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RESEARCH ARTICLE

Transcriptomic response of *Hermetia illucens* L. (Diptera: Stratiomyidae) to wounding and Gram-negative bacterial infection

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Abstract

The larvae of the black soldier fly (BSFL), *Hermetia illucens* L. (Diptera: Stratiomyidae), are of economic interest due to their use as livestock feed component. Unraveling their response to a bacterial infection will allow us to gain a better insight into their biology. In the current study, we used RNA-Seq analysis to unravel the transcriptomic response of BSFL to wounding and infection by a Gram-negative bacterium, *Pseudomonas protegens* Pf-5. Five-day-old BSFL were subjected to three treatments, i.e. untreated, PBS-injection, and bacteria-injection (5000 CFU per larva) and samples were collected at three time points (2 h, 6 h, and 13 h) post- treatment. Wounding induced expression of genes encoding recognition molecules and signaling pathway genes such as *PGRP-SA*, and *Relish*, and antimicrobial peptides (AMPs) such *cecropin*, *defensin*, and *attacin*. At 2 h, wounding resulted in a significant upregulation of immunity-related genes whereas genes encoding for resilin and cuticle proteins were significantly downregulated. At 6 h, the expression of immunity-related genes reduced in response to wounding whereas their expression increased in infected larvae. At 13 h, the expression of immunity-related genes reduced drastically in response to wounding, while their expression increased significantly in infected larvae. Conversely, the expression of metabolism-related genes, such as *trypsin* and *chymotrypsin*, was significantly upregulated in wounded larvae at 13 h, while their expression was significantly reduced in infected larvae. Increased investments in immunity-related processes in infected BSF larvae correlated with the downregulation of genes associated with metabolic processes indicative of a trade-off. Various immunity-related genes, including those encoding *cecropin*, *defensin*, *attacin*, and PGRP-SA, were consistently induced only during pathogen infection, indicating their role in immunity against Gram-negative bacteria. In this study, we report multiple genes that are significantly upregulated post-bacterial infection in BSFL that may be utilized as biomarkers to monitor insect health in mass production facilities.

Keywords

antimicrobial peptide – BSF – development – Gram-negative bacterium – immune response – infection – wounding

1 Introduction

Insects are generally exposed to physical injuries and infections by microbial pathogens in their natural environment. The insect hosts employ multiple defense mechanisms such as encapsulation, phagocytosis, nodulation, melanization, and the production of antimicrobial peptides (AMPs) to deal with wounding or pathogens (Lemaitre and Hoffmann, 2007). Wounding elicits a hemocyte clotting response and activation

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of the wound-repair pathway to maintain homeostasis and limit the spread of pathogens that may have gained entry into the insect hemocoel (Galko and Krasnow, 2004; Haine *et al.*, 2007). Insects possess specific pathways, such as the JNK pathway, which aids wound healing through epidermal spreading and re-epithelialization by activating epidermal cells around the wound (Galko and Krasnow, 2004). The entry of an entomopathogen in an insect's hemocoel triggers a cascade of host-specific immunological responses, initiated by recognition of bacterial peptidoglycans through molecules such as peptidoglycan receptor proteins (PGRPs), Gram-negative bacteria-binding protein (GNBPs), and beta-1,3-glucan-binding proteins (GBPs) that eventually lead to the biosynthesis of effector molecules such as antimicrobial peptides (AMPs) (Hultmark *et al.*, 1980; Wang *et al.*, 2019b). AMPs were initially isolated from the hemolymph of the giant silk moth, *Hyalophora cecropia*, immunized with *Enterobacter cloacae* strain β12 as antibacterial peptide cecropin (Hultmark *et al.*, 1980). Since then, a broad and diverse range of AMPs acting against multiple bacteria, fungi, parasites, and viruses have been identified (Vogel *et al.*, 2022).

