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Immunocompetence of the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae, Scolytinae): Variation between developmental stages and sexes in populations in China

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ABSTRACT

Immune defense imposes fitness costs as well as benefits, so organisms should optimize, not maximize, their immune function through their life cycle. We investigated this issue in the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae, Scolytinae), which is a pine-killing invasive beetle in China, though it is usually considered as a secondary pest in its native range of North America. We hypothesized that pathogen pressure may affect these beetles differently throughout their life history. We measured the insect's immunocompetence throughout life, determining encapsulation ability and phenoloxidase activity in larval stages, pupae and adults. Pupae had the highest encapsulation ability, but encapsulation was not different between final instar larvae and adults. Phenoloxidase (PO) activity was highest in final instar larvae and pupae, followed by the second instar larvae and adults. Total phenoloxidase activity increased significantly from the second instar larval stage to pupae, and then decreased in adults. Although the second instar larvae had the lowest phenoloxidase activity, more than 90% of total PO existed in the hemolymph in the form of the active enzyme, as compared with pupae, in which over 60% of PO occurred as a proenzyme. Both active PO and total PO were much higher in females than in males, though no significant differences were detected between the encapsulation ability of male and female adults. This result suggests the existence of a sexual dimorphism of immunocompetence in *D. valens* adults. Variations in immunocompetence across developmental stages suggest that *D. valens* adopts diverse investment strategies in immunocompetence during different stages. Potential reasons for variation in immunocompetence among developmental stages and between the sexes of *D. valens* are discussed.

1. Introduction

In natural environments, pathogens such as parasites, bacteria, viruses, protozoan and fungi coexist with insects under most circumstances. Insects are generally considered to lack adaptive immunity and to rely solely on innate immunity to fight against the infections of those pathogens (Rolff and Siva-Jothy, 2003; Siva-Jothy et al., 2005). The host defense cascade against parasites might start with adaptive changes in the life history, density-dependent prophylaxis, or altered behavior, minimizing the risk of becoming infected in the first place (Schmid-Hempel, 2005). The outer body wall, the cuticle, and the endothelia represent further formidable barriers (Schmid-Hempel and Ebert, 2003). Once a pathogen has breached these defenses, the immune system is activated (Schmid-Hempel, 2005). When recognition of the parasite has occurred, an appropriate response must be initiated. In insects, major immune responses include opsonization, phagocytosis, melanization, encapsulation, and coagulation (Söderhall and Cerenius, 1998). Insects also release cytotoxic and reactive oxygen chemicals and produce antimicrobial peptides and other defense molecules such as lysozyme, and proteolytic and hydrolytic enzymes (Nappi and Ottaviani, 2000; Tzou et al., 2002).

Immunocompetence is the ability of an organism to mount an immune defense against pathogens; it is usually estimated by measuring one or more components of the immune system (Adamo, 2004). The insect immune defense is an evolved trait which imposes fitness costs as well as benefits (Schmid-Hempel, 2005; Siva-Jothy et al., 2005). Several studies also suggest that insect immune defense is a highly dynamic trait, varying both across and within individuals in response to factors such as age (Adamo et al., 2001; Rolff, 2001; Doums et al., 2002), diet quality (Zuk et al., 2004; Lee et al., 2008), and reproductive effort (Fedorka et al., 2004). This variation in immunocompetence may stem, in part, from the fact that immune defense has costs as well as

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benefits (Zuk and Stoehr, 2002; Schmid-Hempel and Ebert, 2003). Broadly, there are two types of cost brought out by immune defense: (i) costs associated with evolving immunity and (ii) physiological costs of maintaining and utilizing immune effector systems (Rolff and Siva-Jothy, 2003). Recent work in insects indicates that the activation of immune defense may trade-off against other fitness such as reproduction (McKean and Nunney, 2001; Ahmed et al., 2002) and mating-induced reduction of immunocompetence has been documented in several species (Siva-Jothy et al., 1998; McKean and Nunney, 2001).

