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Temporal patterns in immune responses to a range of microbial insults (*Tenebrio molitor*)

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ABSTRACT

Much work has elucidated the pathways and mechanisms involved in the production of insect immune effector systems. However, the temporal nature of these responses with respect to different immune insults is less well understood. This study investigated the magnitude and temporal variation in phenoloxidase and antimicrobial activity in the mealworm beetle *Tenebrio molitor* in response to a number of different synthetic and real immune elicitors. We found that antimicrobial activity in haemolymph increased rapidly during the first 48 h after a challenge and was maintained at high levels for at least 14 days. There was no difference in the magnitude of responses to live or dead *Escherichia coli* or *Bacillus subtilis*. While peptidoglylcan also elicited a long-lasting antimicrobial response, the response to LPS was short lived. There was no long-lasting upregulation of phenoloxidase activity, suggesting that this immune effector system is not involved in the management of microbial infections over a long time scale.

1. Introduction

The success of insects at avoiding and surviving infections may be largely attributable to the way they manage their suite of immune responses. Insect immune effector system upregulation and activity varies both temporally, and according to the type of pathogen challenge (reviewed by Lemaitre et al., 1997; Hultmark, 2003; Siva-Jothy et al., 2005).

The production of antimicrobial peptides following an immune challenge is possibly the major induced component of insect immunity (reviewed in Dunn, 1986; Boman and Hultmark, 1987), and the biochemical pathways and molecular machinery involved in their upregulation have been well studied in some instances (reviewed by Bulet et al., 1999, 2004). In *Drosophila*, the temporal nature of the upregulation of genes involved in the production of antimicrobial peptides is relatively well documented (e.g. Lemaitre et al., 1997; Leulier et al., 2003). The expression of antimicrobial peptide genes begins 1–3 h after an immune challenge (Lemaitre et al., 1997), peaks at 3–12 h after a challenge, and may remain at high levels for relatively long periods, depending on the challenge and the antimicrobial peptide assayed (Lemaitre et al., 1997). Drosomycin expression, for example, is still at high levels 72 h after *D.* melanogaster were challenged with *Beauveria bassiana* (Lemaitre et al., 1997). Antimicrobial peptide gene expression is also detectable in the haemolymph of the moth *Pseudoplusia includens* 2 h after an immune challenge and peaks at 8–24 h post-challenge (Lavine et al., 2005).

In order to understand which factors have had important roles in the evolution of insect antimicrobial peptides, it is important to investigate how antimicrobial peptide expression varies temporally, and according to the type of pathogen challenge received in a number of different insects. The appearance of antimicrobial peptides in insect haemolymph varies. It is detectable in the bumblebee *Bombus terrestris* 2 h after a challenge (Korner and Schmid-Hempel, 2004); 4 h after a challenge in the locust *Locusta migratoria* (Hoffmann (1980); 10 h after challenge in the silkmoth *Samia cynthia* (Faye et al., 1975); 6–12 h after challenge in the beetle *Zophobas atratus* (Bulet et al., 1991); 24–48 h after challenge in *Rhodnius prolixus prolixus* (Azambuja et al., 1986). The duration of antimicrobial activity in insect haemolymph can be long: at least 5 days in the butterfly *Pieris brassicae* and the wax moth *Galleria mellonella* (Jarosz, 1993); up to 9 days in the silkmoth *S. cynthia* (Faye et al., 1975); 11 days in *R. prolixus* (Azambuja et al., 1986) and the locust *L. migratoria* (Hoffmann, 1980); at least 14 days in the bumblebee *B. terrestris* (Korner and Schmid-Hempel, 2004); and is still at high levels 28 days after challenge in the beetle *Z. atratus* (Bulet et al., 1991) and 44 days after the challenge in the dragonfly *Aeschna cyanea* (Bulet et al., 1992).

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In the mealworm beetle *Tenebrio molitor*, the antimicrobial response is maintained for at least 7 days after insult, a trait that is adaptive *via* its effect on resistance against subsequent pathogen insults (Moret and Siva-Jothy, 2003). However, while the fact that insect immune responses are long lasting has been known for a number of years (Boman and Hultmark, 1987), few (if any) studies have systematically sampled haemolymph antimicrobial activity in relation to both time and more than two types of immune challenge (e.g. variation according to challenge only: Kanost et al.,1988; temporal variation only: Dunn et al., 1985 (3 days); Postlethwait et al.,1988 (8 days)), i.e. little is known about how the type of challenge impacts on the temporal nature of the response at the physiological level.

Another important humoral component of insect immune responses is the phenoloxidase (PO) cascade. Pathogen recognition elicits the production of the inactive zymogen prophenoloxidase and its conversion into the enzyme PO (Ashida and Brey, 1995). PO is an enzyme involved in the conversion of phenols to quinones and the subsequent production of melanin. The production of melanin is likely to cut off the pathogen's supply of oxygen and nutrients, eventually killing it. PO is therefore thought to have a major role in insect defences against pathogens (Cerenius and Söderhall, 2004). The PO cascade is upregulated within 1 h after a pathogen challenge, and may continue to be expressed for more than 24 h (Korner and Schmid-Hempel, 2004). Little is known about how the type of challenge impacts upon the temporal nature of PO responses, or whether PO activity trades-off with other immune defences (but see Korner and Schmid-Hempel, 2004).

