Effects of Nucleopolyhedrovirus Infection on the Development of Helicoverpa armigera (Lepidoptera: Noctuidae) and Expression of Its 20-Hydroxyecdysone— and Juvenile Hormone—Related Genes

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Effects of nucleopolyhedrovirus infection on the development of *Helicoverpa armigera* (Lepidoptera: Noctuidae) and expression of its 20-hydroxyecdysone– and juvenile hormone–related genes

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Abstract

In recent years, the interactions between baculoviruses and their insect hosts have become a research focus because baculoviruses can suppress the development and manipulate the behavior of insects. Many studies reported that nucleopolyhedrovirus (NPV) infection might disrupt the hormone balance in insects, but the effect of NPV infection on the development and expression of hormone-related genes in larvae of *Helicoverpa armigera* Boddie (Lepidoptera: Noctuidae) remains unclear. In this study, the mortality, development time, and pupal weight of *H. armigera* were recorded after 4th and 5th instars had been treated per os with different concentrations of *Helicoverpa armigera single NPV* (*HaSNPV*). Results showed that mortality increased and development time was prolonged to different degrees along with increasing concentrations of *HaSNPV*. The pupal weight did not differ between the *HaSNPV*-infected and control insects when 4th instars were infected but was significantly reduced when 5th instars were infected with *HaSNPV* at concentrations of 10^9 and 10^{10} polyhedral inclusion bodies (PIB) per milliliter. Compared with the healthy control group, larval body weight was significantly reduced from the 3rd day after infection when 4th instars had been treated with *HaSNPV* at concentrations of 10^8 and 10^9 PIB/mL. Results from quantitative reverse-transcriptase polymerase chain reaction assays revealed that 20-hydroxyecdysone–related genes (*ECR*, *USP*, *E75*, and *NTF2*) were down-regulated and juvenile hormone–related genes (*MET*, *JHi*, and *HSP90*) were up-regulated after *HaSNPV* infection. This study improves our understanding of the interactions between baculoviruses and host insects.

Key Words: NPV infection; insect hormone; transcript level; qRT-PCR; development

Resumen

En los últimos años, las interacciones entre los baculovirus y sus hospederos de insectos han convertido en un enfoque de investigación porque los baculovirus pueden suprimir el desarrollo y manipular el comportamiento de los insectos. Muchos estudios informan que la infección nucleopoliedrovirus (NPV) podría alterar el equilibrio hormonal en insectos, pero el efecto de la infección por el NPC en el desarrollo y la expresión de genes relacionados con las hormonas en las larvas de *Helicoverpa armigera* Boddie (Lepidoptera: Noctuidae) sigue siendo no clara. En este estudio, se registraron la mortalidad, el tiempo de desarrollo, y el peso de pupa de *H. armigera* después de cuarto y quinto estadios habían sido tratados por vía oral con diferentes concentraciones de *Helicoverpa armigera NPV singular* (*HaSNPV*). Los resultados mostraron que la mortalidad incrementó y el tiempo de desarrollo se prolongó a diferentes grados a lo largo con concentraciones crecientes de *HaSNPV*. El peso de pupa no fue diferente entre los insectos infectados por *HaSNPV* y de control cuando se infectaron el cuarto estadios, pero se redujo significativamente cuando las larvas del quinto estadio fueron infectados con *HaSNPV* en concentraciones de 10^9 y 10^{10} cuerpos de inclusión poliédricos (CIB) por mililitro. En comparación con el grupo de control sano, el peso corporal de las larvas se redujo significativamente desde la tercera día después de la infección cuando las larvas del cuarto estadio habían sido tratados con *HaSNPV* a concentraciones de 108 y 109 CIB/ml. Los resultados de los ensayos de reacción en cadena de la polimerasa cuantitativa la transcriptasa inversa revelaron que los genes relacionados con el 20-hidroxiecdisona (*ECR, USP, E75* y *NTF2*) fueron reguladas hacia abajo y los genes relacionados con las hormonas juveniles (*MET, JHI* y *HSP90*) fueron reguladas después de la infección *HaSNPV*. Este estudio mejora nuestra comprensión de las interacciones entre los baculovirus e insectos huésped.

Palabras Clave: infección VAN; hormona de insectos; nivel de transcripción; QRT-PCR; desarrollo

Baculoviruses are a class of large, double-stranded DNA viruses that infect only invertebrate hosts and have been developed as environmentally safe biological control agents (Park et al. 1993). In recent years, with the improving of people's living standards and the growing environmental consciousness, use and development of high-efficiency, low-toxicity, and pollution-free pesticides have become more

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popular in the public. Nuclear polyhedrosis virus (NPV) is one of the 2 taxonomic groups of baculoviruses and has many advantages including host specificity, excellent control effects, no non-target effects, and low levels of resistance response (Nguyen et al. 2013a). However, there are still many challenges to using NPVs to control pests in agriculture and forestry compared with the commonly applied chemical pesticides, such as lack of entomological expertise and robust automated systems, high production costs, or low potency. To improve the killing speed of NPVs, many studies have been conducted to elucidate the interaction between NPVs and host insects.

