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Mother-derived trans-generational immune priming in the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera, Dryophthoridae)

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Abstract

Rhynchophorus ferrugineus (Coleoptera, Curculionidae) is the most destructive pest of palm trees worldwide containing it invasive areas, such as the southern part of China. It is always emphasized to develop integrated pest management based on biological agents, but their success is not very exciting. Presently, the immune defenses of this pest against biological agents attract scarce attention. It is still unclear whether immune priming also generally occurs in insect pests and in response to different pathogens. Our results indicated that previous challenge of bacteria pathogen enhanced the magnitude of phenoloxidase activity and antibacterial activity in *R. ferrugineus* larvae against the secondary infection. Furthermore, trans-generational immune priming was also determined in this pest, and only challenged *R. ferrugineus* mothers transferred the immune protection to their offspring which suggested males and females of this pest might have evolved different strategies on the investment of delivering immune protection to their offspring. Importantly, our data provide the evidence to suggest that different kinds of biological control agents might be used alternatively or in combination to fight against *R. ferrugineus* because of the existence of immune priming with low species-specific level. On the other hand, for this invasive pest, the immune priming may also facilitate its adaptation and dispersal in the new regions.

Keywords: red palm weevil, insect immunity, trans-generational, antibacterial activity

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Introduction

With the pesticide resistance of pests being widely demonstrated in these decades, biological control is an important and alternative strategy to fight against pests. In the context of pest management, the control efficiency is of great interest. The immune responses of pests against biological agents in the field were always ignored. Recent investigations revealed that the control efficacy of using entomopathogenic fungi against *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) was around 85% under laboratory and semifield conditions (El-Sufty *et al*., 2009; Dembilio *et al*., 2010). Nevertheless, it has also been revealed that some insects had the ability to recover from infection (Parmakelis *et al*., 2008). To our knowledge, increased immune protection following early exposure to bacteria, often being

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defined as immune priming, has been shown in several species of invertebrates (Kurtz & Franz, 2003; Little & Kraaijeveld, 2004; Sadd & Schmid-Hempel, 2006; Roth *et al*., 2009; Yue *et al*., 2013). Therefore, a new problem, if the control efficiency of a biological agent on its target pest will decrease because some infected individuals survived and recovered after being attacked, might present before us.

Growing evidence indicate that insects rely solely on a suite of innate immune responses, which can be classified into two main types, to combat infection by a wide array of pathogens (Cherry & Silverman, 2006). Constitutive defenses that rely on the response of hemocytes and several rapidly activated enzyme cascades such as penoloxidase and lysozyme are always present and ready to act (Siva-Jothy *et al*., 2005; Cerenius *et al*., 2008). Coupled with this line of defense is mediated by humoral factors, especially antimicrobial peptides, which are rapidly produced in the fat body after infection to kill a wide array of microbes (Cherry & Silverman, 2006). It is generally recognized that innate immunity depends on the recognition of broadly conserved molecular moieties and exhibits only weak specificity, such as the ability to distinguish between different structural classes of peptidoglycan (Kaneko *et al*., 2006).

Because insects lack the molecular machinery as the vertebrate adaptive immune system employ, it is thought that they lack the ability to produce lasting and specific immunity. However, in recent years, numerous lines of evidence show that the experience of a microbial infection or parasitic attack can improve the immune response of insects to a subsequent exposure to pathogens or parasitoids (Moret & Schmid-Hempel, 2001; Schmid-Hempel, 2005; Roth *et al*., 2010; Zanchi *et al*., 2011). For instance, immune priming has been demonstrated over an adult's lifetime in bumble-bee *Bombus terrestris* exposed to bacterial pathogens (Sadd & Schmid-Hempel, 2006). Similar results also have been found in flour beetles such as *Tribolium castaneum* (Roth *et al*., 2009) and in *Drosophila melanogaster* (Pham *et al*., 2007). Obviously, these reports are mainly from social insects and some model systems. Despite these studies have advanced our understanding of the abilities of insect immune systems, because of high diversification of insect species and their life history, it is still unclear how general immune priming is and whether immune priming generally occurs in the invertebrates including the destructive invasive pests and in response to different pathogens.

R. ferrugineus is an invasive and extremely devastating pest for many palm species in China and European countries and this makes it advisable to control it as a quarantine pest (Ju *et al*., 2006; EPPO, 2008; Llácer *et al*., 2012*a*). Since *R. ferrugineus* often hides inside palm trunks except for mating and oviposition, it is usually difficult to find adults and larvae, let alone trying to kill them with chemical insecticides. At present, it is a priority to develop integrated pest management based on pheromone traps and biological control (El-Sufty *et al*., 2007; Poorjavad *et al*., 2009). However, the success of two above strategies is still insufficient. In this regard, *R. ferrugineus* is considered as one pest that is very difficult to be controlled, and rare data are still available about the immunity of *R. ferrugineus* against its pathogens. Thus, investigations on the immune defenses of this pest against its pathogens are promising because they could provide some novel suggestions to improve and even develop new control strategies. The purpose of this study is to determine whether previous nonlethal infection of *R. ferrugineus* could affect its immune response when they were under the secondary attack by bacteria pathogen. As a holometamorphosis insect, the larvae, pupae and adult of *R. ferrugineus* may be attacked by different pathogens and have various infection risk. Consequently, if so, is this influence pathogen species-specific and could the parent's infection experience protect their offspring when they were also attacked by the same pathogen as their parents?

