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Midgut immune responses induced by bacterial infection in the silkworm, *Bombyx mori*[*]

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Abstract: Insect gut epithelial cells produce reactive oxygen species (ROS) and antimicrobial peptides (AMPs) to protect hosts from pathogenic microorganisms. In this study, we evaluate the pathogenicity of *Pseudomonas aeruginosa* and *Bacillus bombysepticus* in the silkworm, *Bombyx mori*. Survival curves show that *B. bombysepticus* is deadly when larval silkworms are infected orally. Bacterial infection caused intestinal hydrogen peroxide (H_2O_2) and nitric oxide (NO) levels to increase significantly by 8 and 16 h post-infection (hpi), respectively. Real-time quantitative polymerase chain reaction (qPCR) analysis shows that the transcription levels of dual oxidase (*Duox*) and catalase (*CAT*) are highly up-regulated by *P. aeruginosa* infection at 8 hpi. *P. aeruginosa* infection induced nitric oxide synthase 2 (*NOS2*) expression at 16 hpi, which contributes to the generation of NO. mRNA levels of AMP genes, specifically Glovorin 2 and Glovorin 3, which obviously increase during the early infection stage. These results indicate that invading bacteria elevate intestinal ROS and NO levels and induce AMP gene transcription, which contributes to intestinal immune defense.

Key words: *Bombyx mori*, Midgut, Immune, Hydrogen peroxide, Nitric oxide, Antimicrobial peptide
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1 Introduction

Innate immunity, which exists in all metazoan organisms, is an evolutionarily conserved system for defending the host against microbial invasion. In *Drosophila*, the gut epithelium is the first line of protection for the host against microorganismal invasion and proliferation (Hoffmann and Reichhart, 2002). Two types of immune molecules are involved in *Drosophila* gut defense. First, the production of reactive oxygen species (ROS) and nitric oxide (NO) was demonstrated to kill pathogens in the gut epithelium and to trigger downstream immune responses (Wink *et al*., 2011). *Erwinia carotovora carotovora 15* (*Ecc15*) infection increases the levels of ROS synthesized by dual oxidase (Duox) in the *Drosophila* gut. *Duox*-RNA interference (RNAi) flies showed increased mortality and failed to control *Ecc15* proliferation in the gut, suggesting that Duox is the main enzyme inducing ROS during gut infection (Ha *et al*., 2005b). In *Aedes aegypti*, midgut epithelial cells generate ROS to control bacterial growth (Oliveira *et al*., 2011). Excess ROS is toxic to the host and is degraded by immune responsive catalase (IRC) to maintain homeostatic redox balance. *IRC*-RNAi flies exhibited ROS over-production and increased lethality, indicating that IRC plays an antioxidant role in the host defense system (Ha *et al*., 2005a). NO is generated by nitric oxide synthase (NOS) enzymes,

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including NOS1, NOS2, and NOS3. NOS2 is inducible, while the other two are constitutively expressed (Wink *et al*., 2011). Lipopolysaccharide (LPS) stimulation induced the expression of *NOS* in *Bombyx mori* (Imamura *et al*., 2002).

The second intestinal immune defense is the generation of local antimicrobial peptides (AMPs) via the immune deficiency (IMD) pathway (Tzou *et al*., 2000). Drosomycin and Diptericin are induced in the gut of *Drosophila* after *Erwinia carotovora* infection (Basset *et al*., 2000). In *B. mori*, local AMP genes, including Cecropin A1 (*CecA1*), Gloverin 1 (*Glov1*), *Glov2*, *Glov3*, *Glov4*, and *lysozyme* (*Lys*), are induced by *Staphylococcus aureus*, whereas the expression of *CecA1*, *Glov3* and *Glov4* is sometimes inhibited by *Escherichia coli* infection (Wu *et al*., 2010b).

