Developmental and Comparative Immunology 145 (2023) 104711

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

RNAi-mediated *CrebA* silencing inhibits reproduction and immunity in *Locusta migratoria manilensis*

Ya Tang [1], Yao-Wen Hu [1], Shao-Hua Wang, Min Zhou, Yan-Juan Ding, Si-qi Cai, Bin Tang, Shi-Gui Wang [*]

Hangzhou Key Laboratory of Animal Adaptation and Evolution, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, Zhejiang, 311121, PR China

ARTICLE INFO

Keywords:
Locusta migratoria manilensis
Reproduction
CrebA
Immunity
RNA interference

ABSTRACT

Locusta migratoria manilensis is a major agricultural pest that causes severe direct and indirect damage to several crops. Thus, to provide a theoretical foundation for pest control, the role of *CrebA* in the reproduction and immune regulation of *L. migratoria* was investigated. CrebA is a bZIP transcription factor that critically regulates intracellular protein secretion. In this study, *CrebA* was widely expressed in the brain, fat body, integument, midgut, and reproductive tissues of different maturity stages of adult locusts, especially in the female fat body. RNA interfering (RNAi)-mediated silencing of *CrebA* inhibited locusts ovarian development, and key reproduction gene expressions, *Vgs*, *VgRs*, *Chico*, and *JHAMT* were downregulated. After the locusts were injected with *Micrococcus luteus* or *Escherichia coli*, *M. luteus* activated lysozyme expression, while the *E. coli* activated both phenol oxidase cascade and lysozyme expression. Furthermore, both bacteria stimulated the upregulation of the antimicrobial peptide genes *DEF3* and *DEF4*. However, *CrebA* silencing is fatal to locusts infection with *E. coli*, with a mortality rate of up to 96.3%, and resulted in a significant decrease in the expression of *DEF3* and *DEF4* and changes in the activities of phenol oxidase and lysozyme of locusts infected by bacteria. Collectively, *CrebA* may be involved in diverse biological processes, including reproduction and immunity. *CrebA* inhibited locusts reproduction by regulating JH signaling pathway and inhibits the expression of immune genes *TLR6, IMD*, and *AMPs*. These results demonstrate *CrebA* seems to play a crucial role in reproduction and innate immunity.

1. Introduction

The migratory Locust (*Locusta migratoria manilensis*) is a notorious agricultural pest with a strong ability to reproduce (Sangbaramou et al., 2018.). The development of ovaries and the maturation of oocytes are essential for the reproduction of locusts. The formation of vitellin, the major egg storage protein in oocytes , plays an important role in the development of insect ovaries. The formation of vitellin is closely related to the synthesis and absorption of vitellogenin (Vg) (Ferenz and Aden, 1993; Ullah et al., 2019). During vitellogenesis, Vg is primarily synthesized in the fat body of the insect, transferred to the ovary through the hemolymph, and is then absorbed by the developing oocytes via the vitellogenin receptor (VgR)-mediated endocytosis (Roy et al., 2018). Vitellogenin is deposited in oocytes to form vitellin, which increases ovarian volume and provides adequate nutrition for oogenesis and embryo development (Seidelmann et al., 2016). Therefore, *Vg* and *VgR* play important roles in vitellogenesis (Xiang et al., 2019). Abnormal expression of *Vg* and *VgR* leads to abnormal formation of vitelloprotein, which in turn leads to delayed ovarian development and even infertility in females (Lu et al., 2015; Upadhyay et al., 2016; Shang et al., 2018; Yao et al., 2018). Previous studies have shown that the insulin-like peptide (ILP) signaling, juvenile hormone (JH), and Toll pathways regulate *Vg* gene expression and are important for insect ovarian development (Parthasarathy et al., 2010; Parthasarathy and Palli, 2011; Gijbels et al., 2019; Li et al., 2021; Hu et al., 2022a). In female locusts, JH regulates *Vg* mainly by activating genes in the Vg synthesis pathway. In insects, polyploidy is widely observed in highly metabolically active tissues, such as fat bodies, ovaries, midgut, salivary glands, thoracic glands, and pterygium imaginary discs (Tenlen, 2018; Troha et al., 2018). The downstream genes of the JH pathway can regulate the polyploidization of fat body cells and the transcription, translation, post-translational modification, transfer, and absorption process of vitellogenin (Shinoda and Itoyama, 2003; Shmuel-Galia et al., 2017).

* Corresponding author.
E-mail address: sgwang@hznu.edu.cn (S.-G. Wang).
[1] The Ya Tang and Yao-Wen Hu are contributed equally to this work and share first authorship.

https://doi.org/10.1016/j.dci.2023.104711
Received 6 September 2022; Received in revised form 12 April 2023; Accepted 14 April 2023
Available online 14 April 2023

List of abbreviations

AMP	Antimicrobial peptides
CrebA	Cyclic AMP response element binding protein A
GFP	green fluorescent protein
IMD	pathway immune deficiency pathway
JH	juvenile hormone
PAE	post adult eclosion
PO	phenol oxidase
RNAi	RNA interference
dsRNases	dsRNA-degrading nucleases
RT-qPCR	reverse-transcription quantitative polymerase chain reaction
TLR	Toll-like receptor
Tollip	toll interacting protein
VgA	vitellogenin A
VgB	vitellogenin B
VgR	vitellogenin receptor

Polyploidization of the fat body ensures normal synthesis of Vg. In migratory locusts, JH-dependent Vg synthesis (Song et al., 2014) and uptake (Jing et al., 2018), and polyploidization of fat body cells promote vitellogenesis and oocyte maturation have been reported in related studies (Guo et al., 2014; Wu et al., 2018).

Insects have evolved an effective innate immune system to counter the stresses induced by various pathogens. When the pathogens penetrate the hard body surface of the insect, which is the first line of defense, and enter the hemolymph, they face the hemolymph immunity, which is the second line of defense of the insect, comprising both cellular and humoral immunity. Cellular immunity, which mainly relies on plasma cells and granulocytes, involves phagocytosis and the formation of nodules and cysts. The main features of humoral immunity are the production of antibacterial substances and antimicrobial peptides (AMPs), phenol oxidase (PO) cascade, and activation of the phagocytic reaction (Cerenius et al., 2008; Levitin and Whiteway, 2008; Xia et al., 2018). PO cascade reaction is rapidly activated after pathogens invade the insect, and serine protease cleave the prophenoloxidase (proPO) into active PO, which catalyze melanin formation and mediate coagulation responses, participating in the immune defense response of the body (Hultmark, 1993; Mavrouli et al., 2005). AMPs are short-chain peptides present in insect hemolytic lymph, which exhibit killing activity against Gram-positive and Gram-negative bacteria. Defensins are a class of naturally occurring AMPs and are the most widely characterized antimicrobial molecules in arthropods (Tonk et al., 2015). Defensins are mainly effective against Gram-positive bacteria, but some members are also active against certain Gram-negative bacteria, fungi, and protozoa (Contreras et al., 2020; Ganz and Lehrer, 1994). The mechanisms of antimicrobial activity of defensins may lead to the formation of pores on the microbial membrane, leading to the fatal destruction of integrity and function (Zaalouk et al., 2004). The production of lysozyme, AMPs, and other effector proteins occurs mainly through the Toll and immune deficiency (IMD) pathways (Jo et al., 2019). The Toll pathway has a dual function, involved in both immune regulation and development of insects, whereas IMD functions exclusively in immune regulation (Viljakainen, 2015). Fungi and some Gram-positive bacteria induce AMPs production through the Toll pathway, whereas the IMD pathway is typically induced by Gram-negative bacteria (Ferrandon et al., 2007).

Cyclic AMP response element binding protein A (CrebA) is a transcription factor of the bZip family, which was first discovered in *Drosophila*. It is homologous to mammalian *Creb3L1,* and *Creb3L2* genes (Fox et al., 2010; Panagopoulos et al., 2007). CrebA contains ATB domains and highly conserved BZip segments related to transactivation activity (Barbosa et al., 2013). It plays an important role in cell protein secretion, embryonic development, gene transcription, and regulating insect circadian rhythms (Rose et al., 1997; Fox et al., 2010; Jing et al., 2016; Troha et al., 2018). In *Drosophila*, *CrebA* is necessary for the high expression of all early secretory pathway genes in the salivary glands (Abrams and Andrew, 2005), and expressed in reproductive tissues (Smolik et al., 1992). It directly binds to the enhancer of secretory pathway genes, which is sufficient and necessary to activate the expression of each secretory pathway component gene. In addition, *CrebA* affects the development of larval epidermis, and the absence of *CrebA* can cause larval epidermal defects and mouthpart deformities (Andrew et al., 1997). *CrebA* is a core transcription factor that regulates secretion in the fat body. *Drosophila* infected with microorganisms upregulated the expression of *CrebA* through Toll and IMD signaling pathways (Troha et al., 2018). The stability of the endoplasmic reticulum (ER) environment is a basic necessity for the realization of endoplasmic reticulum function. Inhibition of *CrebA* triggers endoplasmic reticulum stress and significantly increases mortality from microbial infections (Xu et al., 2019). In addition, Creb plays a role in innate immune defense by regulating AMP gene expression in *Antheraea pernyi* challenged by pathogen (Gao et al., 2018). Direct evidence of Creb functions in insect reproduction has not been reported, but a study found that Creb regulates glycogen and lipid metabolism and plays a vital role in energy balance (Iijima et al., 2009; Choi et al., 2011). Energy homeostasis is essential for insects stress adaptation, reproductive processes, and tolerance to pathogen (Arrese and Soulages, 2010). It can be seen that *Creb/CrebA* is essential for insect growth reproduction and immunity. Currently, research on the *CrebA/Creb3-like* gene is mainly focused on mammals and *Drosophila*, but has rarely been studied in other insects. To explore the regulatory effect of *CrebA* on insect reproduction and immunity, further research is necessary.

