

Gene Expression Responses in Larvae of the Fleshfly *Sarcophaga bullata* after Immune Stimulation

(gene expression / immune stimulation / larvae / fleshfly / *Sarcophaga bullata* / sapecin / transferrin / bacteria / injury)

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Abstract. Insect larvae develop in decaying organic matter and their defence against various microorganisms must therefore be highly efficient. In the present study, we explored the transcriptional kinetics and induction levels of eight genes in *Sarcophaga bullata* larvae after infection or aseptic injury. Using real-time PCR, we studied the time-dependent immune response of larvae of the fleshfly *S. bullata*. We compared the mRNA levels of eight selected genes in induced and non-induced larvae. The third-instar larvae of *S. bullata* were induced by injecting a bacterial suspension of *Escherichia coli*, *Staphylococcus aureus* or *Pseudomonas aeruginosa*, or by simple aseptic injury with an entomological pin. We used intact larvae as a control for basal mRNA expression. Total RNA was isolated from the whole body, fat body and haemocytes. We determined the mRNA levels of genes encoding sapecin, transferrin, prophenoloxidase 1 and 2, storage-binding protein, cathepsin L, sarcocystatin, and 26/29 kDa protease. We found that there was massive up-regulation of genes encoding the fleshfly peptide sapecin, as well as

the protein transferrin. We also detected down-regulation of, or no change in, the expression of genes that encode prophenoloxidase 1 and 2, storage-binding protein, cathepsin L, sarcocystatin, and 26/29 kDa protease.

Introduction

Insects and microorganisms co-exist within the biosphere and interact in numerous ways. Since insect larvae often develop in decaying organic matter, they must have an efficient defence against various microorganisms. The first line of defence consists of physical barriers such as the outer exoskeleton and the chitinous linings of the midgut and trachea. At the molecular level, insects have developed an efficient host defence against microorganisms that involves both humoral and cellular mechanisms (Lemaitre and Hoffmann, 2007).

Rapid humoral response reactions are triggered by injury and microbial infection. The humoral response includes production of lectins and rapid *de novo* synthesis of antimicrobial peptides by the fat body, the functional homologue of the mammalian liver, as well as by haemocytes and surface epithelia (Vilmos and Kurucz, 1998; Iwanaga and Lee, 2005). Studies in *Drosophila* mutants have revealed that the Toll and Imd pathways control synthesis of specific antimicrobial peptides. The Toll pathway is activated by, and reacts to, G⁺ bacteria and fungi. The Imd pathway governs defence reactions against G⁻ bacteria (Hoffmann and Reichhart, 2002; Tzou et al., 2002; Hultmark, 2003; Leclerc and Reichhart, 2004). Blood coagulation is one of the most important insect defence reactions, where it prevents the animal from excessive bleeding when injured and aids in limiting the spread of microbes in the haemocoel (Johansson et al., 2005).

Cellular defence in insects is provided by blood cells, haemocytes. In *Drosophila*, there are three classes of haemocytes that have different, specialized functions: plasmatocytes, crystal cells, and lamellocytes. Plasmatocytes are responsible for the phagocytic removal of

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Abbreviations: CFU – colony-forming unit, PPAE – prophenoloxidase-activating enzyme, PPO – prophenoloxidase, RP-HPLC – reverse-phase high-performance liquid chromatography, RT-qPCR – real-time quantitative polymerase chain reaction, SBP – storage-binding protein.

dead cells and microbial pathogens. Lamellocytes have a role in the encapsulation and neutralization of objects that are too large to be phagocytosed. Crystal cells serve as storage cells for a large amount of prophenoloxidase (PPO), stored as a crystal in their cytoplasm, and also contain enzymes for PPO activation (Lemaitre and Hoffmann, 2007).

Recently, we published a study mapping the immune response in larvae of the fleshfly *S. bullata* (Ciencialova et al., 2008). We described the isolation and characterization of antimicrobial compounds from the larval haemolymph. The fleshfly larvae were injected with a bacterial suspension, and the haemolymph from injected and control larvae were compared. The RP-HPLC analysis showed induction of peptides with antimicrobial activities (sapecins). We also isolated *S. bullata* transferrin, which displayed significant antimicrobial activity against *S. aureus* and *E. coli*, but this protein was not induced in the larval haemolymph by microbial infection. This previous study (Ciencialova et al., 2008), however, only determined protein and peptide responses (e.g. sapecin, transferrin, and PPO) and did not analyse mRNA expression patterns after the immune challenge.

