# 20-hydroxyecdysone regulates the expression of antimicrobial peptides through Dorsal and Relish in *Helicoverpa armigera*

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**Abstract**

20-hydroxyecdysone (20E) plays a key role in insect development, not only regulating molting and metamorphosis but also participating in the innate immune responses. The regulation of immunity by 20E varies among different insect species. The pathway of 20E regulates immunity in the cotton bollworm (*Helicoverpa armigera*) remain unclear. In this study, we infected *H. armigera* with Gram-negative bacterium *Micrococcus luteus* and Gram-positive bacterium *Pseudomonas aeruginosa*, respectively, demonstrating that the larvae exhibits enhanced immunity during the larval-to-pupal metamorphosis stage. Through 20E induction assays, we confirmed that the enhanced immunity is regulated by 20E. 20E upregulates the expression of antimicrobial peptides (AMPs), thereby inhibiting the growth of *M. luteus* and *P. aeruginosa* in the larvae. Knockdown of Dorsal suppressed the 20E-induced expression of AMPs, while knockdown of Relish similarly inhibited the expression of AMPs except Moricin. Overexpression of Dorsal was localized at the nucleus and promoted the expression of AMPs. Treatment with 20E enhanced its transcriptional regulation of AMPs. Overexpression of Relish partially localized at the nucleus and upregulated the expression of certain AMPs (*Cecropin D*, *Gloverin-1*, and *Gloverin-2*). Treatment with 20E resulted in the translocation of Relish from the cytoplasm to the nucleus, enhancing the expression of *Cecropin D*, *Gloverin-1*, *Gloverin-2* and *Lebocin*. We further showed that 20E promoted the cleavage and nuclear translocation of Relish. These data indicate that 20E induces the expression of

AMPs in *H. armigera* through Dorsal and Relish, thereby enhancing immunity during the metamorphosis stage.

Key words: 20-Hydroxyecdysone; Antimicrobial peptides; Innate immunity; Dorsal; Relish.

## Abbreviations

AMP, antimicrobial peptide; 20E, 20-hydroxyecdysone; DMSO, dimethyl sulfoxide; CFU, Bacterial Colony Forming Units; dsRNA, double-stranded RNA; GFP, green fluorescent protein; RFP, red fluorescent protein; NF-κb, nuclear factor-κB.

## 1. Introduction

During their survival, insects encounter a constant threat of pathogen invasion (Hoffmann and Reichhart, 2002). Pathogenic infections significantly impact the survival of insects, particularly for holometabolous insects that experience metamorphosis, a transformative process that involves degradation of the larval midgut and consequent release of microorganisms. To mitigate the potential risks posed by pathogenic microbes, insects must enhance their immune defense during this vulnerable developmental stage (Johnston et al., 2019). Insects rely on innate immune responses to defend against pathogens invasion (Lemaitre and Hoffmann, 2007). The innate immunity includes cellular and humoral responses. Cellular immunity depends on phagocytosis and encapsulation of pathogens by hemocytes, while humoral immunity is mainly achieved through the induction of antimicrobial peptides (AMPs) (Buchon et al., 2014). AMPs are primarily synthesized in the fat body of insects and released into the hemolymph, serving as crucial immune effector molecules with broad-spectrum antimicrobial activity (Lehrer and Ganz, 1999; Patyra and Kwiatek, 2023).

The insect immune responses involve the activation of multiple signaling pathways, including Toll and Immune Deficiency (IMD) pathways, which further regulate the expression of AMPs by controlling the activation and nuclear localization of transcription factors, such as Dorsal and Relish (Lemaitre and Hoffmann, 2007). The Toll and IMD signaling pathways both belong to the nuclear factor-κB (NF-κB) signaling pathway family. The Toll pathway is activated by Gram-positive bacterial and fungal infection (Gobert et al., 2003). In *Drosophila melanogaster*, extracellular peptidoglycan recognition proteins (PGRP-SA/SD or GNBP3) recognize pathogens and activate Spätzle, which triggers a signaling cascade that ultimately leads to the induction of AMPs gene transcription by the transcription factor Dorsal. In contrast, the IMD pathway serves as the primary defense mechanism against Gram-negative bacterial infections (Christophides et al., 2002). This pathway is activated when the PGRP-LC receptor recognizes Gram-negative bacteria. The subsequent signaling

cascade results in the phosphorylation and cleavage of the transcription factor Relish. The activated Relish then translocates to the nucleus to promotes the expression of AMPs (Lemaitre and Hoffmann, 2007).