Materials and methods

Insect rearing

A laboratory population of red palm weevil was established by the larvae which were collected from the naturally infested *Washingtonia filifera* trees on October, 2009 in Fuqing City (25.73°N, 119.39°E), Fujian Province of China. In the laboratory, the larvae were reared on sugarcane stem in the climatic chamber at 27 ± 1°C, 75% relative humidity and a photoperiod of 24 h night. The development of the different larval instars was observed by detecting the molted head capsules. After the adults emerged, they were individually transferred into 100 ml plastic bottles with perforated lids and also fed with sugarcane stem under the same conditions as for the larvae except for a photoperiod of 12: 12 h (L:D).

Immune challenge for the larvae, adults and their offspring

As few good bacteria pathogen are isolated and identified from *R. ferrugineus* (Manachini *et al*., 2011), Gram-negative bacteria *Escherichia coli* DH5α was used as the pathogen to challenge the individuals in this study. This bacteria species was cultured at 30°C overnight with $OD_{600} = 1$ in Luria-Bertani's (LB) medium (1000 ml distilled water, 10 g tryptone, 5 g yeast extract, 10 g NaCl and pH 7.2). Approximately 1 ml of the cultured bacteria solution was centrifuged with the velocity of 12,000 *g* under room temperature and discarded the supernatant and then washed the cell pellet using sterilized phosphate buffer saline (being abbreviated as PBS thereafter, distilled water 500 ml, NaCl 4 g, KCl 100 mg, Na_2HPO_4 720 mg, K_2HPO_4 720 mg, pH 7.2) for three times. Finally, the cell pellet was resuspended with 1 ml sterilized PBS and stored at 4°C for late use.

To test whether the previous infection influenced the immune responses of *R. ferrugineus* when they encountered the same pathogen, the fifth instar larvae were firstly injected with 1 µl *E. coli* solution or PBS. After 24 h, these larvae were infected by *E. coli* again. All the injections in this study were completed with 10 µl Hamilton syringe (Reno, Nevada, USA). Twenty-four hours after the second challenge, the hemolymph of each individual was collected to be assayed in the following experiments. PBS injection and no challenge groups served as controls. Ten individuals were treated as one replication and four replications were completed in each treatment.

To detect whether trans-generational immune priming (TGIP) occurs in *R. ferrugineus*, male and female adults 15 days after emergence experienced the following immune challenge treatments: injection of *E. coli* (E), PBS (P) or no challenge (naïve, N). Twenty-four hours after being challenged, the following breeding pairs were established (15 pairs per treatment, total $n = 90$ pairs): (1) male Ec (E), female naïve (N); (2) male PBS (P), female naïve (N); (3) male naïve (N), female *Ec* (E); (4) male naïve, female PBS; (5) male Ec (E),

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Table 1. Dynamic analysis on the PO activity (V_{max}) of *R. ferrugineus* larva after being treated in different patterns on multiple time points.

Treatment	PO activity of larvae after being infected at different time points (h)						
	0.5	1	2	3	4	5	6	7
EE	6.51 ± 0.41a (D)	15.76 ± 0.77a (AB)	15.50 ± 0.65a (ABC)	15.36 ± 0.58a (ABC)	17.33 ± 0.89a (A)	13.61 ± 0.74a (BC)	13.19 ± 0.55a (C)	7.46 ± 0.80b (D)
PE	7.90 ± 1.10ab (D)	8.82 ± 0.73b (CD)	13.33 ± 0.61b (B)	16.28 ± 0.61a (A)	17.50 ± 0.81a (A)	12.50 ± 1.00a (B)	11.38 ± 0.69a (BC)	9.45 ± 0.61a (CD)
PP	5.26 ± 0.51bc (D)	8.45 ± 0.65b (BC)	9.70 ± 0.86c (AB)	10.69 ± 0.73b (A)	9.79 ± 0.85b (AB)	7.54 ± 0.81b (C)	7.11 ± 0.68b (CD)	6.70 ± 0.68b (CD)
NN	3.74 ± 0.35c (BC)	2.94 ± 0.27c (C)	3.09 ± 0.32d (C)	3.58 ± 0.38c (BC)	4.34 ± 0.57c (B)	5.35 ± 0.43c (A)	2.92 ± 0.19c (C)	2.95 ± 0.26c (C)

Notes: EE-two injections of Ec solution, PE-successive injections of PBS and Ec solution, PP-two injections of PBS, NN-larvae without being challenged. The different lowercase and capital letters indicated the statistical significance was determined in the columns or rows by *post hoc* test, respectively.

female Ec (E); and (6) male naïve (N), female naïve (N). Each pair was fed alone in a single plastic container. The breeding pairs were allowed to oviposit for 21 days (the oviposition peak) and the eggs of each pairs were transferred into the Petri dish (9 cm in diameter) with a humid cotton layer every 3 days. After eggs hatched and developed into fifth instar larvae, they were randomly designated into the three following treatments: (1) *Ec* injection, (2) sterilized PBS and (3) naïve. Ten individuals were treated as one replication and each treatment contained four replications. After 24 h, the hemolymph of each larva was collected individually to be assayed in the following experiments.

