stars indicate significant difference at p value < 0.05 (\*), 0.01 (\*\*), or 0.001 (\*\*\*) using t test. NS, no significance. The 10-day-old ovaries were shown (F-J). The oviposition was observed 0 to 20 days post eclosion (K). A presumptive model of the molecular regulation of 20E signal for choriogenesis in the two Coleopteran species (L). EcR/USP complex activates the expression of chitin biosynthesis genes, such as UAP and ChS1, or may indirectly acts on these genes, to regulate the supply of chitin-like substance (CLS). Meanwhile, EcR and USP may respectively regulate a specific subset of chorion protein genes, dependent or independent of EcR/USP complex, to synthesize constructive proteins for the formation of eggshells (L).

### 3. Discussion

#### 3.1. 20E Signal Triggers Choriogenesis

After initiation of choriogenesis, chorion proteins and other structural components are deposited on eggshells. Whether 20E signal activates choriogenesis remains an opening question [9].

In the present paper, four pieces of experiment evidence were attained to indicate that 20E signal initiates choriogenesis in the two Coleopterans. Firstly, both EcR and usp were actively expressed immediately after adult eclosion. The accumulated EcR and USP proteins may mediate 20E signaling and activate oogenesis. As a result, oocytes are continuously matured and the oviposition persists in mature *H. vigintioctopunctata* females [21]. Likewise, ecdysone response gene transcripts (E75, E74, BrC, HR3, HR4, and FTZ-F1) are mostly upregulated in the ovary during the first three days post blood meal in *Rhodnius prolixus* [24].

Secondly, our data showed that the EcR and usp transcripts were present in the ovaries in both *H. vigintioctopunctata* (Figure 1, 2, 3) and *L. decemlineata* (Figure 1, 6) females, similar to those in *R. prolixus* [24,25].

Thirdly, knockdown of either EcR or usp significantly repressed oviposition in both *H. vigintioctopunctata* (Figure 2, 3) and *L. decemlineata* (Figure 6) females. In accordance with our data, knockdown of EcR, usp, E75, E74 or FTZ-F1 decreases mature eggs, and severely reduces the laid eggs and their hatching rate in *R. prolixus* [24,25]. The reduced oviposition in 20E signal repressed females has also been documented in *T. castaneum* [7], *D. melanogaster* [26], *Nilaparvata lugens* [27,28], and *Cimex lectularius* [29].

Lastly, we discovered that RNAi of either EcR or usp destroyed the structure of eggshells and deformed oocyte shapes. Consistently, some of the eggs have an irregular shape and smaller volume laid by the EcR, usp, E75, E74 or FTZ-F1 RNAi *R. prolixus* females [24,25]. The deformation of chorion structure and oocyte shape gives a solid piece of experiment evidence that 20E cascade triggers choriogenesis, in addition to activation of vitellogenesis in both *H. vigintioctopunctata* and *L. decemlineata* females [22].

### 3.2. 20E Signal Triggers the Production of a CLS

In the current article, our findings suggest that 20E signal triggers the production of a chitin-like substance (CLS), a component critical for the formation of oocyte chorion during choriogenesis in the two Coleoptera species.

Firstly, UAP and ChA catalyze the last two biochemical steps for chitin biosynthesis in *L. decemlineata* [16,17] and *H. vigintioctopunctata* [14,15]. In the present paper, expression analysis showed that their encoding genes were actively expressed in the adult ovaries (Figure 1, 4, 5, 6, 7). Moreover, the expression of either UAP or ChS is strictly coordinated with the transcription of 20E receptor gene EcR in both Coleopterans (Figure 1). Similar expression patterns between chitin biosynthesis and 20E cascade genes have widely been documented during larval development in representative insect species [17–20].