In insects, The molting hormone 20-hydroxyecdysone (20E) regulates many physiological traits, including juvenile-to-adult developmental transitions (Texada et al., 2020), reproductive maturation (Uryu et al., 2015), energy metabolism (Koyama et al., 2020)， behavior (Ganter et al., 2011), stress responses (Simon et al., 2003), and longevity (Tricoire et al., 2009). 20E also is involved in the innate immune processes (Nunes et al., 2021b). 20E activates hemocytes by regulating both actin and the tubulin cytoskeleton, enhancing their phagocytic and motility capabilities in *Drosophila* (Lanot et al., 2001; Regan et al., 2013). 20E also triggers release of Growth-Blocking Peptide Binding protein (GBPB) from oenocytoid-like cells, inhibiting spreading and encapsulation activities of hemocytes (Zhuo et al., 2018). In addition to regulating cellular immunity, 20E also modulates the expression of AMPs. The regulation of AMP expression by 20E varies significantly among different insect species. In *Drosophila*, 20E upregulates the expression of PGRP-LC, a receptor of the IMD pathway, and cooperates with peptidoglycan to induce the expression of AMP genes (Rus et al., 2013). In *Locusta migratoria*, 20E up-regulates the expression of PGRP-SA through its nuclear receptor EcR, activating the Toll signaling pathway and promoting the expression of AMPs (Han et al., 2017). The regulation of AMP expression by 20E requires synergistic action with peptidoglycan in *D. melanogaster* (Rus et al., 2013). However, there is a distinct effect of 20E on AMPs in the silkworm *Bombyx mori*. The early study showed that 20E treatment down-regulates AMPs in the fat body of *B.mori*. Recent studies have shown 20E upregulates AMPs in larva-to-pupa metamorphosis by BmMD-2A, which is involved in the Toll signaling pathway (Zhang et al., 2023). In the cotton bollworm *Helicoverpa armigera*, 20E also modulates the expression of immune-related genes (Wang et al., 2014). However, the mechanism of 20E-mediated innate immune responses is unclear.

In this study, we aim to reveal the molecular mechanisms of 20E-mediated regulation of innate immunity in *H. armigera*. We found that as the larvae enter

wandering stage, increased concentration of 20E enhances the innate immune response. 20E upregulates the expression of Dorsal and Relish, activates Dorsal and promotes the cleavage and nuclear translocation of Relish, thus leading to expression of AMP genes.

## 2. Material and Methods

### *2.1 Insects and HaEpi cells*

The Cotton bollworms *H. armigera* were reared in an artificial climate chamber under the following conditions: temperature at 26 ± 1°C, relative humidity at 60%, and a photoperiod of 14 hours light: 10 hours dark. The larvae were fed with an artificial diet, while the adults were provided with a 10% honey water solution (Zhou et al., 2023). HaEpi cells form epidermis of H.armigera were developed as a loosely attached monolayer and were maintained at 27°C with Grace's insect cell culture medium containing 10% Fetal Bovine Serum (Bioind).

### *2.2 Bacterial infection*

The Gram-positive bacterium *M. luteus* and the Gram-negative bacterium *P. aeruginosa* were cultured in LB medium at 37°C with shaking until the $OD_{600}$ value reached one. The cultured bacterial suspension was centrifuged at 8,000 g for 5 minutes and the supernatant was discarded. The pelleted bacterial cells were resuspended and washed three times using a sterilized 0.85% NaCl solution. After washing, the bacterial cells were collected and resuspended in a sterilized 0.85% NaCl solution. *M. luteus* was diluted to a concentration of $2 \times 10^9$ CFU/mL, and *P. aeruginosa* was diluted to $10^8$ CFU/mL. 30 larvae were chosen and placed on ice for anesthesia for 15-20 minutes. Each larva was injected with 5 μL of bacterial suspension. During injection, the needle was kept as horizontal as possible to the body of the larva and inserted gently. After injection, the needle was left in place briefly before being slowly withdrawn. A control group was injected with an equal volume of 0.85% NaCl solution.