The migration of large swarms of locusts not only causes huge economic losses but also cause serious ecological imbalances (Sangbaramou et al., 2018). Therefore, screening and discovering new locust control targets and enriching information and methods to control locust plaque are of great practical significance (Mohandkaci and Doumandji-Mitiche, 2012), while RNAi technology and RNAi-mediated crop protection for pest control have develop quickly (Zhang et al., 2021; Ullah et al., 2022; Wei et al., 2023). This study elucidated the expression patterns of *CrebA* in different organs of locusts and maturation stages after eclosion via fluorescence quantification. Furthermore, the influence of *CrebA* on ovarian development of locusts and the effects caused by Gram-positive and Gram-negative bacteria were also observed. In addition, the immune response was analyzed, which revealed that the *CrebA* transcription factor plays an important role in the reproduction and immunity in locusts. This study provides a potential target site for future pest biological control and laying a theoretical foundation for pest control.

2. Materials and methods

2.1. Insects

L. migratoria eggs were purchased from a locust farm in Huaibei, Anhui Province. Locust eggs (50 g) were placed in a box (10 cm × 15 cm × 20 cm) with a layer of wet sand (2–3 cm) and were reared at 30 ± 2 °C and 80% RH, with a 14 h: 10 h (L:D) photoperiod. The eggs were covered with vermiculite to maintain moisture. After hatching, the locusts were fed a mixture of fresh wheat seedlings and wheat germ. Approximately 200–300 individuals in each cage were placed in an insect cage (50 cm × 50 cm × 50 cm) in an artificial climate chamber. The feeding and temperature conditions were the same as those described above.

2.2. RNA extraction and RT-qPCR

Total RNA was extracted from the different tissue samples using Trizol reagent (Invitrogen, Carlsbad, California, USA), according to the manufacturer's instructions. The RNA integrity was assessed bands

using gel electrophoresis. When the 18S and 28S bands were visible, the RNA bands were considered complete. The concentration and purity of total RNA were measured using a nucleic acid-protein micrometer (ND2000, Thermo Fisher Scientific, Massachusetts). Reverse transcription (RT) reactions were carried out using the PrimeScript RT reagent Kit (Takara, Dalian, China), and then each cDNA template was diluted 5-fold for reverse-transcription quantitative PCR (RT-qPCR) analysis.

RT-qPCR was performed with a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, CA, USA). The qPCR assays were carried out in a total volume of 20 μL using SYBR Premix Ex Taq reagents (Takara, Dalian, China). All RT-PCR primers were designed using Primer 5.0 software (Table 1). The thermal profile for RT-qPCR was: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The thermal melting profile was assessed using a final PCR cycle of 95 °C for 30 s, with the temperature constantly increasing from 60 to 95 °C. The expression levels of the selected genes were normalized to *actin* expression. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, with three replicates per sample.

2.3. Tissue and developmental expression analysis of CrebA gene

To analyze the spatiotemporal expression profiles of *CrebA* gene, brain, fat body, integument, midgut, and reproductive tissues from different developmental stages of adult locusts were dissected and collected, including the early eclosion phase (12 h post adult eclosion, 0.5 day PAE), sexual maturity phase (4 days PAE), mating phase (8 days PAE), and spawning phase (16 days PAE). Each sample included three biological replications of five locust individuals each. The samples were snap-frozen in liquid nitrogen and stored in a refrigerator at −80 °C for subsequent extraction of total RNA. The spatiotemporal expression profiles of *CrebA* were analyzed with RT-qPCR.

2.4. Synthesis and injection of dsCrebA

The full-length sequence of *CrebA* was obtained from *the L. migratoria manilensis* genome database, and the specific primers used for *CrebA* PCR amplification and dsRNA synthesis were designed using Primer5.0 software (Table 1). The green fluorescent protein (*GFP*) gene was used as the negative control. The ds*CrebA* (392 bp) and ds*GFP* (365 bp) were synthesized *in vitro* using T7RiboMAX Express RNAi System (Promega Corporation, Madison, WI) and was purified using the method previously described by Tenlen (Tenlen (2018).

Injection of dsRNA Based on the results of the preliminary experiments, female locusts at the early eclosion phase were selected as the test insects. Twenty micrograms per unit of dsRNA was injected into the second and third abdominal node using a microinjector (Sangon, Shanghai, China). The control group was injected with ds*GFP*, and the tissue samples were collected on the 5th day, and ovarian morphology was photographed using a dissecting microscope (Leica EZ4). The tissue samples were snap-frozen in liquid nitrogen and stored in a refrigerator at −80 °C. The hemolymph was collected and stored in a refrigerator at −20 °C.

2.5. Induction of the immune system response by bacterial infection

Micrococcus luteus and *Escherichia coli* strains were purchased from Beijing Biobw Biological Company and were activated before the experiment. The strain was cultured until $OD600_{nm} = 0.9$ was reached and stored at 4 °C for later use.

Bacteria injection. At sexual maturity the locusts were injected with either 10 μL of *M. luteus* or *E. coli* solution; the control group was injected with an equal volume of the corresponding bacterial medium. Dissection was performed 24 h after the injection of the bacterial solution. The samples were snap-frozen in liquid nitrogen and stored in a refrigerator at −80 °C, and the hemolymph was stored at −20 °C for subsequent detection of related gene expression and enzyme activity. For survival analysis, female locusts at sexual maturity stage were selected to be injected with bacteria, and mortality was recorded every 24 h for 7 days. Each treatment included 75 individuals.

Combined injection of bacteria and dsRNA. Based on the results of the preliminary experiments, the RNA interference efficiency was the highest on the fifth day after injection of dsRNA. The female locusts were injected with 20 μg/head of dsRNA at the early eclosion stage. After 4 days, the locusts were injected 10 μL of either *M. luteus,* or *E. coli* was injected; the control group was injected with an equal volume of the corresponding strain of the liquid medium. Dissection was performed 24 h after the injection of the bacterial solution, and they were processed as described above. To estimate bacterial load, the hemolymph was collected in sterile 1.5 mL centrifuge tube, and subsequently diluted to an appropriate concentration with sterile PBS. One hundred μL of each dilution was inoculated into solid medium, the colony forming units (CFU) were counted after inoculated at 37 °C for 24 h (Saha et al., 2017). For survival analysis of bacteria infection after *CrebA* silencing, the mortality rate of *LmCrebA* knocked down was recorded every 24 h for 7 days after bacteria infection as described above. Each treatment included 43 to 70 individuals.

2.6. Determination of phenol oxidase and lysozyme activity

For experiments measuring PO and lysozyme activity, the

Table 1
Primers for PCR.

Primer name	F-Primer sequence (5′–3′)	R-Primer sequence (5′–3′)	
GFP	CTTCTCGTTGGGGTCTTTGC	TCCAGGAGCGCACCATCTT	cDNA clones
CrebA	CCATCCAGACGCCACTCATC	ACTATTCCTCGCTGCCCTTG	
Lm-actin	GACGAAGAAGTTGCCGCTC	TCCCATTCCCACCATCACA	RT-qPCR
CrebA	CCATCCAGACGCCACTCATC	GGAATTGGGTATCCTTCAGC	
Chico	TTTTGCGTGGCGAGTCAG	TTTTGGGAGGATGATGGGAT	
Met	GCGGTCACCTCTTGTCAATAAT	CACTTTCTGATGCTGCCCTAA	
JHAMT	GAGACTCGCCTTCCAGAACA	AGAGGACATCGCCATTGACT	
Toll-like receptor-6	AGTGGACTCGTTCGTCTCGT	ATGGCGTCTATTCCGTTGTG	
Toll receptor-9	CGTGCTCCAGTTCAGATAATGC	GATGAACCTGACCTTGCCCTA	
LmDEF3	AGGGCTACAAGGGAGGACAC	CATCAGCAGGCAAGAAACAAG	
LmDEF4	GAAACCTCGTGCGAGCAGA	CGATGCCAGCAGTGGTAAGG	
IMD-like protein	GAACAAGTGGCAACGCTAAC	TCTTCTGAATCATGCGAGTC	
Lm-VgA	CTCTTTCGTCCAACAGCCG	CTCGCAACCATTCCCTTCA	
Lm-VgB	GAACTCAAGGGTCGTGGCA	TGGGTGAAGCAGCGGAAT	
Lm-VgR1	ATAAAGGTCTACCATCCAGCCC	GACAGGCACAGGTGTAGGAGTT	
Lm-VgR2	TGACTCCAAAGTGAAGAAGACC	ATTGCCCTGTAGCACCATT	
dsGFP	TAATACGACTCACTATAGGGAGA CTTCTCGTTGGGGTCTTTGC	TAATACGACTCACTATAGGGAGATCCAGGAGCGCACCATCTT	dsRNA synthesis
dsCrebA	TAATACGACTCACTATAGGGAGA CCATCCAGACGCCACTCATC	TAATACGACTCACTATAGGGAGA ACTATTCCTCGCTGCCCTTG	

hemolymph samples were collected from females. The samples were then centrifuged at 4 °C for 20 min at 3500 g to remove hemocytes. Subsequently, the supernatant was diluted 5 times to determine lysozyme activity, 10 times to determine PO activity, and 50 times to determine total protein content. The lysozyme and PO activities were assessed using commercially available kits (Nanjing Jiancheng Biological Company, China) following our previously described method (Hu et al., 2022b), with appropriate modifications. Determination of PO activity: 20 μL serum diluent was added to 40 μL phosphate-buffered saline (PBS) (pH 6.4) buffer and 50 μL L-3, 4-dihydroxyphenylalanine (L-dopa) (3 g/L). In a blank control, 60 μL of PBS and 60 μL of 3 g/L L-dopa were used. The reaction mixture was sonicated at 25 °C for 30 min in a water bath, thereafter absorbance was measured at 490 nm against a blank containing 50 μL (PBS) buffer (pH = 6.4) and 50 μL L-3, 4-dihydroxyphenylalanine (L-dopa) (3 g/L). The total absorbance was calculated as follows: $\triangle OD_{490\ nm} = OD_{490\ nm}$ of sample − $OD_{490\ nm}$ of blank. Total protein content was determined using the Coomassie bright blue protein kit (Nanjing Jiancheng Biological Company, China) following the manufacturer's instructions. PO enzyme activity (U_p) was calculated as follows: $U_p = \triangle OD_{490\ nm}$/min/mg protein.