In this study, we demonstrate that intestinal hydrogen peroxide (H_2O_2) and NO levels are elevated after bacterial infection and that the mRNA transcription levels of ROS-related genes and AMP genes are also up-regulated. These results indicate that ROS and AMP have vital defense roles in the midgut of silkworms.

2 Materials and methods

2.1 Silkworm rearing

Silkworm larvae (*Nistari* strain) were reared on mulberry leaves at 27 °C, 70% relative humidity, and a 12-h light:12-h dark photoperiod.

2.2 Oral infection

Pseudomonas aeruginosa and *Bacillus bombysepticus* were cultured overnight in Luria-Bertani (LB) medium at 37 °C. The bacterial pellet was collected by centrifugation at 8000*g* for 15 min and washed three times with 0.85% (8.5 g/L) NaCl. The harvested bacterial cells were suspended in 400 μl 0.85% NaCl to an optical density at 600 nm ($OD_{600\ nm}$) of 40 and used for silkworm oral infection. Fresh mulberry leaves were cut into 1 cm×1 cm pieces and coated with bacterial suspensions. Day 3 fifth instar larvae were starved for 12 h before feeding them bacteria. A group of 20 larvae were used for oral infection. Each larva was fed 20 μl bacteria or 0.85% NaCl as a control. Midguts were collected at different time points (4, 8, 16, and 24 h) after feeding.

2.3 Mortality recording and colony forming unit (CFU) assay

A group of 20 larvae were infected as described above to evaluate mortality. The number of surviving larvae was recorded every 24 h. Another nine larvae were infected for a bacterial persistence assay. At 0.5, 12, and 24 h post-infection, larvae were dissected, and the peritrophic membranes and their contents (PMC) were collected. Gut contents from individual larvae were separated from the PMC by centrifugation at 500*g* for 10 min. The supernatant was diluted 100-fold with fresh LB and incubated on a LB agar plate with ampicillin (100 μg/ml) at 37 °C for 12 h. The numbers of colony forming units (CFUs) were counted. Three larvae were selected for each time point, and the experiment was repeated three times.

2.4 H_2O_2 level measurement in the midgut

After oral infection, five larvae were dissected to collect the PMC at different time points. Gut contents were separated from the PMC by centrifugation at 13 000*g* for 10 min. The supernatants were transferred to Amicon Ultra 10K filters (Millipore, Billerica, MA, USA) and centrifuged at 13 000*g* for 5 min. The flow-through samples were used in H_2O_2 assays using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.5 Gene expression analysis using real-time quantitative PCR

Real-time quantitative polymerase chain reaction (qPCR) was used to evaluate the expression levels of ROS-related genes and AMP genes. Total RNA was extracted from the midguts of silkworms after various treatments and purified using the Direct-zol™ RNA MiniPrep Kit (Zymo, Irvine, CA, USA). First strand complementary DNA (cDNA) was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. *B. mori* initiation factor 4α (*IF4α*) was used as an internal control to normalize the expression of target genes (Wu *et al*., 2010a). All specific primers for qPCR are listed in Table 1. qPCR was performed using a FastStart Essential DNA Green Master mix (Roche, Indianapolis, IN, USA) with the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). qPCR was performed

using an initial denaturation at 95 °C for 10 min, followed by 39 cycles of amplification (95 °C for 10 s, 55 °C for 20 s, and 72 °C for 30 s), and the melting curve analysis was performed from 65 to 95 °C. The relative expression levels of target genes were analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). All experiments were repeated independently three times.

