

The mechanism of sublethal chlorantraniliprole exposure causing silkworm pupation metamorphosis defects

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Abstract

BACKGROUND: Chlorantraniliprole (CAP) is widely used in agriculture and forestry to prevent and control pests. The effects of environmental CAP residue on non-target insect metamorphosis have not been reported. Our research aimed to investigate the sublethal effect of CAP on larva–pupa transformation in silkworm, and explore the mechanism of sublethal CAP exposure-mediated pupation metamorphosis defects.

RESULT: Sublethal CAP exposure affected the growth and development of silkworm larvae and caused defects in pupation metamorphosis. After CAP exposure, formation of prepupa procuticle, ecdysial membrane and new epidermis was inhibited. Also, the level of 20-hydroxyecdysone (20E) and mRNA levels of the 20E signaling pathway-related genes *EcR*, *USP*, *E74*, *E75* and *Ftz-f1* were significantly reduced. Moreover, genes involved in chitin synthesis, such as *ChsA*, *CDA1* and *CDA2*, were downregulated. Injection of 20E led to the upregulation of chitin synthesis-related genes and increased formation of new epidermis in CAP-treated silkworm. However, injection of 20E failed to prevent downregulation of *Ftz-f1* and the defects in pupation metamorphosis.

CONCLUSION: Our results suggested that 20E is a target hormone of CAP exposure-mediated epidermis formation phenotype. *Ftz-f1* was silenced by CAP and might be a direct target gene of sublethal CAP exposure. Our study provided new evidence of the effects of sublethal CAP exposure on insect development and metamorphosis.

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Keywords: *Bombyx mori*; chlorantraniliprole; metamorphosis defects; 20E; *Ftz-f1*; chitin

1 INTRODUCTION

The anthranilic diamides chlorantraniliprole (CAP) and cyantraniliprole, and the phthalic diamide flubendiamide belong to a novel class of insecticides that activate ryanodine receptors to release and deplete intracellular Ca^{2+} .¹ These insecticides can lead to feeding cessation, lethargy and muscle paralysis, and cause the death of target insects including lepidopteran, coleopteran, dipteran and hemipteran pests.^{2–4} CAP has a low degradation rate in the environment and its half-life in soil is 16.0 days.⁵ Low-dose CAP treatment did not result in immediate death, but led to abnormal development of pupae in *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) (0.001 mg L^{-1}),⁴ and could significantly reduce adult fecundity and offspring survival rates in *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) Quarter lethal concentration (LC_{25}).^{6,7} Recently, a commercialized formulation of CAP has been registered and widely used in China.⁸ Improper and extensive use of insecticide not only pollutes cultivated soil and groundwater, but also leads to drug accumulation in aquatic plants, resulting in sublethal exposure.^{9–11} Moreover, pesticides, particularly insecticides, can also affect non-target organisms such as predators of the target pest, economic insects

and beneficial organisms with sublethal effects.¹² These sublethal effects include disruption of molting and cuticle formation, reduction in adult longevity and fecundity, sex ratio change, and population decline.^{13,14} CAP can reduce tibia length in *Macropsophus basicornis* (Stål) (Hemiptera: Miridae),^{15,16} influence the number of eggs laid and change the sex ratio of *Bracon nigricans* (Szépligeti) (Hymenoptera: Braconidae) offspring.^{17,18} However, the mechanism behind the sublethal effect of CAP residue on insect pupation metamorphosis has not been reported.

Bombyx mori (Linnaeus) (Lepidoptera: Bombycidae) is not only an important economic insect in China, with a history of silk

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production of > 5000 years, but also a model organism of Lepidoptera, the second biggest order of insects. Because of its relatively clear genetic background and complete genomic information, the species is a good organism for studying insect development and metamorphosis.^{19,20} The fat body is the central organ integrating and coordinating different hormonal signals for regulating insect development and metamorphosis.²¹ Silkworm growth and development are mainly regulated by juvenile hormone and 20-hydroxyecdysone (20E). A 20E signal can initiate major developmental transitions in insects, including larval–larval molting and larval–pupal–adult metamorphosis.^{22,23} The level of 20E increases before molting and pupation metamorphosis, leading to upregulation of 20E response genes.²⁴ The ecdysone receptor (EcR) and ultraspiracle (USP) form the functional nuclear receptor complex of 20E. After binding to EcR–USP, 20E can activate a small set of early response genes encoding several transcription factors, which further activate a large set of late response genes.²³ Ecdysone-induced *E74*, *E75* and *Ftz-f1* are thought to function as transcription factors and play essential roles in initiating the 20E-induced gene expression cascade.^{24–26} *Ftz-f1* functions as a ligand-dependent transcription factor and is involved in diverse biological processes in *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae).²⁶ Knockdown of *BmβFTZ-F1* in *B. mori* results in abnormalities in larva to pupa transition.²⁷