For example, when the ecdysteroid UDP-glucosyltransferase (*EGT*) gene was deleted from *Lymantria dispar multicapsid NPV* (*LdMNPV*), the killing speed of the recombinant viral strain was significantly faster than that of the wild type virus in 5th instars of *Lymantria dispar* L. (Lepidoptera: Noctuidae) (Slavicek et al. 1999). Transcriptome analyses and microarray methods were widely used to compare different aspects of the virus–host interactions, including infections at different time-points (Salem et al. 2011; Nguyen et al. 2013b), infection of fat body versus hemocytes (Bao et al. 2010), and characteristics of uninfected versus infected cells (Gatehouse et al. 2009; Sagisaka et al. 2010; Breitenbach et al. 2011; Nguyen et al. 2012). Many genome-scale analyses of differential mRNA expression between virus-infected and non-infected hosts were conducted, such as *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) insect cells infected with *Helicoverpa armigera single nucleopolyhedrovirus* (*HaSNPV*) (Nguyen et al. 2013b), *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) larvae infected by active *Autographa californica multiple nuclear polyhedrosis virus* (*AcMNPV*) (Choi et al. 2012), or the hemocytes of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) larvae infected by baculovirus (Breitenbach et al. 2011). Results from these studies showed that the transcript levels of many host genes (responsible for detoxification, anti-virus peptide production, energy generation, hormone activity, etc.) were changed after virus infection. To evade anti-viral responses by host insects, viruses target the apoptotic genes, steroid hormones, and the host immune system.

The holometabolous cotton bollworm *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) is an omnivorous and widespread pest that causes enormous economic loss in the cotton, corn, vegetable, and other crop industries throughout Asia (Wu et al. 2008; Lu et al. 2012). The steroid 20-hydroxyecdysone (20E) and the sesquiterpenoid juvenile hormone (JH) are 2 major hormones that regulate the metamorphosis and development of *H. armigera* (Riddiford et al. 2003). In the signal transduction pathway of 20E, 20E initially binds to its receptor, a heterodimeric transcription complex including ecdysteroid receptor (ECR) and ultraspiracle protein (USP) (Lan et al. 1999), and then initiates the 20E primary response genes *HR3* (Koelle et al. 1992), "early" ecdysone-induced transcription factor (*E75*) (Segraves & Hogness 1990), and Broad-Complex (*BR*) (Karim et al. 1993), and these transcription factors then induce the expression of other late genes, such as nuclear transport factor 2 (*NTF2*) (He et al. 2010). In the JH signal transduction pathway, JH initially binds to an intracellular receptor candidate methoprene-tolerant (MET) and then regulates the expression of the transcription factors *BR* and *Kr-h1* (Jindra et al. 2013; Liu et al. 2013). The JH inducible (*JHi*) gene is also used as an indicator for the JH signaling pathway (Dubrovsky et al. 2004). The heat shock protein HSP90 also was reported to be induced by JH in *H. armigera* (Liu et al. 2013). Juvenile hormone epoxide hydrolase (JHEH) is a vital metabolism enzyme that catalyzes JH to metabolize (Gilbert et al. 2000). These genes involved in 20E and JH signal transduction play vital roles in the growth and development of *H. armigera* (Riddiford et al. 2003; Liu et al. 2013). However, it is seldom reported how the expression of these genes varies after virus infection to disrupt the hormone balance in *H. armigera* larvae.

In order to better elucidate the interactions between baculoviruses and insects, we examined the effects of *HaSNPV* infection on the development and transcriptional profile of 20E- and JH-related genes in *H. armigera* larvae. The results may help to further develop efficient biopesticides and explain the mechanisms of host behavior alteration manipulated by baculoviruses.

Materials and Methods

INSECTS AND VIRUS

The *H. armigera* colony was obtained from the Integrated Pest Management (IPM) laboratory of the Entomology department at Chinese Agricultural University (Beijing, China) and reared on artificial diet (Wu & Gong 1997) at 26 ± 1 °C, 75 ± 10% RH, and a 16:8 h L:D photoperiod. Larvae were individually reared in separate glass tubes (5.5 cm in length × 2.0 cm in diameter) after the 3rd instar to prevent cannibalism.

The raw powder of *HaSNPV* (5×10^{11} PIB/g) was bought from Henan Jiyuan Baiyun Industry Co., Ltd (Jiyuan, China) and stored at 4 °C for later use.

INFECTION EXPERIMENT

HaSNPV powder was diluted with sterile water to 6 concentrations (10^5, 10^6, 10^7, 10^8, 10^9, and 10^{10} PIB/mL). Then, 10 µL *HaSNPV* suspension at different concentrations were dispensed onto artificial diet pieces (0.8 cm L × 0.8 cm W × 0.5 cm H). The artificial diet for control treatments received an equal amount of sterile water. One piece of the treated diet and one newly molted 4th or 5th instar were placed into a glass tube, and normal diet was replenished once the diet with NPV had been consumed (Zhang et al. 2015).

In the experiment examining physiological indicators, 4th instars received *HaSNPV* at 10^5, 10^6, 10^7, 10^8, and 10^9 PIB/mL, and 5th instars received *HaSNPV* at 10^6, 10^7, 10^8, 10^9, and 10^{10} PIB/mL based on the above method. In the gene expression experiment, all virus-treated larvae received *HaSNPV* at 10^7 PIB/mL. Each treatment in each experiment included 60 larvae tested in 3 replications (i.e., 20 larvae per replicate treatment).