Secondly, biochemical analysis demonstrated that the CLS was present in *L. decemlineata* ovaries (Figure 6), similar to those on *A. aegypti* eggshells [13] and in *R. prolixus* ovaries [30]. Moreover, the Calcofluor stain [23] showed that two layers of CLS were observed in *H. vigintioctopunctata* ovaries, one lining the follicle cells and the other in the eggshells (Figure 4, 5). Follicle cells are ectodermally-derived epidermal tissue. Thus, our data are in agreement with the common notion that ChS1 locates in the ectodermally-derived epidermal cells while ChS2 in midgut cells [14–17].

Thirdly, our findings showed that knockdown of either EcR or usp significantly lowered the expression levels of UAP and ChS1, but not ChS2, in the two Coleopterans (Figure 4, 5, 6). Consistently, suppression of 20E signal reduced the CLS contents in *L. decemlineata* ovaries (Figure 6) and decreased the positive reaction to Calcofluor stain in *H. vigintioctopunctata* follicle cells and eggshells (Figure 4, 5). Similarly, a CLS has been reported to be present in oocytes in a Diptera species [13]. Recently, a specific mucin protein has been documented to contribute to the formation of eggshell in *N. lugens* [31,32] and *Spodoptera exigua* [33]. Moreover, enzymes involving in chorion tanning are reported in *D. melanogaster* [34,35] and *N. lugens* [36] eggshells. Given that intestinal mucins exhibit strong association with chitin [37] and tanning process involves the oxidative conjugation and cross-linking of cuticular proteins and chitin by quinones [38], it is reasonably assumed that many insect eggshells contains chitin. Further researches will shed light on this issue.

Fourthly, knockdown of LdUAP1 and LdChS1, rather than LdUAP2 and LdChS2, caused similar defects on *L. decemlineata* oocytes to those in the EcR or usp RNAi beetles (Figure 7). We accordingly propose that dysfunction of EcR or USP inhibits CLS accumulation on the eggshells in *L. decemlineata*. As a result, the structure of eggshells is destroyed and the oocyte shapes are deformed (Figure 2, 3, 6). Interestingly, dsChS treatment affects oviposition in *R. prolixus* [30]. Knockdown of ChS1 disrupts adult fecundity in *Aphis gossypii* [39]. Moreover, dietary introduction of chitin synthesis inhibitors declines fecundity in *A. aegypti* [13]. In addition, RNAi of LsChS1 in pre-adult I results in deformation of ovaries and oocytes in adult *Lepeophtheirus salmonis* [40]. This result suggests the likelihood that CLS may also be present in eggshells and deficiency of CLS causes misshapen eggs in other arthropods. Nevertheless, whether 20E regulation of CLS deposition on

oocyte chorion is a widely conservative way among arthropods deserves further investigation to determine.

In the current paper, we injected dsLdUAP1 and dsLdChS1 into 4-day-old female adults, the target mRNAs were significantly reduced three days after treatment (Figure 7). Our previous observation showed that some oocytes have completed vitellogenesis and the patency among the follicle epithelium cells was closed in 5- to 7-day-old *H. vigintioctopunctata* females [21], similar to the oocyte maturation processes in other Coleopterans [2]. Moreover, after vitellogenesis oocytes are often arrested in prophase I of meiosis, showing very modest synthetic activity [41]. Therefore, it appears that only follicle epithelium cells can actively generate CLS in the 5- to 7-day-old *H. vigintioctopunctata* females and only the CLS biosynthesized by follicle epithelium cells can deposit on eggshells. Our findings support a common notion that the chorion is formed by the surrounding follicular epithelial cells during oocyte maturation [1].

### 3.3. Does 20E Signal Tune the Production of Chorion Proteins?

Comparison of the data in the current survey displays two dissimilarities. Firstly, even though inhibition of chitin deposition affected eggshell structure in the two Coleopterans after disruption of 20E signal, the defects of matured eggs are different. *L. decemlineata* eggs in treated females looked normal and could be deposited (Figure 6, 7), whereas *H. vigintioctopunctata* eggs were seriously deformed and failed to lay out (Figure 2, 3). Secondly, within *H. vigintioctopunctata* the influence of chitin deposition was more serious in the *usp* RNAi females (Figure 3), whereas the destruction of chorion and the delay of oviposition were more severe in the *EcR* RNAi females (Figure 2). The two dissimilarities suggest that 20E signal may also tune the production of chorion proteins. In accordance with the suggestion, *EcRB1* isoform and *USP* are responsible for 20E-mediated transcriptional activation of the eggshell gene *VM32E*, which produces a protein component of the vitelline membrane and endochorion layers [42]. Similarly, silence of *EcR*, *usp*, or ecdysone biosynthesis gene *shade* significantly reduces the expression of chorion protein gene transcripts (*Rp30* and *Rp45*) in *R. prolixus* [25].