Insect metamorphosis includes formation of a new epidermis and separation of the old epidermis. Chitin is the main component of insect epidermis, and its production involves the dynamic balance between chitin synthase (CHS) and chitin degradation enzyme. In *D. melanogaster*, the *ChsA* gene encodes chitin in epidermis.^{28,29} Chitin deacetylase (CDA) is a hydrolytic enzyme that catalyzes hydrolysis of the acetamido group in *N*-acetylglucosamine units of chitin and chitosan, thus generating glucosamine and acetic acid.³⁰ CDA results in proper deacetylation of chitin microfilaments in the assembly area to help chitin fold correctly.³¹ Inhibition of chitin synthetase can prevent the synthesis of chitin in both larval and adult insects of *Stomoxys calcitrans* (Linnaeus) (Diptera: Muscidae).³² In *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), knockdown of either *CDA1* or *CDA2* affects all types of molts, including larval–larval, larval–pupal and pupal–adult.³³ Chitin synthase and CDA are the key regulatory enzymes for chitin synthesis and excretion in insects. Thus, these insect growth regulators are also specific targets of insecticides that can inhibit the synthesis of chitin such as diflufenuron and lufenuron.^{33–35}

In this study, we investigated the developmental and metamorphosis effects of sublethal CAP exposure in silkworm larvae. We also analyzed the underlying mechanisms from the aspects of 20E signaling pathway and chitin synthesis. This study provides a reference for safety evaluation of sublethal CAP exposure in silkworm.

2 MATERIALS AND METHODS

2.1 Insect strains and chemicals

Larvae of *B. mori* (Jingsong × Haoyue) were stocked in our laboratory, the National Engineering Laboratory for Modern Silk, Soochow University. Larvae were fed fresh mulberry (60 larvae/box) three times per day in a plastic box (45 × 31 × 16 cm) (Lock & Lock, Seoul, Korea), at 25 ± 1 °C, 12:12 h light/dark photoperiod, and 75 ± 5% relative humidity. CAP (20% SC, Rynaxypyr™, Dupont, Shanghai, China) was purchased from Shanghai Shengnong Biochemical Products Co., Ltd. 20E (95%, reagent grade) was purchased from Sangon Biological Technology and Services Co., Ltd (Shanghai, China).

2.2 CAP exposure and 20E rescue experiment

CAP was delivered via the leaf-dipping method as described previously.³⁶ The sublethal concentration (0.01 mg L^{−1}) was determined by preliminary experiments, which ensured 100% survival after 24 h CAP feeding of the fifth-instar larvae.³⁶ CAP was dissolved in double-distilled (dd)H₂O (200 mg L^{−1}) to form the stock solution and the working solution was prepared by diluting the stock solution with ddH₂O.^{36,37} Silkworm larvae at the third day of fifth instar were randomly selected for CAP treatment. The treatment group was fed sublethal CAP-treated mulberry leaves, and the control group was fed ddH₂O-treated leaves. There were three replicates in each group. After 24 h of feeding, CAP-treated insects were fed the same mulberry leaves as the control group. Twenty insects per replicate were collected to measure the body weight, cocooning rate and good pupa rate. 20E was dissolved in dimethyl sulfoxide to a final concentration of 0.4 μg μL^{−1}. Thirty CAP-treated insects (ten per replicate) were injected with 20E solution (5 μL per larva) through the intersegmental membrane using a micro-injector at prepupa stage (192 h after CAP exposure).

2.3 Sample preparation

Silkworms from different treatments were dissected on ice at the prepupa stage (216 h after CAP exposure). Hemolymph, fat body and epidermis were collected from different treatment conditions and stored at −80 °C. Hemolymph specimens were used for measuring 20E levels. Fat body specimens were used for quantifying the transcription level of 20E signaling pathway-related genes. Epidermis specimens were collected for histopathological examination, scanning electron microscopy (SEM) and quantification of transcription level of chitin synthesis-related genes.