DATA COLLECTION

The 4th and 5th instars treated with *HaSNPV* at different concentrations or with sterile water were checked daily for mortality, molting, and pupation, upon which the pupae were weighed. The cumulative mortality before pupation, the development time, and the pupal weight were recorded (Tables 1 and 2). Furthermore, the body weight of the 4th instars treated with *HaSNPV* or water was recorded daily until pupation.

PRIMER DESIGN

The expression levels of ecdysone-related genes (*ECR*, *USP*, *E75*, *BR*, *HR3*, and *NTF2*) and JH-related genes (*MET*, *JHEH*, *HSP90*, and *JHi* [Liu et al. 2011]) were determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The ribosomal protein L32 (*RPL32*) gene in *H. armigera* was used as an internal control for qRT-PCR normalization. The selected gene sequences were obtained from GenBank of the National Center for Biotechnology Information (NCBI). The primers used in qRT-PCR were designed with DNAClub software (http://www.softpedia.com/get/Science-CAD/DNA-Club.shtml) according to gene sequences. All primer pairs were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) (Table 3).

Table 1. Effect of different *HaSNPV* concentrations on the mortality, development time, and pupal weight of *Helicoverpa armigera* larvae treated in the 4th instar.

		Development time (d)[a,b]			
Concentration (PIB/mL)	Mortality (%)	4th instar	5th instar	4th instar to pupa	Pupal weight (g)[a,b]
0	3.33	2.58 ± 0.07 a	5.40 ± 0.62 a	8.00 ± 0.11 a	0.2173 ± 0.0038 a
10^5	18.33	2.73 ± 0.08 a	5.92 ± 0.91 b	8.65 ± 0.15 ab	0.2184 ± 0.0047 a
10^6	45.00	3.15 ± 0.08 b	5.82 ± 0.73 b	9.12 ± 0.17 bc	0.2203 ± 0.0057 a
10^7	78.33	3.33 ± 0.10 b	6.46 ± 0.52 c	9.77 ± 0.28 c	0.2118 ± 0.0061 a
10^8	100	3.55 ± 0.13 bc	n/a	n/a	n/a
10^9	100	3.78 ± 0.15 c	n/a	n/a	n/a
Summary statistics					
df	n/a	300 (5, 295)	152 (3, 149)	152 (3, 149)	152 (3, 149)
F	n/a	20.77	9.34	17.32	0.25
P	n/a	< 0.0001	< 0.0001	< 0.0001	0.86

In total, 60 larvae were treated per concentration in 3 replicate experiments.
[a]n/a = not applicable.
[b]The data in the table are means (± SE). Means within the same column followed by a different letter are significantly different at $P \leq 0.05$, Turkey's HSD test.

SAMPLE COLLECTION AND TOTAL RNA EXTRACTION

To analyze the temporal expression profile of 20E- and JH-related genes in *H. armigera* larvae upon *HaSNPV* infection, larvae were treated with 10 µL *HaSNPV* suspension (10^7 PIB/mL) according to the above method. Then, at least 10 larvae were collected at each of 6 time points (0, 24, 48, 72, 96, and 120 h), quickly frozen in liquid nitrogen, and immediately placed at −80 °C for later use. The larvae fed with artificial diet pretreated with an equal amount of sterile water were simultaneously collected as controls.

To avoid contamination with RNase, thawed larvae were placed into RNase-free micro tissue grinders that contained 1 mL Trizol reagent (Invitrogen, Gaithersburg, Maryland, USA) and ground for 5 min until the samples were completely homogenized. Then the total RNA was extracted by transferring 400 µL larval homogenate into a 2 mL RNase-free centrifuge tube that contained 600 µL Trizol reagent and following the manufacturer's instructions (Zhang et al. 2015). The purity and concentration of RNA samples were determined twice with an ultraviolet spectrophotometer (Thermo Scientific NanoDrop 2000, Rockford, Illinois, USA). The 1st-strand complementary DNA (cDNA) was synthesized in triplicate from 1 µg total RNA of each sample according to PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Kyoto, Japan), and the resulting products were immediately stored at −80 °C for later use (Bustin et al. 2009; Zhang et al. 2015).

QUANTITATIVE REAL-TIME PCR ANALYSIS

Real-time PCR amplification and analysis were performed using SYBR green supermix (TaKaRa) following the manufacturer's instructions on a Bio-Rad CFX Connect™ Real-Time PCR System (Bio-Rad, Hercules, California, USA), and the final reaction volume obtained was 20 µL. The real-time PCR was ran in triplicate for each cDNA sample (Zhang et al. 2015). The amplification conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The specificity of amplified products was further confirmed by melting curve analysis from 65 to 95 °C and 2% agarose gel electrophoresis. The mRNA expression of target genes was quantified using the comparative Cross Threshold (CT, the PCR cycle number that crosses the signal threshold) method (Livak & Schmittgen 2001). The CT value of the reference gene was subtracted from the CT value of the target gene to obtain ΔCT. The normalized fold changes of the target gene mRNA expression were expressed as $2^{-\Delta\Delta CT}$, where ΔΔCT is equal to $\Delta CT_{treated\ sample} - \Delta CT_{control}$.