Hemolymph collection

Before collection, the individuals were immobilized on ice for not over 10 min and then their body surface was sterilized with 75% alcohol. Subsequently, the first back segment was pricked with a sterilized insect needle (1#) and then lightly squeezed the larva body to push the hemolymph out. The hemolymph bubble was immediately transferred by 2.5 µl pipette (Eppendorf North America, Hauppauge, NY, USA) into 94 µl cold PBS for phenoloxidase (PO) activity assay and 2 µl PBS supplemented with Phenylthiourea to inhibit melanization) for antibacterial activity assay, respectively. All the collected hemolymph samples were saved under −80°C condition for late assays.

PO activity assay

PO activity was measured using a spectrophotometric assay (Shi & Sun, 2010). After being taken out from the −80°C refrigerator, the hemolymph samples were placed into the ice bath box to dissolve. After centrifugation (4°C, 10,000 rpm, Sigma 5084R Centrifuge), 20 µl of the supernatant were mixed with 140 µl ultrapure water, plus 20 µl cold PBS and 20 µl *L*-Dopa (Acros Organic, 4 mg ml^{-1} ultrapure water) as a substrate. The reaction was allowed to proceed at 30°C in a microplate reader (SpectraMax, Molecular Devices Corp., USA) for 30 min. Readings were taken every 30 s at 490 nm. For each sample, two independent assays were performed and an average V_{max} (the slope value of the reaction curve) was determined from the two reactions to measure the enzyme activity.

Antibacterial activity assay

Both *E. coli* and *Staphyloccocus aureas* were grown under the above conditions in LB medium. The hemolymph samples were taken out from the −80°C refrigerator, dissolved as above and then diluted into the volume of 220 µl with cold PBS. Our preliminary assays found that it was optimal to the antibacterial assays of hemolymph samples after the culture was serially diluted by 500 times with fresh LB medium. Antibacterial activity assays were performed in sterile 96-well plates with a final volume of 140 µl. An aliquot of 100 µl of diluted culture, or sterilized PBS or tetracycline solution (1 mg ml^{-1}) was added to 40 µl of hemolymph samples. The latter treatments served as the negative and positive controls, respectively. Subsequently, plates were incubated at 30°C for 12 h. Growth of bacteria was measured as the cell concentration which was determined by the absorbance value at 600 nm using a SpectraMax reader (Molecular Devices Corp., USA).

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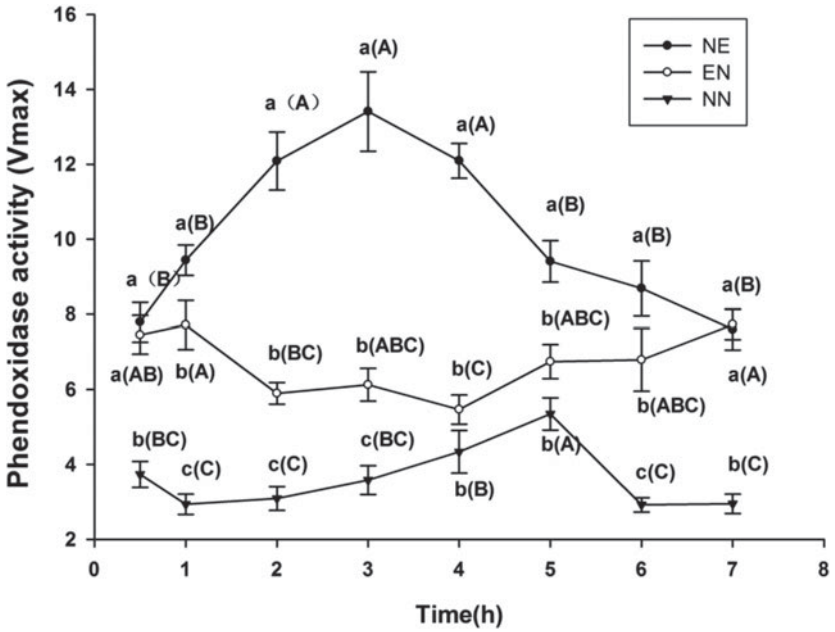

Fig. 1. Dynamic changes in the PO activity of *R. ferrugineus* larvae after being injected at different time points

Statistical analysis

A general linear model was used to determine the effect of immune challenge on the immune response of *R. ferrugineus* larvae. Least-significant difference or Tamhane's T2, determined by the results of the test of homogeneity of variances, was used as the multiple comparison procedure to determine cohort group differences. A confidence level of 95% ($P<0.05$) was maintained for all tests. All statistical analyses were performed with SPSS 12.0 for Windows (SPSS Inc.: Chicago, IL, USA, 2003).