The larvae of the black soldier fly (BSFL), *Hermetia illucens* L., are increasingly utilized as livestock feed ingredients due to their rich nutritional profile and their ability to process low-quality organic streams (Broeckx *et al.*, 2021; Van Huis, 2020; Veldkamp *et al.*, 2021). In mass-rearing operations, BSFL may be fed on various organic streams derived from agriculture or supermarket leftovers (Isibika *et al.*, 2019; Lalander *et al.*, 2013; Lalander *et al.*, 2019). The organic streams harbor diverse microorganisms, some of which could be opportunistic pathogens of BSFL. Although no pathogens that cause mortality in BSF are known (Joosten *et al.*, 2020), the recent study by She *et al.* (2023) reported that vegetative cells of *Paenibacillus thiaminolyticus* GX6 resulted in approx. 30% mortality in BSFL larvae in mass-rearing conditions. However, the mortality of BSFL to *P. thiaminolyticus* GX6 cells depends on temperature and substrate moisture, with insect mortality increasing at high temperature and low substrate moisture. While infection may increase BSFL mortality, sub-lethal infections could negatively affect the development and welfare of insect larvae. The use of entomopathogenic microorganisms on crops as biological control agents, such as *Pseudomonas protegens* Pf-5 (Garrido-Sanz *et al.*, 2023), could make their way into the diet of BSF larvae and result in adverse effects on larval health. The bacterium, *P. protegens* Pf-5, produces multiple insecticidal toxins (Flury *et al.*, 2017; Loper *et al.*, 2016) that are capable of causing oral toxicity in insects such as *D. melanogaster*, *Musca domestica*, and *Galleria mellonella* (Loper *et al.*, 2016; Ruiu *et al.*, 2022; Ruiu and Mura, 2021).

Despite the commercial importance of BSFL, only a few studies have explored various facets of its immune response to wounding and experimental infections so far (Bruno *et al.*, 2021; Vogel *et al.*, 2018; Von Bredow *et al.*, 2022; Zdybicka-Barabas *et al.*, 2017). The effects of wounding on hemocyte count (Von Bredow *et al.*, 2022), phenoloxidase activity (Bruno *et al.*, 2021; Zdybicka-Barabas *et al.*, 2017), and effect of infection with an *Escherichia coli* – *Micrococcus luteus* mixture on the expression of selected AMP genes through qPCR analysis have been identified (Bruno *et al.*, 2021). Despite the commercial importance of BSFL, only two studies have so far has looked at the overall transcriptomic response of BSFL to bacterial infection (Cho and Cho, 2024; Vogel *et al.*, 2018). Vogel *et al.* (2018) observed a significant induction of AMP genes in BSFL, 72 h after oral exposure of fifth-instar larvae to a bacterial mix of Gram-positive and Gram-negative bacteria. Similarly, Cho and Cho (2024) observed a significant upregulation of AMP genes 12 h post-injection with the Gram-negative bacterium *Escherichia coli*. Both studies assessed the transcriptomic response at a single time point. To gain insight into the temporal dynamics and phases of the immune response, it is imperative to determine the transcriptional response pattern at different time points and elucidate the underlying mechanisms in BSFL generated as a response to wounding and infection.

In this study, we investigated the transcriptomic response of five-day-old BSFL to wounding and infection with the Gram-negative bacterium *P. protegens* Pf-5. The BSF larvae were subjected to three treatments: Untreated, PBS injection, and bacteria injection, to investigate the effects of wounding (comparing PBS-injected with untreated larvae) and infection (comparing bacteria-injected with PBS-injected larvae). Differentially expressed genes, pathways, and biological processes affected by wounding and bacterial infection in BSFL have been identified and are discussed.