2.4 ELISA analysis of ecdysone

The ecdysone level in hemolymph was determined by an enzyme-linked immunosorbent assay (ELISA; Meimian, Nanjing, China). The antibody was detected using a horseradish peroxidase chromogenic assay. After incubation with the substrate 3,3',5,5'-tetramethylbenzidine, ecdysone content was measured by optical density at 450 nm. The final ecdysone level was presented as the mean ± SD of three replicates. All procedures were performed according to the manufacturer's instructions.

2.5 RNA extraction and cDNA synthesis

Total RNA was extracted from fat body and epidermis specimens using TRIzol reagent (Takara, Dalian, China), followed by DNase treatment to remove genomic DNA contamination. RNA was quantified by NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and its quality was assessed by agarose gel (1.5%) electrophoresis. First-strand cDNA was synthesized with M-MLV reverse transcriptase and an oligo(dT) primer (Takara) based on the manufacturer's instructions.

2.6 Quantitative RT-PCR

All the sequences were found in National Center for Biotechnology Information (NCBI). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) primers were designed using Primer 6.0 software (Premier Biosoft, Palo Alto, CA, USA), and synthesized by Sangon. The qRT-PCR primers used in this study are listed in Table 1. Actin-3 was used as the internal reference gene. Real-time qPCR was performed using the Viia 7 Real-time PCR System (Applied Biosystems, Foster, CA, USA) with SYBR Premix Ex Taq™ (Takara). The amplification cycle was as follows:

Table 1. Primer sequences used in quantitative RT-PCR

Gene name	NCBI gene ID	Prime sequences (5' to 3')	Length of product (bp)
<i>Actin-3</i>	100145915	F:CGGCTACTCGTTCACTACC R:CCGTCGGGAAGTTCGTAAG	147
<i>EcR</i>	692756	F:CGGCGAATCAGAAGTCTC R:TCCTCGTCTCTTCATCC	121
<i>USP</i>	693034	F:GTCGGTAACTGCGTTGAT R:GGTGTGGTTGAAGGTGTAG	126
<i>E74</i>	693011	F:CCTTCTACCAGCATCATCA R:CGCCGTAACCATATCCATA	111
<i>E75</i>	692595	F:ACCAGCAGTGTAGTATCCT R:GAATCGCACAGCATCTCT	101
<i>Ftz-f1</i>	693070	F:ATGGTCTGTTATGCTGGTT R:ATTGAAGTGGTCGGCTAAT	145
<i>ChsA</i>	100884166	F:ACAAGAGGCTCGCATAGCAG R:CCAGACCACGTGAAGCTGAT	136
<i>CDA1</i>	732885	F:TCAGTTGTGCGACGGTAGAC R:GGTGACGCTGTTCTCTAGGC	106
<i>CDA2</i>	732885	F:ACAAGATGACGACGGAGCTG R:ACCTGACCTAGTGCACCTGA	130

denaturation at 95 °C for 1 min, followed by 45 cycles of 95 °C for 5 s, 55 °C for 10 s, and 72 °C for 10 s. Each biological replicate had three technical replicates.

2.7 Histopathological examination

All histopathological examinations were performed following standard laboratory procedures. Epidermis was embedded in paraffin blocks, sliced into thin sections (5 µm), and placed on glass slides. After hematoxylin–eosin staining, the sections were examined blind by a pathologist using a fluorescence inverse microscope (Nikon eclipse TE2000-U, Nikon, Tokyo, Japan).

2.8 Scanning electron microscopy

A Hitachi S-4700 SEM (Hitachi, Tokyo, Japan) was used in this study. Prepupal epidermis specimens were cleaned and dried with critical point dryer (Labconco, Kansas, USA), and then mounted using double-sided tape on SEM stubs. After coating twice with gold in a polaron sputter-coater E-1010 (Hitachi), specimens were examined at an accelerating voltage of 10–15 kV. Photos were taken by SEM (Hitachi).

2.9 Statistical analysis

All the presented data were expressed as the mean \pm SE of three biological replicates. Growth curves were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test, other data were analyzed using SPSS 19.0 (SPSS, Chicago, IL, USA) with Student's *t*-test. $P < 0.05$ was considered statistically significant compared with the control. All figures were drawn using Origin 9.1E (Origin Lab, Northampton, MA, USA).

