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Developmental modulation of immunity: Changes within the feeding period of the fifth larval stage in the defence reactions of *Manduca sexta* to infection by *Photorhabdus*

I. Eleftherianos[a,*], H. Baldwin[a], R.H. ffrench-Constant[b], S.E. Reynolds[a]

[a]*Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK*
[b]*Centre for Ecology and Conservation, University of Exeter, Cornwall Campus, Penryn TR10 9EZ, UK*

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Abstract

In insect pathogen interactions, host developmental stage is among several factors that influence the induction of immune responses. Here, we show that the effectiveness of immune reactions to a pathogen can vary markedly within a single larval stage. Pre-wandering fifth-stage (day 5) larvae of the model lepidopteran insect *Manduca sexta* succumb faster to infection by the insect pathogenic bacterium *Photorhabdus luminescens* than newly ecdysed fifth-stage (day 0) caterpillars. The decrease in insect survival of the older larvae is associated with a reduction in both humoral and cellular defence reactions compared to less developed larvae. We present evidence that older fifth-stage larvae are less able to over-transcribe microbial pattern recognition protein and antibacterial effector genes in the fat body and hemocytes. Additionally, older larvae show reduced levels of phenoloxidase (PO) activity in the cell-free hemolymph plasma as well as a dramatic decrease in the number of circulating hemocytes, reduced ability to phagocytose bacteria and fewer melanotic nodules in the infected tissues. The decline in overall immune function of older fifth-stage larvae is reflected by higher bacterial growth in the hemolymph and increased colonization of *Photorhabdus* on the basal surface of the insect gut. We suggest that developmentally programmed variation in immune competence may have important implications for studies of ecological immunity.

Keywords: *Manduca sexta*; *Photorhabdus luminescens*; Humoral response; Cellular response; Immunocompetence

1. Introduction

Innate immunity is an animal's first line of defence against infection. Recognition of pathogen-derived substances relies on pattern recognition receptors. In both mammals and insects, activation of the innate immune system through these receptors results in the induction of cellular and humoral responses (Gillespie et al., 1997; Kimbrell and Beutler, 2001; Mushegian and Medzhitov, 2001; Hoffmann and Reichhart, 2002; Lemaitre and Hoffmann, 2007). In insects, cellular responses result in phagocytosis, encapsulation of invaders, the production of reactive nitrogen and oxygen radicals, and the activation of proteolytic cascades leading to blood coagulation and wound healing (Lavine and Strand, 2002). Humoral responses are characterised by the transcriptional activation of a panoply of genes encoding antimicrobial peptides that attack microorganisms generally by interaction with the cellular membrane followed by displacement of lipids, alteration of membrane structure and in some cases entry of the peptide into target cells (Zasloff, 2002; Uvell and Engström, 2007).

The immune response to infection is not constant, however. Insect immunity may be temporarily enhanced after previous exposure to microorganisms, even those that are not pathogenic (e.g., Eleftherianos et al., 2006a). Further, the efficacy of immune responses may vary in a predictable way according to the insect's developmental stage and/or age. Age-related variation in immunity in the

*Corresponding author. Present address: CNRS-UPR9022, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg Cedex, France. Tel.: +33 388 417065; fax: +33 388 606922.
E-mail address: Ioannis.Eleftherianos@ibmc.u-strasbg.fr (I. Eleftherianos).

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adult stage has frequently been reported (DeVeale et al., 2004). A number of recent studies using *Drosophila* have revealed age-dependent tissue-specific increase in the expression of recognition proteins and the downstream antimicrobial effectors indicating a global activation of the immune response in old adult flies, which led to the conclusion that *Drosophila* innate immunity declines with age (Pletcher et al., 2002; Seroude et al., 2002; Landis et al., 2004; Zerofsky et al. 2005). In mosquitoes, it has also been shown that there is age-associated mortality in *Aedes aegypti* following immune challenge with *Escherichia coli*, which is correlated with a decrease in hemocyte numbers and a reduced ability to kill the bacteria (Hillyer et al., 2005).