In the present study, we decided to explore the transcriptional kinetics and induction levels of eight genes in *S. bullata* larvae after infection or injury. We determined the mRNA levels of genes encoding sapecin, transferrin, and PPO 1 and 2 proteins. Simultaneously, we analysed RNA levels for storage-binding protein (SBP), cathepsin L, sarcocystatin, and 26/29 kDa protease, proteins for which cDNA sequences in *S. bullata* were available, in order to investigate the possible involvement of these proteins in the insect immune response.

Transferrin is a protein involved in the vertebrate and invertebrate immune response (Yoshiga et al., 1999), where it is supposed to inhibit bacterial growth by reducing free iron levels (Law, 2002; Gomme et al., 2005). Recently, we demonstrated (Ciencialova et al., 2008) that transferrin isolated from *S. bullata* haemolymph displayed a potent antimicrobial activity against *S. aureus* and *E. coli*.

Sapecins, or defensins, are well-studied insect antimicrobial peptides. Sapecin was the first identified defensin in *Sarcophaga peregrina*, and it was isolated from the embryonal cell line NIH-Sape-4 by Matsuyama and Natori (Matsuyama and Natori, 1988a).

The most immediate immune response in insects occurs within minutes and consists of the activation of prophenoloxidase activating enzyme (PPAE), which converts enzymatically inactive PPO into active phenoloxidase. PPAE also exists as an inactive zymogen that is activated by a serine protease (Royet, 2004; Leclerc et al., 2006; Lemaitre and Hoffmann, 2007). Phenoloxidase is an enzyme involved in melanin biosynthesis and is also important in cuticular sclerotization, wound healing and encapsulation of foreign material (Chase et al., 2000).

Storage-binding protein (SBP), also known as arylphorin-binding protein, is believed to participate in the

selective uptake of arylphorin from the haemolymph into the pupal fat body during metamorphosis. Arylphorin is a storage protein with many aromatic amino acids, and it is used as a source of amino acids and energy to construct adult structures during insect metamorphosis. SBP mRNA was found to increase several-fold during pupation (Chung et al., 1995).

Cathepsin L is a lysosomal cysteine protease that may be involved in the phagocytosis of invading microorganisms (De Gregorio et al., 2001; Kocks et al., 2003).

Saito et al. (Saito et al., 1992; Natori et al., 1999) found that the 26/29 kDa protease is secreted by haemocytes into the haemolymph when the larvae of *S. peregrina* are injected with foreign cells or after simple injury. They also predicted that new mRNA synthesis, and therefore probably new protein synthesis, is not needed for secretion of this cysteine protease, and that direct interaction between haemocytes and foreign substances probably triggers release of the stored protein into the haemolymph.

Sarcocystatin is a low-molecular-mass inhibitor of cysteine proteases and was identified in the haemolymph of *S. peregrina* larvae (Suzuki and Natori, 1985). The authors showed that sarcocystatin levels progressively increased in third-instar larvae and continued to increase after pupation. In haemocytes, sarcocystatin selectively inhibits activity of the cysteine proteases, which digest most of the larval tissues during metamorphosis. Thus, sarcocystatin is thought to protect developing adult tissues in pupae from attack by cysteine proteases (Saito et al., 1989). Sarcocystatin may also modulate the activity of cysteine proteases involved in the degradation of foreign proteins.

Since northern blot analysis and microarray experiments have mainly been used to monitor gene expression profiles in *Drosophila*, we used real-time quantitative PCR in this study for a more precise estimation of the comparative transcriptional rates of our selected genes.

Material and Methods

S. (Neobellieria) bullata Parker fleshflies were reared under laboratory conditions as previously described (Zdarek, 1980). We used third-instar larvae in their wandering period. Briefly, larvae of *S. bullata* were kept at 200–300 specimens per batch on beef liver in small, open, disposable packets made from aluminum foil at 25 ± 1 °C.

Induction of larvae

Before induction, larvae were immobilized by chilling on ice. A bacterial suspension in physiological saline was injected (1 µl with about 2×10^6 cells) into the abdomen of larvae during their third-instar wandering period using a calibrated glass capillary with a finely drawn tip. Larvae were also induced by simple injury with a sterile entomological pin. Neither the injection nor the mechanical injury had any apparent toxic or lethal effect on the larvae.