STATISTICAL ANALYSES

All experiments were performed in triplicate, and the results were expressed as the means ± standard error (SE). The results of development time and pupal weight were analyzed by ANOVA followed by Turkey's HSD multiple comparison test in SPSS 17.0 software for statis-

Table 2. Effect of different *HaSNPV* concentrations on the mortality, development time, and pupal weight of *Helicoverpa armigera* larvae treated in the 5th instar.

Concentration (PIB/mL)	Mortality (%)[a]	Development time (d)[b]	Pupal weight (g)[b]
0	0	5.23 ± 0.08 a	0.2167 ± 0.0032 a
10^6	25.00	5.38 ± 0.12 a	0.2136 ± 0.0045 a
10^7	41.67	5.94 ± 0.19 ab	0.2111 ± 0.0041 ab
10^8	56.67	6.35 ± 0.26 bc	0.2092 ± 0.0045 ab
10^9	68.33	6.84 ± 0.21 bc	0.1926 ± 0.0050 bc
10^{10}	75.00	7.20 ± 0.22 c	0.1762 ± 0.0085 c
Summary statistics			
df	n/a	196 (191, 5)	196 (191, 5)
F	n/a	20.67	7.59
P	n/a	< 0.0001	< 0.0001

In total, 60 larvae were treated per concentration in 3 replicate experiments.
[a]n/a = not applicable.
[b]The data in the table are means (± SE). Means within the same column followed by a different letter are significantly different at $P \leq 0.05$, Turkey's HSD test.

Table 3. Primer pairs used in qRT-PCR for gene expression analysis.

Gene name	Annotation	Accession number	Sequence (5'-3')[a]	Product length (bp)
ECR	*H. armigera* ecdysone receptor	EU526831.1	F: 5'-GCAGTCAGATCAGATCACGT-3' R: 5'-GGAGTACATGCACCGACAG-3'	196
USP	*H. armigera* ultraspiracle isoform 1	EU526832.1	F: 5'-CTGACTGAAGAGCGAGACG-3' R: 5'-GATAGCACGCGGTCGAAGATC-3'	146
E75	*H. armigera* transcription factor E75B	EU526834.1	F: 5'-CGCCAACTGATTCTGGCAT-3' R: 5'-ACAGGCATGTCGTCGGCT-3'	145
BR	*H. armigera* broad complex isoform Z2	KC316049.1	F: 5'-CTGATGTTGGCAGGGATGTC-3' R: 5'-CATGCCGCAGTAAGGACAGTC-3'	123
HR3	*H. armigera* molt-regulating transcription factor 3	AF337637.3	F: 5'-GCTCGTCTATATCTGGCATG-3' R: 5'-CAGGTGATGACGCCATAGTG-3'	127
NTF2	*H. armigera* nuclear transport factor 2	DQ875254.2	F: 5'-GCTCAATCCACAATACGATGC-3' R: 5'-GCACCTTGCAACTGTACTCC-3'	150
MET	*H. armigera* methoprene tolerant protein 1	KJ184572.1	F: 5'-CTATCCAGTGCAATGCCACCTC-3' R: 5'-CTTCTTCTGTCATGTAGCC-3'	136
JHi	*H. armigera* JH inducible gene	none	F: 5'-GAAGTCAGCGGAGAACAG-3' R: 5'-CAGTCATAATACGGTGGGT-3'	see Liu et al. (2011)
JHEH	*H. armigera* juvenile hormone epoxide hydrolase	FJ602793.2	F: 5'-CTGGCTACGGATTCTCAGATG-3' R: 5'-GAAGGTAGCCAAGGTGGTGC-3'	158
HSP90	*H. armigera* heat shock protein 90	FJ986209.1	F: 5'-CATGTCCCTGATCATCAACAC-3' R: 5'-CTGTCCAGCTTCGATGGGTC-3'	129
RPL32	*H. armigera* ribosomal protein L32	JQ744274.1	F: 5'-CATCAATCGGATCGCTATG-3' R: 5'-CCATTGGGTAGCATGTGAC-3'	152

[a]F and R indicate forward primer and reverse primer, respectively.

tically significant differences between different groups ($P < 0.05$). The differences in gene expression between control and *HaSNPV*-infected larvae were compared using Student's *t*-tests ($P < 0.05$) in SPSS 17.0 software.

Results

MORTALITY, DEVELOPMENT TIME, AND PUPAL WEIGHT AFTER VIRUS INFECTION

When 4th instars were treated with *HaSNPV* at different concentrations, the mortality of control larvae (fed artificial diet with sterile water) was 3.3% and that of virus-treated larvae increased with increasing *HaSNPV* concentrations; all larvae treated with 10^8 and 10^9 PIB/mL died before pupation (Table 1). The development time of larvae treated with *HaSNPV* increased significantly compared with control larvae. When the larvae were infected with *HaSNPV* at 10^8 and 10^9 PIB/mL, the development time of 4th instars increased by 37.6 and 46.5%, respectively. At 10^7 PIB/mL, the development time of 4th and 5th instars to pupation increased by 2.1 and 1.8 d, respectively, compared with control larvae (Table 1), whereas the pupal weight was similar between *HaSNPV*-infected and control insects.