Many ecological and evolutionary studies of insect immunology have focused on two generalized immune effector traits: phenoloxidase (PO) activity and encapsulation ability. Prophenoloxidase (proPO) is an inactive enzyme stored in the hemolymph, and is activated to PO once infection occurs. PO is an oxidoreductase that catalyses the oxidation of phenols to quinones, which then polymerize non-enzymatically to melanization (Sugumaran, 1996). Quinones as well as melanin are toxic to microorganisms (Nappi and Ottaviani, 2000). PO is also involved in the encapsulation process, and it is thus one important component of insect immunity that is often used to estimate immune function in insects. PO activity is frequently correlated with host resistance (Adamo, 2004). PO activity is usually measured by measuring conversion of a selected substrate to melanin using hemolymph or tissue samples as a source of enzyme (Schmid-Hempel, 2005). Encapsulation occurs when the immune system encounters abiotic antigens or biotic ones, which cannot be phagocytosed (Klemola et al., 2007). Previous studies have indicated that encapsulation ability is positively correlated with resistance to viral infection, parasitoids and parasites (Wilson-Rich et al., 2008).

Studies on insect ecological immunity have focused on scorpionflies, moths, crickets, dragonflies, fruit flies, damseflies, and *Tribolium* flour beetles (Lindsey and Altizer, 2009), but no such studies have been conducted on bark beetles, a group of subcortical insects that feed as larvae and adults in the phloem of trees and woody shrubs (Seybold et al., 2006). Many bark beetles are significant economic and ecological pests, and an examination of their immunocompetence could provide insights on possible management techniques for these species.

The red turpentine beetle *Dendroctonus valens* LeConte (Coleoptera: Curculionidae, Scolytinae), is a serious, invasive killer of pines in China, though it is generally considered as a secondary pest in its native range of North America (Yan et al., 2005). *D. valens* has four developmental stages: eggs, larvae, pupae and adult. Eggs are always laid in an elongate mass along the side of the gallery and partitioned off from the adult gallery by a wall of pitchy borings. The larvae hatch from eggs often feed gregariously under the bark, and large number of larvae within a few square cm is usually found in the field. There are four larval instars, and after the larvae complete their feeding, they construct separate pupal cells in which they develop into adults (Smith, 1971).

Since parasite transmission is often density-dependent, group living is normally thought to lead to an increased exposure to parasitism. As a consequence, it is predicted that animals living in groups will invest more resources (energy, time, risk, etc.) in parasite defense than those living solitarily (Wilson et al., 2003). Based on the previous reports and the unique biology of *D. valens*, we hypothesize that variation in population density of this insect could result in variation in immunocompetence across developmental stages. Due to differences in life habit and behavioral traits, it is expected that larvae and pupae will have higher physiological immunocompetence than adults. Because immune defense always imposes some cost, the correlation between body mass and immunocompetence was also investigated. Cellular and humoral immunocompetence were examined to determine if *D. valens* immunocompetence varied with developmental stage. Encapsulation ability, PO activity and total PO activity were assayed as the indicators of physiological immunocompetence.

2. Methods

2.1. Specimen collection

Newly emerged adult *D. valens* dispersing from overwintering sites were collected daily from the traps baited with the standard *D. valens* pheromonal lure ((+)-(3)-carene:(−)-β-pinene:(+)-α-pinene = 1:1:1) from May to June, 2009 in a *Pinus tabulaeformis* stand at Tunlanchuan Forest Farm (37°48′N, 111°44′E; average elevation 1400 m), Gujiao City, Shanxi Province. Sexes were separated by the stridulation of live males (Lyon, 1958), and were held separately in Petri dishes with moist filter paper at 4 °C and utilized within 7 days after capture. *D. valens* larvae were collected from 10 fresh stumps in the same area in mid-September, 2009. The larvae were placed into a plastic box containing artificial diet (100 g phloem powder of Chinese pine, 2 g vitamin C, 10 g agar, 2 g methylparaben, 1 g sorbic acid, 12 drops linolic acid, 200 ml distilled water) and brought into a climate incubator (20 °C, L:D = 0:24). 2- or 3-day-old pupae were used in the experimental treatments.