We used *E. coli* DBM 3001, *S. aureus* DBM 3002 and *P. aeruginosa* DBM 3081 bacteria to induce the larvae. Bacteria used for induction were cultivated in Luria-Bertani broth at 37 °C or 28 °C for 12 h to the mid-exponential phase of growth, with a final concentration of cells at 10⁹ cfu.ml⁻¹. After cultivation, the cells were centrifuged at 3000 g for 10 min and washed twice with 0.1 M phosphate buffer, pH 7.0. The final suspension of cells in physiological saline (0.9% NaCl) was diluted to an absorbance reading of 1.5–1.8 at 550 nm, and the final concentration of viable cells was determined from the number of cfu (about 2 x 10⁹ cfu.ml⁻¹).

Isolation of haemocytes from larval haemolymph

The haemolymph from induced and control larvae (100–150 µl of haemolymph from five larvae in each isolation tube) was collected on ice at 3, 6, and 22 h after induction. The haemocytes were separated from the haemolymph by centrifugation for 10 min at 1000 g at 4 °C. The haemocytes were washed twice using 50 mM Hepes buffer, pH 7.4, supplemented with 90 mM NaCl and then deep frozen.

Isolation of larval fat bodies

The fat bodies were isolated from induced and control larvae at 0.5, 6, and 22 h after induction. All larvae were anesthetized with CO₂. The anterior and posterior tips of the third-instar larvae were cut off with fine scissors. The fat body was excised from the larvae under a binocular microscope and placed into a physiological saline-containing Petri dish on ice. The Malpighian tubules and tracheas were carefully removed using tweezers.

Homogenization of whole larval bodies

The whole bodies of induced or control larvae were collected at 3, 6, 12, and 22 h after induction. At least two larvae for each time point and induction challenge were used. The larvae were deep-frozen using liquid nitrogen and then homogenized to a powder with a pre-cooled mortar and pestle.

RNA extraction

Total RNA from haemocytes, fat bodies or whole bodies of *S. bullata* fleshflies was extracted using 300 µl

(for haemocytes and fat bodies) or 1000 µl (for whole bodies) of TriReagent (Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's instructions.

RNA samples were treated with a DNA-free kit (Ambion, Inc., Austin, TX) to avoid genomic DNA contamination. The RNA concentration was determined by UV spectrophotometry at 260 nm, and the quality of the RNA samples was analysed by electrophoresis through 1.5% agarose gels stained with ethidium bromide. The RNA samples were stored at -80 °C.

cDNA preparation

cDNA was synthesized using 1 µg total RNA, 10 pmoles of oligo25-dT and 10 pmoles of random hexamers. The mixture was incubated at 72 °C for 10 min. One hundred U of MMLV reverse transcriptase (Promega Corporation, Madison, WI), 12 U of RNasin (Promega) and 5 nmol dNTPs were then added to a total volume of 10 µl, and incubation was continued at 37 °C for 70 min. The reactions were further diluted in water to 100 µl and frozen (-20 °C).

Real-time quantitative PCR (RT-qPCR)

Primers for *S. peregrina* sapecin, transferrin, cathepsin L, SBP, sarcocystatin, 26/29 kDa protease and *S. bullata* 18S ribosomal RNA, PPO1 and PPO2 were designed using Primer3 software. The nucleotide sequences of these primers are shown in Table 1. After optimization of qPCR reactions, the amplicon sizes for all genes were verified by electrophoresis of the products through 2% agarose gels. Efficiencies of all RT-qPCR assays were determined to be close to 100 % using standard curves.

The RT-qPCR mixtures, in a final volume of 25 µl, contained 2 µl cDNA, SYBRGreen solution (Life Technologies Corporation, Carlsbad, CA, diluted 200-fold with dimethyl sulphoxide and then diluted 20-fold with water), forward and reverse primers (0.4 mM solution of each), 0.3 mM dNTPs, 3 mM MgCl₂ and 1 U Taq polymerase (Promega). qPCR reactions were performed with an Opticon2 instrument (Bio-Rad, Hercules, CA) using the following cycling conditions: 95 °C for 3 min, 40 cycles at 95 °C for 15 s, 60 °C for 20 s and 72 °C for 30 s. A melting curve analysis was performed in order to