3 RESULTS

3.1 Effect of sublethal CAP on the pupation metamorphosis of silkworm

After 48 h of CAP treatment, the body weight of silkworm decreased significantly ($P < 0.05$), with average body weights at 48, 72, 96 and 120 h of 92.92% ($P < 0.05$), 88.47% ($P < 0.001$), 85.45% ($P < 0.001$) and 81.70% ($P < 0.001$) of the control group,

respectively (Fig. 1B; Table S1). After CAP treatment, the cocooning rate was decreased by 7.22% ($P < 0.05$) (Fig. 1C). Moreover, there were abnormal pupae (Fig. 1Af), and the good pupa rate was significantly reduced by 33.33% ($P < 0.01$) (Fig. 1C).

Pathological examination of epidermis at the prepupa stage showed that the epidermis and epithelial cells were separated, the cuticular protein and chitin fiber were accumulated in the procuticle with a certain thickness, and the old outer epidermis was dissolved in the control group (Fig. 2A (a,c)). We could also observe obvious ecdysial membrane during the prepupa period (Fig. 2Ac). Conversely, in the CAP exposure group, the epidermis and epithelial cells were stuck together, no obvious ecdysial membrane was formed at the prepupa stage, and formation of new epidermis and dissolution of the old epidermis were minimal (Fig. 2Ab,d). SEM results showed that, in the control group, development and formation of new epidermis at the prepupa stage were relatively complete and regular, and new cortex was clearly separated from old cortex (Fig. 2Ba). In the CAP exposure group, the formation of new epidermis was deficient, and separation between old and new epidermis was incomplete (Fig. 2Bb).

3.2 Effect of sublethal CAP on the transcription levels of 20E signaling pathway related genes

20E is an important endogenous hormone regulating development and metamorphosis. To study the mechanism of CAP exposure on silkworm development and metamorphosis, we performed qRT-PCR to detect the transcription levels of 20E-related genes in the fat body. The results showed that mRNA levels of *EcR*, *USP*, *E74*, *E75* and *Ftz-f1* in CAP-treated insects were reduced to 43.14% ($P < 0.05$), 10.26% ($P < 0.05$), 26.18% ($P < 0.05$), 64.59% ($P < 0.001$) and 29.05% ($P < 0.01$) of levels in the control group (Fig. 3).

3.3 Effect of sublethal CAP exposure on 20E content and the rescue experiment with 20E injection

20E content in hemolymph at the prepupa stage was analyzed by ELISA. The results showed that the hormone level in the CAP-treated group was 188.66 ng L⁻¹, which was 23.88% ($P < 0.01$)