2 Materials and methods

Insect rearing

BSF larvae used in the experiments were grown from eggs derived from an adult *H. illucens* colony maintained at the Laboratory of Entomology, Wageningen

University (the Netherlands) in a climate room (27 ± 1 °C, 70 ± 10% R.H., L12:D12). The genetic background of this strain has been described by Khamis *et al.* (2020). Eggs laid within six hours in clean corrugated cardboard sheets were collected from a cage harbouring adult flies of different ages. Two egg clutches (~1,500 eggs) were selected at random and were provided with a chickenfeed diet (150 g, Kuikenopfokmeel 1; Kasper Faunafood, Woerden, the Netherlands), and 300 ml water in a circular plastic container (15.5 × 10.5 × 6 cm). The chickenfeed composition was evaluated at Mérieux NutriSciences (Ede, the Netherlands), and contained 21% protein, 40% carbohydrates, 4.4% fats, 6.6% ash, 12.2% water, 4.4% crude fibre and 12.1% other dry matter (minerals and lignin). The container was closed with a plastic lid, had a rectangular ventilation hole (7 × 5.5 cm), and was covered with nylon of 1 mm mesh size.

Larval infection and sample collection

In previous experiments (Shah *et al.*, 2024a), we experimentally determined that infection with a dose of ~5,000 CFU of *P. protegens* Pf-5 per BSF larva resulted in 100% mortality, for which LT_{100} (time to 100% mortality) was 16-18 h at 27 °C. Based on qPCR experiments in the same study, the expression of five immunity-related genes was determined at five time points (i.e. 2 h, 6 h, 10 h, 16 h, and 21 h), sample collection was pre-determined at 2 h, 6 h, and 13 h post-infection to assess the effect of early, mid, and late phases of the transcriptomic response of BSFL to wounding and bacterial infection, respectively.

Sterile PBS buffer was prepared by dissolving 1× Oxoid™ Phosphate Buffered Saline Tablets (Thermo Scientific, Waltham, MA, USA) in 100 mL distilled water and autoclaved at 121 °C for 15 min. For the preparation of the bacterial suspension, colonies of *P. protegens* Pf-5 stored at −80 °C were transferred to Petri dishes containing King Agar B medium (Merck Millipore, Burlington, MA, USA) and stored inverted inside a climate cabinet maintained at 27 °C for 48 h. An isolated single CFU was transferred from the Petri dish to sterile LB broth and placed overnight in a rotary incubator at 24 °C and 180 rpm. The bacterial culture was diluted (OD600 = 0.25 corresponding to 5×10^8 CFU/ml) in sterile PBS buffer to generate a 5,000 CFU/µL concentration.

Five-day-old BSFL were surface-sterilized in 70% ethanol for 5 s followed by two consecutive rinses in sterile water for 5 s each time. Surface-sterilized larvae were randomly sampled and subjected to one of the following treatments: (a) untreated, (b) injection with 1 µL sterile PBS buffer, and (c) injection with 1 µL of *P. protegens* Pf-5 suspension, ~5,000 bacterial cells per larva. Treatments a, b, and c will be referred to as 'Untreated', 'PBS', and 'Bacteria', respectively. Forty larvae, provided with chickenfeed (50 g + 100 mL water), were housed per container (volume 550 mL, height 11 cm, top-width 10 cm, bottom width 8.5 cm; the lid (9.5 cm × 5 mm) had a circular vent of 5 cm diameter covered with nylon of 1 mm mesh size) for each treatment. At each time point, five biological replicates (of two pooled larvae each) were collected for each treatment across three sampling time points (2 h, 6 h, and 13 h).

Sample preparation for RNA-Seq

Total RNA was extracted from the samples as described in Shah *et al.* (2023). 200 µL TRI-Reagent® (Sigma-Aldrich, St. Louis, MO, USA) and two 5-mm glass beads were added per 1.5 mL Eppendorf Safe-Lock tube and snap-frozen in liquid nitrogen for each biological replicate. Each sample was homogenized using a bead-mill homogenizer (TissueLyser II, Qiagen, Venlo, the Netherlands) at 30 oscillations/s for 5 min. After that, all samples were treated with TURBO™ DNase (ThermoFisher Scientific) per the manufacturer's instructions to remove any DNA contamination. Samples were additionally treated with phenol: chloroform: isoamyl alcohol (25:24:1; Sigma-Aldrich) solution to remove protein impurities. Purified total RNA was re-suspended in sterile RNase-free water (Qiagen) and stored at −80 °C until further use.