2.2. Encapsulation assay

The magnitude of the cellular encapsulation reaction against an artificial antigen is an informative and simple way to collectively assay the innate immune resistance (Siva-Jothy et al., 2005). The artificial antigen, a 2-mm-long piece of nylon monofilament with a diameter of 0.20 mm, was rubbed with sandpaper, knotted at one end, and rinsed in pure ethanol before insertion into the abdominal haemocoel of *D. valens* through a needle-puncture in the middle of the abdomen. After 6 h incubation at room temperature, the insects were frozen and stored at −20 °C. Before implantation, the body mass of each insect was measured using an analytical balance (METTLER TOLEDO, AL204, Shanghai, China). Subsequently, the nylon monofilament was recovered and photographed at 100× magnification using an Olympus BX51 microscope with a digital imaging system DP 72 under the same illumination; the pictures were analyzed using an image analysis program (Image J 1.34s, National Institute of Mental Health, USA; Rasband, 2007). A grey-scale image ranges in brightness from 0 to 255, where 0 is black and 255 is white. 16 final instar (old) larvae, 8 pupae, and 69 adults (33 males, 36 females) were successfully treated in this experiment, respectively.

2.3. Phenoloxidase activity

Before hemolymph extraction, the body mass of each insect was measured as above. Because the haemocoel of *D. valens* contains only a small volume of hemolymph, compared to that of typical Lepidoptera larvae, after the cuticle was sterilized by 95% ethanol, the larvae and pupae were cut open using two sterilized forceps, and an extract of hemolymph made by putting the insect into a 0.5 ml microcentrifuge tube containing 500 μl cold phosphate buffer saline (distilled water 500 ml, NaCl 4 g, KCl 100 mg, Na_2HPO_4 720 mg, K_2HPO_4 720 mg, pH 7.2). The adults were similarly treated as above after removal of the elytra. All hemolymph samples were mixed individually by Vortexer for several seconds and stored at −20 °C for 72 h and an additional 24 h at 4 °C. For each sample, the activity of naturally activated PO enzymes only (hereafter "PO activity"), the additional activatable activity of the proenzyme ("proPO") and the sum total activity of all PO activity ("total PO activity") were measured using a spectrophotometric assay (Cornet et al., 2009). PO activity was

quantified without further activation, while total PO activity required the activation of the proPO into PO with trypsin. After centrifugation (4 °C, 10,000 rpm, Sigma 1-15PK Centrifuge), 30 μl of the supernatant were mixed with either 110 μl of ultrapure water to measure PO activity only, or 110 μl of trypsin solution (Amrexco, 2 mg/ml of ultrapure water), plus 30 μl phosphate buffer saline, and 30 μl L-Dopa (Acros Organic, 4 mg/ml ultrapure water) as a substrate. The reaction was allowed to proceed at 30 °C in a microplate reader (VersaMax, Molecular Devices Corp., USA) for 30 min. Readings were taken every 30 s at 492 nm. For each individual, we performed two independent measurements and determined an average V_{max} (the slope value of the reaction curve) for the two reactions to measure the enzyme activity. The PO ratio was calculated by PO activity/total PO activity. Because PO is often stored in the hemolymph as the proenzyme, PO ratio was calculated to evaluate the investment in the proPO system of *D. valens* across different stages. The number of specimens treated in the experiments was as follows: 23 second instar (young) larvae, 45 final instar (old) larvae, 17 pupae and 81 adults (39 females, 42 males).

2.4. Data analysis

The normality of the data was tested by One-Sample Kolmogorov–Smirnov Test, and in all except one case was found to be normally distributed. Only the data for PO activity of *D. valens* in different stages was not normally distributed. Therefore, PO activity was compared among developmental stages using the Kruskal–Wallis test followed by pairwise comparisons using Mann–Whitney *U* tests. A general linear model (GLM) was used to analyze differences in the means of body mass and encapsulation score among developmental stages. Levene's homogeneity of variance test was performed on body mass and encapsulation score to check if data transformation was needed before GLM could be performed. If a significant *F*-test statistic (at $P \leq 0.05$) was obtained from GLM, differences of least squares means were used as the multiple comparison procedure for determining cohort group differences. Immunocompetence variations between sexes were detected using an independent samples *t*-test. Pearson correlation analysis was used to investigate the relationship between immunocompetence and body mass. All tests were performed with the statistical software SPSS for windows (v 11.5).