Table 1. Forward and reverse primers used for qPCR analysis

Gene	GenBank Accession Number	Forward primer	Reverse primer
Sapecin	J04053	TCGCTGTTACCTTGTGCTTG	TAAGGCATGCAAACCATCAA
Transferrin	D28940	AGCTCGCGATGGAAAACCTTA	GATGGGCATGCTTTGTAGGT
PPO1	AF161260	TTCATGGAAAAGGGCAAAC	CTTTTCGCCAAAACGTGATT
PPO2	AF161261	TCACTGGCACTGGCATTTAG	CCTTGCCAATATCTGGTGGT
Cathepsin L	D297741	GTACCCAAATCGGTGGATTG	GCGGAAATGTTGTCCCTCTA
SBP	D16533	CGCATTGCAGAACGTAGAAA	CTGTTGTCCCTGTTGGACCT
Sarcocystatin	J02847	GTCGGTTGTCCCAGTGAAGT	TGTGTGGTGGCTGAGTTGAT
26/29kDa protease	AB011375	GGCAAACCATCCCTTACAA	ATGGCCAATAGTGCCAAAAG
18S-rRNA	AF322419	CCTGCGGCTTAATTTGACTC	AACTAAGAACGGCCATGCAC

confirm the unique and specific qPCR product for each reaction. Controls containing no template gave no signal, or only primer-dimers appeared at Ct (cycle of threshold) values (> 35).

We were not able to find and validate *S. bullata* sequences for frequently used reference genes such as β -actin (Wang et al., 2006) or α -amylase (Thompson et al., 2003). Only the sequence for 18S rRNA was accessible, and therefore comparison of 18S rRNA levels between the induced and control samples was used as an internal control for the reliability of total RNA isolation, DNase treatment, cDNA synthesis and RT-qPCR quantification. Since 18S rRNA is highly abundant when compared to the studied genes, it was not used as a classical reference gene (Bustin and Nolan, 2004; Bonefeld et al., 2008). As expected, the level of 18S rRNA remained nearly constant after induction. Therefore, we concluded that normalization to 18S rRNA would not significantly change the results.

The Ct values of the induced and control groups were analysed using Excel software (Microsoft). The ratios for gene expression of induced to control larvae were calculated using the dCt method (ratio = $2^{(Ct(\text{induced})-Ct(\text{control}))}$).

Two biological duplicates were taken when collecting the whole larvae, haemocytes or fat bodies at all time points and treatment conditions. The means of two experimental values (columns) and standard error (error bars) of the representative biological duplicate samples (N = 2) are shown in Figures 1–3. The results of parallel biological duplicates agreed well and followed the same trend as the displayed results (Figs. 1–3).

Results

Isolation of RNA from larvae

Total RNA was isolated from the whole bodies, haemocytes and fat bodies of induced and control larvae. Agarose gel electrophoresis of the total RNA showed that there was one broad band containing the 18.7, 18.0 and 17.4S rRNAs, and one weaker band that contained the 5.8S rRNA (data not shown). The 18.7S and 17.4S fragments result from cleavage of the 26S rRNA (French et al., 1981).

Comparison of gene expression levels in induced and control larvae by qPCR

Whole body

Gene expression levels in the whole bodies of *S. bullata* larvae were compared between induced and control larvae. Figure 1 shows the expression of the eight genes that encode sapecin, transferrin, PPO1, PPO2, SBP, cathepsin L, sarcocystatin, and 26/29 kDa protease at 3, 6, 12 and 22 h after induction. The data are presented as ratios of gene expression between the induced and control larvae. Larvae were induced using four different stimuli, the G⁺ bacteria *S. aureus*, two different G⁻ bac-

teria, *E. coli* and *P. aeruginosa*, or injury with a sterile entomological pin.

According to their different responses to immune stimulation we can divide the genes studied into two groups. The transcription of sapecin and transferrin genes was rapidly increased after all four types of induction. The expression of transferrin mRNA gradually increased, reaching a maximum at 22 h after induction, and mRNA levels increased approximately 25–50-fold. Sapecin mRNA expression was increased 125–250-fold 12 h after induction.

In contrast, the expression of the genes encoding PPO1, PPO2, cathepsin L, SPB and sarcocystatin was suppressed or fluctuated around the normal level. The expression ratio of PPO1 and PPO2 transcripts decreased 2–5-fold in induced larvae compared to controls after stimulation. The expression of the genes that encode cathepsin L, SPB and sarcocystatin also decreased slightly. mRNA levels of the 26/29 kDa protease remained essentially unchanged.

The expression profiles for all genes studied showed no significant differences between G⁺ infection, G⁻ infection, or mechanical aseptic injury. Therefore, we used only induction with *E. coli* in further experiments.