Table 1 Primers for qPCR

Gene	Primer sequence
Duox	F: 5' GCTTCGTCGTATAACTCTGTGG 3'
	R: 5' TGCAGGGTGGAAGTTTGG 3'
CAT	F: 5' GGGAGCGTATTCCAGAAC 3'
	R: 5' GAGGGTCACGAACAGTATCA 3'
NOS1	F: 5' AGTTGGCTTGGCGTAATG 3'
	R: 5' TACCGTCTGTGCGTTGTG 3'
NOS2	F: 5' CGGGAAAGACCCTGACTA 3'
	R: 5' AAACGCATACTGGAGACG 3'
Att2	F: 5' TTCAAACAGAAGGTGGGC 3'
	R: 5' GACGGAGATTGGAACAGG 3'
CecB6	F: 5' TCCTTCGTCTTCGCTCTG 3'
	R: 5' GATGCCGTCACGGATGTT 3'
CecD	F: 5' CTCCCGGCAACTTCTTCA 3'
	R: 5' CGAACCCTCTGACCCATT 3'
Glov2	F: 5' ACGGACCTTCTGATTACGC 3'
	R: 5' CATTCTTGTTCGCCCAGT 3'
Glov3	F: 5' GACACGAGAATGGGAGGAG 3'
	R: 5' AAGACCCTGGTGCCGTAA 3'
Mor	F: 5' CAAGGCCATTAAGACTGT 3'
	R: 5' TTTCTTTTCTTCGGTTTC 3'

F: forward; R: reverse

2.6 Statistical analysis

All data are presented as mean±SD. The unpaired Student's *t*-test was used to compare expression differences between control and infection conditions. Bonferroni's correction was used to determine the critical significance level. The log-rank test was used to analyze the survival rate using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3 Results

3.1 *B. bombysepticus* infection in the silkworm

When silkworms were infected with *P. aeruginosa* or *B. bombysepticus*, death rarely occurred during the first 5 d. Afterwards, the survival rate after *B. bombysepticus* infection was reduced remarkably, reaching approximately 20% by Day 8, while only 10% mortality was seen by Day 8 after *P. aeruginosa* infection (Fig. 1). These results suggest that *B. bombysepticus* is more pathogenic to silkworms.

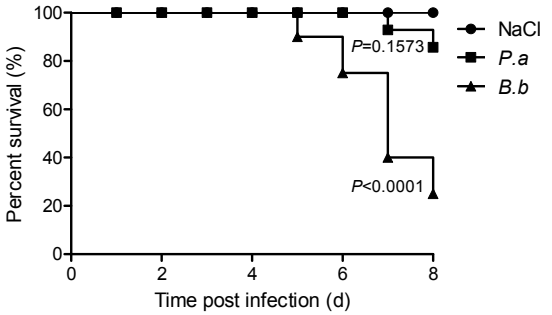

Fig. 1 Survival rate of silkworm larvae after bacterial feeding
NaCl: control; *P.a*: *Pseudomonas aeruginosa*; *B.b*: *Bacillus bombyseptieus*. Twenty larvae were used for each treatment

3.2 Bacterial numbers in infected larvae

To investigate the persistence of ingested bacteria in infected larvae, we examined the viability of bacteria in the gut. At 0.5 h post oral infection, compared with the control, *P. aeruginosa* was more persistent, at 100-fold CFUs higher, than *B. bombysepticus*, but by 12 h and 24 h, no *P. aeruginosa* cells were growing on the plates. In contrast, *B. bombysepticus* persisted and proliferated in the gut with CFUs increasing gradually and reaching a maximum at 24 h (Fig. 2). These findings suggest that the silkworm employs different mechanisms to combat different invading bacteria. Alternatively, *B. bombysepticus* and *P. aeruginosa* may have different abilities to strive against silkworm immune responses.