Results

Immune protection from nonlethal early infection in the larvae of R. ferrugineus

Statistical analysis revealed that there were no significant differences in the antibacterial activity between sterilized PBS-injected groups and blank groups (antibacterial activity: $P>0.05$), which suggested that the injection treatment itself could not induce the antibacterial activity of *R. ferrugineus*. However, this is not the case for PO activity of *R. ferrugineus*. The PO activity of individuals injected by sterilized PBS was markedly stronger than that of the naïve ones ($P<0.001$), which indicated that injection itself could up-regulate the insect's PO activity. Compared with the sterilized PBS-injected individuals, both the PO activity and the antibacterial ability of *E. coli* solution-injected ones were dramatically enhanced ($P<0.001$). Therefore, the injection of *E. coli* solution could mount the immune system of *R. ferrugineus*. As shown in fig. 1, 24 h later after being injected, the PO activity of *R. ferrugineus* larvae was relatively stable and maintained at markedly lower level. Thus, the second injection was completed 24 h after the first immune challenge.

The PO activity of *E. coli*-injected larvae was dramatically higher than that of those PBS-injected ones and the larvae without injection (ANOVA: $F_{3,156}=61.355$, $P<0.001$). Time-course analysis revealed that the PO cascade was activated within 0.5 h after being infected, and 1 h later, the PO activity of larvae with previous infection by *E. coli* was always markedly higher than that of PBS-injected ones and blank treatments. Especially, the PO activity at 1 h was two times higher than that of larvae previously injected with PBS (table 1). These data revealed that the previous exposure to *E. coli* could enhance the PO activity of larvae dramatically when they were infected by this species of bacteria again.

The infection by injecting *E. coli* into the hemocoel of *R. ferrugineus* dramatically induced the antibacterial ability of their hemolymph (ANOVA: $F_{3,156}=61.355$, $P<0.001$). As shown in fig. 2, the concentration of *E. coli* cells after being incubated with the hemolymph of the larvae immune priming with *E. coli* was obviously lower than other treatments. These data suggested that the antibacterial activity of the larvae immune priming with *E. coli* was significantly stronger than that of immune priming with PBS (fig. 2a). Interestingly, the hemolymph of larvae immune priming with *E. coli* could also inhibit the propagation of Gram-positive bacteria *S. aureas* (fig. 2b). After being uniformized with the negative control in the antibacterial activity assays, the ratio of inhibiting *E. coli* (0.47) was lower than that of inhibiting *S. aureas* (0.51). It indicated that the hemolymph of *Ec*-infected individuals presented higher inhibition ability on the growth of *E. coli* than that of *S. aureas* and this priming performed with low species-specific level.

Mother-derived trans-generational immune protection in R. ferrugineus

Our data demonstrated that the PO activity of offspring was different across treatments (ANOVA: $F_{5,439}=43.825$, $P<0.001$) and parental immune priming caused up-regulated PO activity in the hemolymph of their offspring under the

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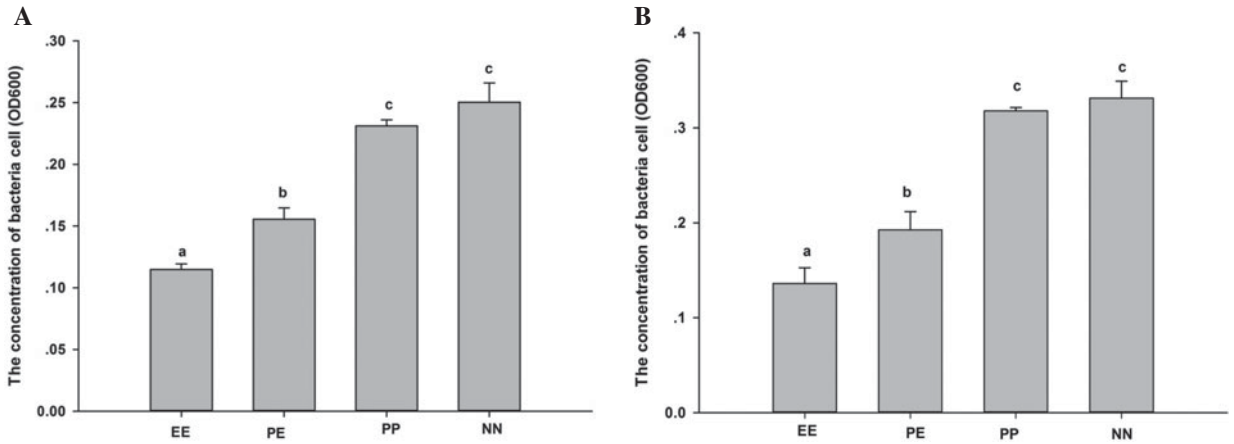

Fig. 2. The antibacterial activity of *R. ferrugineus* larvae against *E. coli* (a) and *Staphyloccocus aureus* (b) in the secondary infection Statistical significance was given by the different lowercase letters above the histograms ($P < 0.05$).