If 20E signal stimulates the production of chorion proteins, as suggested above, the difference of eggshell phenotypic defects between *EcR* and *usp* RNAi females implies that *EcR* and *USP* respectively regulate a specific subset of chorion protein genes, dependent or independent of *EcR/USP* complex (Figure 7L). In this context, both *EcR* and *USP* exert specific atypical function independent of *EcR/USP* complex. For *EcR*, it may dimerize with other partners, form homodimers [43] and/or form protein complexes that have yet to be described [44]. In *D. melanogaster* male adults, for example, *EcR*-depleted (but not *USP*-depleted) accessory glands fail to make seminal proteins and have dying cells. The active receptor may be a homodimer [45]. A similar paradigm is observed in the expression of genes encoding glue proteins in the salivary glands [44]. Interestingly, *EcR* apparently does not require *USP* as a heterodimeric partner in scorpions [46]. As for *USP*, it is able to form homomeric complexes in living cells [43]. It is also bound to other nuclear receptors [47]. In fact, some functions are independent of *EcR* have been explored in *D. melanogaster* and *A. aegypti* *USPs* [47,48]. In *A. aegypti*, for instance, the interaction of *Svp* and *USP* rather than binding competition for the *Vg* ecdysteroid response element accounts for the inhibition of *Vg* expression after a batch of eggs is laid [49]. This issue deserves further investigations.

## 4. Materials and Methods

### 4.1. Insect Rearing

Both *H. vigintioctopunctata* and *L. decemlineata* beetles were cultured in laboratory at  $28 \pm 1^\circ\text{C}$ , 16 h:8 h light–dark photoperiod and 50–60% relative humidity conditions, using the fresh potato leaves, using foliage at the vegetative growth or young tuber stages from potato plants (*Solanum tuberosum* L) growing in insecticide-free soil. Under this feeding protocol, the duration from laying eggs to emergence adults of the next generation was about one month.

#### 4.2. Synthesis of dsRNAs

For *H. vigintioctopunctata*, a highly efficient and specific siRNA fragment originated from HvEcR or Hvusp was selected using the siRNA online design website (<http://sidirect2.rnai.jp/>). Then, a pair of primers was designed using the software primer premier 5.0 (Table S1) to amplify a cDNA including the siRNA fragment. The targeted cDNA sequence was further BLASTN searched against the *H. vigintioctopunctata* transcriptome data to identify any possible off-target sequences that had an identical match of 20 bp or more. For *L. decemlineata*, the same dsLdEcR and dsLdusp were used [50,51]. Moreover, a cDNA fragment was derived from enhanced green fluorescent protein (egfp) in *Aequorea victoria*. These fragments were respectively amplified by PCR using specific primers (Table S1) conjugated with the T7 RNA polymerase promoter. The dsRNAs were synthesized using the MEGAscript T7 High Yield Transcription Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Subsequently, the synthesized dsRNA (at a concentration of 5-8 µg/µl) was determined by agarose gel electrophoresis and the Nanodrop 1000 spectrophotometer (data not shown) and kept at -80°C until use.

#### 4.3. Injection of dsRNAs

The same method described previously was used to inject dsRNA into 0-day-old *H. vigintioctopunctata* and *L. decemlineata* female adults [52,53]. Briefly, an aliquot (0.1 µl) of the solution including 400 ng dsRNA was injected into the newly emerged female adults. Negative control newly emerged female adults were injected with the same volume of dsegfp solution.