Determination of lysozyme activity: 20 μL serum diluent was added to 200 μL of the bacterial liquid medium prepared according to the manufacturer's instructions. After mixing, the reaction mixture was added to the enzyme label plate, and absorbance (A_0) was determined at a wavelength of 530 nm. The reaction mixture was incubated at 37 °C for 30 min and then placed on ice for 10 min. Thereafter, absorbance (A) was determined at a wavelength of 530 nm. Ly enzyme activity was calculated as follows: $U = (A_0 - A)/A$.

2.7. Data statistics and analysis

Statistical differences were analyzed using the SPSS 26.0 software. The spatiotemporal expression data of *CrebA* gene and the enzyme activity data after bacterial injection were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test, and the different letters above the error bars indicate significant differences ($p < 0.05$), data are expressed as mean ± standard error (SE) of three replicates. Kaplan-Meier survival analysis was used to assess variables that affect locusts survival. The rest of the statistics were analyzed using Student's *t*-test (*$P < 0.05$, **$P < 0.01$), data are expressed as mean ± SE of three replicates.

3. Results

3.1. Spatiotemporal expression profiles of CrebA gene

To investigate the relative expression of *CrebA* in different tissues and different maturity stages, we examined their transcript abundance in different tissues at 12 h, 4, and 8, 12, and 16 days after adult eclosion by RT-qPCR. Obviously, The *CrebA* gene exhibited the difference spatiotemporal expression patterns in male and female locusts. For analysis of *CrebA* expression patterns in male locusts, expression level in the brain 12 h after eclosion (0.5 PAE) was considered as a control. In the selected five stages, we observed that the expression level of *CrebA* after eclosion first increased and then decreased (Fig. 1A). Interestingly, in all selected tissues, the relative expression level of *CrebA* was the highest in the mating period, that is, the 8th day of eclosion (Fig. 1A). For analysis of *CrebA* expression patterns in female locusts, expression level in the brain 12 h after eclosion (0.5 PAE) was considered as a control, and it was observed that the *CrebA* expression level in the fat body was the highest in four out of the five-time points measured (Fig. 1B). The spatiotemporal expression profiles of *CrebA* in female were shown, a continuous upward trend was observed in the expression level of *CrebA* in female locusts from the beginning of eclosion to 16 days after eclosion, except for the expression in ovarian tissue. The expression level of *CrebA* was highest during the egg-laying period, especially in the fat body, and was significantly different from those in the brain, ovary, integument, and midgut (Fig. 1B). The expression level of *CrebA* between tissues at each period were showed, *CrebA* expression in the fat body was significantly higher than those of other tissues at 12 h, 8 days, and 16 days after adult eclosion (Fig. 1B).

3.2. The effect of interference with CrebA gene on locusts reproduction

The results of RT-qPCR showed that the interference of *CrebA* significantly reduced the expression of target genes in the fat body. It was observed that the knockdown efficiency of *CrebA* was approximately 80% (Fig. 2A), however, no effect on the mortality rate was observed during the process. To explore the effect of *CrebA* silencing on locusts reproduction, reproduction-related gene expression levels and ovarian development were examined. The results showed that the mRNA levels of *JHAMT*, *Chico*, *VgA*, *VgB* and *VgR1* in fat body were significantly downregulated when *CrebA* expression decreased (Fig. 2B and C), indicating that vitellogenin synthesis was blocked. In addition, ovarian weight was significantly reduced and ovarian development was severely atrophied, and oocytes were poorly developed compared with

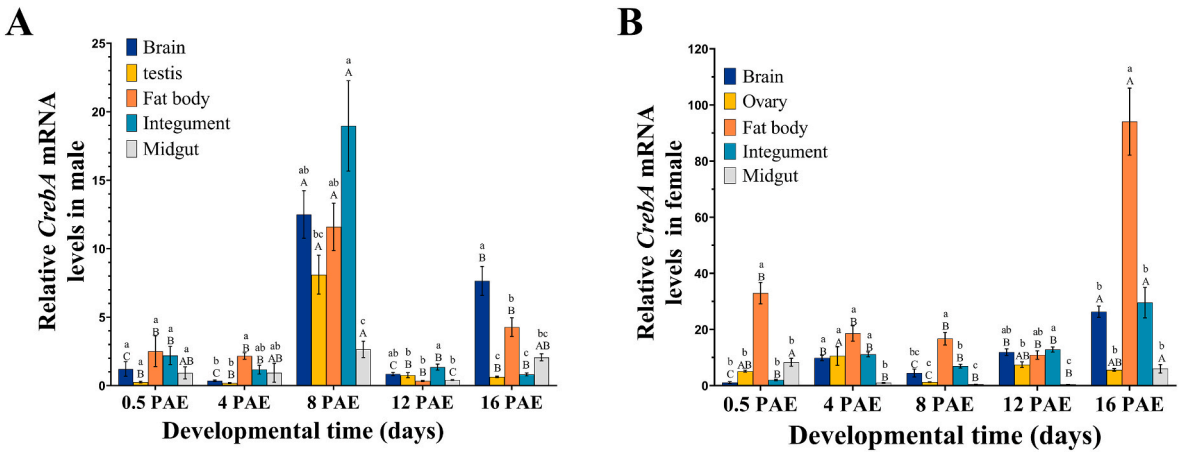

Fig. 1. Relative expression of *CrebA* in different tissues and maturity stages of male locusts (A) and female locusts (B). Each value is presented as mean ± SE. Different uppercase letters above the error bars indicate significant differences between the same tissue at different developmental stages, and different lowercase letters indicate differences between different tissues at the same developmental stage (one-way ANOVA followed by Tukey's multiple comparisons test, $P < 0.05$). Each group included three biological replicates of five locust individuals each. PAE, post adult eclosion.

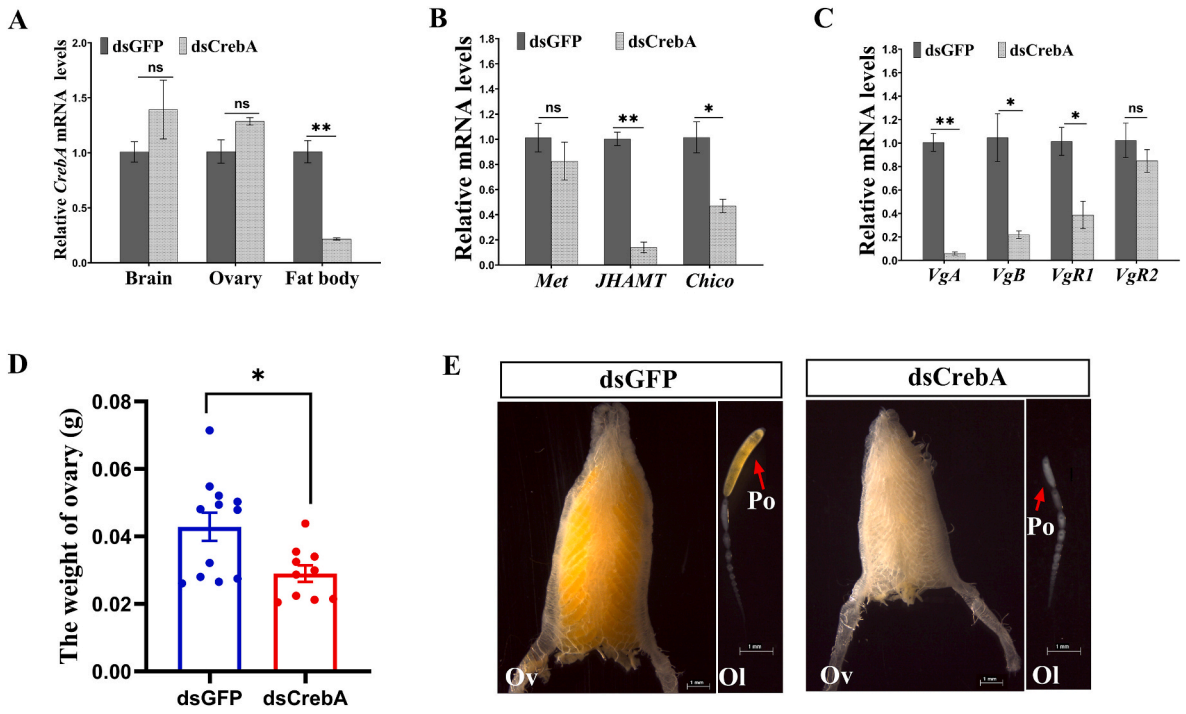

Fig. 2. The effect of knockdown of *CrebA* gene on the expression of reproduction-related genes and ovarian development. (A) *CrebA* silence efficiency in different tissues. (B) The effect of knockdown *CrebA* gene on the expression of Juvenile hormone (JH) pathway related genes. (C) The knockdown of effect with *CrebA* gene on the expression of *Vg*s and *VgR*s. (D) Changes in ovarian weight after *CrebA* silencing. Each value is presented as mean ± SE, and significant differences are indicated by * ($P < 0.05$) and ** ($P < 0.01$) between the control group (Student's t-test). Each group included three biological replicates of five locust individuals each. (E) Changes in ovarian morphology after *CrebA* silencing. Ov, Ol, and Po represent ovaries, ovarioles, and primary oocytes, respectively. Ovarian morphology was photographed using dissecting microscope at scale = 1 mm.

the control group (Fig. 2D and E). These results indicate that low expression of *CrebA* can inhibit ovarian development.