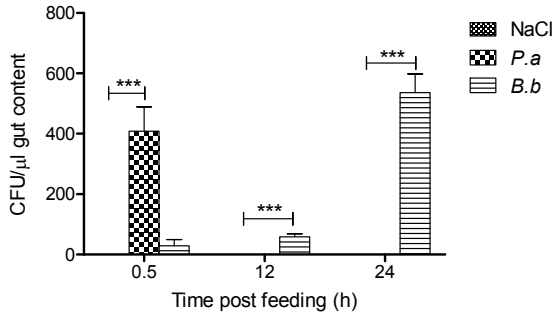

Fig. 2 CFU changes after the bacterial feeding
NaCl: control; *P.a*: *Pseudomonas aeruginosa*; *B.b*: *Bacillus bombyseptieus*. Each value is given as the mean±SD of three replicates. *, **, and *** indicate statistical significance at $P<0.05$, $P<0.01$, and $P<0.001$, respectively. Three larvae were used at each time point, and the experiment was repeated three times

3.3 H_2O_2 levels in the gut and expression of genes involved in H_2O_2 metabolism after bacterial infection

We next measured the H_2O_2 concentration in the gut after bacterial infection. Both *P. aeruginosa* and *B. bombysepticus* infection significantly increased intestinal H_2O_2 levels at 8 and 16 h (Fig. 3a). The *Duox* and catalase (*CAT*) transcription levels were highly up-regulated by *P. aeruginosa* infection and down-regulated by *B. bombysepticus* infection at 8 h. At other post-infection times, the expression levels of these two genes were lower than those of the control (Figs. 3b and 3c). These data suggest that H_2O_2 is an important defense molecule in response to bacterial challenge.

3.4 Changes in the level of NO in the gut and the transcription of genes related to NO metabolism after bacterial infection

We also measured intestinal NO levels after bacterial infection. After *P. aeruginosa* infection, the NO concentration was lower than that of the control at 8 h, followed by a significant increase at 16 h and then a decrease at 24 h; whereas after *B. bombysepticus* infection, the NO level was slightly elevated only at 24 h (Fig. 4a). qPCR results showed that the *NOS1* gene was down-regulated by both *P. aeruginosa* and *B. bombysepticus* infection from 4 to 24 h (Fig. 4b). The *NOS2* expression level was up-regulated at 8 h by *B. bombysepticus* infection and at 16 h by *P. aeruginosa* infection (Fig. 4c). These results imply that NO may play an important role in host defense and that NOS2 is involved in NO generation.

3.5 Expression changes in AMP genes in response to bacterial challenge

We measured the expression levels of six AMP genes (Attacin 2 (*Att2*), Cecropin B6 (*CecB6*), Cecropin D (*CecD*), Glovorin 2 (*Glov2*), Glovorin 3 (*Glov3*), and Morricin (*Mor*)) in the midgut using qPCR. All AMP genes were highly up-regulated from 4 to 24 h post-infection except for *Mor* (Fig. 5). *Glov2* and *Glov3* were the two most induced genes. At 4 h after *P. aeruginosa* and *B. bombysepticus* infection, the expression of *Glov2* mRNA increased approximately 90-fold compared with the control, while the transcription level of *Glov3* induced 500- and 650-fold by *P. aeruginosa* and *B. bombysepticus* infection,