Fat body

Larval fat bodies were isolated from larvae induced by injection with *E. coli*. RNA was isolated 0.5, 6 and 22 h after induction. Expression of the eight genes was measured in induced and control larvae.

The levels of sapecin and transferrin in the induced subjects were increased almost 80- and 35-fold, respectively, compared to the control larvae (Fig. 2A). PPO1, PPO2 and SBP expression decreased about 2.5-fold upon induction (Fig. 2B). The levels of sarcocystatin and cathepsin L mRNA decreased even more significantly (Fig. 2B). The mRNA levels of the 26/29 kDa protease remained unchanged (Fig. 2B).

Haemocytes

Time-dependent expression analysis in the haemocytes after induction with the *E. coli* suspension is shown in Fig. 3. The expression levels of the eight genes of interest were measured at 3, 6 and 22 h after induction. Sapecin and transferrin expression increased 13- and 11-fold, respectively, 6 h after induction. PPO1 expression decreased approximately 2-fold. The expression of the genes that encode PPO2, SBP, cathepsin L, sarcocystatin, and 26/29 kDa protease were not significantly affected by the induction.

Discussion

In this study, we investigated the transcriptional kinetics and induction levels of eight selected *S. bullata* larva genes after infection of the larvae with different bacteria (*E. coli*, *S. aureus* and *P. aeruginosa*) or after mechanical injury with a sterile entomological pin. We investigated the mRNA levels of the genes