*2.3 Quantitative real-time reverse transcription PCR (qRT-PCR)*

The fat body of the *H. armigera* was dissected and total RNA was extracted using the Tripure Isolation Reagent (Roche). Subsequently, cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Specific primers for the genes of interest were designed for qRT-PCR (Table 1). qRT-PCR was performed by Fast qPCR Master mix and the program was 95℃ 5 min; 95℃ 10 s, 60℃ 15 s, 72℃ 15 s; 40 cycles; 65-95℃ analysis dissolution curve, interval 0.5 ºC. After obtaining the cycle threshold (Ct) value, the relative mRNA expression levels were calculated using the formula $2^{-\Delta\Delta Ct}$. The *ACTB* (*β-actin*) gene was used as housekeeping gene.

*2.4 20E titers quantification*

Larvae of the *H.armigera* at 6 h, 24 h, 72 h, and 96 h of the 6th instar stage were individually selected and anesthetized by placing them on ice. Hemolymph (100 mg) was collected and then freeze-dried using a lyophilizer. The dried powder was dissolved in 500 μL of 80% methanol solution and thoroughly homogenized on ice. The homogenate was centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant obtained after centrifugation was air-dried at room temperature. The dried residue was reconstituted in 100 μL of enzyme immunoassay (EIA) buffer. Each sample was diluted 1000-fold with EIA buffer, and the experiment was conducted according to the instructions provided with the 20-Hydroxyecdysone Enzyme Immunoassay Kit (Cayman). The absorbance of the samples was measured at a wavelength of 414 nm using a full-wavelength microplate reader. The concentration of 20E in the hemolymph was calculated based on a standard curve of known concentrations.

*2.5 20E Induction in larvae and HaEpi Cells*

Larvae: 20E was dissolved in dimethyl sulfoxide (DMSO) at a storage concentration of 20 mM (9.6 mg/ml). 20E was diluted to 100 ng/μL using sterile 1 × phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4) buffer and injected 5 μL (500 ng) into the 6 th-6 h larval hemocoel for

6 h,12h and 24 h. The protein or total RNA was extracted from the larval fat body for qRT-PCR or western blot.

HaEpi Cells: HaEpi cells were incubated in culture dishes with fresh Grace's medium with 10% FBS for 24 h and reached 80% confluence. Cells were incubated in culture medium containing 20E (5 μM) for 12 h. An equivalent volume of DMSO was used to treat the cells for the same durations as a control. The total RNA was extracted for qRT-PCR.

### *2.6 Bacterial Colony Forming Units (CFUs) counting*

After infecting the *H.armigera* with *M.luteus* and *P.aeruginosa*, the larvae were anesthetized on ice. The surface of the larvae was cleaned with 75% ethanol, and the legs were cut off to collect hemolymph. Collected hemolymph and 5 μL of 1% phenylthiourea (PTU) was added to prevent melanization. The hemolymph was then diluted to an appropriate concentration using sterile 0.85% NaCl. The diluted hemolymph was evenly spread onto Petri dishes containing LB solid medium. The plates were incubated at 37°C until bacterial colonies became clearly visible and bacterial colonies were counted.

### *2.7 RNA interference in larvae*

Double-stranded RNA (dsRNA) was synthesized using the T7 RiboMAX Express RNAi System (Promega) following the manufacturer's instructions. The synthesized dsRNA was diluted to an appropriate concentration using sterile, nuclease-free 1× PBS. Sixth-instar larvae at 12 hours post-molting were placed on ice for 15 minutes to anesthetize them. A volume of 5 μL of sterile dsRNA was injected into the hemocoel of the larvae. dsRNA dsGFP was used as control group.

### *2.8 RNAi in HaEpi cells*

HaEpi cells were cultured in Grace's medium with 10% FBS at 27°C to about 80% confluence in 6 well plates, and then 2 μg of dsRNA was transfected into the cells using the QuickShuttle-enhanced transfection reagent (Biodragon Immunotech,

Beijing, China) in 2 mL Grace's medium with 10% FBS for 24 h. Control cells were prepared using the same amount of dsGFP.

### *2.9 Overexpression of Dorsal and Relish*

The pIEx-4-GFP/RFP-His vector that was fused with green (GFP) or red fluorescent protein (RFP) was used for the experiments in the insect cell line. The open reading frames (ORFs) of the *dorsal* and *relish* were amplified using primers (Table 1) and inserted into the vector. The recombinant plasmids (5 mg) were transfected into HaEpi cells using the Quick Shuttle enhanced transfection reagent (Biodragon Immunotech). Cell fluorescence was observed using laser scan confocal microscope (OLYMPUS).