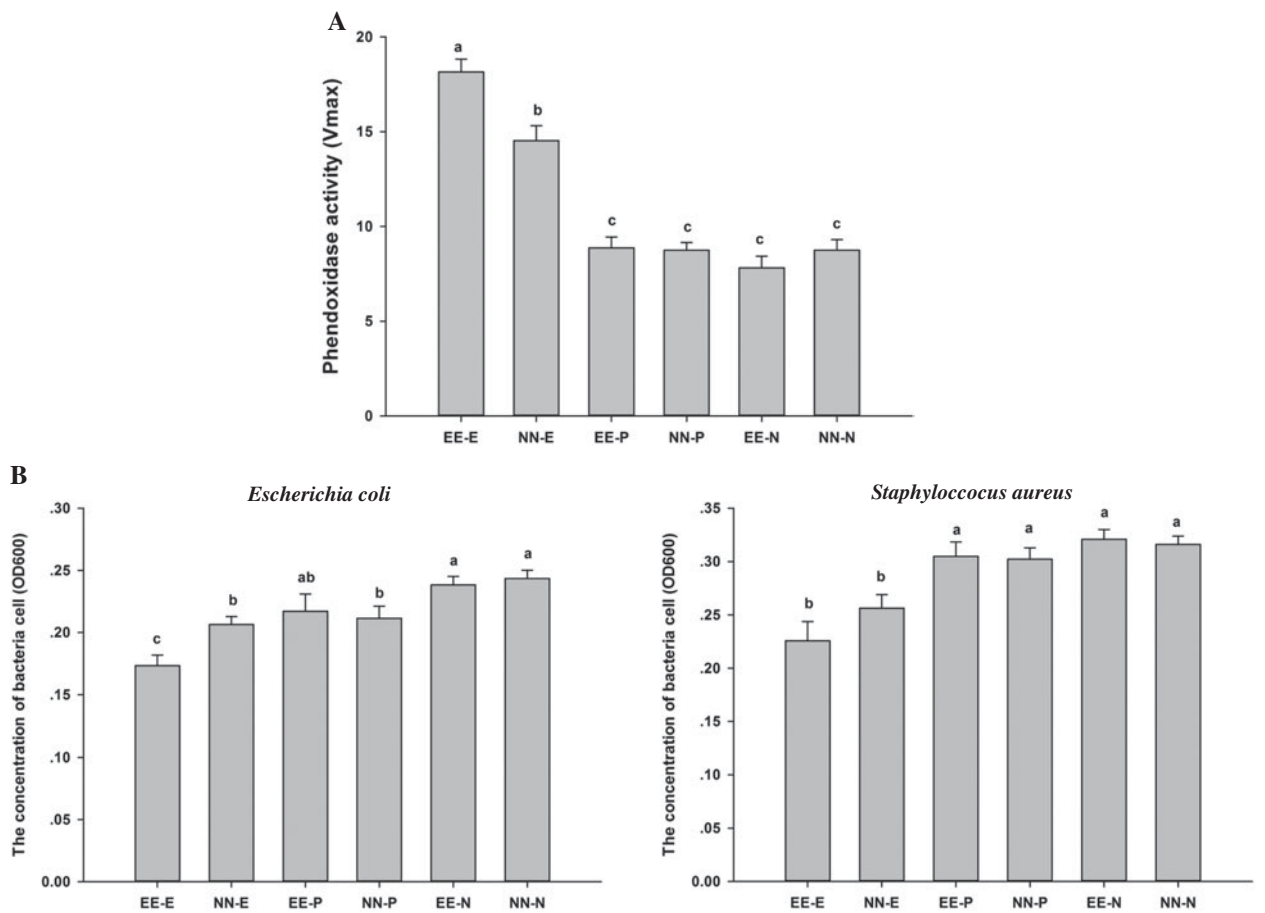

Fig. 3. The influences of parental immune challenge on the PO activity (a) and antibacterial activity (b) of their Ec-challenged offspring. Statistical significance was given by the different lowercase letters above the histograms ($P < 0.05$).

secondary exposure of *E. coli*, but no significant differences were detected in these larvae when they were not challenged (fig. 3a). However, no significant difference was detected by *post hoc* test between the offspring from *Ec*-challenged and PBS-challenged fathers ($P = 0.781$), which showed that paternal immune priming with *E. coli* and PBS had no main