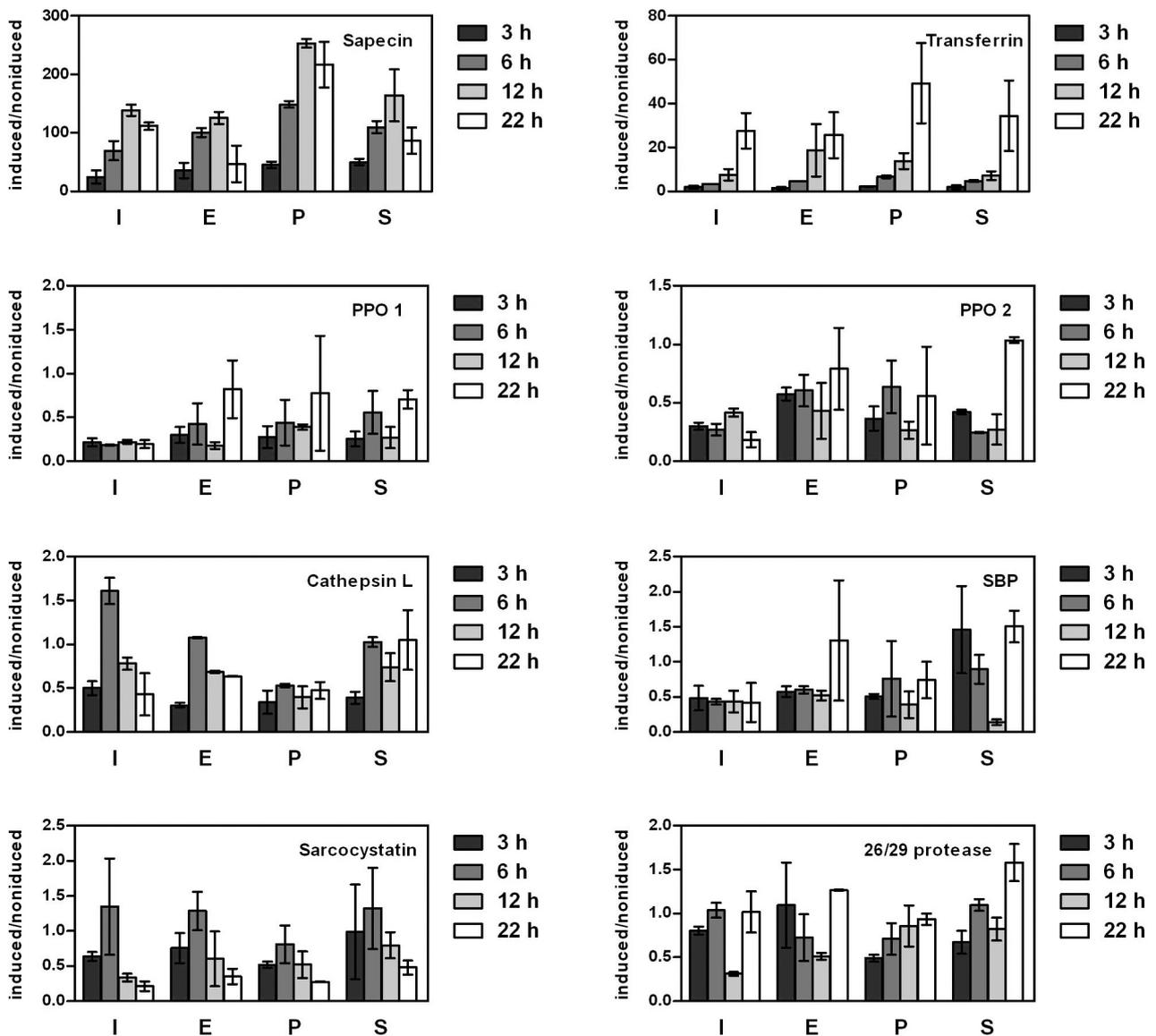


Fig. 1. Gene expression time-course from the whole body of *S. bullata* larvae after induction. The expression of genes for following proteins was studied: sapecin, transferrin, PPO1 and 2, SBP, cathepsin L (CatL), sarcocystatin (Sarc) and 26/29 kDa protease (26/29). The induction of larvae was done with aseptic injury (I), *E. coli* (E), *S. aureus* (S) and *P. aeruginosa* (P). The means of two experimental values (columns) and standard error (error bars) of the representative biological duplicate samples ($N = 2$) are shown. For details see Methods.

in the whole body, fat body and haemocytes of *S. bullata* larvae.

We observed no significant differences in the expression profiles of all studied genes between induction with G^- bacteria (*E. coli* and *P. aeruginosa*) and with G^+ bacteria (*S. aureus*) in the larval whole bodies. The sensitivity of the larvae or adult insect immune response to bacteria may certainly be influenced by the pathogenicity of the bacterial strains or species (Lazzaro et al., 2006). Irving et al. (2001) also found that a G^+ infection had an effect similar to a G^- infection in their study of adult *Drosophila* immune responses. In our experiments with the larval whole bodies, the expression of the analysed genes was also similarly affected by different bacteria and by mechanical aseptic injury of the larval body wall

without bacterial infection. Lemaitre et al. (1997) also observed a similar expression level for antimicrobial peptides (diptericin, attacin, cecropin A or drosocin) in adult *Drosophila* after induction with aseptic injury or with the G^+ bacteria *Micrococcus luteus*. We believe that the main reason for similar gene expression responses to immune challenges with different bacteria or with aseptic injury in our experiments is caused by the fact that we worked with *S. bullata* larvae and not with adult flies. In most studies, scientists have focused upon the systemic immune response in adult flies (e.g., *Drosophila*). However, during their development, flies pass through distinct larval stages and live in a highly septic environment, such as decaying organic matter. Adult flies are exposed to these kinds of environments to a

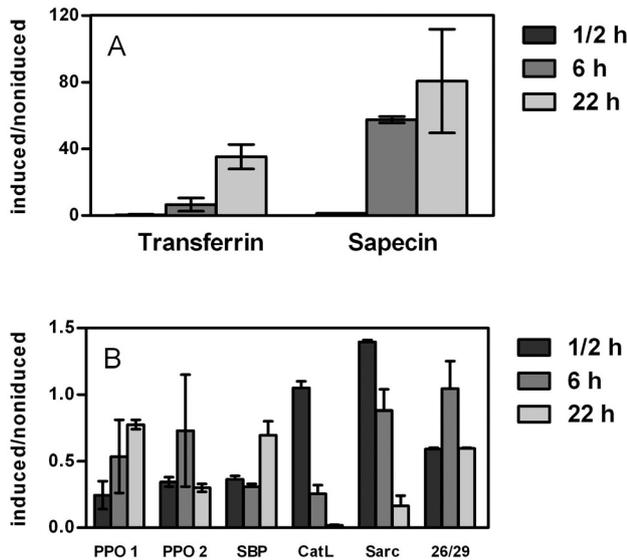


Fig. 2. Time-dependent analysis of gene expression in the fat body of *S. bullata* larvae after infection with *E. coli*. **A.** Expression of genes for sapecin and transferrin. **B.** Expression of genes for PPO1 and 2, SBP, cathepsin L (CatL), sarcocystatin (Sarc) and 26/29 kDa protease (26/29). The means of two experimental values (columns) and standard error (error bars) of the representative biological duplicate samples (N = 2) are shown. For details see Methods.

lesser extent. Although a systemic immune response also exists in larvae, there are features specific to this developmental stage that cannot be extended to adults. These include a fat body distinct from that of adults (Ferrandon et al., 2007), hormonal control of antimicrobial peptide gene expression (larvae must be accurately staged to avoid artefacts), and haemocytes that are more numerous in larvae than in adults. All these aspects of fly larvae (Ferrandon et al., 2007) might cause the high sensitivity of the larvae to simple aseptic injury or the same response to challenge with G⁺ or G⁻ bacteria.