When 5th instars were treated with *HaSNPV* at different concentrations, mortality increased with increasing *HaSNPV* concentration, the development time increased after treatment with 10^8, 10^9, and 10^{10} PIB/mL compared with control larvae, and the weight of pupae infected with 10^9 and 10^{10} PIB/mL was significantly less than that of control pupae (Table 2).

FLUCTUATION OF BODY WEIGHT IN 4TH INSTARS UPON INFECTION

In general, the body weight of 4th instars gradually increased, and fast growth occurred from the 2nd day after treatment in every group (Fig. 1A). The body weight of infected larvae decreased with increasing *HaSNPV* concentration. Weight (Fig. 1A) and size (Fig. 1B) of larvae treated with 10^8 and 10^9 PIB/mL were significantly reduced from the 3rd day onward.

TRANSCRIPTION ANALYSIS OF 20E-RELATED GENES

Effects of *HaSNPV* infection on 20E-related genes in *H. armigera* larvae at the transcript level were analyzed by real-time PCR. The results showed that *HaSNPV* infection significantly inhibited the expression levels of 20E receptor *ECR* and its copartner *USP* after virus infection at 48, 72, 96, and 120 h, but had no obvious effect at 24 h (Fig. 2). The transcript levels of *E75*, a 20E early responsive gene, significantly decreased after virus infection at 24, 72, 96, and 120 h but was not different from controls at 48 h (Fig. 2). *HaSNPV* infection induced the expression of the two 20E early responsive genes *BR* and *HR3* (Fig. 2), which significantly increased 48, 96, and 120 h after virus infection. The transcript levels of *NTF2* increased after virus infection at 24 and 48 h, but decreased at 96 and 120 h (Fig. 2).

TRANSCRIPTION ANALYSIS OF JH-RELATED GENES

As shown by real-time PCR, *MET*, which is a JH candidate receptor gene, was significantly up-regulated at 24, 72, and 120 h after virus infection, with no noticed expression difference to controls at 48 and 96 h (Fig. 3). The transcript levels of *JHi* and *HSP90* were significantly up-regulated at 24, 48, 72, and 120 h and at 24, 48, 72, and 96 h, respectively, after virus infection (Fig. 3). The *JHEH* gene was significantly up-regulated by virus infection at 24 and 120 h and down-regulated at 48 and 96 h (Fig. 3).

Discussion

In order to enhance their transmission, baculoviruses cause the host insects to develop slower or to change their behavior (Kamita et al. 2005; Liu et al. 2006; Hoover et al. 2011). Parasites of invertebrates and vertebrates mainly target 4 physiological systems (endocrine,