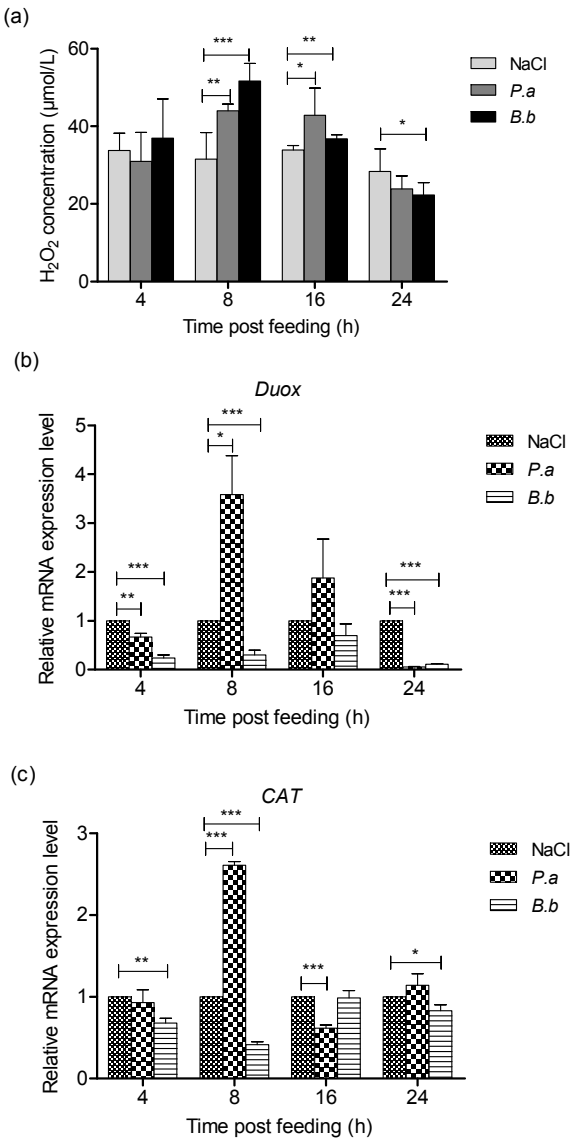

Fig. 3 H_2O_2 concentrations (a) in the gut after bacterial feeding and *Duox* (b) and *CAT* (c) expression levels after bacterial feeding

In (b) and (c), shown are the relative expression levels of *Duox* and *CAT* compared to *IF4α*. NaCl: control; *P.a*: *Pseudomonas aeruginosa*; *B.b*: *Bacillus bombyseptieus*. Each value is given as the mean±SD of three replicates. *, **, and *** indicate statistical significance at $P<0.05$, $P<0.01$, and $P<0.001$, respectively. Three larvae were used at each time point, and the experiment was repeated three times

respectively. The transcription level profile of *Mor* differed from the other AMP genes. It was only significantly up-regulated at 8 and 24 h by *P. aeruginosa* infection. At other time points, both *P. aeruginosa* and *B. bombysepticus* infection caused a reduction in the *Mor* expression. At 24 h, only *Att2* and

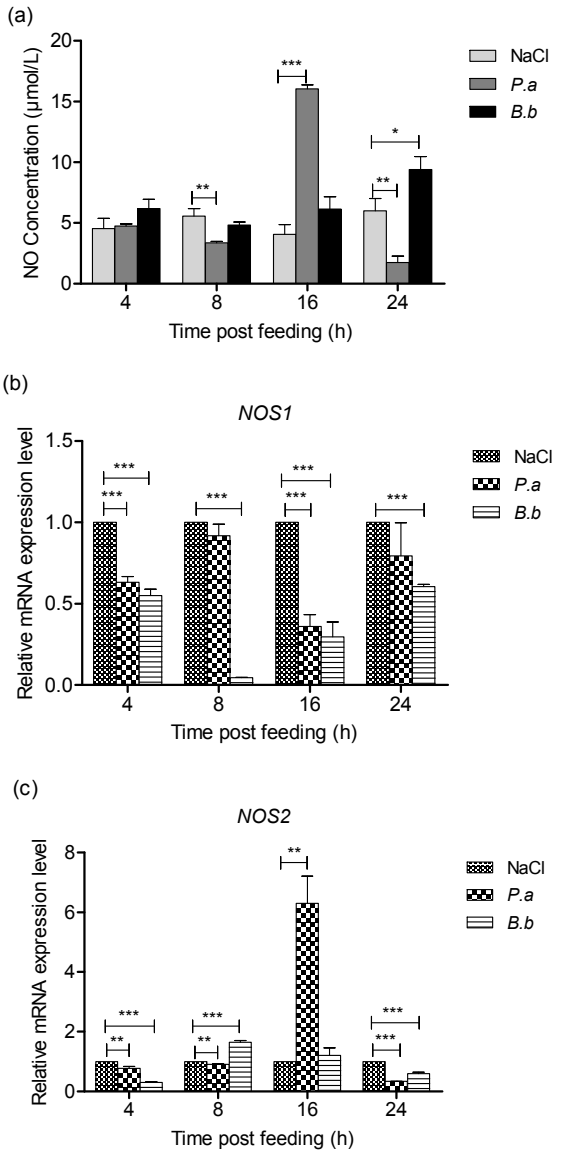

Fig. 4 NO concentrations (a) in the gut content after bacterial feeding and *NOS1* (b) and *NOS2* (c) expression levels after bacterial feeding