In this study, we observed approximately a 50-fold increase of transferrin expression 22 h after bacterial infection or injury in the larval whole body, and a 35-fold increase in the fat body. We also detected up-regulation of transferrin in larval haemocytes, where transferrin expression was increased 11-fold. Our results are in agreement with findings of other groups working with *Drosophila* transferrin (the sequence identity with *S. peregrina* transferrin is about 63.5 %). Yoshiga et al. (1999) demonstrated that when adult *Drosophila* were inoculated with bacteria (*E. coli*), transferrin mRNA synthesis was markedly increased relative to controls both in sterile needle- or bacteria-challenged flies. The effect was monitored by northern blot analysis 6, 12 and 24 h after induction. Genome-wide analysis of the *Drosophila* immune response using oligonucleotide microarrays detected an 11-fold induction of transferrin after septic injury (De Gregorio et al., 2001). A quantitative PCR assay confirmed a significant increase in termite transferrin expression following infection. These results

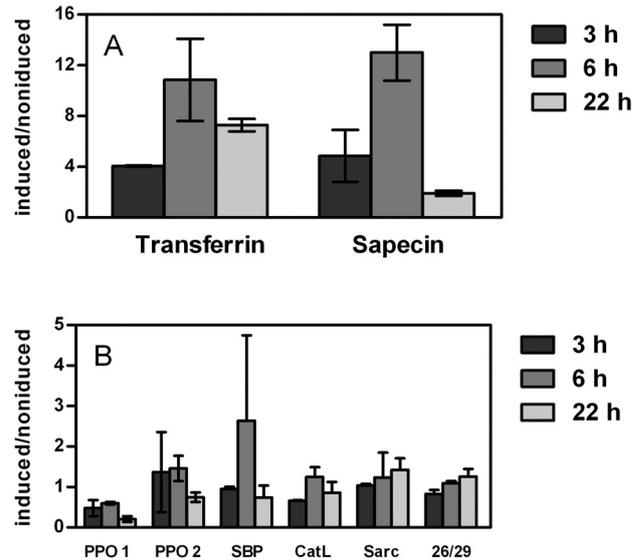


Fig. 3. Time-dependent analysis of gene expression in haemocytes of *S. bullata* larvae after infection with *E. coli*. **A.** Expression of genes for sapecin and transferrin. **B.** Expression of genes for PPO1 and 2, SBP, cathepsin L (CatL), sarcocystatin (Sarc) and 26/29 kDa protease (26/29). The means of two experimental values (columns) and standard error (error bars) of the representative biological duplicate samples (N = 2) are shown. For details see Methods.

indicated a 2.5–3.5-fold increase in termite transferrin expression levels 84 h after fungal infection (Thompson et al., 2003). Transferrin is abundant in insect haemolymph and is generally believed to be a product of the fat body (Jamroz et al., 1993; Ampasala et al., 2004). Our results suggest that transferrin is expressed more actively in other organs than in the fat body, because the increase in expression was higher for the whole body than for the fat body. In this study, we did not investigate other organs that may express transferrin more actively than the fat body. The larval gut and the Malpighian tubules are possible producers, and targets for future work. In our previous study (Cienicalova et al., 2008) we did not detect induction of the *S. bullata* transferrin protein in larval haemolymph (free of haemocytes) after the immune challenge. This result conflicts with our present observation of massive transferrin mRNA induction. A possible explanation for this discrepancy could be that while there may be increased levels of transferrin in the fat body or haemocytes, they are not increased in the haemolymph.