3.3. Effects of CrebA silencing on mortality and bacterial load

To investigate the importance of *CrebA* in tolerance against bacteria, we first examined the effects of Gram-positive bacteria *M. luteus* and Gram-negative bacteria *E. coli* on locusts mortality. The day of injection was considered as the first day, and mortality rate data were recorded for 7 days. The results showed that the survival rates of locusts infected with *M. luteus* and *E. coli* were 37.5% and 22.3% compared to media injected control, respectively (Fig. 3A and B). After injection of *M. luteus*,

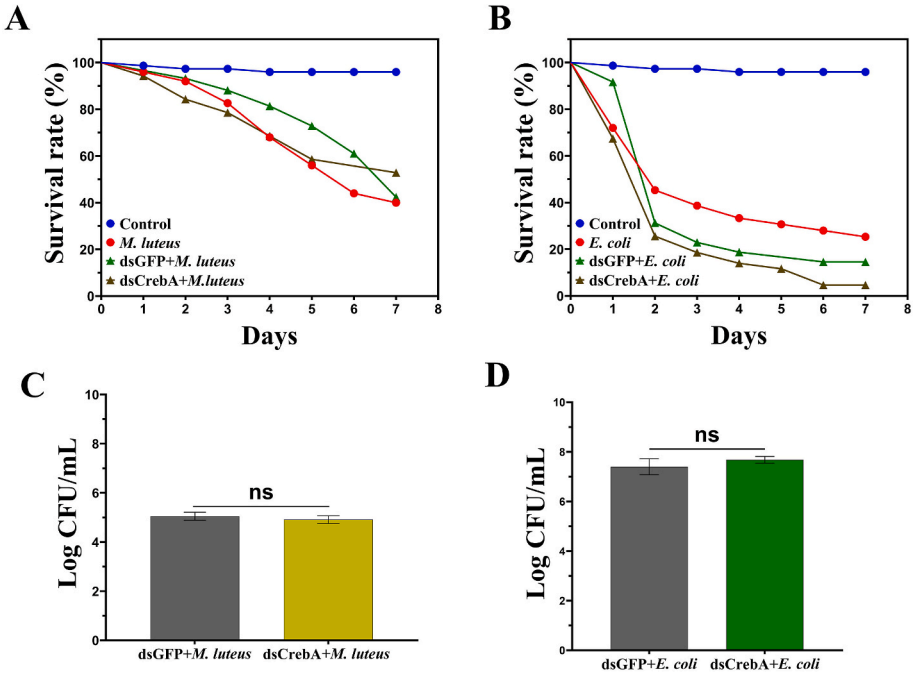

Fig. 3. *M. luteus* and *E. coli* significantly reduced the survival rate of locusts, and *CrebA* silencing increased susceptibility to *E. coli*. (A) Survival rate of *M. luteus* and *CrebA RNAi* + *M. luteus* to locusts. (B) Survival rate of *E. coli* and *CrebA RNAi* + *E. coli* to locusts. Each treatment included 43 to 75 individuals (Kaplan-Meier survival analysis). To calculate the bacterial counts (CFUs), locusts were injected with ds*CrebA* in the early stage of emergence, after 4 days, the locusts were injected *M. luteus* (C) or *E. coli* (D). Each value is presented as mean (log-transformed) ± SE. Each group included three biological replicates of five locust individuals each.

the mortality rate was lower in the early stage and higher in the middle and late stages of infection. Furthermore, the mortality rate was the highest on the fourth day after infection (Fig. 3A). After injection of *E. coli*, the mortality rate was as high as 28.7% in the early stages of infection (1 day after injection); however, the mortality rate reduced in the middle and late stages of infection (Fig. 3B). We then investigated the effects of *CrebA* silencing on bacterial load and mortality, and found that compared with ds*GFP* injection control, there was a significant increase in mortality after *E. coli* infection ($P = 0.0338$) (Fig. 3B), and a decrease after infection with *M. luteus*, but there was no significant difference ($P = 0.6483$) (Fig. 3A). Reduced mortality and bacterial loads were observed in *M. luteus*-infected group and increased in *E. coli*-infected group after *CrebA* silencing compared to the control group injected with ds*GFP* (Fig. 3).

3.4. Changes in enzyme activity of locusts after bacterial infection

To explore the role of *CrebA* in locust immunity, we tested the vitality of the important enzymes that participate in immune defense, namely PO and lysozyme. The PO (Fig. 4A) and lysozyme (Fig. 4D) activity levels of the control group injected with ds*GFP* and the experimental group injected with ds*CrebA* were consistent. This indicated that in the absence of an immune response, the decrease in *CrebA* expression level did not affect the PO and lysozyme activities in locusts. In the immune response triggered by infection with *M. luteus*, no significant change was observed in the PO activity of locust injected with ds*GFP* (Fig. 4B); however, lysozyme activity significantly increased (Fig. 4E); furthermore, it was observed that the low expression of *CrebA* inhibited the expression of PO (Fig. 4B), but did not affect lysozyme activity (Fig. 4E). In the immune response triggered by infection with *E. coli*, the activities of PO and lysozyme in locusts significantly increased; however, the low expression of *CrebA* did not have any significant effect compared to the ds*GFP* control on the expression of PO and lysozyme (Fig. 4C and F).

3.5. Changes in expression of immune-related genes in locusts after bacterial infection

In insect humoral immunity, the invading organism is recognized by the pattern recognition protein that triggers the secretion of antibacterial substances through the Toll and IMD pathways. To evaluate the impact of bacterial infection on locusts immunity, the key immune-related genes *TLR6*, *TLR9*, *Tollip*, *IMD*, *DEF3*, and *DEF4* mRNA levels were measured by RT-qPCR. In the absence of an immune response, the decrease in *CrebA* expression resulted in downregulation of *DEF3* and *DEF4*, and that of *Tollip* was up-regulated, while *TLR6*, *TLR9* and *IMD* showed no significant difference (Fig. 5A). After infection with *M. luteus*, *IMD* was downregulated, *TLR6*, *DEF3*, and *DEF4* genes were significantly upregulated (Fig. 5B and C). However, after infection with *E. coli*, *CrebA*, *TLR9* and *IMD* genes were downregulated, while *DEF3* and *DEF4* were significantly upregulated. The expression levels of *CrebA*, *TLR* and *IMD* showed extremely significant differences (Fig. 5D, E and F).

3.6. Changes in expression of immune-related genes in locusts infected with bacteria after CrebA knockdown

To further verify the hypothesis that *CrebA* affects the expression level of antimicrobial peptides via the Toll/IMD pathway, RNAi technology was used to reduce the expression level of *CrebA* in locusts. It was

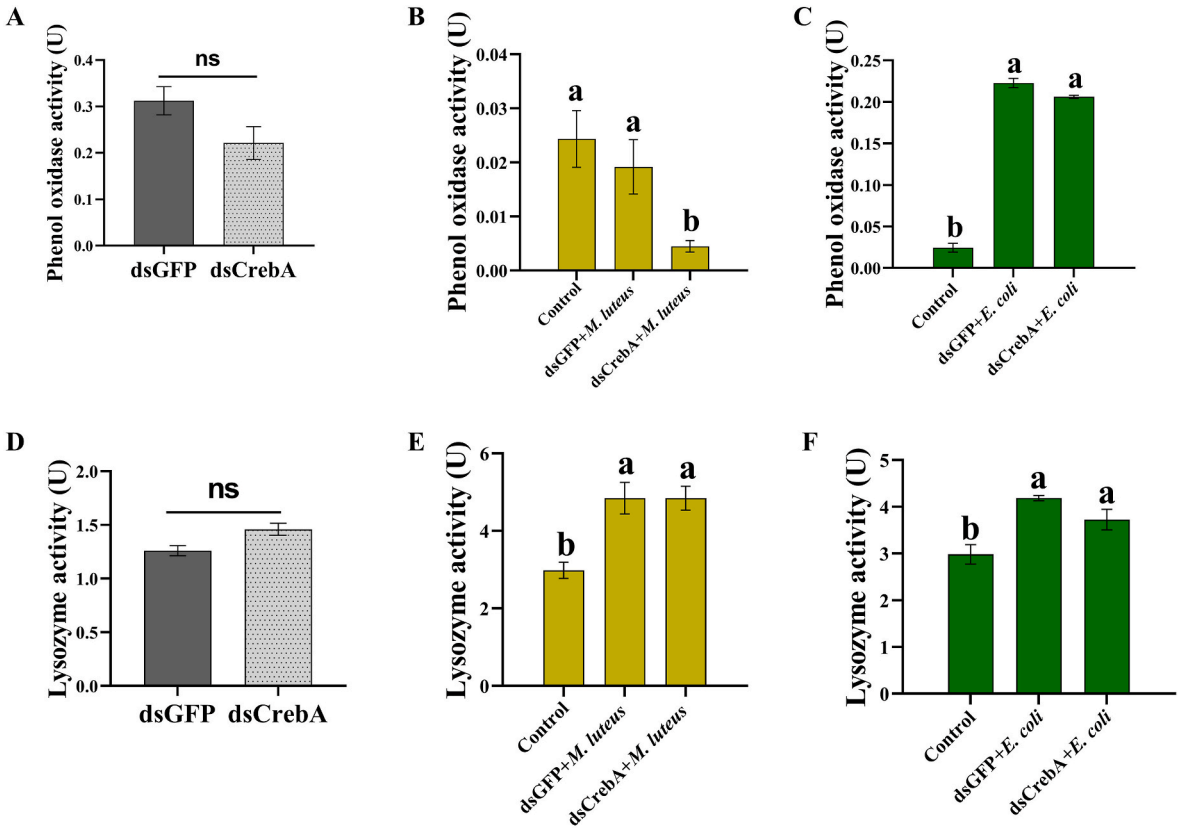

Fig. 4. Changes in PO and Lysozyme activities after infection with *M. luteus* and *E. coli*. Effect of *CrebA* Silencing on the activities of PO (A) and Lysozyme (D). Effect of *CrebA* Silencing on the activities of PO (B) and Lysozyme (E) in *M. luteus* Infection. Effect of *CrebA* Silencing on the activities of PO (C) and Lysozyme (F) in *E. coli* Infection. The control group was injected with *M. luteus* and *E. coli*, respectively. Each value is presented as mean ± SE, and different letters above the error bars indicate significant differences among treatments (one-way ANOVA followed by Tukey's multiple comparisons test, $P < 0.05$). Each group included three biological replicates of five locust individuals each.