3. Results

3.1. Body mass of D. valens in each developmental stage

Significant differences were detected in body mass across developmental stages ($F_{3,166} = 82.826$, $P < 0.001$). Second instar (young) larvae were the lightest, with a mean of 5.62 ± 0.48 mg (Fig. 1). Body mass of *D. valens* increased with development. No significant differences were found between the body mass of the final instar larvae and pupae, with means of 38.10 ± 1.37 mg and 35.51 ± 2.21, respectively. Body mass of adult decreased after emergence, with a mean of 31.13 ± 0.99 mg. There were no significant differences between the body mass of female (32.06 ± 1.06 mg) and male (29.59 ± 0.81 mg) adults (*t*-test: $t = 1.853$, $df = 148$, $P = 0.066$).

3.2. Encapsulation ability

D. valens pupae encapsulated nylon implants to a markedly greater degree than final instar larvae and adults ($F_{3,92} = 7.095$, $P < 0.001$. Fig. 2). No significant differences in encapsulation scores were detected between final instar larvae and adults. Furthermore, encapsulation scores were similar for adult females and males, with means of 164.54 ± 0.33 and 164.68 ± 0.33, respectively (*t*-test: $t = -0.302$, $df = 67$, $P = 0.764$).

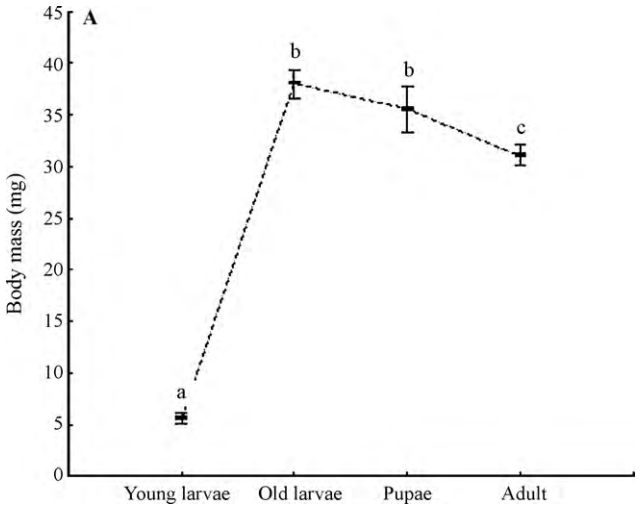

Fig. 1. The variations of body mass in *Dendroctonus valens* larvae, pupae and adult. The results were presented with mean ± S.E., and significant differences were indicated with various letters.

3.3. Phenoloxidase activity

Significant differences were detected in PO activity (Kruskal–Wallis test, $df = 3$, $P < 0.001$), total PO activity (Kruskal–Wallis test, $df = 3$, $P < 0.001$) and PO ratio (Kruskal–Wallis test, $df = 3$, $P < 0.001$) of *D. valens* across different life stages. Final instar larvae and pupae had the highest PO activity, followed by second instar larvae and adults (Fig. 3A). Total PO activity increased significantly as the second instar larvae progressed through the final instar and then increased further as they transformed into pupae, but decreased in the adult stage (Fig. 3B). Although the second instar larvae had the lowest PO activity, the PO ratio in their hemolymph was the highest (Fig. 3C). Pupae had the lowest PO ratio, which suggested that the majority of PO existed as the proenzyme in the hemolymph of pupae.