Among the eight genes studied, in the whole body, in the fat body and in haemocytes we observed the highest induction for sapecin (a member of the defensin family). We detected the highest sapecin expression in the whole body of *S. bullata* larvae. In our previous study (Cienicalova et al., 2008) we detected significant induction of the sapecin protein in the larval haemolymph and we found that isolated sapecins were active against different bacteria. Our present results indicate that sapecin may also be expressed in other larval tissues apart from

the fat body and haemocytes. The defensins in adult *Drosophila* are synthesized predominantly in the fat body and are secreted into the haemolymph (Leclerc and Reichhart, 2004). In adult *Drosophila*, antimicrobial peptides are also expressed in several epithelia that are potentially in contact with the environment. These include the respiratory tract, oral region, digestive tract, Malpighian tubules, and the male and female reproductive tracts. Expression of *Drosophila* defensin can also be induced in the oral region of larvae (Tzou et al., 2000). Matsuyama and Natori (1988b) investigated expression of the gene for the sapecin precursor protein by northern blot analysis 6 h after damage of the *S. peregrina* larval body wall. Their results showed that haemocytes, together with the fat body, respond to the injury, and that haemocytes synthesize sapecin and secrete it into the haemolymph. In the whole body we detected the highest expression of the sapecin gene after 12 h, but in haemocytes the highest expression level was after 6 h, and in the fat body the highest expression was only detected after 22 h. These differences may reflect different activation mechanisms for sapecin expression in various tissues. It was shown, by northern blot analysis and RT-PCR, that the *Drosophila* defensin gene can be induced by various microorganisms, as well as by simple injury (Dimarcq et al., 1994; Lemaitre et al., 1997). A time course of defensin transcription over 0–48 h after inoculation with two different bacteria in the housefly *Musca domestica* showed that the induction pattern of the defensin gene in housefly was different than that observed in *Drosophila* (Wang et al., 2006). Defensin transcription reached its highest level at 3 h in *Drosophila* but at 36–48 h in *Musca domestica*. Our present results indicate that the timing and localization of sapecin expression may be different in different organisms.

In our previous study (Ciencialova et al., 2008) we detected very high levels of the PPO protein in both immune challenged and control *S. bullata* larvae haemolymph, but it was not clear whether PPO was induced or not. We have also shown that isolated PPO does not display any antibacterial activity. In this study, the expression of genes encoding PPO 1 and 2 in *S. bullata* larvae was slightly decreased or unchanged in the whole body, and also in the fat body and haemocytes. Steady levels of PPO mRNA have been observed in the *Drosophila melanogaster* haemocyte-like cell line mbn-2 after induction with crude lipopolysaccharides or *E. coli* (Johansson et al., 2005). In agreement with our present finding, steady mRNA levels for the three PPO genes in *Drosophila* have been seen in microarray analysis of immunized adult *Drosophila* (De Gregorio et al., 2001). Our results suggest that novel PPO1 and PPO2 expression is not necessary for an acute immune response.

In our study, we found that third-instar larvae display a slight decrease in the expression of SBP after immune challenge. It is possible that an efficient response to infection, which involves intense synthesis of transcripts from a high number of genes, requires repression of dispensable metabolic pathways.

In our experiments, the level of cathepsin L expression decreased in the whole body, and this effect was even more pronounced in the fat body. This is in contrast to the results of De Gregorio et al. (2001), who observed up-regulation of the cathepsin L gene in adult *Drosophila* challenged by septic injury. Liu et al. (2006) reported higher cathepsin L gene expression levels in the larvae of the lepidopteran *Helicoverpa armigera* during moulting. Philip et al. (2007) recently characterized a cathepsin L-like protease in *S. peregrina* that is involved in differentiation of imaginal discs. It is probable that cathepsin L-like proteases have a distinct role in the development of fly larvae and its gene expression may be suppressed during bacterial infection or injury.

In our experiments, the mRNA level of the 26/29 kDa protease remained nearly unchanged in haemocytes, fat body and in the whole larval body after the immune challenge. Our real-time PCR data seem to support the findings of Saito et al. (1992).

In our study, the expression of the gene that encodes sarcocystatin decreased in the whole body and in the fat body, but it was not significantly affected by induction in haemocytes. It cannot be excluded that the expression of cysteine protease inhibitors (e.g. sarcocystatin) is closely related to the expression of targeted proteases and that these genes follow the same trend upon bacterial infection.

In conclusion, we found that there was massive up-regulation of genes encoding the fleshfly peptide sapecin and the protein transferrin in the whole larval body, as well as in the fat body and haemocytes. We also detected either down-regulation or no change in the expression of the genes that encode PPO 1 and 2, SBP, cathepsin L, sarcocystatin, and 26/29 kDa protease. We hope that our study will help to shed more light on the complex processes of immune responses in *S. bullata* larvae.

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