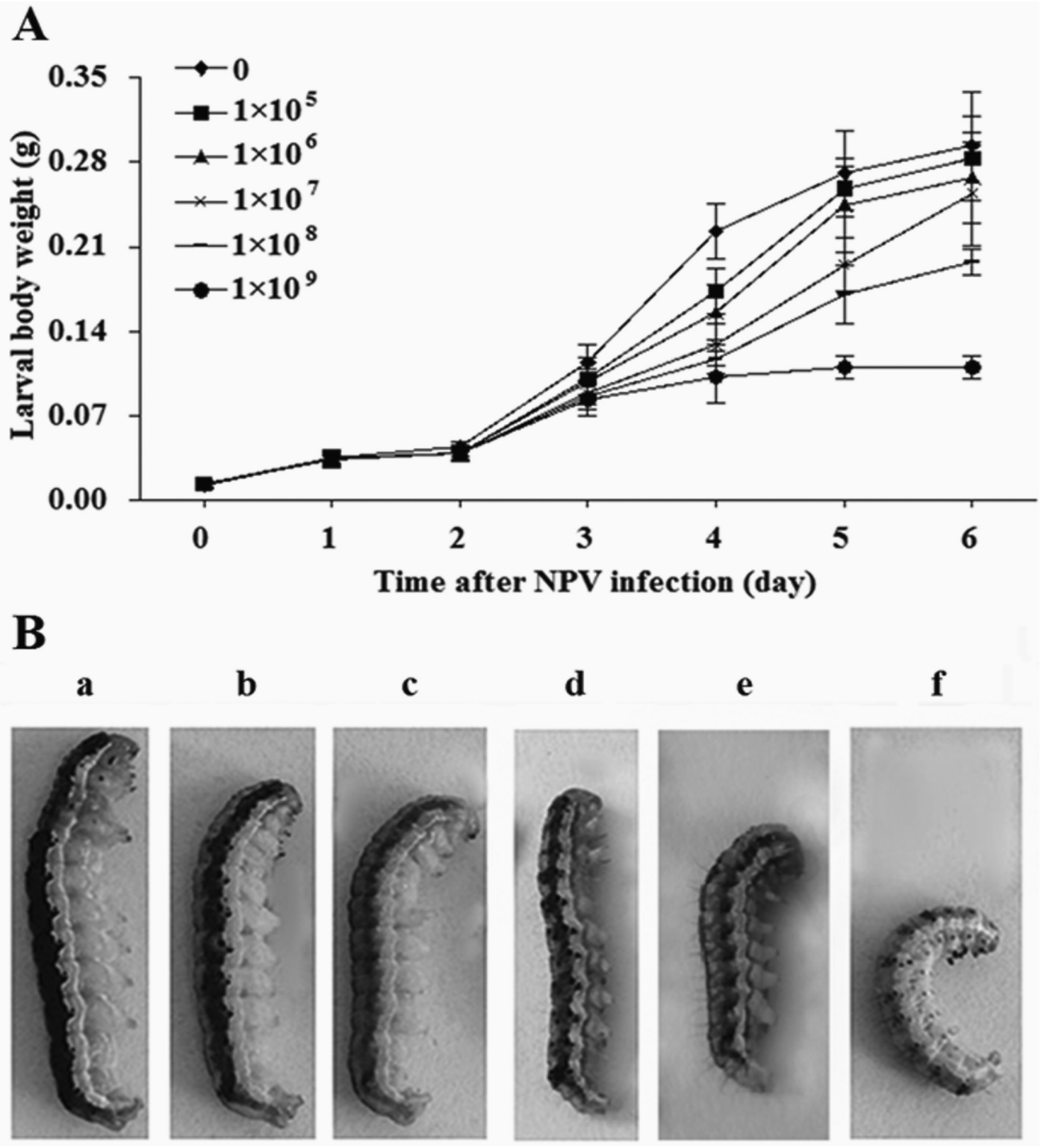

Fig. 1. Effect of *HaSNPV* infection on the larval body weight of *Helicoverpa armigera*. (A) ♦, ■, ▲, ×, —, and ● indicate larvae infected with *HaSNPV* at the concentrations 0, 10^5, 10^6, 10^7, 10^8, and 10^9 PIB/mL, respectively. (B) a, b, c, d, e, and f show *H. armigera* larvae on the 5th day after infection with *HaSNPV* at the concentrations 0, 10^5, 10^6, 10^7, 10^8, and 10^9 PIB/mL, respectively.

neural, immunomodulatory, and neuromodulatory) to induce behavioral changes (Beckage 1993; Adamo 2002; Thomas et al. 2005; Helluy 2013). Understanding how these systems connect and communicate is important for theoretical as well as practical reasons.

In our study, *HaSNPV* showed a high virulence and pathogenicity to 4th and 5th instars of *H. armigera*. The development time of virus-infected larvae was prolonged compared with larvae of the healthy control group. Our results are consistent with former studies showing that the molting and pupation of larvae were blocked by virus infection via controlling host insect ecdysone levels (O'Reilly & Miller 1989; Liu et al. 2006). Although the levels of ecdysone, which regulates the molting and metamorphosis in insects, have been shown to decline after