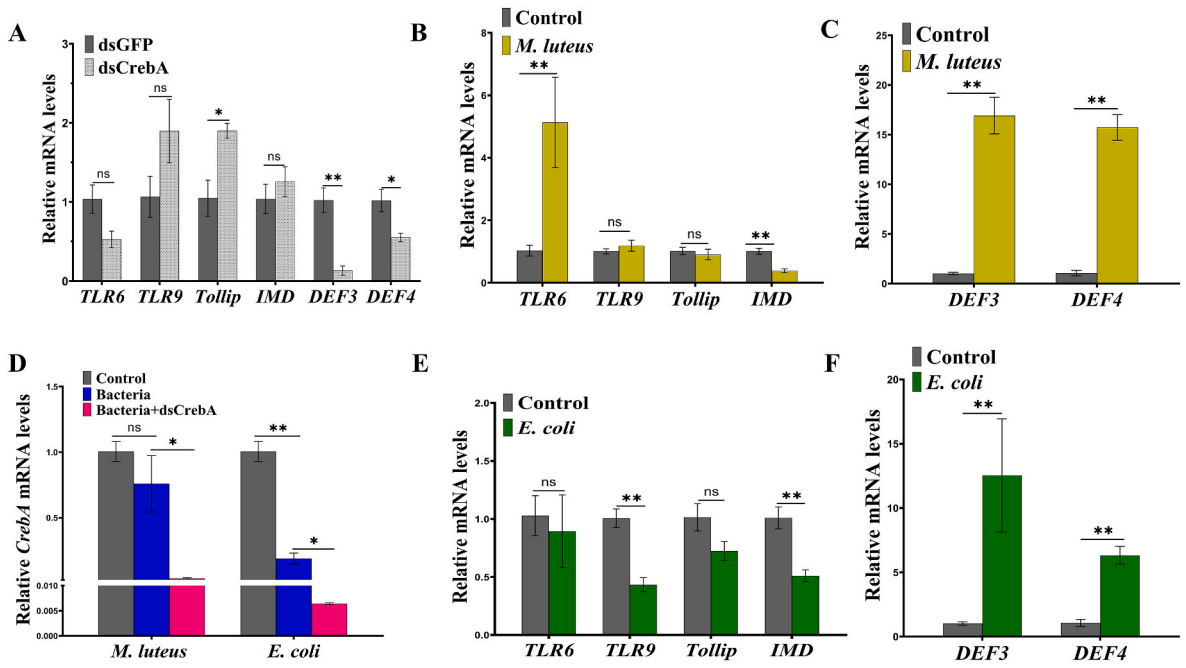

Fig. 5. Effect of bacterial infection on the relative expression of immune-related genes in *L. migratoria manilensis*. (A) Expression of immune-related genes after *CrebA* silencing. Changes in mRNA levels of Toll/IMD related genes (B) and defensins(E) induced by *M. luteus*. Changes in mRNA levels of Toll/IMD related genes (C) and defensins(F) induced by *E. coli*. (D) Effect of RNAi of *CrebA* combined with bacteria injection on the expression of *CrebA*. The control group was injected with an equal volume of the corresponding bacterial medium. Each value is presented as mean ± SE, and significant differences are indicated by * ($P < 0.05$) and ** ($P < 0.01$) (Student's t-test). Each group included three biological replicates of five locust individuals each.

observed that the functions of *CrebA* vary depending on the immune responses induced by infection with *M. luteus* and *E. coli*. After injection of *M. luteus*, the expression levels of *TLR6*, *DEF3*, and *DEF4* were significantly downregulated in locusts with low *CrebA* expression, whereas no significant changes were observed in the expression levels of *TLR9*, *Tollip*, and *IMD* (Fig. 6A and B). After infection with *E. coli*, the expression levels of *IMD*, *DEF3*, and *DEF4* were downregulated in locusts with low *CrebA* expression, whereas no significant change in the expression levels of *TLR6*, *TLR9*, and *Tollip* was observed (Fig. 6C and D).

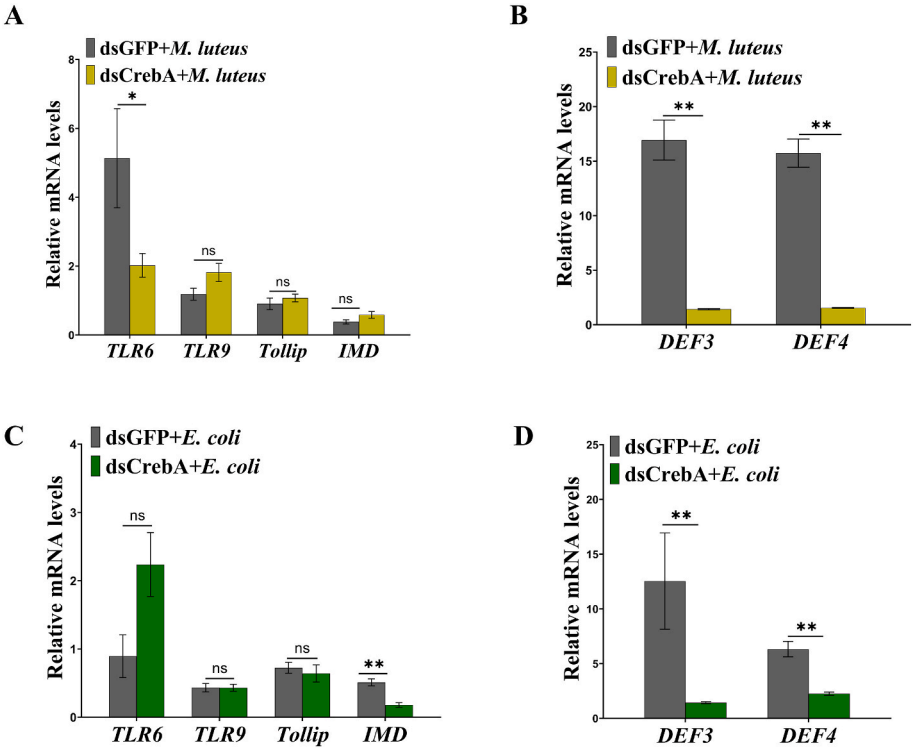

Fig. 6. Effects of bacterial infection on immune-related genes expression with reduced *CrebA* expression in *L. migratoria manilensis*. Changes in mRNA levels of Toll/IMD related genes (A) and defensins (C) induced by *M. luteus* after *CrebA* silencing. Changes in mRNA levels of Toll/IMD related genes (B) and defensins (D) induced by *E. coli* after *CrebA* silencing. Each value is presented as mean ± SE, and significant differences are indicated by * ($P < 0.05$) and ** ($P < 0.01$) (Student's t-test). Each group included three biological replicates of five locust individuals each.

4. Discussion

Amazing fecundity is one of the key factors in the outbreak of the locust plague. The JH pathway affects insect reproduction by inducing the synthesis and uptake of vitellogenin (Verlinden et al., 2009; Roy et al., 2018). The insulin receptor substrate, CHICO, participates in several key processes of insect growth and development mediated by the IIS/TOR pathway and affects the survival and metamorphosis of insect larvae by modifying JH signal transduction (Böhni et al., 1999; Nässel et al., 2015; Zhou et al., 2022). *JHAMT*, a juvenile hormone acid methyltransferase, is essential for the synthesis of JH. The function of *JHAMT* was first studied in silkworms, and it was confirmed to be related to the synthesis of JH (Kinjoh et al., 2007). The necessity of *JHAMT* in JH synthesis has been reported in other insects, such as *D. melanogaster* and *Aedes aegypti* (Diptera) (Shinoda and Itoyama, 2003), *Tribolium castaneum* (Coleoptera) (Xu et al., 2013), *Spodoptera frugiperda* (Lepidoptera) (Jia et al., 2022), and *Schistocerca gregaria* (orthoptera) (Marchal et al., 2011). *CrebA* is widely expressed in various tissues of both male and female locusts, and is highly expressed in female fat body, suggesting that *CrebA* may be involved in a variety of biological processes, including reproduction and immunity (Fig. 1). At the early eclosion phase of locusts, which was also the time point of dsRNA injection, *CrebA* was expressed significantly higher in female locusts fat body than in other tissues, including brain, ovary, integument, and midgut (Fig. 1B). We speculate that low expression in the brain and ovary may resulted in the lack of *CrebA* interference efficiency (Fig. 2A). After RNAi-mediated knockdown of *CrebA*, we detected significant reductions in the expression levels of *Chico* and *JHAMT* in the fat body (Fig. 2B). The fat body is a unique organ in insects that are involved in the secretion of a variety of proteins and has high metabolic activity (Arrese and Soulages, 2010), and is closely involved in various key processes during the life cycle of locusts such as, in the reproduction, immunity, growth, and development. The fat body synthesizes several proteins, such as storage proteins, which are secreted into the hemolymph, antibacterial proteins related to immune pathways, and vitellogenin, which affects the reproductive ability of insects by affecting vitellogenesis (Arrese and Soulages, 2010). The polyploidization of adipocytes and JH promotes the large-scale expression of *Vg*, which is required for the maturation of oocytes and accelerates the production of other proteins required for egg production (Wu et al., 2018). The observation of the length of basal oocytes of the desert locust indicated that the knockdown of *JHAMT* led to a delay in sexual maturation (Marchal et al., 2011). In addition, studies have shown that previtellogenic wild-type *Drosophila* ovaries transplanted into a *Chico* mutant of a sterile *Drosophila* insulin receptor protein can induce vitellogenesis, whereas *Chico* mutant ovaries transplanted into wild-type *Drosophila* cannot produce Yolk protein (Richard et al., 2005; Parthasarathy and Palli, 2011). These results indicate that the low expression of both *Chico* and *JHAMT* can inhibit insect ovarian development, which is consistent with our findings (Fig. 2B). In female locusts that interfere with *CrebA*, vitellogenin gene expression was significantly reduced (Fig. 2C), and the development of locust ovaries was inhibited (Fig. 2D and E). These findings confirm the negative impact of *CrebA* deficiency on locust reproduction.