In (b) and (c), shown are the relative expression levels of *NOS1* and *NOS2* compared to *IF4α*. NaCl: control; *P.a*: *Pseudomonas aeruginosa*; *B.b*: *Bacillus bombyseptieus*. Each value is given as the mean±SD of three replicates. *, **, and *** indicate statistical significance at $P<0.05$, $P<0.01$, and $P<0.001$, respectively. Three larvae were used at each time point, and the experiment was repeated three times

Glov3 were induced at higher levels (15- and 100-fold, respectively) by *P. aeruginosa* and *B. bombysepticus* infection. These findings suggest that *Glov2* and *Glov3* are the major AMPs used to cope with early stage bacterial invasion.

4 Discussion

To defend against ingested harmful microorganisms, the insect gut has evolved an effective immune system depending on the local production of AMPs and ROS (Lemaitre and Hoffmann, 2007).

Drosophila showed high lethality under *P. aeruginosa* infection, which activated both the Toll and IMD pathways (Lau *et al*., 2003). *P. aeruginosa* exhibited higher pathogenicity to flies in the hemocoel than in the intestine (Chieda *et al*., 2005). *B. bombysepticus*, which produces spores and parasporal crystals, was reported to be a highly pathogenic bacterium in silkworms. *B. bombysepticus* oral infection could provoke strong host immune responses (Huang *et al*., 2009). In our study, we rarely found live *P. aeruginosa* in the gut at 12 h after oral infection (Fig. 2), which is likely caused by the orally invading *P. aeruginosa* being eliminated by the silkworm intestinal immune system; whereas the proliferation of *B. bombysepticus* increased gradually from 0.5 to 12 h (Fig. 2). The higher CFU of *B. bombysepticus* in the gut at 24 h is consistent with the higher silkworm mortality after infection (Fig. 1). Furthermore, our results showed that the expression of *Duox* and *CAT* was induced by *P. aeruginosa*, but not by *B. bombysepticus* (Figs. 3b and 3c), though both bacteria increased local ROS levels. We found that *B. bombysepticus* infection induced ROS production only at 8 h (Fig. 3a). Afterwards, ROS levels, *Duox* and *CAT* expression were barely increased after 8 h (Figs. 3b and 3c). We speculate that proliferation of *B. bombysepticus* is able to rapidly overcome the silkworm intestinal immune system and gradually cause epithelial cells damage, and finally block further ROS generation. The high level of ROS at 8 h might not be produced by the gut epithelia, but from the haemolymph instead.

In *Drosophila*, Attacin, Diptericin, Defensin, and Mechtnikowin genes were induced in the gut after *Ecc15* oral infection (Buchon *et al*., 2009). In our study, among the six AMP genes induced in the midgut by *P. aeruginosa* and *B. bombysepticus* infection, *Glov2* and *Glov3* were significantly upregulated at 4 h (Fig. 5), while local ROS levels showed no changes between infected and control individuals (Fig. 3a). These results imply that *Glov2* and *Glov3* may serve as the main intestinal defense molecules during the early infection stage.