### *2.10 Protein extraction and western blotting*

The total protein of tissues was extracted using cell lysis buffer (Beyotime) contains 1 mM phenylmethanesulfonyl fluoride (PMSF). After thorough homogenization of the tissue samples, the homogenate was centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant was collected, and the protein concentration was determined using the Bicinchoninic Acid (BCA) assay method. To extract HaEpi cells protein, the cells were collected and lysed using radio immunoprecipitation assay buffer (Beyotime) plus protease inhibitors (Roche). an appropriate amount of loading buffer was added to the lysate and then the samples were boiled for 10 min. 50 μg of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated at room temperature in blocking buffer (5% fat-free powdered milk) for 1 h. The primary antibodies were diluted with blocking buffer, followed by overnight incubation at 4 °C. The membrane was subsequently washed three times with TBST (0.02% Tween-20 in TBS) for 10 min each time, followed by the addition of the Horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody, diluted 1:10000 with the same blocking buffer. The protein signal was observed using a chemiluminescence reagent kit (Advansta).

### *2.11 Statistical methods*

All data were plotted using GraphPad Prism 9 software. For qRT-PCR data, statistical significance was determined using Student's t-test. The *p* value was calculated through paired and two tailed analysis (*$p$<0.05, **$p$<0.01 and ***$p$<0.001). The value indicated as the mean ± SD (standard deviation), n ≥ 3. For mortality data, differences between groups were analyzed using the log-rank test. Three biological replicates and three technical replicates were performed for all experiments.

## 3. Results

### *3.1 The antibacterial activity of H. armigera is enhanced during the metamorphic molting stage*

The larvae of *H. armigera* enter the sixth instar after undergoing five molts. The last instar larvae pass through three distinct phases: the feeding stage (from molting to 72 hours), the wandering stage (from 72 to 96 hours), and the pre-pupal stage (from 96 hours to pupation) (Gao et al., 2023). To assess the antimicrobial capabilities of *H. armigera* at different developmental stages, we infected feeding-stage (6th-12h) and wandering-stage (6th-72h) larvae with the Gram-negative bacteria *P. aeruginosa* and the Gram-positive bacteria *M. luteus*, respectively, and subsequently recorded their survival rates. Compared to the NaCl-injected groups, the survival rates of larvae at the same instar decreased after infection with either *M. luteus* or *P. aeruginosa*. However, the survival rates of wandering-stage larvae infected with either bacterium were significantly higher than those of feeding-stage larvae, particularly after *M. luteus* infection (Fig.1 A and B). Subsequently, we analyzed the expression levels of AMPs, including *Attacin*, *Defensin*, *Cecropin D*, *Gloverin-1*, *Gloverin-2*, *Moricin*, and *Lebocin*, as well as the transcription factors *Dorsal* and *Relish* at different stages. The results demonstrated that the expression levels of AMPs in the fat body of *H. armigera* larvae during the wandering and pre-pupal stages were significantly higher than those in the feeding-stage. The expression of *Dorsal* and *Relish* were also

upregulated during these stages (Fig. 1C). These findings suggest that when entering the wandering stage, the larvae of *H. armigera* show a stronger immune response, particularly elevated expression of AMPs.

### *3.2 20E regulates the expression of AMPs*

20E plays a crucial role in the molting and metamorphosis of insects. Therefore, we measured the 20E titers in the hemolymph of feeding larvae (6th-6 h and 6th-24 h) and wandering larvae (6th-72 h and 6th-96 h). As expected, 20E titers were low in the feeding larvae and increased rapidly in the wandering larvae (Fig. 2A). Whether the elevated expression of AMPs during this stage is induced by 20E? To answer this question, we injected 20E into 6th-6 h larvae and examined the expression of AMPs in their fat body. The results showed that the expression of all examined AMPs was up-regulated at 12 h and 24 h post-injection (Fig. 2B). Further validation in HaEpi cells revealed that, similar to the results in larvae, the expression of AMPs was also up-regulated at 12 h after addition of 20E in the culture (Fig. 2C).