Infection with *M. luteus* and *E. coli* triggered different immune responses in the locusts. The lethal effects of the two pathogenic bacteria were different. After injection of *E. coli*, a high mortality rate was observed in the early stage of infection, whereas it considerably reduced in the later stages of infection, *CrebA* RNAi further weakened locust tolerance against *E. coli* (Fig. 3B). As a similar result, low expression of *CrebA* increased mortality after infection with the Gram-negative bacteria *Pseudomonas entomophila* (Ragheb et al., 2017). In contrast, after infection with *M. luteus*, the majority of the locusts were still alive after 24 h of infection, and a higher mortality rate was observed in the middle and late stages than in the early stage of infection. However, there was no significant effect on the survival rate after *CrebA* silencing (Fig. 3A). Our finding also found that bacterial load was not affected by *CrebA* RNAi, which may be by promoting tolerance to infection rather than resisting bacterial proliferation (Troha et al., 2018). Accurate evaluation of the energy invested in immunization is difficult. The composition of the immune system is complex, and pathogens invade insects to trigger a complex immune response. In this series of complex immune responses, some immune indicators are positively correlated, while some are negatively correlated. Therefore, the reduction of a single immune component cannot be regarded as a reduction in immune investment; immunity should be comprehensively evaluated by integrating various indicators. This study demonstrated that the PO or lysozyme activity, expression of AMP, and immunity of the locusts increased after injection of pathogenic bacteria (Figs. 3 and 4).

Immune defense response was activated after the challenge of *M. luteus* and *E. coli*. Quantitative data of AMP indicated that both the pathogenic bacteria can stimulate the expression of *DEF3* and *DEF4* (Fig. 5C and F). AMPs are produced mainly through the Toll and IMD pathways. To explore the mechanism of upregulation of AMP genes, we measured the expression of genes related to the Toll/IMD pathway. Toll-like receptors (TLRs) are L-type transmembrane proteins that play a crucial role in the innate immune response by recognizing pathogen-associated and damage-associated molecular patterns (Li et al., 2013). TLR activation requires a ligand specific dimerization state/-dimerization state specific to a particular ligand, e.g., lipoprotein acid (LTA) from Gram-positive bacteria is used for TLR2/6 heterodimerization (Shmuel-Galia et al., 2017), and DNA with CpG motifs derived from bacteria and viruses is used for *TLR9* II aggregation (Ohto et al., 2018). A recent evidence showed that TLRs play a role in insect innate immune responses to exogenous stimulus (Zhao et al., 2020), our previous data found that locusts infected with bacteria upregulated Immune-related genes in the Toll signaling pathway, including *TLR6*, *TLR9*, and *Tollip* (toll interacting protein) (Wang et al., 2019). In the current study, after injection of *M. luteus*, the expression of *TLR6* as a pattern recognition protein of the Gram-positive bacteria was significantly upregulated (Fig. 5B). Excessive activation of the Toll pathway may cause serious damage to the host (Wang et al., 2013). It is necessary to terminate immune signal transduction after pathogen elimination (Wang and Xia, 2018). IMD is a signaling molecule in cells (Lemaitre and Hoffmann, 2007). Most Gram-negative bacteria are mediated through the IMD pathway, and the injection of *M. luteus* also triggers down-regulation of *IMD* expression (Fig. 5E). The immune regulation of insects does not act alone, but a network regulation mode affects each other. Therefore, the down-regulation of *IMD* expression may have been triggered by activation of the Toll pathway. This result is consistent with the findings of Liu and colleagues, that the knockdown of *Toll* in shrimp caused the down-regulation of *IMD* expression (Liu et al., 2016). *E. coli* injection triggered significant down-regulation of *TLR9* and *IMD* (Fig. 5E). The *IMD* acts as an adaptor molecule in the antibacterial defense system of insects, which can recognize Gram-negative bacteria and activate the transcription of antibacterial peptide genes (Myllymäki et al., 2014). After injection of *E. coli* in *Tribolium castaneum*, the expression of genes to reduce *IMD* increased within 12 h, and the *IMD* expression peaked at 6 h after injection (Jo et al., 2019). This result is inconsistent with the results of the present study, wherein a decrease in the *IMD* expression was observed after injection of *E. coli*. However, the expression of AMP genes was significantly upregulated and did not decrease with the down-regulation of *IMD* (Fig. 5E and F). This may be because the sampling time in this experiment was 24 h after the infection. It was observed that the *IMD* pathway was correspondingly faster than the Toll pathway, the locusts were already in the late stage of immune regulation, and the *IMD* expression level had already recovered. The immune response triggered by the two pathogens did not cause any significant change in the expression of *Tollip*; this may be because the pathogens were still persistent in the insects, and the Toll pathway was still activated to respond to infection of foreign organisms (Fig. 5B and E).

CrebA plays a different role in the selected two bacteria-induced

immune responses, *M. luteus* increased the activity of lysozyme but did not stimulate PO activity by simple bacteria challenge (Fig. 4B and E). In contrast, in the immune response triggered by injection of *E. coli*, the enzyme activity of PO was nine times that of the uninitiated immune response, and the activity of lysozyme was also stimulated (Fig. 4C and F). Similar results were obtained by Mohamed et al. (2016) who reported that in desert locusts, lysozyme was upregulated after injection of *E. coli*. This result indicates that Gram-positive bacteria only activate lysozyme in locusts, while Gram-negative bacteria activate both PO and lysozyme simultaneously. After *CrebA* RNAi, only a decrease in PO activity infected with *M. luteus* was observed. (Fig. 4B). Previous studies have reported the immune response triggered by injection of *E. coli* in *Drosophila* upregulated the expression of *CrebA* (Troha et al., 2018). However, in the present study, *CrebA* expression decreased after injection of *M. luteus* and *E. coli* for 24 h (Fig. 5D), which may be that the expression of *CrebA* differs due to the difference in the time of bacterial infection, which can be explained by the fact that in *Antheraea pernyi*, infection with *M. luteus* and *E. coli* upregulated the expression of *CrebA* within 12 h and was downregulated after 24 h (Gao et al., 2018). Locusts with low *CrebA* expression showed a significant downregulation of *DEF3* and *DEF4* expression and reduced PO activity after *M. luteus* infection (Figs. 4B and 6B), but the survival rate was not different from the control (Fig. 3A). Locust has many AMP genes, such as defensin, attacin and lectin, and the lack of one AMP can be overcome by the expression of other AMPs, as described by Magalhaes et al. (2010). However, in *E. coli*-injected locusts, low expression of *CrebA* suppressed the expression of *DEF3*, *DEF4* and *IMD*, resulting in increased mortality (Figs. 3B and 6C and D), suggesting specificity of *CrebA* towards pathogens. The silencing of IMD severely impaired the bacterial in the hemolymph and down-regulation of AMP gene expression (Zhou et al., 2018). This indicates that after knockdown of *CrebA* in insects/locusts the reduction in immunity triggered by injection of Gram-negative bacteria down-regulated the expression of AMP genes through the IMD pathway.

This study clarified the expression pattern of *CrebA* in different organs and maturation stages of locusts. In male locusts, the highest expression of *CrebA* was observed during the mating stage, while in the female locusts, the highest expression of *CrebA* was observed during the egg-laying stage. Furthermore, the highest expression of *CrebA* was observed in the fat body of female locusts, and was significantly higher than in other tissues. After the knockdown of the *CrebA* gene, the reproductive ability decreased, which was indicated by the decrease in *Chico* and *JHAMT* expression levels and down-regulation of *Vg*s and *VgR*s gene expression. In locusts infected with *E. coli*, *CrebA* plays a vital role in innate immune system response, by producing defensins and regulating of PO and lysozyme activity against pathogenic infections. Thus, these results demonstrate the positive role of *CrebA* in locust immunity and reproduction. However, whether *CrebA* directly affects *Vg* gene expression or indirectly regulates *Vg* by down-regulating the expression of *Chico* and *JHAMT* to affect the titers of JH in the hemolymph is still unknown. In addition, whether *CrebA* in the transcription of antibacterial substances can be directly controlled in the immune regulation caused by pathogenic bacteria remains to be further studied. Our results also provide a foundation for further exploration of the molecular mechanism of *CrebA* in regulating locusts reproduction and immune trade-offs in *Locusta migratoria manilensis*.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (Grant No. 2017YFD0201000), National Natural Science Foundation of China (Grant Nos. 30970473 and 31270459), and the Project of Zhejiang Qian-Jiang Talents Program (2010R10093).