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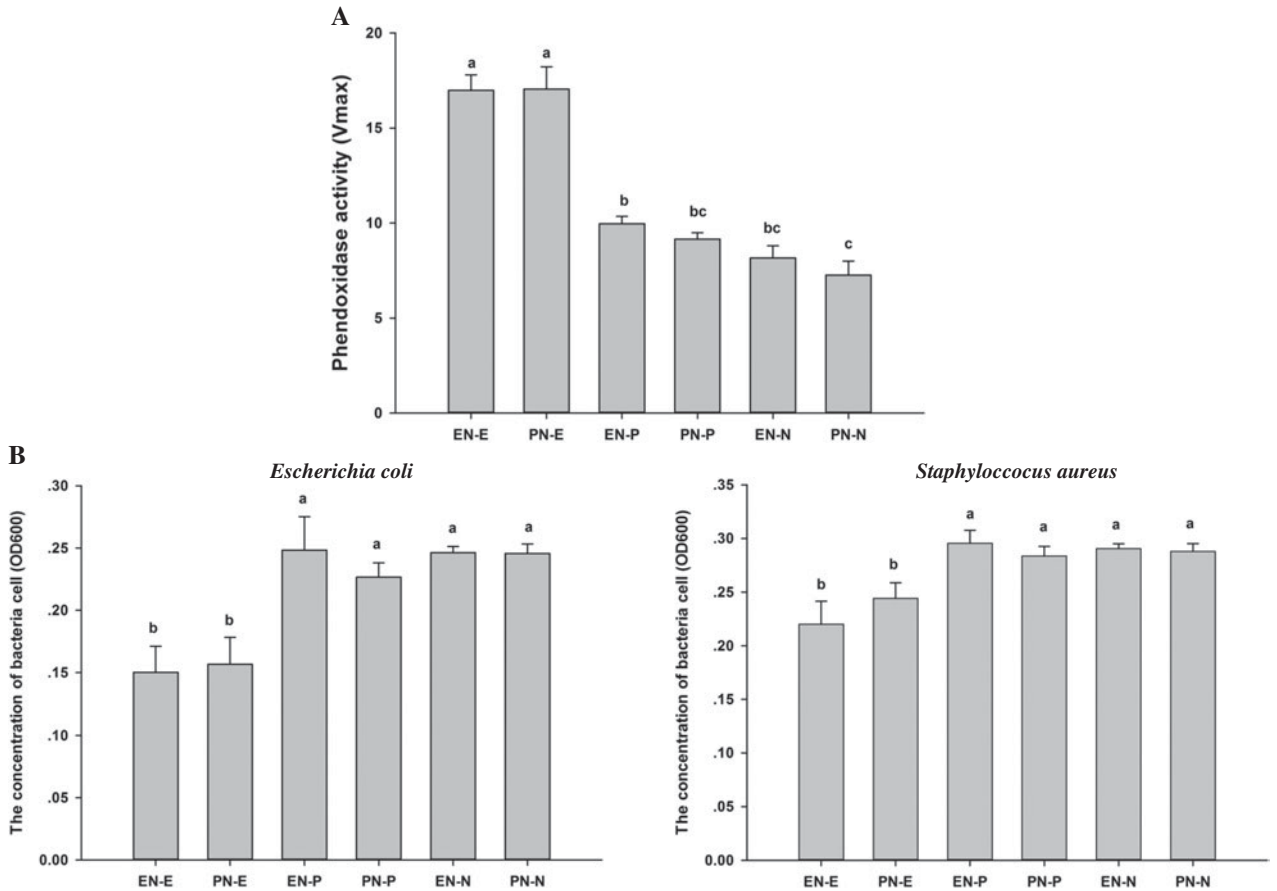

Fig. 4. Effects of paternal infection experience on the PO activity (a) and antibacterial activity (b) of their Ec-challenged offspring. Statistical significance was given by the different lowercase letters above the histograms ($P<0.05$).

effect on levels of the PO activity of their offspring (fig 4a). Only maternal priming produced the significant influence on the activation of PO cascade in their offspring. When their offspring were under the secondary exposure, the PO activity of their hemolymph was dramatically higher than that in other treatments (fig 5a).

Antibacterial activity of larvae varied across our six designated treatments (ANOVA: $F_{5,239}=7.711$, $P<0.001$). Under no challenge conditions, no significant differences were found in the antibacterial activity between the offspring from parents priming with *E. coli* and PBS, respectively. However, after being infected with *E. coli*, the antibacterial activity in the offspring of *E. coli*-challenged parents was stronger than that from parents without infection experience (fig. 3b). It showed that previous infection experience of parents could alter the magnitude of immune defense of their offspring when they were attacked. No statistic difference was detected in the antibacterial activity of larvae between the groups with only fathers being infected and controls (fig. 4b). As shown in fig. 5b, the concentration of bacteria which was incubated with the hemolymph of larvae from only mother being primed was statistically lower than that of larvae from control groups. Furthermore, the hemolymph of their offspring did not only inhibit the growth of *E. coli* that attacked their parents but also retarded the propagation of *S. aureas* (figs 3b, 4b and 5b). These data suggested when parents were challenged with bacteria, only mothers transferred immune protection to their offspring. In other word, paternal priming produced no main effects on their offspring's immune response when the offspring were under infection situation.