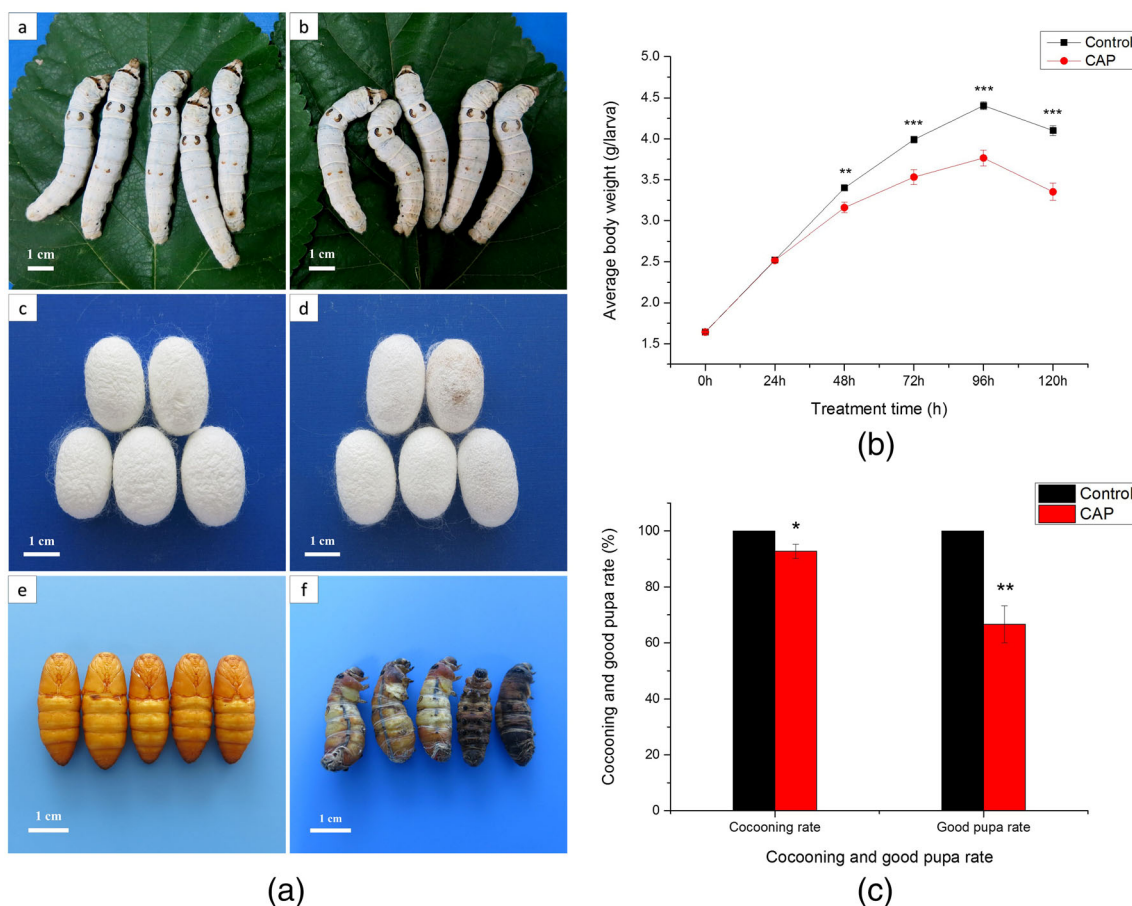


Figure 1. The effects of sublethal chlorantraniliprole (CAP) on the development of silkworm. (A) Fifth-instar larvae, cocoon and pupa of the control (a, c, e) and CAP-treated (b, d, f) groups. (B) Effects of CAP on the growth of *Bombyx mori* larvae. (C) Cocooning and good pupa rate. Statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$. All data are presented as mean \pm SE ($n = 20$).

lower than in the control group (247.86 ng L^{-1}). To further investigate the effect of ecdysone titer on pupation metamorphosis after CAP exposure, we injected 20E into silkworms at the pre-pupa stage and measured the ecdysone content at 24 h after injection. The results showed that the hormone level in the CAP +20E group was 635.35 ng L^{-1} (Fig. 4A), confirming the efficacy of the injection. Interestingly, the transcription level of *Ftz-f1* in the CAP +20E group was still significantly lower than the control group (0.30 times control; $P < 0.01$), and not different from the CAP-treated group (Fig. 4B). These results suggested that 20E injection could significantly increase the level of ecdysone in pre-pupa, but was not able to rescue the *Ftz-f1* downregulation caused by CAP exposure.

3.4 Effect of 20E injection on the transcription levels of chitin synthesis-related genes and pupation metamorphosis

To study the effect of 20E injection on chitin synthesis, we conducted qRT-PCR to measure the relative transcription levels of chitin synthesis-related genes after CAP exposure. The results showed that *ChsA*, *CDA1* and *CDA2* were significantly downregulated by CAP treatment (Fig. 5A). As expected, after 20E injection, mRNA levels of these three genes increased significantly (Fig. 5A), indicating that 20E injection can upregulate the transcription of chitin synthesis-related genes. Moreover, we found increased formation of new epidermal of prepupa in the CAP +20E group,

Nevertheless, pupation metamorphosis was still incomplete in the CAP +20E group (Fig. 5Bc).

4 DISCUSSION

In this study, we used sublethal CAP to treat the fifth-instar silkworm larvae and found that pupation metamorphosis at the pupa stage was deficient, leading to incomplete pupation and abnormal pupa. Moreover, 20E levels at the prepupa stage were decreased, and the primary 20E response genes *EcR*, *USP*, *E74* and *E75* were downregulated. One of the secondary 20E response genes, *Ftz-f1*, which is closely related to metamorphosis and pupation, was also downregulated. Transcription levels of chitin synthesis-related genes were reduced as well. Taken together, our results suggest that sublethal CAP exposure not only affected the formation of epidermis, but also impeded larval–pupal metamorphosis development.

As an active form of ecdysone, 20E coordinates the major developmental transitions in insects. Fluctuation of the 20E level is important for coordinating metamorphosis and molting, making both ecdysteroid biosynthesis and inactivation of physiological relevance.^{24,27} Reduction of the ecdysone concentration at the pupal stage may kill the pupa or delay its maturation.³⁸ In this study, 20E content at the prepupa stage was decreased after CAP treatment, leading to downregulation of a series of 20E response genes.