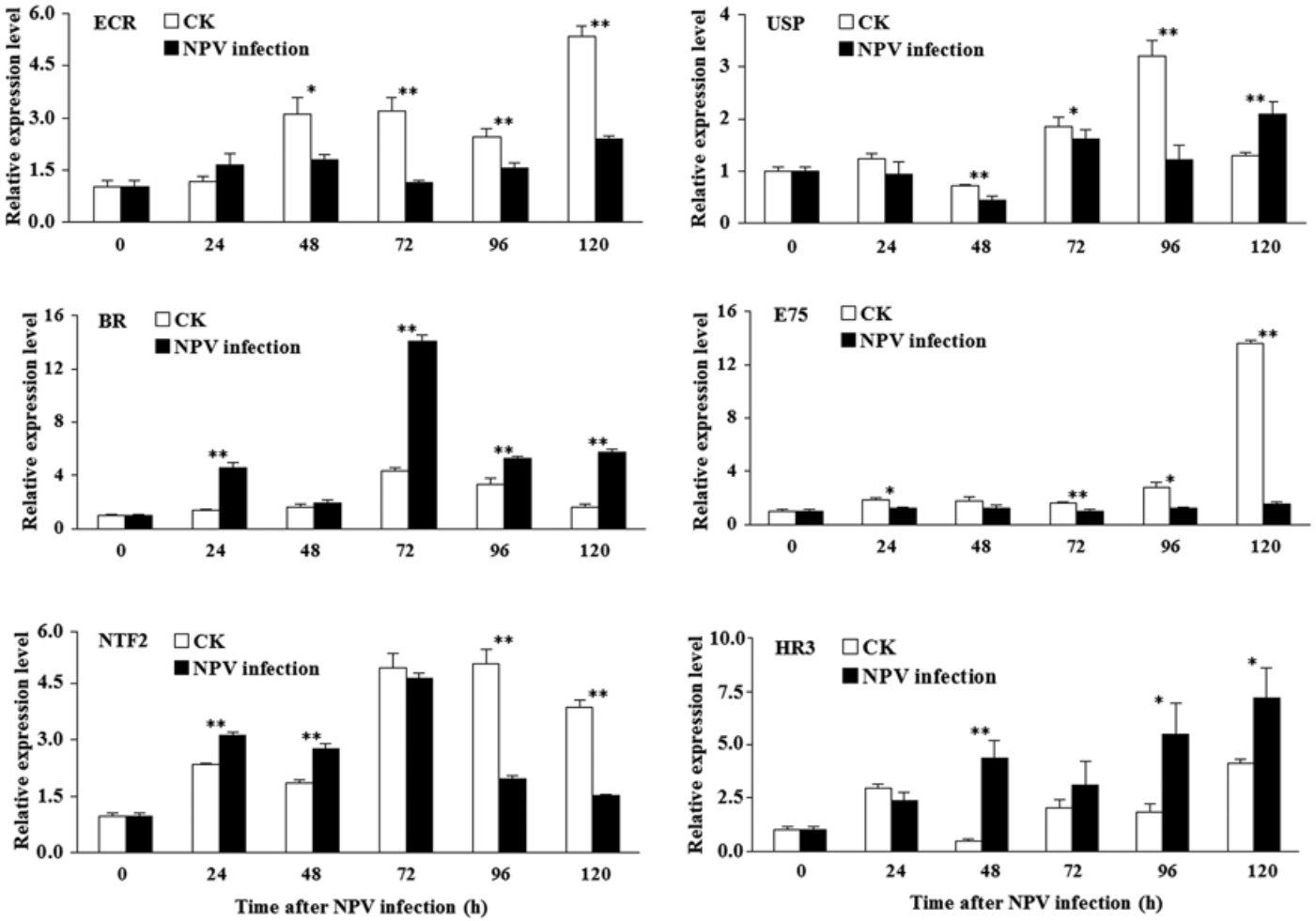

Fig. 2. Expression analysis by qRT-PCR of the 20E related genes *ECR*, *USP*, *E75*, *BR*, *HR3*, and *NFT2* in *Helicoverpa armigera* larvae after *HaSNPV* infection at 0, 24, 48, 72, 96, and 120 h. The blank bars represent the larvae infected with sterile water (CK). The black bars represent the larvae infected with NPV at the concentration of 10^7 PIB/mL (NPV infection). The data represent the mean ± SD of 3 biological replicates. Statistically significant differences from gene expression are denoted by * ($0.01 < P \leq 0.05$) and ** ($P \leq 0.01$) as determined by the pairwise Student's *t*-test analysis in SPSS 17.0 software.

virus infection, the expression changes of ecdysone-related genes are little investigated. Therefore, we selected 6 ecdysone-related genes, namely *ECR*, *USP*, *E75*, *BR*, *HR3*, and *NTF2*, to study the effect of *HaSNPV* infection on the larval 20E signal in *H. armigera*. Transcript levels of two 20E receptors (*ECR* and *USP*) and an early transcription factor (*E75*) were down-regulated after virus infection, which agrees with former results that *HaECR* transcript levels declined 72 h after *HaSNPV* infection (Jayachandran et al. 2013). Interestingly, the transcript levels of *BR* and *HR3* genes were up-regulated after virus infection, which is consistent with previous research showing that *HR3* was up-regulated nearly 8-fold in response to baculovirus infection (Breitenbach et al. 2011). The vital roles of *BR* have been demonstrated in metamorphic processes and embryogenesis of insects, but whether it is involved in neural, endocrine, and muscular coordination remains unclear (Piulachs et al. 2010). *HR3*, which is a probable nuclear hormone receptor and metamorphosis-related gene, plays key roles during metamorphosis (Xiong et al. 2013), but whether the up-regulation of *HR3* after virus infection implies other functions remains to be investigated. The transcript levels of *NTF2* markedly increased after virus infection at 24 and 48 h, but then decreased at 96 and 120 h. The reason for this fluctuation may be that *NTF2* and small GTPase Ran are involved not only in the 20E signal transduction pathway but also in the nucleo-cytoplasm transport of macromolecules (He et al. 2010). Hence, our results showed that virus infection altered the transcription of 20E-related genes that may relate to the biological and physiological changes observed in infected larvae.

From the 3rd day of virus infection, the body weight and size of larvae treated with 10^8 and 10^9 PIB/mL were reduced compared with healthy larvae. The fluctuation of body weight was closely related to molting and development time because the exoskeleton limits the continuous growth of insects (Riddiford et al. 2003). It is possible that virus infection may suppress the growth and development of host insects by disturbing the hormone balance via influence of the viral *Egt* gene. The virus *Egt* gene encodes an enzyme that modifies a hydroxyl group on 20E, thereby inactivating the molting hormone and resulting in a delay or in the absence of molting in infected larvae (O'Reilly et al. 1992; Chen et al. 1997; Slavicek et al. 1999).

It is known that JH is a central hormone that regulates insect development and growth (Dubrovsky 2005), but the specific interactions between JH and virus infection remain unclear. Generally, it is hypothesized that inactivating 20E and maintaining the JH titer at status quo level are beneficial to the reproduction of the virus because the infected insects continue to feed and produce more occlusion bodies (polyhedra) (Chen et al. 1997). In *Adoxophyes honmai* (Lepidoptera: Tortricidae), JH esterase activity had no peak in the final instar of entomopoxvirus-infected larvae, suggesting that JH titers in virus-infected larvae remained high (Nakai et al. 2004). In *Apis mellifera* L. (Hymenop-