### *3.3 Injection of 20E suppresses bacterial proliferation in the hemolymph*

To further confirm the relationship between 20E and the immune response of *H. armigera*, exogenous 20E was injected into 6th-12 h larvae, followed by infection with *M. luteus* and *P. aeruginosa*, and the CFU in the hemolymph at different time post-infection were quantified. The results showed that, compared to the DMSO control group, there was no significant difference in CFU counts within 12 h post-infection by *M. luteus* and 24 h post-infection by *P. aeruginosa*. However, at 24 h post-infection by *M. luteus* and 36 h post-infection by *P. aeruginosa*, the CFU counts in the hemolymph of 20E-treated larvae were significantly reduced, indicating that the bacterial proliferation was suppressed (Fig. 3A and B). These findings demonstrate that 20E induces antibacterial activity in *H. armigera*.

### *3.4 20E regulates the expression of AMPs via Dorsal and Relish*

The transcription factors Dorsal and Relish directly regulate the transcription of

AMPs (Buchon et al. 2014). To investigate the roles of Dorsal and Relish in the 20E induced AMPs expression, we reduced the expression of *Dorsal* (Fig. 4A-a) and *Relish* (Fig. 4A-b) in *H. armigera* by RNA interference. After injection of *dsDorsal* or *dsRelish* for 12 h, 20E was injected into hemocoel, and the expression of AMPs in the fat body was measured 12 h later. The results showed that the 20E-induced expression of these AMPs was suppressed after *Dorsal* knockdown (Fig. 4B). After *Relish* knockdown, the expression of AMPs (except *Moricin*) decreased (Fig. 4C). We further validated these results in HaEpi cells. After transfecting dsRNA for 24 hours, the cells were treated with 20E, and the expression of AMPs was assayed. The results indicated that 20E-induced expression of AMPs was suppressed after *Dorsal* knockdown (Fig. 4D). Similarly, after *Relish* knockdown, the 20E-induced expression of AMPs (except *Moricin*) was inhibited too (Fig. 4E). These results were consistent with the *in vivo* experiments. The above findings suggest that 20E regulates the expression of AMPs including Attacin, Defensin, Cecropin D, Gloverin-1, Gloverin-2, and Lebocin through both Dorsal and Relish, while the expression of Moricin is solely regulated by Dorsal.

To further investigate the roles of Dorsal and Relish in the expression of AMPs, Dorsal and Relish were overexpressed in HaEpi cells respectively (Fig. 5A and C), followed by an examination of the regulatory effects of 20E on AMPs expression. The expression of AMPs was upregulated in cells transfected with pIEx-4-Dorsal-RFP compared to those transfected with pIEx-4-RFP after treatment with DMSO, overexpression of Dorsal alone can induce AMPs expression, and expression of AMPs was further enhanced in cells overexpressing Dorsal after incubation with 20E (Fig. 5B). Similarly, overexpression of GFP-Relish led to up-regulation of AMPs including *Cecropin D*, *Gloverin-1*, and *Gloverin-2* after DMSO treatment, compared to overexpression of GFP. After 20E treatment, the GFP-Relish overexpression group also showed up-regulation of some AMPs, including *Cecropin D*, *Gloverin-1*, *Gloverin-2*, and *Lebocin*, compared to overexpression of GFP (Fig. 5D). These results suggested that increased expression of Dorsal and Relish promotes the expression of *Cecropin D*, *Gloverin-1*, and *Gloverin*-2, and that 20E can enhance this process.

Interestingly, expression of *Attacin*, *Defensin*, and *Moricin* responded positively only to overexpressed Dorsal (Fig. 5B), but not to overexpressed Relish (Fig. 5D). Additionally, the up-regulation of *Lebocin* expression mediated by Relish seemed to depend on the presence of 20E (Fig. 5D).

### *3.5 20E promotes expression and activation of Dorsal and Relish*

To examine the expression of Dorsal and Relish during the development of *H. armigera*, we measured their protein levels in the fat bodies of larvae from early 5th instar to late 6th instar. Western blot results indicated that the expression of Dorsal remarkably increased after the larvae enter the metamorphosis molting stage (6th-72 h) (Fig. 6A). The expression of Relish gradually increased from 6th-6h larvae, and its cleaved form also increased after the larvae entered the metamorphosis molting stage (Fig. 6B).