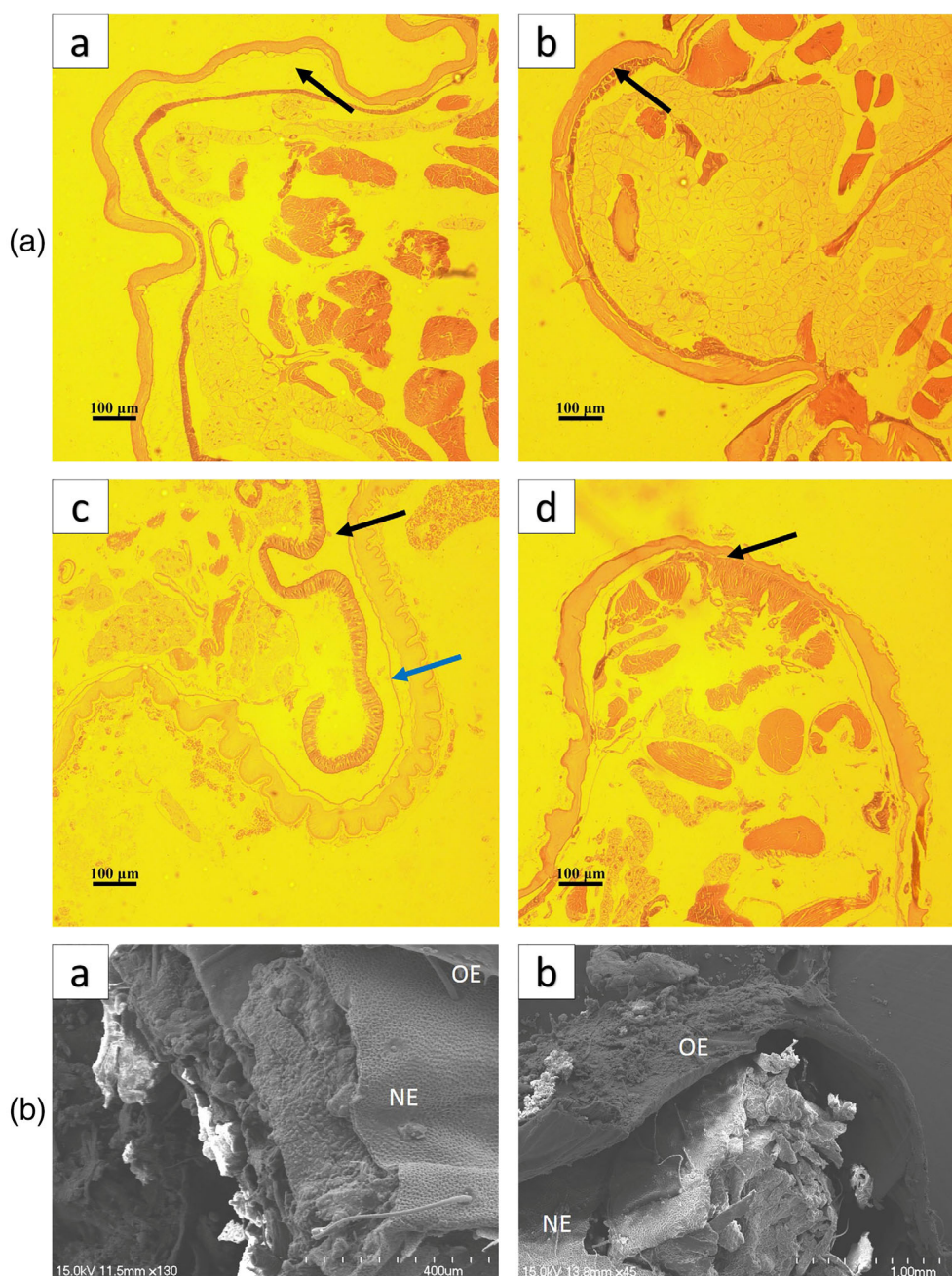


Figure 2. Histopathological and SEM examination on the effects of sublethal chlorantraniliprole (CAP) on silkworm prepupa epidermis. (A) Epidermis section of prepupa stage in the control group (a, c) and the CAP exposure group (b, d). Black arrow, accumulated procuticle; blue arrow, ecdysial membrane secreted by epithelial cell. (B) SEM images of prepupa epidermis in the control group (a) and the CAP exposure group (b). NE, new epidermis; OE, old epidermis.