References

Abrams, E.W., Andrew, D.J., 2005. CrebA regulates secretory activity in the *Drosophila* salivary gland and epidermis. Development 132 (12), 2743–2758.
Andrew, D.J., Baig, A., Bhanot, P., Smolik, S.M., K.D, 1997. The Drosophila dCREB-A gene is required for dorsal/ventral patterning of the larval cuticle. Development 124 (1), 181–193.
Arrese, E.L., Soulages, J.L., 2010. Insect fat body: energy, metabolism, and regulation. Annu. Rev. Entomol. 55, 207–225.
Barbosa, S., Fasanella, G., Carreira, S., Llarena, M., Fox, R., Barreca, C., Andrew, D., O'Hare, P., 2013. An orchestrated program regulating secretory pathway genes and cargos by the transmembrane transcription factor CREB-H. Traffic 14 (4), 382–398.
Böhni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., Hafen, E., 1999. Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4. Cell 97 (7), 865–875.
Cerenius, L., Lee, B.L., Söderhäll, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 29 (6), 263–271.
Choi, S., Kim, W., Chung, J., 2011. *Drosophila* salt-inducible kinase (SIK) regulates starvation resistance through cAMP-response element-binding protein (CREB)-regulated transcription coactivator (CRTC). J. Biol. Chem. 86 (4), 2658–2664.
Contreras, G., Shirdel, I., Braun, M.S., Wink, M., 2020. Defensins: transcriptional regulation and function beyond antimicrobial activity. Dev. Comp. Immunol. 104, 103556.
Ferenz, H.J., Aden, E., 1993. Ovarian control of juvenile hormone biosynthesis in *Locusta migratoria* (Orthoptera: acrididae). Entomol. Gen. 18 (1–2), 9–17.
Ferrandon, D., Imler, J.L., Hetru, C., Hoffmann, J.A., 2007. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. Nat. Rev. Immunol. 7 (11), 862–874.
Fox, R.M., Hanlon, C.D., Andrew, D.J., 2010. The CrebA/Creb3-like transcription factors are major and direct regulators of secretory capacity. J. Cell Biol. 191 (3), 479–492.
Ganz, T., Lehrer, R.I., 1994. Defensins. Curr. Opin. Immunol. 6 (4), 584–589.
Gijbels, M., Lenaerts, C., Broeck, J.V., Marchal, E., 2019. Juvenile Hormone receptor Met is essential for ovarian maturation in the Desert Locust, *Schistocerca gregaria*. Sci. Rep. 9. Article 10797.
Gao, J., Sun, Y., Sun, Y.X., Chen, C., Kausar, S., Tian, J.W., Zhu, B.J., Liu, C.L., 2018. Identification and function of cAMP response element binding protein in Oak silkworm *Antheraea pernyi*. J. Invertebr. Pathol. 151, 14–20.
Guo, W., Wu, Z.X., Song, J.S., Jiang, F., Wang, Z.M., Deng, S., Walker, V.K., Zhou, S.T., 2014. Juvenile hormone-receptor complex acts on *Mcm4* and *Mcm7* to promote polyploidy and vitellogenesis in the Migratory Locust. PLoS Genet. 10 (10). Article e1004702.
Hu, Y., Yang, Z.Z., Gong, C., Wu, Q.J., Wang, S.L., Li, C.R., Zhang, Y.J., Guo, Z.J., 2022a. Molecular characterization of insulin-like peptide genes and their important roles in Bemisia tabaci reproduction. Entomol. Gen. 42 (6), 977–986.
Hu, Y.W., Wang, S.H., Tang, Y., Xie, G.Q., Ding, Y.J., Xu, Q.Y., Tang, B., Zhang, L., Wang, S.G., 2022b. Suppression of yolk formation, oviposition and egg quality of locust (*Locusta migratoria manilensis*) infected by *Paranosema locustae*. Front. Immunol. 13. Article 848267.
Hultmark, D., 1993. Immune reactions in *Drosophila* and other insects: a model for innate immunity. Trends Genet. 9 (5), 178–183.
Iijima, K., Zhao, L., Shenton, C., Iijima-Ando, K., 2009. Regulation of energy stores and feeding by neuronal and peripheral CREB Activity in *Drosophila*. PLoS One 4 (12). Article e8498.
Jia, Z.Q., Zhan, E.L., Zhang, S.G., Wang, Y., Song, P.P., Jones, A.K., Han, Z.J., Zhao, C.Q., 2022. Broflanilide prolongs the development of fall armyworm *Spodoptera frugiperda* by regulating biosynthesis of juvenile hormone. Entomol. Gen. 42 (5), 761–769.
Jing, Y.P., An, H.L., Zhang, S.J., Wang, N.B., Zhou, S.T., 2018. Protein kinase C mediates juvenile hormone-dependent phosphorylation of Na+/K+-ATPase to induce ovarian follicular patency for yolk protein uptake. J. Biol. Chem. 293 (52), 20112–20122.
Jing, Y.P., Wang, D., Han, X.L., Dong, D.J., Wang, J.X., Zhao, X.F., 2016. The Steroid hormone 20-Hydroxyecdysone enhances gene transcription through the cAMP response element-binding protein (CREB) signaling pathway. J. Biol. Chem. 291 (24), 12771–12785.
Jo, Y.H., Patnaik, B.B., Hwang, J., Park, K.B., Ko, H.J., Kim, C.E., Bae, Y.M., Jung, W.J., Lee, Y.S., Han, Y.S., 2019. Regulation of the expression of nine antimicrobial peptide genes by *Tm*IMD confers resistance against Gram-negative bacteria. Sci. Rep. 9 (1). Article 10138.
Kinjoh, T., Kaneko, Y., Itoyama, K., Mita, K., Hiruma, K., Shinoda, T., 2007. Control of juvenile hormone biosynthesis in *Bombyx mori*: cloning of the enzymes in the mevalonate pathway and assessment of their developmental expression in the corpora allata. Insect Biochem. Mol. Biol. 37 (8), 808–818.
Lemaitre, B., Hoffmann, J., 2007. The host defense of *Drosophila melanogaster*. Annu. Rev. Immunol. 25, 697–743.
Levitin, A., Whiteway, M., 2008. *Drosophila* innate immunity and response to fungal infections. Cell Microbiol. 10 (5), 1021–1026.