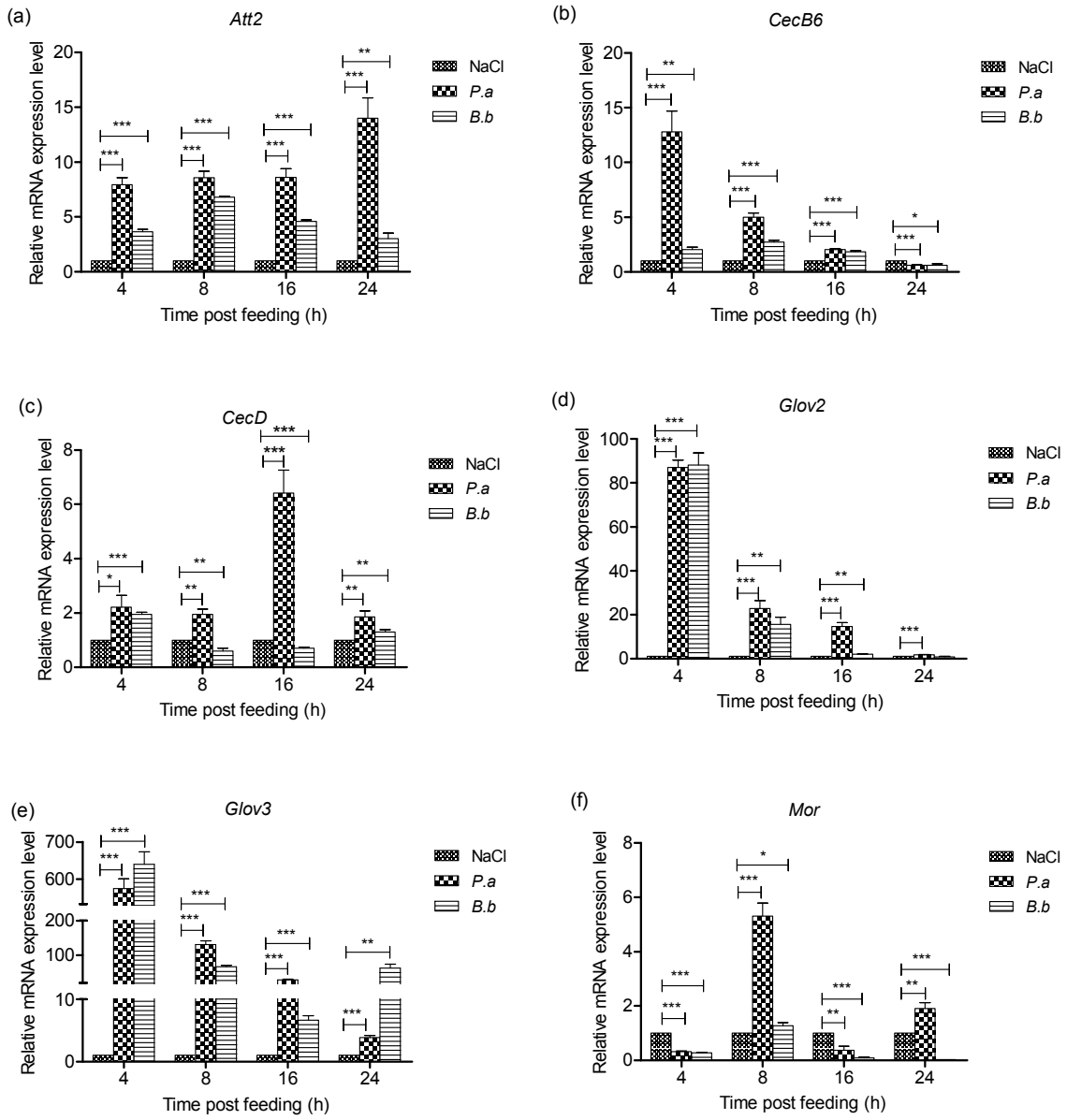

Fig. 5 mRNA expression levels of AMP genes after bacterial feeding

Shown are the relative expression levels of AMP genes in relation to *IF4α*. Each value is given as the mean±SD of three replicates. *, **, and *** indicate statistical significance at $P<0.05$, $P<0.01$, and $P<0.001$, respectively. Three larvae were used at each time point, and the experiment was repeated three times

Natural gut infection also activates the IMD pathway to induce local AMP gene transcription, which plays complementary roles in combating ROS-resistant microbes (Ryu *et al*., 2006). In our study, at 24 h, intestinal ROS levels were relatively low after *P. aeruginosa* and *B. bombysepticus* infection (Fig. 3a). However, the expression levels of AMP genes were still high. These results indicate that local AMP has more persistent antibacterial activity than ROS.