In adults, females had greater PO activity and total PO activity than males (*t*-test, PO: $t = 2.634$, $df = 79$, $P < 0.05$; total PO: $t = 2.604$, $df = 79$, $P < 0.05$). The mean of PO activity and total PO activity of females were 68.99 ± 1.84 and 100.64 ± 4.00, while those of males were 61.40 ± 1.49 and 90.70 ± 2.32 (Fig. 4). The mean PO ratio of females and males was 67.58 ± 1.99% and 68.34 ± 1.26%,

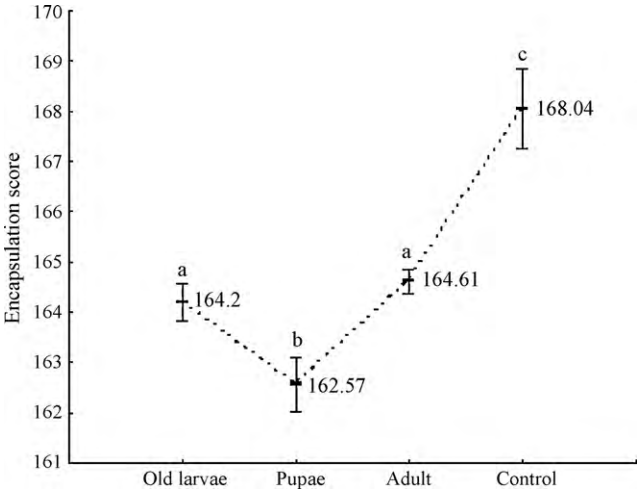

Fig. 2. Cellular encapsulation rate in the final instar larvae, pupae and adult of *Dendroctonus valens*. The results were presented by mean ± S.E., and significant differences were indicated with various letters. The unimplanted nylons were the "control".