For 0-day-old *H. vigintioctopunctata* female adults, two biologically independent experiments were carried out using different generations, each bioassay setting two treatments: (1) dsegfp and (2) dsHvEcR or dsHvusp. A group of 10 injected newly emerged female adults was set as a replicate. The resultant newly emerged female adults were allowed to feed potato foliage until the death. Each treatment had 24 replicates. A total of 9 replicates were harvested 3, 10 and 20 days after treatment to dissect their ovaries. Three replicates were sampled 3 days after injection for qRT-PCR to test RNAi efficacy in the ovaries. The other 6 replicates were used to measure the expression levels of genes involved in chitin biosynthesis (*HvChS1* and *HvChS2*) in the ovaries. Three replicates were used to record oviposition, to measure body size and to detect fresh weight. Another 12 replicates were collected, dissected for observation under a microscope or stained using HE or Calcofluor 10, 20, 30 or 40 days after injection.

For 0-day-old *L. decemlineata* female adults, two biologically independent experiments were carried out using different generations, with two treatments: (1) dsegfp and (2) dsLdEcR or dsLdusp. A group of 10 injected newly emerged female adults was set as a replicate. The resultant newly emerged female adults were allowed to feed potato foliage until the death. Each treatment had 12 replicates. A total of 6 replicates were harvested 3 and 10 days after treatment to dissect their ovaries. Three replicates were sampled 3 days after injection for qRT-PCR to test RNAi efficacy in the ovaries. Three replicates at the age of 10 days old were used to measure the expression levels of genes involved in chitin biosynthesis (*LdUAP1*, *LdUAP2*, *LdChS1* and *LdChS2*) in the ovaries. Three replicates were used to record oviposition. Another three replicates were collected 10 days after injection, dissected the ovaries to determine chitin content.

For 4-day-old *L. decemlineata* female adults, a bioassay was performed with five treatments: (1) dsegfp, (2) dsLdUAP1, (3) dsLdUAP2, (4) dsLdChS1 and (3) dsLdChS2. A group of 10 injected female adults was set as a replicate, and were allowed to feed potato foliage. Each treatment had 12 replicates. Three replicates were harvested 3 days after treatment for qRT-PCR to test RNAi efficacy, and for measurement of the expression levels of genes involved in chitin biosynthesis (*LdUAP1*, *LdUAP2*, *LdChS1* and *LdChS2*) in the ovaries. Three replicates were used to record oviposition. Another six replicates were collected 10 days after injection, dissected the ovaries, and determined chitin content in the ovaries.

#### 4.4. Quantitative Real-Time PCR

For temporal expression analysis, RNA templates were derived from 0-9 and 0-13 days old in *L. decemlineata* and *H. vigintioctopunctata* female adults respectively. Each sample contained 20-30 individuals and was repeated three times. For analysis of the effects of treatments, total RNA was extracted from treated female adults. Each sample contained 10 individuals and was repeated three times. The RNA was extracted using SV Total RNA Isolation System Kit (Promega, USA). Purified RNA was subjected to DNase I to remove any residual DNA according to the manufacturer's instructions. Quantitative mRNA measurements were performed by qRT-PCR in technical triplicate, using 2 stably-expressed internal control genes (for *L. decemlineata*, *LdRP18* and *LdRP4*; for *H. vigintioctopunctata*, *HvRPS18* and *HvRPL13*; the primers listed in Table S1) according to the published results [54,55]. An RT negative control (without reverse transcriptase) and a non-template negative control were included for each primer set to confirm the absence of genomic DNA and to check for primer-dimer or contamination in the reactions, respectively. The primer pair for each gene was tested with a 5-fold logarithmic dilution of a cDNA mixture to generate a linear standard curve (crossing point plotted vs. log of template concentration), which was used to calculate the primer pair efficiency.

All primer pairs amplified a single PCR product with the expected sizes, showed a slope less than  $-3.0$  and exhibited efficiency values ranging from 2.4 to 2.7. Data were analyzed by the  $2^{-\Delta\Delta CT}$  method, using the geometric mean of the four internal control genes for normalization.