Changes in immunity also occur during pre-adult development, however. Although numerous published examples show that the expression of immune-related genes changes both between and within larval stages, the consequences of this variation for immune efficacy has rarely been examined. For example, there is a significant increase in phenoloxidase activity in hemolymph plasma sampled from the wandering and pre-pupal larval stages compared to the sixth larval stage of *Spodoptera exigua* (Hung and Boucias, 1996). In *Manduca sexta*, measurements of lysozyme activity in the midgut of fifth-stage larvae revealed little or no activity in homogenates of the midgut epithilium during the first 5 days of the stadium. However, beginning on day 6, which coincides with the end of feeding and the onset of wandering, a progressive increase in gut lysozyme activity was observed (Russell and Dunn, 1996). The immunoglobulin family protein Hemolin is expressed in larval *M. sexta* at only a low level in uninfected feeding larvae, but mRNA levels in both fat body and gut increase markedly at the time of wandering in preparation for pupation, and Hemolin protein is subsequently found in hemolymph during all of pupal and adult life (Yu and Kanost, 1999). Similarly, Jiang et al. (2004) found that β-1,3-Glucan Recognition Protein-2 (BGRP-2) is not expressed in feeding fifth-stage larvae of *Manduca*, but is strongly upregulated at both mRNA and protein levels at the time of wandering.

Lepidoptera have recently received considerable attention as tractable experimental models of infection and immunity processes in insects (Silva et al., 2002; Kanost et al., 2004). While investigating the *Manduca* immune response against the insect pathogenic bacterium *Photorhabdus luminescens* (Eleftherianos et al., 2006a, b), we observed that there was appreciable increase in the susceptibility of fifth-stage larvae to this pathogen during the feeding stage. Older larvae were considerably more susceptible to a standard dose of the pathogen compared to younger fifth-stage larvae. In order to investigate this phenomenon further, we looked at several aspects of the humoral and cellular immune response of newly ecdysed (day 0 (D0)) and pre-wandering (day 5 (D5)) fifth-stage caterpillars. We found that increased susceptibility to *Photorhabdus* in older larvae was associated with reduced transcription of microbial pattern recognition protein and antibacterial effector genes, reduced phenoloxidase (PO) activity in the hemolymph plasma together with a decrease in total number of hemocytes, less phagocytic ability of hemocytes and reduced number of melanotic nodules in the infected tissues. This dramatic decrease in immune function of D5 fifth-stage larvae led to faster *Photorhabdus* colonization in *Manduca* tissues and rapid insect death. Therefore, we suggest that small age changes within the fifth larval stage of *Manduca* play a critical, yet underestimated, role in bacterial immune challenge.

2. Materials and methods

2.1. Insects and bacteria

Larvae of the tobacco hornworm, *M. sexta* (L.) (Lepidoptera: Sphingidae), were maintained individually as described (Reynolds et al., 1985).

The insect pathogenic bacterium *P. luminescens* subsp. laumondii strain TT01 was cultured as previously described (Eleftherianos et al., 2006a, b).

2.2. Survival experiments

For insect injection, 50 μL of a phosphate-buffered saline (PBS; 0.15 M sodium chloride, 10 mM sodium phosphate buffer, pH 7.4) suspension containing 1×10^3 washed *Photorhabdus* cells were injected directly into the hemocoel of *Manduca* larvae using a 100 μL disposable syringe with a 30-gauge needle. PBS-injected insects served as controls. Larvae were held at 28 °C and mortality was scored at intervals up to 48 h following the pathogen injection. Ten insects were used for each treatment and each bioassay was replicated three times.

2.3. RNA extraction and RT-PCR

At 18 h after injection insects were chilled on ice for 15 min, surface sterilised with 70% ethanol and then dissected or bled to collect the fat body (100 mg) or hemocytes (20 mg), respectively, as described by Eleftherianos et al. (2006a, b). To isolate total RNA, extracted tissues were homogenised in TRI reagent (Sigma, UK). Single step reverse transcription (RT)-PCR was performed with the 'OneStep' RT-PCR kit (Qiagen, UK). Each reaction was carried out in a 50 μL volume containing 0.6 μM of forward and reverse gene primers and 2 μg of RNA template (Eleftherianos et al., 2006a, b). All primers were gene-specific and their sequences have been given before (Eleftherianos et al., 2006a; 2007a). Amplifications were performed on a PTC-100 thermal controller (MJ Research, USA) under cycling conditions previously described (Eleftherianos et al., 2006b). RT-PCR control reactions for ribosomal protein S3 (*rpS3*) (Jiang et al., 1996) were performed as before (Eleftherianos et al., 2006b).