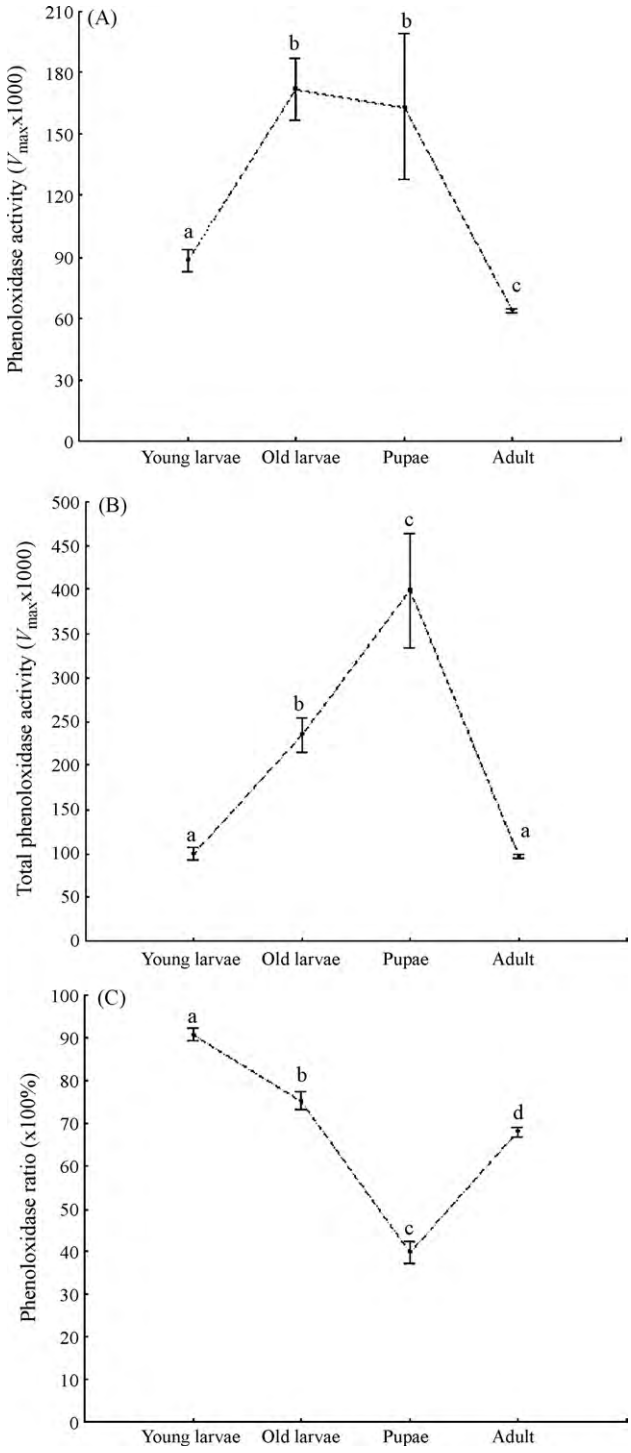

Fig. 3. The phenoloxidase activity (A), total phenoloxidase activity (B) and phenoloxidase ratio (C) in the different development stage of RTB. The results were presented by mean ± S.E., and different letters in the figures indicated significant differences.

respectively, and they did not differ significantly (*t*-test: $t = -0.328$, $df = 79$, $P = 0.744$).

3.4. Correlation analysis between immunocompetence and body mass in each developmental stage

In each developmental stage of *D. valens*, there was no significant correlation between encapsulation ability and body mass. In the final instar (old) larvae and also in adults, however, PO

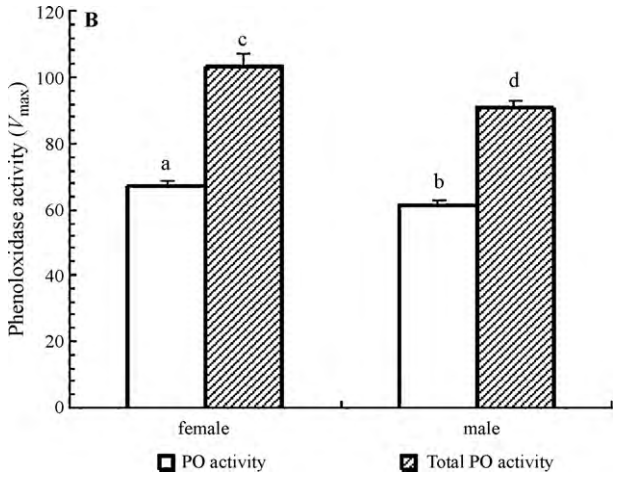

Fig. 4. The phenoloxidase activity (PO) and total phenoloxidase (total PO) activity in female and male adults of RTB, and the results were performed with mean ± S.E. Various letters in the figures indicated significant differences. 39 females and 42 males were sampled in this experiment, respectively.

activity and total PO activity were both positively correlated with body mass. For pupae, only total PO activity had a positive correlation with body mass (Table 1). In female and male adults, there were no significant correlations between encapsulation ability and body mass. Total PO activity in females and PO activity in males were positively correlated with body mass, respectively (Table 2).

4. Discussion

Holometabolous insects have significant differences in body structure, physiology and behavioral trait across developmental stages. Each developmental stage also will have different pathogens and infection risk (Schmid-Hempel, 1998; Schmid-Hempel

Table 1
Correlation analysis between body mass and encapsulation, phenoloxidase activity (PO), total PO activity (phenoloxidase + prophenoloxidase) in the different developmental stage of *Dendroctonus valens*.

		Encapsulation	PO	Total PO
Young larvae	Pearson's *r*	–	0.345	0.347
	P	–	0.107	0.105
Old larvae	Pearson's *r*	0.038	0.712	0.673
	P	0.889	0.000**	0.000**
Pupae	Pearson's *r*	−0.294	0.371	0.638
	P	0.480	0.143	0.006*
Adult	Pearson's *r*	−0.109	0.335	0.372
	P	0.371	0.002*	0.001*

* Significance at $P < 0.05$.
** Significance at $P < 0.001$.

Table 2
Correlation analysis between body mass and encapsulation, phenoloxidase activity (PO), total PO activity (phenoloxidase + prophenoloxidase) in females and males of *Dendroctonus valens*.