To determine whether the increased expression of Dorsal and Relish during the metamorphosis molting stage, and the activation of Relish, are regulated by 20E, we treated 6th-12 h larvae with 20E. Western blotting showed that the expression of both Dorsal and Relish was upregulated in the fat body, and Relish was processed (Fig.6C). Functioning as nuclear transcriptional factors, Dorsal and Relish need to translocate to the nucleus to carry out their regulatory roles. To investigate the effect of 20E on the localization of these transcription factors, we treated HaEpi cells overexpressing RFP, Dorsal-RFP, and RFP-Relish with 20E, respectively. We found that overexpressed RFP was uniformly distributed in both the nucleus and cytoplasm after DMSO or 20E treatments. Dorsal-RFP localized in the nucleus under both DMSO and 20E treatments. In contrast, RFP-Relish was distributed in both the nucleus and cytoplasm when treated with DMSO, but moved to the nucleus after 20E treatment (Fig. 6D).

## 4. Discussion

In holometabolous insects, their larval tissue degradation and adult tissue formation happen during larvae to pupa development stage (Di et al., 2020; Zhang and

Edgar, 2022). With the degradation of the midgut, a large number of microorganisms are released, exposing the insects directly to the risk of pathogenic infection (Hakim et al., 2010). Studies show that insects defend against pathogens through enhancing their innate immune response. In *Bactrocera dorsali*s and *Spodoptera frugiperda*, the pro-phenoloxidase (PPO), which plays a key role in melanization of insect, is highly expressed during larvae to pupa (Bai et al., 2014; Eychenne et al., 2022). In the tobacco hornworm *Manduca sexta*, expression of lysozyme and AMPs *cecropin A*, *cecropin B* and *hemolin* increases dramatically during metamorphosis (Russell and Dunn, 1996; Yu and Kanost, 1999). In *D. melanogaster*, higher expression of AMPs was observed in pupariation (Nunes et al., 2021a; Thurmond et al., 2019). In this study, we found that the expression of AMPs is up-regulated before entering the pupal stage and the antimicrobial activity is enhanced in *H. armigera*.

It is well known that 20E regulates growth and development in insects (Malita and Rewitz, 2021). In mammals, the steroid hormones participate in regulation of immune responses and enhancement of anti-inflammatory reactions (Baschant and Tuckermann, 2010). 20E also has been shown to enhance innate immune responses in insects and reduce mortality caused by pathogenic infections (Keith, 2023). In normal circumstances, pathogen infection induces the expression of AMPs. However, 20E can regulate AMPs at specific developmental stages regardless the presence of pathogen (Nunes et al., 2021b; Regan et al., 2013). The 20E titer in insects is strictly regulated, which enables its precise control over the immune system. This mechanism allows insects to preemptively prepare for potential infections. Furthermore, upon infection, in addition to directly activating immune responses, insect may further upregulate the expression of AMPs by promoting the synthesis of 20E (Sun et al., 2016). In this study, we demonstrated that during the metamorphic molting stage of *H. armigera*, the titer of 20E increases, upregulating the expression of AMPs, and significantly enhancing the antibacterial capacity of the larvae. Our results are consistent with previously reported transcriptome sequencing data (Wang et al. 2014). The expression levels of AMPs induced by 20E during the feeding stage are significantly lower than those during the metamorphic molting stage. This might be

due to the release of pathogens during the metamorphic molting stage.

The Imd and Toll pathways play key roles in the synthesis of AMPs in insects. AMPs are regulated by conserved NF-κB signalling cascades (Buchon et al. 2014). Dorsal is associated with the classical Toll pathway, while relish belongs to the IMD pathway in *Drosophila* (Myllymaki et al. 2014). 20E regulates the expression of AMPs through different pathways in different insect species. 20E activates the IMD pathway in *Drosophila*, whereas activates the Toll pathway in *B. mori* and *L. migratoria* (Han et al., 2017; Rus et al., 2013; Zhang et al., 2023). AMPs are also directly regulated by the 20E signaling pathway, independent of the IMD or Toll pathways (Ma et al., 2019; Mai et al., 2017). Which pathway of 20E regulates AMPs in *H. armigera* remains unclear. In present study, we demonstrated that 20E up-regulates the expression of AMPs through Dorsal and Relish. This result implies 20E can activate both the Toll and IMD pathways. In the IMD pathway, Relish requires cleavage to become activated. Upon cleavage, the C-terminal part (Rel-49) remains in the cytoplasm, and the active N-terminal part (Rel-68) translocates to the nucleus to initiate transcription of AMPs (Stöven et al. 2003). In current study, we also found that 20E promotes the expression, cleavage and nuclear translocation of Relish. However, the overexpressed Dorsal located to nuclei directly to perform the transcriptional function when 20E or pathogens is absent in HaEpi cells. This phenomenon may be attributed to the deficiency of IκB homologue Cactus in the cells. Cactus is present in the cytoplasm and form a complex with Dorsal protein, preventing Dorsal movement to the nucleus (Belvin and Anderson, 1996).