Knockdown of the genes involved in 20E signaling pathway could reduce the expression of pupal-specific cuticle protein genes, and prevent growth and differentiation of the wing discs, which eventually leads to dead larvae or abnormal pupae in different insect species.^{24, 38–40} *Ftz-f1* is one of the 20E response genes, that regulates molting and metamorphosis by initiating the expression of a series of downstream genes. The exact timing of its action on the ecdysone response genes is critical for proper development.^{27,41} Knockdown of *Ftz-f1* in insects at the pupal stage caused phenotypic defects in eyes, antennal segments, wings and legs, and affected

emergence behavior.^{42,43} Also, silencing *Ftz-f1* can cause larval death prior to pupation, developmental arrest, and ecdysis failure.^{44–47} In this study, the transcription level of *Ftz-f1* was decreased under CAP exposure, which might mediate abnormal molting and metamorphosis at the prepupa stage. Ecdysteroid injection could rapidly promote adult development.⁴⁸ In addition, the transcription level of *Ftz-f1* gene in silkworm was rapidly increased after 20E injection.⁴⁹ In this research, the transcription level of *Ftz-f1* gene did not increase under 20E rescue in CAP-treated insects, suggesting *Ftz-f1* might be a target gene of CAP. Thus, sublethal CAP exposure reduced

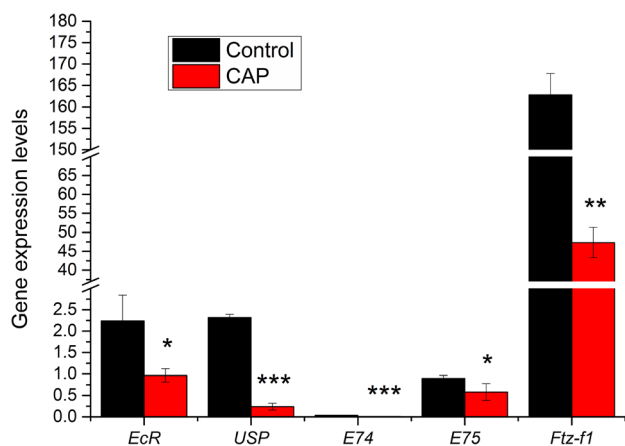


Figure 3. Transcriptional levels of 20-hydroxyecdysone (20E) responding genes in the fat body of prepupa stage. Statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$. Data are expressed as mean \pm SE ($n = 3$).

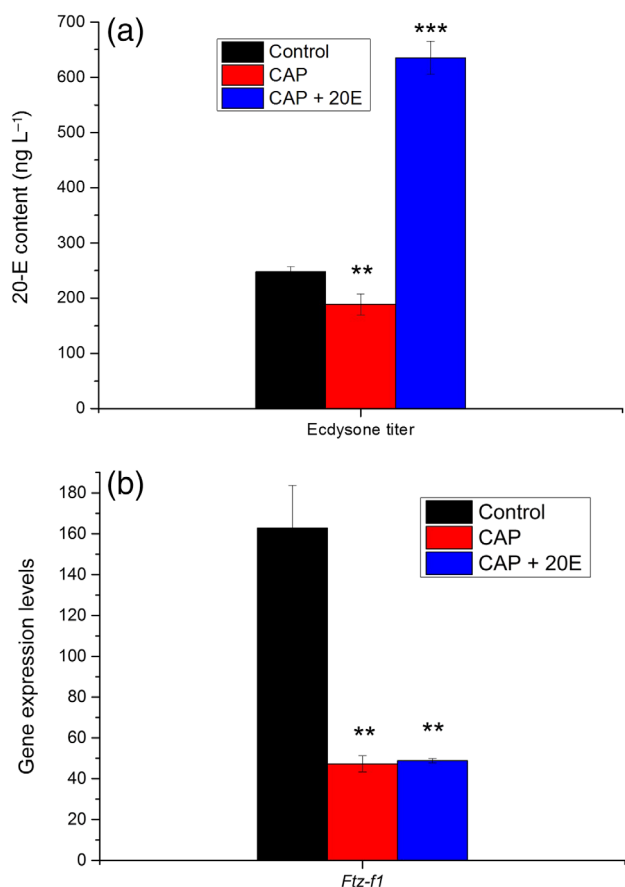


Figure 4. Effect of 20-hydroxyecdysone (20E) injection on hormone and *Ftz-f1* mRNA levels. (A) 20E concentration of hemolymph at prepupa stage. (B) Transcription level of *Ftz-f1* gene of the fat body in prepupa stage. Statistical significance is indicated by ** $P < 0.01$ or *** $P < 0.001$. Data are expressed as the mean \pm SE ($n = 3$).

transcription of *Ftz-f1*, and affected metamorphosis and pupation, leading to pupation defects.