Discussion

In the present work, we provide the evidence that priming with *E. coli* could enhance the magnitude of the immune response of *R. ferrugineus* larvae against subsequent bacterial infections. The induced changes cover two aspects of the immune response containing PO activity and the antibacterial activity. This study gives further support to the existence of immune priming in the innate immune defense of invertebrates (Kurtz & Franz, 2003; Little *et al*., 2003; Sadd & Schmid-Hempel, 2006). *R. ferrugineus* is considered as an invasive and destructive pest for palm trees in China now. This species is a concealed tissue borer that lives inside the palm and the larvae is the main lethal factor for the palm trees because the larvae often destroy the trunk of palm to cause its death. In the context of pest management, the larvae stage is the main target to be controlled. The mortalities of *R. ferrugineus* larvae caused by bacterial pathogens, including *Pseudomonas aeruginosa* (Bunerjee & Dangar, 1995) and three species of *Bacillus* (Salama *et al*., 2004) ranged between 40 and 60%.

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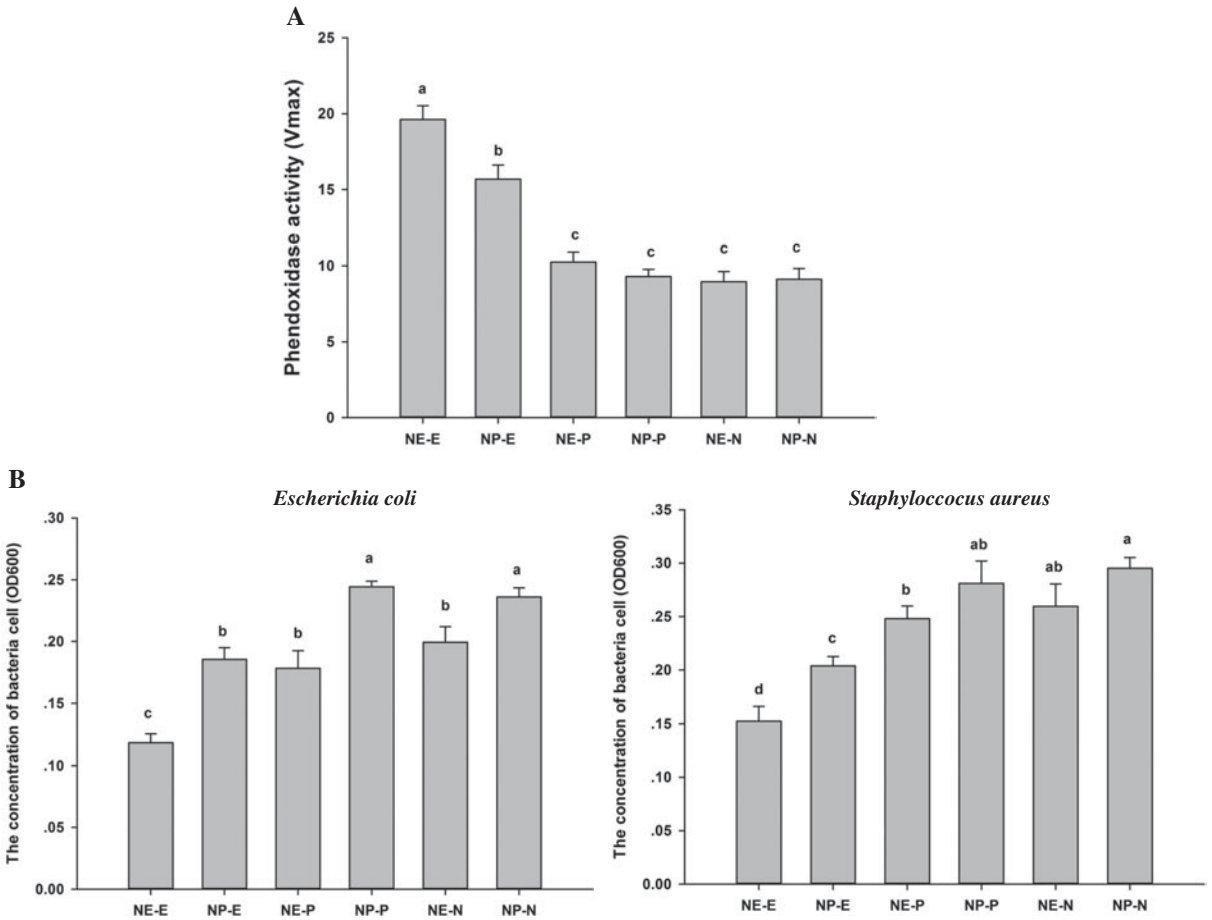

Fig. 5. Effects of maternal infection experience on the PO activity (a) and antibacterial activity (b) of their Ec-challenged offspring. Statistical significance was given by the different lowercase letters above the histograms ($P < 0.05$).