ROS signaling triggers local NO production by inducing intestinal *NOS* transcription in *Drosophila* (Wu *et al*., 2012). NO is induced and serves as an innate immune signal in response to gram-negative bacteria challenge (Foley and O'Farrell, 2003). NO is generated by inducible NOS2 (Wink *et al*., 2011). NO is thought to induce AMP gene expression by activating the IMD pathway in bacterially challenged *Drosophila* (Nappi *et al*., 2000). In the silkworm, Ceropin D was specifically activated by the IMD

pathway after *Escherichia coli* and *Bacillus subtilis* infections (Tanaka *et al*., 2009). In our study, the expression of *NOS2* was significantly induced by *P. aeruginosa* at 16 h, leading to an increase of the NO level in the gut (Figs. 4a and 4c). It is notable that the transcription of *CecD* was highly up-regulated by *P. aeruginosa* infection at 16 h (Fig. 5c). It is possible that the elevated NO serves as a signal to induce *CecD* expression. Given the facts that there was no living *P. aeruginosa* in the gut after 12 h and the H_2O_2 level increased earlier than NO after bacterial challenge, we speculate that H_2O_2 induces NO generation in *Bombyx* as in *Drosophila* (Wu *et al*., 2012), though this hypothesis needs further investigation.

Taken together, we investigated the intestinal immune defenses after oral bacterial infection in the silkworm. Our results show that ROS and AMP play important roles in protecting the host against bacterial infection.

Compliance with ethics guidelines

Lei ZHANG, Yan-wen WANG, and Zhi-qiang LU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：细菌感染引起的家蚕中肠免疫反应研究

目　的：探索经喂食细菌感染引起的家蚕肠道内免疫反应变化情况。

创新点：证明了家蚕肠道内的活性氧（ROS）、一氧化氮（NO）及抗菌肽在肠道免疫反应中的重要作用。

方　法：通过绿脓杆菌（*Pseudomonas aeruginosa*）及黑胸败血菌（*Bacillus bombysepticus*）喂食感染家蚕以后，统计家蚕死亡率、检测感染后不同时间肠道内过氧化氢（H_2O_2）及 NO 的水平变化；同时利用实时荧光定量聚合酶链反应（qPCR）检测中肠组织中活性氧相关基因及抗菌肽基因的转录情况。

结　论：死亡率结果显示，黑胸败血菌比绿脓杆菌具有更强的致病性。活性氧检测结果显示，喂食细菌感后 8 h 到 16 h,家蚕肠道内 H_2O_2 及 NO 水平显著升高。通过 qPCR 研究 ROS 相关基因的表达变化的结果显示，*P. aeruginosa* 感染后 8 h 可诱导肠道内双氧化酶（*Duox*）及过氧化氢酶（*CAT*）的转录上调，而感染后 16 h，*P. aeruginosa* 可诱导 NO 合成关键基因（一氧化氮核酶 2，*NOS2*）的上调表达，喂食细菌感染同样可以诱导家蚕中肠抗菌肽基因的上调表达，而抗菌肽 Glovorin 2 及 Glovorin 3 在感染初期转录上调最为明显。实验结果进一步证明 ROS、NO 及 AMP 的产生在家蚕肠道免疫防御中的重要作用。

关键词：家蚕；中肠；免疫；过氧化氢；一氧化氮；抗菌肽