Li, J., Wang, X.H., Zhang, F.C., Yin, H., 2013. Toll-like receptors as therapeutic targets for autoimmune connective tissue diseases. Pharmacol. Ther. 138 (3), 441–451.
Li, Y., Gao, H., Zhang, Y.W., Lin, X.D., 2021. Role of the transcription factor Taiman in moulting and ovarian development of *Nilaparvata lugens*. Entomol. Gen. 41 (2), 169–177.
Liu, Y.J., Song, L., Sun, Y.H., Liu, T., Hou, F.J., Liu, X.L., 2016. Comparison of immune response in Pacific white shrimp, *Litopenaeus vannamei*, after knock down of *Toll* and *IMD* gene *in vivo*. Dev. Comp. Immunol. 60, 41–52.
Lu, K., Shu, Y.H., Zhou, J.L., Zhang, X.Y., Zhang, X.Y., Chen, M.X., Yao, Q., Zhou, Q., Zhang, W.Q., 2015. Molecular characterization and RNA interference analysis of vitellogenin receptor from *Nilaparvata lugens* (stål). J. Insect Physiol. 73, 20–29.
Magalhaes, T., Leandro, D.C., Ayres, C.F., 2010. Knock-down of REL2, but not defensin A, augments *Aedes aegypti* susceptibility to *Bacillus subtilis* and *Escherichia coli*. Acta Trop. 113 (2), 167–173.
Marchal, E., Zhang, J.R., Badisco, L., Verlinden, H., Hult, E.F., Wielendaele, P.Van, Yagi, K.J., Tobe, S.S., Broeck, J.Vanden, 2011. Final steps in juvenile hormone biosynthesis in the desert locust, *Schistocerca gregaria*. Insect Biochem. Mol. Biol. 41 (4), 219–227.
Mavrouli, M.D., Tsakas, S., Theodorou, G.L., Lampropoulou, M., Marmaras, V.J., 2005. MAP kinases mediate phagocytosis and melanization via prophenoloxidase activation in medfly hemocytes. Biochim. Biophys. Acta 1744 (2), 145–156.
Mohamed, A., Zhang, L., Dorrah, M.A., Elmogy, M., Yousef, H.A., Bassal, T.T., Duvic, B., 2016. Molecular characterization of a c-type lysozyme from the desert locust, *Schistocerca gregaria* (Orthoptera: acrididae). Dev. Comp. Immunol. 61, 60–69.
Mohandkaci, H., Doumandji-Mitiche, B., 2012. Evidence of the entomopathogenic capacity of *Bacillus sphaericus* against the migratory locust *Locusta migratoria* (Orthoptera:Acrididae). Entomol. Gen. 33 (4), 289–300.
Myllymäki, H., Valanne, S., 2014. The *Drosophila* Imd signaling pathway. J Immunol. 192 (8), 3455–3462.
Nässel, D.R., Liu, Y.T., Luo, J.N., 2015. Insulin/IGF signaling and its regulation in *Drosophila*. Gen. Comp. Endocrinol. 221, 255–266.
Ohto, U., Ishida, H., Shibata, T., Sato, R., Miyake, K., Shimizu, T., 2018. Toll-like receptor 9 contains two DNA binding sites that function cooperatively to promote receptor dimerization and activation. Immunity 48 (4), 649–658.e4.
Panagopoulos, I., Möller, E., Dahlén, A., Isaksson, M., Mandahl, N., Vlamis-Gardikas, A., Mertens, F., 2007. Characterization of the native CREB3L2 transcription factor and the FUS/CREB3L2 chimera. Genes Chromosomes Cancer 46 (2), 181–191.
Parthasarathy, R., Palli, S.R., 2011. Molecular analysis of nutritional and hormonal regulation of female reproduction in the red flour beetle, *Tribolium castaneum*. Insect Biochem. Mol. Biol. 41 (5), 294–305.
Parthasarathy, R., Sun, Z., Bai, H., Palli, S.R., 2010. Juvenile hormone regulation of vitellogenin synthesis in the red flour beetle, *Tribolium castaneum*. Insect Biochem. Mol. Biol. 40 (5), 405–414.
Ragheb, R., Chuyen, A., Torres, M., Defaye, A., Seyres, D., Kremmer, L., Fernandez-Nunez, N., Tricoire, H., Rihet, P., Nguyen, C., Röder, L., Perrin, L., 2017. Interplay between trauma and *Pseudomonas entomophila* infection in flies: a central role of the JNK pathway and of CrebA. Sci. Rep. 7 (1). Article 16222.
Richard, D.S., Rybczynski, R., Wilson, T.G., Wang, Y., Wayne, M.L., Zhou, Y., Partridge, L., Harshman, L.G., 2005. Insulin signaling is necessary for vitellogenesis in *Drosophila melanogaster* independent of the roles of juvenile hormone and ecdysteroids: female sterility of the *chico*^{1} insulin signaling mutation is autonomous to the ovary. J. Insect Physiol. 51 (4), 455–464.
Rose, R.E., Gallaher, N.M., Andrew, D.J., Goodman, R.H., Smolik, S.M., 1997. The CRE-binding protein dCREB-A is required for *Drosophila* embryonic development. Genetics 146 (2), 595–606.
Roy, S., Saha, T.T., Zou, Z., Raikhel, A.S., 2018. Regulatory pathways controlling female insect reproduction. Annu. Rev. Entomol. 63, 489–511.
Saha, S.S., Suzuki, J., Uda, A., Watanabe, K., Shimizu, T., Watarai, M., 2017. Silkworm model for *Francisella novicida* infection. Microb. Pathog. 113, 94–101.
Sangbaramou, R., Camara, I., Huang, X.Z., Shen, J., Tan, S.Q., Shi, W.P., 2018. Behavioral thermoregulation in *locusta migratoria manilensis* (orthoptera: Acrididae) in response to the entomopathogenic fungus, *beauveria bassiana*. PLoS One 13 (11), 0206816.
Seidelmann, K., Helbing, C., Göbeler, N., Weinert, H., 2016. Sequential oogenesis is controlled by an oviduct factor in the locusts *Locusta migratoria* and *Schistocerca gregaria*: overcoming the doctrine that patency in follicle cells is induced by juvenile hormone. J. Insect Physiol. 90, 1–7.
Shang, F., Niu, J.Z., Ding, B.Y., Zhang, Q., Ye, C., Zhang, W., Smagghe, G., Wang, J.J., 2018. Vitellogenin and its receptor play essential roles in the development and reproduction of the brown citrus aphid, *Aphis* (*Toxoptera*) *citricidus*. Insect Mol. Biol. 27 (2), 221–233.
Shinoda, T., Itoyama, K., 2003. Juvenile hormone acid methyltransferase: a key regulatory enzyme for insect metamorphosis. Proc. Natl. Acad. Sci. U. S. A. 100 (21), 11986–11991.
Shmuel-Galia, L., Klug, Y., Porat, Z., Charni, M., Zarmi, B., Shai, Y., 2017. Intramembrane attenuation of the TLR4-TLR6 dimer impairs receptor assembly and reduces microglia-mediated neurodegeneration. J. Biol. Chem. 292 (32), 13415–13427.
Smolik, S.M., Rose, R.E., Goodman, R.H., 1992. A cyclic AMP-responsive element-binding transcriptional activator in *Drosophila melanogaster*, dCREB-A, is a member of the leucine zipper family. Mol. Cell Biol. 12 (9), 4123–4131.
Song, J.S., Wu, Z.X., Wang, Z.M., Deng, S., Zhou, S.T., 2014. Krüppel-homolog 1 mediates juvenile hormone action to promote vitellogenesis and oocyte maturation in the migratory locust. Insect Biochem. Mol. Biol. 52, 94–101.
Tenlen, J.R., 2018. Microinjection of dsRNA in tardigrades. Cold Spring Harb. Protoc. 11 https://doi.org/10.1101/pdb.prot102368.
Tonk, M., Knorr, E., Cabezas-Cruz, A., Valdés, J.J., Kollewe, C., Vilcinskas, A., 2015. *Tribolium castaneum* defensins are primarily active against Gram-positive bacteria. J. Invertebr. Pathol. 132, 208–215.
Troha, K., Im, J.H., Revah, J., Lazzaro, B.P., Buchon, N., 2018. Comparative transcriptomics reveals *CrebA* as a novel regulator of infection tolerance in *D. melanogaster*. PLoS Pathog. 14 (2). Article e1006847.
Ullah, E., Gul, H., Tariq, K., Hafeez, M., Desneux, N., Gao, X.W., SongL, D.L., 2022. RNA interference-mediated silencing of ecdysone receptor (EcR) gene causes lethal and sublethal effects on melon aphid, *Aphis gossypii*. Entomol. Gen. 42 (5), 791–797.
Upadhyay, S.K., Singh, H., Dixit, S., Mendu, V., Verma, P.C., 2016. Molecular characterization of vitellogenin and vitellogenin receptor of *Bemisia tabaci*. PLoS One 11 (5). Article e0155306.
Ullah, F., Gul, H., Desneux, N., Tariq, K., Ali, A., Gao, X.W., Song, D.L., 2019. Clothianidin-induced sublethal effects and expression changes of vitellogenin and ecdysone receptors genes in the melon aphid, *Aphis gossypii*. Entomol. Gen. 39 (2), 137–149.
Verlinden, H., Badisco, L., Marchal, E., Van Wielendaele, P., Vanden Broeck, J., 2009. Endocrinology of reproduction and phase transition in locusts. Gen. Comp. Endocrinol. 162 (1), 79–92.
Viljakainen, L., 2015. Evolutionary genetics of insect innate immunity. Brief Funct Genomics 14 (6), 407–412.
Wang, F., Xia, Q.Y., 2018. Back to homeostasis: negative regulation of NF-κB immune signaling in insects. Dev. Comp. Immunol. 87, 216–223.
Wang, P.H., Gu, Z.H., Wan, D.H., Zhu, W.B., Qiu, W., Chen, Y.G., Weng, S.P., Yu, X.Q., He, J.G., 2013. Litopenaeus vannamei Toll-interacting protein (LvTollip) is a potential negative regulator of the shrimp Toll pathway involved in the regulation of the shrimp antimicrobial peptide gene *penaeidin-4* (*PEN4*). Dev. Comp. Immunol. 40 (3–4), 266–277.
Wang, S.H., Liu, X.J., Xia, Z.J., Xie, G.Q., Tang, B., Wang, S.G., 2019. Transcriptome analysis of the molecular mechanism underlying immunity- and reproduction trade-off in *Locusta migratoria* infected by *Micrococcus luteus*. PLoS One 14 (8). Article e0211605.
Wei, D., Wang, S., Niu, J.Z., 2023. RNAi-based pesticides: current knowledge and potential applications for Integrated Pest Management. Entomol. Gen. 43 (1), 1–4.
Wu, Z.X., Guo, W., Yang, L.B., He, Q.J., Zhou, S.T., 2018. Juvenile hormone promotes locust fat body cell polyploidization and vitellogenesis by activating the transcription of *Cdk6* and *E2f1*. Insect Biochem. Mol. Biol. 102, 1–10.
Xia, X.F., You, M.S., Rao, X.J., Yu, X.Q., 2018. Insect C-type lectins in innate immunity. Dev. Comp. Immunol. 83, 70–79.
Xiang, M., Sang, D., Dong, B., Hu, H.X., Ji, R., Wang, H., 2019. Molecular features and expression patterns of vitellogenin receptor in *Calliptamus italicus* (Orthoptera: acrididae). J. Insect Sci. 19 (6), 15.
Xu, F.K., Kula-Eversole, E., Iwanaszko, M., Lim, C.H., Allada, R., 2019. *Ataxin2* functions via CrebA to mediate Huntingtin toxicity in circadian clock neurons. PLoS Genet. 15 (10). Article e1008356.
Xu, J.J., Sheng, Z.T., Palli, S.R., 2013. Juvenile hormone and insulin regulate trehalose homeostasis in the red flour beetle, *Tribolium castaneum*. PLoS Genet. 9 (6). Article e1003535.
Yao, Q., Xu, S., Dong, Y.Z., Que, Y.L., Quan, L.F., Chen, B.X., 2018. Characterization of vitellogenin and vitellogenin receptor of *Conopomorpha sinensis* Bradley and their responses to sublethal concentrations of Insecticide. Front. Physiol. 9, 1250.
Zaalouk, T.K., Bajaj-Elliott, M., George, J.T., McDonald, V., 2004. Differential regulation of beta-defensin gene expression during *Cryptosporidium parvum* infection. Infect. Immun. 72 (5), 2772–2779.
Zhang, Y., Feng, Z.J., Chen, Z.S., Wang, X.X., Cong, H.S., Fan, Y.L., Liu, T.X., 2021. Connection between cuticular hydrocarbons and melanization in *Harmonia axyridis* revealed by RNAi-mediated silencing of the CYP4G79. Entomol. Gen. 41 (1), 83–96.
Zhao, J.Q., Wang, Y.J., Li, X., Gai, Z.C., 2020. Genome-wide identification and characterization of Toll-like receptors (TLRs) in housefly (*Musca domestica*) and their roles in the insecticide resistance. Int. J. Biol. Macromol. 150, 141–151.
Zhou, C., Yang, X.B., Yang, H., Gong, M.F., Long, G.Y., Jin, D.C., 2022. Role of insecticide-mediated transcription of the TOR and JH signaling pathway-related genesin the regulation of reproduction in *Sogatella furcifera*. Entomol. Gen. 42 (5), 771–779.
Zhou, Y.L., Wang, L.Z., Gu, W.B., Wang, C., Zhu, Q.H., Liu, Z.P., Chen, Y.Y., Shu, M.A., 2018. Identification and functional analysis of *immune deficiency* (IMD) from *Scylla paramamosain*: the first evidence of IMD signaling pathway involved in immune defense against bacterial infection in crab species. Fish Shellfish Immunol. 81, 150–160.