#### 4.5. Hematoxylin-Eosin (HE) Staining

HE staining was performed to observe defective phenotypes in *H. vigintioctopunctata* ovaries. Briefly, the ovaries in the *dsefp*- and *dsHousp*-treated larvae were dissected 10, 20 and 30 days after the initiation of bioassay, and were then fixed in 4% paraformaldehyde and embedded in paraffin. The two embedded tissues were subsequently cut into 6- $\mu$ m thick sections. The sections were stained using Mayer's H&E (Yeasen, Shanghai, China) following a routine staining procedure and observed with an Olympus BH-2 light microscope (Olympus, Tokyo, Japan).

#### 4.6. Chitin Staining with CALCOFLUOR

The *H. vigintioctopunctata* ovary sections prepared above were also stained with Calcofluor according to a previously described method [23]. Briefly, section preparations were incubated in 0.001% Calcofluor in 100 mM Tris-HCl, pH 9 for 30 min at room temperature. After washing off the excess Calcofluor, the fluorescence was recorded using light of appropriate excitation and emission wavelengths.

#### 4.7. Chitin Analysis

The same method as described [16,17] was used to test chitin contents in 10-day-old *L. decemlineata* ovaries. Briefly, the samples were individually mixed with 0.5 g zirconium beads (0.7 mm diameter, BioSpec Products, Bartlesville, OK) and 0.5 ml 6% KOH, and were homogenized. The homogenates were then heated at 80 °C for 90 min, and were centrifuged at 12000 g for 20 min and the supernatants were removed. The pellets were individually suspended in 1 ml PBS, and centrifuged again at 12000 g for 20 min and the PBSs were discarded. Each pellet was then resuspended in 200  $\mu$ l McIlvaine's buffer (0.1 M citric acid, 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, pH 6) and 5  $\mu$ l of *Streptomyces plicatus* chitinase-63 (5 mg/ml in PBS) was added to hydrolyze chitin to GlcNAc by incubation for 40 h at 37 °C.

GlcNAc concentrations were individually measured by a modified Morgan-Elson assay[56]. In a 0.2 ml PCR tube, 10  $\mu$ l 0.27 M sodium borate and 10  $\mu$ l of sample supernatant (12000 g, 1 min centrifugation) were combined. In a thermocycler, samples were heated to 99.9 °C for about 60 s, mixed gently, and incubated at 99.9 °C for 10 min. Immediately upon cooling to room temperature, 100  $\mu$ l of diluted dimethylaminobenzaldehyde (DMAB) solution (10% w/v DMAB in 12.5 ml concentrated HCl and 87.5 ml glacial acetic acid stock, diluted 1 : 10 with glacial acetic acid) was

added, followed by incubation at 37 °C for 20 min. Eighty µl of each sample was transferred to 96-well low-evaporation microtitre dishes, and the absorbance at 585 nm was recorded. Standard curves were prepared from stocks of 0.075-2.0 mM GlcNAc.

#### 4.8. Data Analysis

The SPSS for Windows (Chicago, IL, USA) was used for statistical analyses. The averages ( $\pm$ SE) were compared using t-test.

### 5. Conclusions

Accordingly, a presumptive model is proposed for summarization of the regulation of 20E signal to chorionogenesis in two representative Coleoptera species (Figure 7L). Our findings highlighted that 20E cascade triggers the deposition of chorion chemical components on the developing oocytes. Moreover, we uncovered that a CLS is critical for oocyte maturation. Our research provides a solid foundation for future studies on hormone regulation of reproduction in beetles.

**Supplementary Materials:** The following supporting information can be downloaded at: [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Table S1: Primers used in dsRNA synthesis and qRT-PCR.

**Author Contributions:** G.Q.L. developed the concepts. X.J.Z., L.J., K.Y.F. and W.C.G. performed the experiments. X.J.Z., K.Y.F. and L.J. analyzed the data. G.Q.L. and X.J.Z. discussed and wrote the manuscript.

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