	Female			Male		
	Encapsulation	PO	Total PO	Encapsulation	PO	Total PO
Pearson's *r*	−0.156	0.234	0.408	−0.012	0.426	0.289
P	0.363	0.152	0.010*	0.946	0.005*	0.064

* Significance at $P < 0.05$.

and Ebert, 2003), and these infections can have substantial effects on fitness (Bradley and Altizer, 2005). Our results demonstrated significant differences in encapsulation ability, PO activity and total PO activity across developmental stages of *D. valens*, indicating that each developmental stage adopts a different investment strategy in immunocompetence to fight against possible infections. *D. valens* pupae had greater encapsulation ability, higher PO activity and total PO activity than other stages. Pupae are not mobile and have no behavioral ability to escape invasive pathogens, so their need for immune defenses would be expected to be higher than for larvae. As *D. valens* larvae develop, the walls between neighbouring larval feeding galleries are obliterated. Later stage larvae are therefore located in large, communal feeding areas, and pathogens could easily spread between individuals. Therefore, it may be predicted that later instar larvae should invest more resources in immune defenses than early instars, a trend confirmed by our results. Adults have a hard exoskeleton which is often regarded as the first defense line to pathogens (Schmid-Hempel and Ebert, 2003). This morphology allows adults to invest more of their limited resources into structures and behaviors related to reproduction and less in those related to immune defense. Our data suggest that immunocompetence, as well as the body structure and behavior of *D. valens*, varies across developmental stages, and that this variation may be important at the population level from an evolutionary perspective.

PO catalyzes the formation of quinones that are important intermediates for melanin synthesis (Sugumaran, 1996). Except killing of entrapped parasites or pathogens, quinones may also be involved in cuticle sclerotization and wound healing (Wang and Jiang, 2004). Pupae have a PO activity similar to final instar larvae, though because they have more proPO, their total PO activity is much higher. This increase in proPO would provide a good foundation for cuticle sclerotization during the transformation into the adult stage, but may also result in the increase encapsulation ability of pupae. The total PO activity in *D. valens* decreased when adult emerged, which might be driven by melanization of the cuticle.

The significantly higher PO activity and total PO activity in *D. valens* females as compared to males is in accordance with other studies showing gender differences in PO activity (Schwarzenbach et al., 2005). Sexual differences in the immunocompetence of insects have been detected in many studies and using many different indicators, such as PO activity (Schwarzenbach et al., 2005), encapsulation ability (Klemola et al., 2007), hemocyte load (Hazarika and Gupta, 1989; Lindsey and Altizer, 2009). Sexual dimorphism in immune function is a common pattern in vertebrates and also in a number of invertebrates. Most often, females are more immunocompetent than males, and this is consistent with the application of "Bateman's principle" to immunity, with females maximizing fitness by lengthening lifespan through greater investment in immune defenses (Nunn et al., 2009). In vertebrates, this dimorphism is usually attributed to the immunosuppressant effects of steroids, primarily testosterone (Folstad & Karter, 1992). The mechanisms underlying the sexual difference in immunocompetence of insects are poorly understood because insects lack testosterone and other male hormones, but sexually transmitted parasites might contribute to this dimorphism (Abbot and Dill, 2001). The sex differences in immunity between *D. valens* females and males may be due to the physiological requirements of different behaviors. Female *D. valens* are the pioneer sex; they locate and attack suitable host trees and construct the galleries. Males only enter pine trees via a preexisting gallery constructed by a female (Liu et al., 2006). Therefore, females might face a higher potential infection rate and require higher immunocompetence.

Insect immune defense is a highly plastic trait, varying both across and within individuals in response to factors such as age, diet quality and quantity, and reproductive effort (Stoehr, 2007). *D. valens* in China is an invasive pine-killing bark beetle, and its success may result from an escape from the natural enemies and pathogens in its native range. The beetles might experience a lower infection risk in China, and may therefore be able to allocate a greater proportion of resources for growth and reproduction to facilitate invasion. However, *D. valens* in China may also be subject to novel pathogens. A comparison of immunocompetence between *D. valens* from North America and China could help explain the invasive success of this insect in China. Furthermore, these results may guide integrated management of this pest. For example, knowledge of the immune defenses could aid in the selection of biocontrol methods and determining which stage of *D. valens* is most easily targeted.

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