Pathogen infection induces expression of specific AMPs. The IMD pathway is activated by Gram-negative bacteria, up-regulating the expression of *Diptericin*, *Attacin*, *Drosocin* and *Cecropin* in *Drosophila* (Asling et al., 1995; Dimarcq et al., 1994). Conversely, the Toll pathway is mainly activated by Gram-positive bacteria and fungi, up-regulating expression of *Defensin*, *Metchnikowin*, and *Drosomycin* (Dimarcq et al., 1994; Fehlbaum et al., 1994; Staczek et al., 2023). There are several AMPs have only been reported in lepidopteran insects, such as Moricin, Gloverin and Lebocin (Yi et al., 2014). 20E induces expression of a variety of AMPs in *Drosophila*

and *B. mori* (Rus et al., 2013; Zhang et al., 2023). In this study, we found that 20E up-regulates expression of *Attacin*, *Defensin*, *Cecropin D*, *Gloverin-1* and *2,* and *Lebocin* via Dorsal and Relish. However, 20E up-regulates expression of *Moricin* via Dorsal only. It has been reported that Moricins are regulated via Toll pathway in *M. sexta* and *B. mori*(Huang et al., 2018; Zhong et al., 2012). 20E also regulates Moricin through Toll pathway in *B. mori* (Zhang et al., 2023). Our results are consistent with these reports. Besides, we also found that 20E inhibits bacterial proliferation more rapidly after *M. luteus* infection than *P. aeruginosa* infection. It revealed that the enhancement of insect immunity induced by 20E exhibits varying efficacy in resisting different types of bacteria. The similar results have been observed in S2 cells (Ghassah et al., 2024).

## 5. Conclusion

Our study systematically assessed the impact of 20E on cotton bollworm innate immunity and uncovered the associated regulatory mechanisms (Fig. 7). During the developmental from the final instar larva to the pupa in *H. armigera*, the titer of 20E increases. In the fat body, 20E promotes the cleavage and nuclear translocation of the transcription factor Relish. The activated Relish, together with Dorsal, regulates expression of AMPs, including Attacin, Defensin, Cecropin D, Gloverin-1 and 2, and Lebocin. In contrast, the expression of Moricin is solely regulated by Dorsal. 20E up-regulates the expression of Dorsal and Relish. The up-regulated Dorsal promotes expression of AMPs throuth a positive feedback. However, up-regulation of Relish only partially contributes to the 20E-mediated expression of AMPs, specifically Cecropin D, Gloverin-1, Gloverin-2, and Lebocin. Following the overexpression of Relish, the Lebocin expression is dependent on 20E induction. 20E enhances the innate immune response of *H.armigera* during the metamorphic molting stage by mediating the expression of these AMPs.

## Author contributions

Caihua Chen: Investigation, Methodology, Data curation, Writing- Original draft preparation.

Youying Yan: Investigation, Methodology.

Jiaxin Wang: Methodology.

Lizhen Zhou: Methodology.

Zhiqiang Lu: Conceptualization, Project administration, Writing - review & editing.

## Declaration of competing interest

The authors declare no conflicts of interest with the contents of this article.


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## Data availability

All the data are contained within the manuscript.

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**Figure legends**

**Figure 1. Assessment of immune responses during larval development.** (A) The survival rate of larvae following infection with *M. luteus.* 6th-12h, 6th-72h represent sixth instarlarvae at the corresponding hours. (B) The survival rate of larvae following infection with *P. aeruginosa.* The log-rank test method was employed to analyze the significance of the survival curves, *** $p < 0.001$. n=90. (C) Expression levels of immune-related genes in fat body. The bars indicate the mean ± SD. Significant differences were calculated using Student's t test (*$p < 0.05$, **$p < 0.01$) according to three biological replicates. GenBank NO.: Attacin: AY948540.1 Defensin: ALT16899.1 Cecropin D: AAX51193.1 Gloverin-1: PZC75293.1 Gloverin-2 ： ALT16898.1 Moricin: GU182911.1 Lebocin: XP_021187065.2