Mounting any long-lasting immune response is potentially costly in terms of the metabolic expense of producing the defensive compound(s) and the potential to cause self harm (e.g. Poulsen et al., 2002; Armitage et al., 2003; Sadd and Siva-Jothy, 2006; Meylaers et al., 2007). Despite these costs, insects upregulate a suite of cytotoxic and antimicrobial responses upon immune insult, and they do this over a long period of time (reviewed in Boman and Hultmark, 1987). This strongly suggests that there is an adaptive advantage to mounting long-lasting responses.

The aim of this study was to measure the temporal variation in antimicrobial and PO activities in response to challenges by different groups of live and dead bacteria and their associated immune elicitors. We used the mealworm beetle *T. molitor* as a model insect for this study because (a) it is known to mount long-lasting immune responses (Moret and Siva-Jothy, 2003), but relatively little is understood about the shape of the response curve(s); (b) it incurs different types of costs directly as a result of mounting an immune response (Armitage et al., 2003; Sadd and Siva-Jothy, 2006); and (c) its relatively large size makes it easy to measure immune activity in the haemolymph of individuals. This study quantifies the temporal nature of the levels of immune activity in response to different challenges at the physiological level of individuals, rather than examining the qualitative nature of the response (i.e. differential gene expression in response to pathogen recognition). In addition, by comparing the production of one induced and one constitutive component of the insect immune system, we aim to generate data that may reveal any correlations between different immune-responsive pathways.

2. Materials and methods

Before solution preparation, all glass and plasticware were rinsed in 1% (w/v) E-Toxa Clean solution (Sigma) several times before autoclaving, and all solutions were made up in endotoxin-free water. This preparation served to minimize any contaminating lipopolysaccharide or peptidoglycan remnants before addition of LPS, peptidoglycan or Ringer. All injections were performed through the pleural membrane between the second and third abdominal sternites using sterilized (see earlier) glass capillaries that had been pulled out to a fine point with an electrode puller (Narishige PC-10). All experiments were performed at ambient temperature ($20 \pm 1\,^{\circ}C$) unless otherwise stated. The size of all animals was measured as wet mass.

2.1. Insect culturing

Experimental *T. molitor* beetles from a stock culture maintained at the University of Sheffield were reared and maintained in an insectory at $25 \pm 2\,^{\circ}C$ with a light/dark (LD) 12:12 h photo cycle, and supplied with an *ad libitum* diet of rat chow and water, supplemented by apple. Newly eclosed pupae were collected, sexed and weighed. Only individuals weighing between 140 and 170 mg were used for the experiments and were maintained individually in grid box containers. At adult eclosion, beetles were allocated to one of the eight treatment groups ('naïve', 'Ringer', 'LPS', 'Peptidoglycan', 'Dead *Escherichia coli*', 'Live *E. coli*', 'Dead *Bacillus subtilis*' and 'Live *B. subtilis*'. All treatments were performed 7 days post-adult eclosion, and all individuals were virgin. All experimental beetles were therefore controlled for differences in age, gender, reproductive status and size.

2.2. Immune challenges

Our experimental protocol consisted of the following eight treatments. 'Naïve' animals did not undergo any treatment. Individuals in the 'Ringer' treatment were injected with 5 μl of sterile insect Ringer solution (128 mM NaCl, 18 mM $CaCl_2$, 1.3 mM KCl, 2.3 mM $NaHCO_3$). Individuals in the "LPS" treatment were injected with 0.5 mg/ml purified LPS (Sigma L4524) in 5 μl of sterile Ringer solution. LPS is a non-pathogenic surface molecule derived from *E. coli*. It is a highly immunogenic component of Gram-negative bacteria and elicits the production of antimicrobial peptides (Lemaitre et al., 1997; Soderhall, 1982; Ratcliffe et al., 1985). Commercial LPS often contains contaminating peptidoglycan fragments so only LPS that had been purified by both phenol extraction and ion exchange chromatography was used for this study. The 'peptidoglycan' treatment involved injection of 0.5 mg/ml peptidoglycan derived from *B. subtilis* (Fluka 69554) in 5 μl sterile Ringer solution; peptidoglycan is a highly immunogenic component of Gram-positive bacterial cell walls. The final four treatments involved injection of dead or live *B. subtilis* A1 or *E. coli* 54.8T. To kill bacteria, 1 ml of freshly grown overnight culture was heat-killed at 95 °C for 30 min, centrifuged for 10 min at $10{,}000 \times g$, rinsed with sterile Ringer solution and finally resuspended in 1 ml of sterile Ringer. Five microlitres of this solution (approximately 10^6 bacteria per ml) was then used for dead bacteria challenges. For live bacteria treatments, the bacteria were grown as described earlier but were not subjected to the heat treatment. The bacterial dose used was non-lethal, to ensure that the challenged individuals survived at least 14 days so that we could measure haemolymph antimicrobial activity over time. It was also in the range used in other invertebrate immunology studies (e.g. Sadd and Schmid-Hempel, 2006). Five beetles of each gender were assigned to each treatment group at each of the nine time intervals; an additional five beetles for each gender were included in the 'naïve' and 'Ringer' treatments to make up for samples lost during assay calibrations.