Insects need to exuviate the old epidermis and form new epidermis when they grow to a certain stage. Larva-pupa

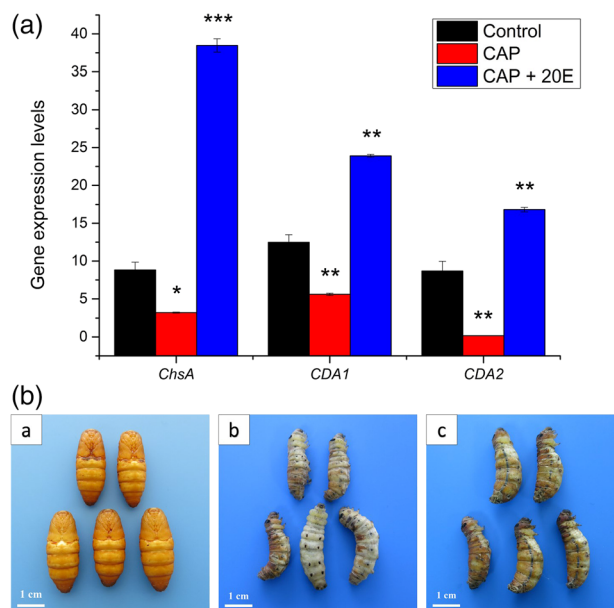


Figure 5. Effect of 20-hydroxyecdysone (20E) injection on the formation of pupa epidermis. (A) mRNA levels of chitin-related genes in epidermis at prepupa stage. (B) (a) Control group pupa, (b) chlorantraniliprole (CAP)-treated pupa, and (c) CAP + 20E treated pupa. Statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Data are expressed as the mean \pm SE ($n = 3$).

transformation involves both chitin synthesis and degradation pathways.^{50–52} Insecticides like dichlorbenzuron, chlorfluazuron and flufenoxuron can affect insect development and metamorphosis by inhibiting chitin synthesis.⁵³ Thus, chitin has been considered as a selective target of pesticides for pests.³⁶ CAP can affect chitin synthesis in several pest species, such as *Locusta migratoria* (Meyen) (Orthoptera: Locustidae), *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and *Mythimna separata* (Walker) (Lepidoptera: Noctuidae).^{54–56} CAP can also affect chitin synthesis in pest predators, like *M. basicornis*.^{15,16} However, the effects of sublethal CAP on chitin synthesis and pupation metamorphosis are still unclear. In this study, sublethal CAP exposure caused defects in epidermis formation at the prepupa stage. Insect growth and development are strictly dependent on the capability to remodel chitinous structures. Chitin synthesis A is mainly responsible for the synthesis of insect epidermis chitin.^{57,58} Knockdown of *ChsA* in *T. castaneum* showed abnormal development in larvae, pupae and even adults, and the chitin content in abnormal prepupa was reduced.^{59,60} Inhibition the transcription of *ChsA* caused delayed formation of pupa in *Oxya chinensis* (Thunberg) (Orthoptera: Acridoidea),⁶¹ and also led to abnormal molting in other insects.⁶² In this study, three chitin synthesis-related genes were significantly downregulated after CAP exposure. After injection of 20E, the transcription levels of these genes were significantly increased, and new epidermis was formed. These results suggested that 20E was the target hormone of sublethal CAP exposure. Our study provides novel insights into the mechanism of sublethal CAP exposure leading to failure in metamorphosis and pupation in insects.

Overall, our results suggested that 20E is a target hormone of sublethal CAP exposure, and *Ftz-f1* might be a direct target gene of sublethal CAP exposure. Future study will focus on the mechanism of how 20E regulates expression of *Ftz-f1* under sublethal

CAP exposure. This study explored the mechanism of sublethal CAP exposure-mediated pupation metamorphosis defects in silkworm, and provided evidence for the safe evaluation of CAP on a non-target economic organism. Moreover, this study also provides a reference for evaluating the toxicity of environmental CAP residues in pest pupation metamorphosis.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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