**Figure 2. The expression of AMP genes induced by 20E.** (A) The changes of 20E titers in the hemolymph of larvae at different developmental stages. 6th-6 h and 6th-24 h represent the 6th instar larvae during the feeding stage, while 6th-72 h and 6th-96 h represent the larvae during the metamorphic molting stage. (B) Expression levels of AMPs in fat body following 20E injection. qRT-PCR detected the expression of AMPs at 6 h, 12 h, and 24 h post 20E injection. (C) Expression levels of AMPs in HaEpi cells after 20E treatment (5 μM, 12 h). *RPL27* as a negative control. The bars indicate the mean ± SD. Significant differences were calculated using Student's t test for all qRT-PCR according to three biological replicates (*$p < 0.05$, **$p < 0.01$).

**Figure 3. The effect of 20E on CFUs within the larvae.** (A) Larvae were infected with *M. luteus* at 12 h post 20E injection and CFUs were counted within 24 h. (B) Larvae were infected with *P. aeruginosa* at 12 h post 20E injection and CFUs were counted within 36 h. DMSO as a control. Significant differences were calculated using Student's t test (*$p < 0.05$). n=15.

**Figure 4. The effects of 20E on AMPs expression after *Dorsal* or *Relish***

**knockdown. (A)** Detection of the interference efficiency of *Dorsal* (a) and *Relish* (b) after *dsDorsal* and *dsRelish* injection at 6th-6h. Knockdown of *Dorsal* (B) or *Relish* (C) using *dsDorsal* or *dsRelish* in fat body, followed by injection with 20E for 12 h. *dsGFP* and DMSO were used as negative controls. The values are the mean ± SD. Expression of AMPs. (D and E) The expression of AMPs was detected after 20E (5 μM) treatment for 12 h in *Dorsal* (C) or *Relish* (D) knockdown cells. The values are the mean ± SD. Significant differences were calculated using Student's t test from three biological repeats (*$p < 0.05$, **$p < 0.01$).

**Figure 5. The influence of 20E on AMPs expression levels after Dorsal or Relish overexpression.** (A) Dorsal-RFP was overexpressed for 48 h, qRT-PCR detected the expression levels of *Dorsal*. (B) Dorsal-RFP Overexpressed cells were treated with 5 μM 20E or same volume DMSO for 12 h. qRT-PCR detected the expression levels of AMPs. (C) GFP-Relish was overexpressed for 48 h, qRT-PCR detected the expression levels of *Relish*. (D) GFP-Relish Overexpressed cells were treated with 5 μM 20E or same volume DMSO for 12 h. qRT-PCR detected the expression levels of AMPs. dsGFP and DMSO as negative controls. The values are the mean ± SD. Significant differences were calculated using Student's t test from three biological repeats (*$p < 0.05$, **$p < 0.01$).

**Figure 6. The effects of 20E on the expression and intracellular localization of Dorsal and Relish.** (A and B) Expression profiles of Dorsal (A) and Relish (B) in fat body. Protein was extracted from the fat bodies of larvae at different developmental stages, and the expression of Dorsal and Relish was detected by Western blot using anti-Dorsal and and anti-Relish antibodies. *β*-Actin was used as the protein quantity control. 5F, fifth instar feeding larvae; 5M, fifth instar molting larvae; F, feeding; MM, metamorphic molting. (C) After injecting DMSO or 20E for 24 h, fat body proteins were extracted and the expression of Dorsal and Relish was detected by Western blot. *β*-Actin was used as the quantity control. (D) Detection of the location of dorsal and Relish after DMSO or 20E treated Dorsal-RFP and

RFP-Relish-overexpressed cells for 6 h by using the laser confocal microscopy. The blue signals indicate the cell nuclei stained by DAPI. Scale bar: 10 μm. Cy: cytoplasm, Nu: nuclei.

**Figure 7. Schematic diagram showing the mechanism of 20E regulates AMPs expression in *H.armigera*.** The elevated 20E acts on the fat body cells, up-regulating expression of Dorsal and Relish, and promoting the activation and nuclear translocation of Dorsal and Relish proteins, thereby leading to the transcription of antimicrobial peptides through distinct mechanisms in metamorphic molting larvae.