That is also the case for *Beauveria bassiana* against this pest (Llácer *et al*., 2012*b*). Here, our data suggested that the previous infection experience of *R. ferrugineus* larvae elevated its immune defense magnitude when they were under secondary exposure and this immune priming was not species-specific to bacteria species. According to this result, if only one biological agent was often employed to fight against *R. ferrugineus*, the control efficiency might be decreased because of the existence of immune priming in this pest. Thus it is possible to sustainably control this pest by the combination of different control agents and strategies.

On the other hand, we also demonstrated that after parental exposure to *E. coli* infection, the immunocompetence of their offspring was statistically higher than that of offspring from the control treatment on the second contact with *E. coli*. These data suggested that the infection experience of parents altered the immune response of offspring in *R. ferrugineus* upon secondary attack. To discriminate whether the immune protection is from father or mother or both, we have designed six kinds of breeding pairs and checked up the immune response of their offspring under the secondary exposure. Interestingly, all the offspring produced by primed mothers had elevated immune defense upon secondary exposure to *E. coli*, but no primed immune was detected in the offspring from challenged fathers. This result indicates that only mothers can transfer biochemical information that primes the immune system of the offspring to target the pathogens already experience by the mother's immune system. Our results contrast to those of Roth *et al*. (2010) and Zanchi *et al*. (2011) who showed that TGIP occurred through fathers as well as mothers in red flour beetle *T. castaneum* and yellow mealworm beetle *Tenebrio molitor*, respectively. We think that the above difference in the pattern of TGIP might be partly explained by the following reasons. Firstly, two beetle species are both stock pests characterized by overlapping generations and relatively low dispersal which should favor persistence of infection across generations while this is not the case for *R. ferrugineus* with high dispersal ability. Secondly, previous work suggests that TGIP is likely to be cost the parents and costs for father-derived TGIP could be larger than the costs for mother-derived TGIP (Zanchi *et al*., 2011). Taken together, no father-derived immune protection was detected in *R. ferrugineus* because of high dispersal of this pest and larger potential cost. It also supports the opinion that males are the ideal candidate to be used as vector to disperse *B. bassiana* (Llácer *et al*., 2012*b*). Our finding of mother-derived immune priming in *R. ferrugineus* has important evolutionary and ecological consequences such as affecting life-history trait evolution and protecting offspring against pathogens and disease. From this perspective, immune priming in this invasive pest may be

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beneficial to fight against pathogens to facilitate its successful dispersal in new regions.

A striking result of our study is that the magnitude of immune response upon the exposure to *S. aureas* in the offspring from *Ec*-challenged parents is significantly greater than that of the offspring from PBS-challenged and control parents. However, species-specific and strain-specific immune priming have been documented in *D. melanogaster* (Pham *et al*., 2007) and *T. castaneum* (Roth *et al*., 2009), respectively. As suggested by Roth *et al*. (2009), the phenomenon of specific priming depends on the type of pathogen involved. So the nonspecific immune priming documented in *R. ferrugineus* might be caused by *E. coli*, an opportunist-generalist pathogen that we used in the present study. As far as is known, insects lack somatic rearrangement of immune receptors as found in vertebrates. Although recent advances have shown that immunological priming was detected in over ten species of insect, including *Galleria mellonella, T. castaneum, T. molitor, Aedes aegypti, Periplaneta americana* and *B. terrestris* (Kurtz, 2005), little is still known about the mechanism underlying the immune priming of insect now. Recent evidence showed that phagocytosis (Pham *et al*., 2007; Roth & Kurtz, 2009), hemocyte differentiation (Rodrigues *et al*., 2010) and maternal transfer of bacteria to the developing eggs (Freitak *et al*., 2014) mediated the primed immune response in *D. melanogaster, Anopheles gambiae* mosquitoes and *G. mellonella*, respectively. Other interesting alternative mechanisms, such as genomic imprinting (Moore & Haig, 1991) and microRNAs that copy the transcriptomic and epistatic information are delivered to the offspring (Krawetz, 2005), might mediate TGIP as well. However, the evidence to support the above views is still scarce. Therefore, the mechanistic background underlying the immune priming of insect remains to be further investigated.

In summary, our study demonstrated that priming *R. ferrugineus* larvae with *E. coli* could protect them against the secondary challenge of *E. coli* and *S. aureas* by induced an increased activity of PO cascade and antibacterial ability. Furthermore, we also revealed that only challenged mothers delivered the immune protection to the offspring in the second contact with pathogen, which suggests that different strategies might be employed by females and males to the immune protection of offspring in the aspect of TGIP. Our study comes in support of previous work on the existence of immune priming and maternally derived immune priming for offspring in insects (Roth *et al*., 2010; Zanchi *et al*., 2011; Trauer & Hilker, 2013). More importantly, we provide the evidence that more types of biological agents might be used alternatively or in combination to fight against this destructive pest because of the existence of immune priming.

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