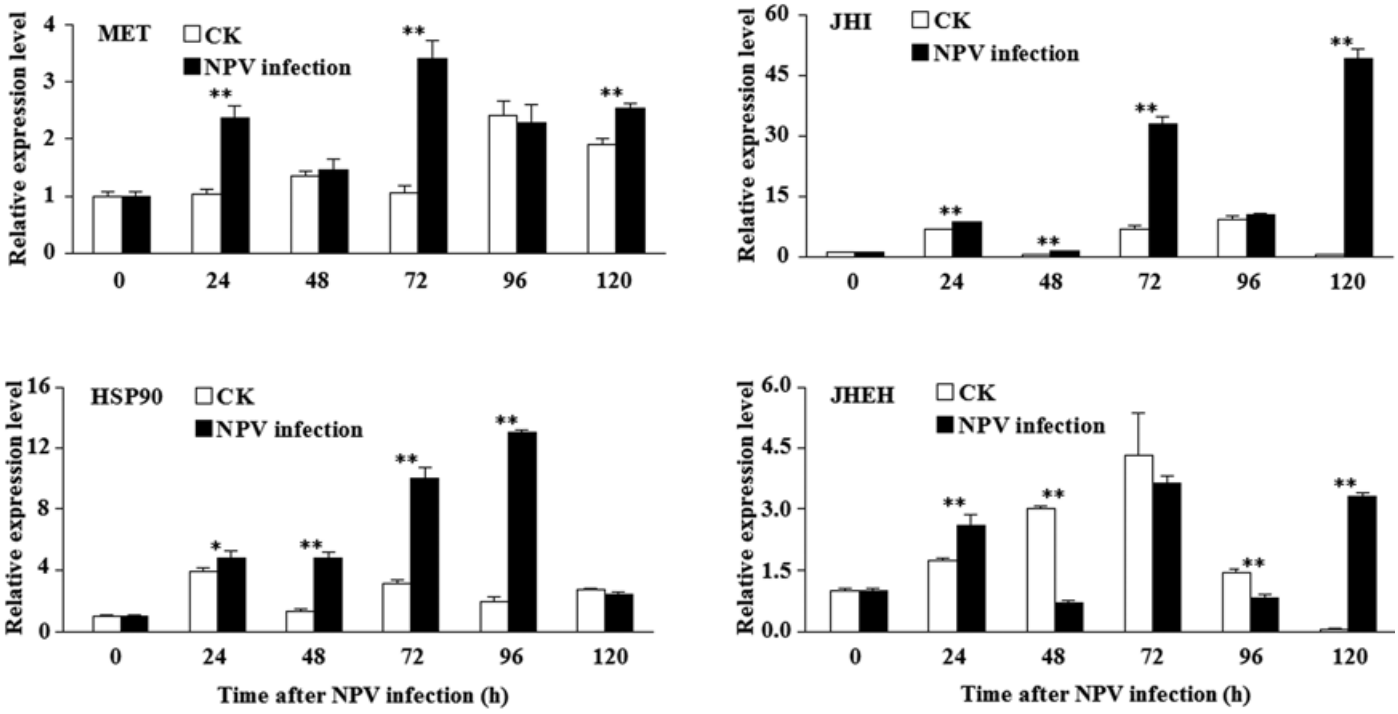

Fig. 3. Expression analysis by qRT-PCR of the JH related genes *MET*, *JHEH*, *HSP90*, and *JHi* in *Helicoverpa armigera* larvae after *HaSNPV* infection at 0, 24, 48, 72, 96, and 120 h. The blank bars represent the larvae infected with sterile water (CK). The black bars represent the larvae infected with NPV at the concentration of 10^7 PIB/mL (NPV infection). The data represent the mean ± SD of 3 biological replicates. Statistically significant differences from gene expression are denoted by * ($0.01 < P \leq 0.05$) and ** ($P \leq 0.01$) as determined by the pairwise Student's *t*-test analysis in SPSS 17.0 software.

tera: Apidae), nurse-aged bees had an elevated JH titer that peaked at 8 d of age after infection with *Nosema ceranae* Fries, Feng, Feng, da Silva, Slemenda & Pieniazek (Dissociodihaplophasida: Nosematidae) (Goblirsch et al. 2013). We selected 4 JH-related genes, namely *MET*, *JHI*, *HSP90*, and *JHEH*, to study the effect of *HaSNPV* infection on JH. The transcript levels of *MET*, *JHI*, and *HSP90* were significantly up-regulated following virus infection, implying that virus infection might induce a JH titer that prolongs the larval stage. *JHEH* was up-regulated after virus infection at 24 and 120 h but down-regulated at 48 and 96 h. The role of *JHEH* in the JH metabolic reaction is to decrease JH levels (Yang et al. 2011). Altered expression of a gene involved in the removal of JH would contribute to delayed pupation and allow the virus to propagate (Breitenbach et al. 2011).

Overall, this study directly determined the transcript levels of several genes involved in the 20E and JH pathways in *H. armigera* larvae after *HaSNPV* infection and investigated the inhibitory effect of *HaSNPV* on the growth and development of *H. armigera* larvae. We hypothesized that the expression levels of most 20E-related genes would decrease to varying degrees after virus infection, possibly because the viral *Egt* gene inactivated the normal ecdysone hormone metabolism in the diseased host larvae. Why JH-related genes were up-regulated after virus infection remains unclear. It is possible that JH may be involved in the host defense against virus infection to suppress the viral life cycle. The particular mechanism by which 20E and JH jointly respond to virus infection is worth of further study.

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