RNA-sequencing and read processing

The quality and quantity of total RNA were assessed using a DS-11 series spectrophotometer (DeNovix, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent, CA, USA). RNA samples were used to prepare cDNA libraries using TruSeq Stranded Total RNA Library Preparation (Illumina 1.9) at BaseClear B.V. (Leiden, the Netherlands). The cDNA libraries were sequenced on a NovaSeq-6000 instrument with paired-end 150 nt sequencing protocol to an average depth of 20 million reads per sample. FASTQ read sequence files were generated using bcl2fastq2 version 2.20 (Illumina 1.9), which included Illumina Chastity quality filtering with default settings. Subsequently, reads containing (partial) adapters were clipped up to a minimum read length of 50 bp. PhiX control signals were removed using an in-house filtering protocol at BaseClear B.V. (Leiden, the Netherlands). An additional quality assessment was performed on the remaining reads using the FASTQC quality control tool version 0.11.8, and reads with a Q-score of >35 were retained.

The quality of reads was assessed using fastQC (Andrews, 2010) and multiQC (Ewels *et al.*, 2016). A genome index (with overhang = 99) was created with the reference genome and gene annotation file (gtf) of *H. illucens* L. (Generalovic *et al.*, 2021) from the NCBI database using STAR (Dobin *et al.*, 2013). The filtered sequences were aligned to the genome index in the quantMode function to obtain read counts per gene directly as output. On average, 78% of the reads (with average GC% = 39%) mapped uniquely to the reference genome (Supplementary Table S1). The raw sequences and processed data have been deposited in NCBI's Gene Expression Omnibus (GEO) public repository and are available under accession code GSE263745. Additionally, the study can be accessed with NCBI's Bioproject (PRJNA1099151) using the following URL: https://www.ncbi.nlm.nih.gov/bioproject/1099151.

Principal component analysis and DESeq2 analysis

The gene counts per sample were transformed to a $\log_2$ scale via restricted logarithmic transformed counts (rLog) using the DESeq2 package in R (Love *et al.*, 2014). rLog counts data was used as input to generate a principal component analysis (PCA) plot using the ggplot2 package in R (Wickham, 2016). Genes in the top and bottom loadings for PC1 and PC2 were extracted using the pcaexplorer package (Marini and Binder, 2019) in RStudio (Posit team, 2024).

Genes with low counts (≤20) were filtered out across all samples before DESeq2 analysis to avoid false positives, resulting in 11,900 genes. The differences in sample library sizes were normalized using the estimateSizeFactors function. A DESeq model with design = Treatment x time was used to determine relevant temporal differences between and within treatments (i.e. time points between Untreated, PBS, and Bacteria treatments).

Differentially expressed genes (DEGs)

Comparisons of gene expression between (1) PBS versus Untreated, and (2) Bacteria versus PBS were made for the three-time points using the result function of the DESeq2 package to identify differentially expressed genes (DEGs) and evaluate the effect of wounding and infection on BSFL, respectively. Genes were identified as DEGs when their false discovery rate or Benjamini-Hochberg adjusted *P*-value was lower than 0.001, and their $\log_2$-fold change was less than −2 (downregulated) or greater than 2 (upregulated). The number of DEGs derived from these comparisons was plotted on a stacked bar plot to display the number of up-and-down-regulated genes resulting from wounding or infection.

DEG cluster analysis and gene ontology (GO) enrichment analysis

DEG cluster analysis

DEGs were divided into distinct clusters via k-means clustering (Pearson correlation matrix) based on their expression over treatment and time. The scaled gene count (row z-score) of these DEGs was plotted on the expression heatmap using the pheatmap package (Kolde, 2019) in R to determine temporal patterns of DEG expression between treatments.

Gene ontology (GO) enrichment analysis

A list containing associated GO-terms for each gene of the reference *H. illucens* genome was accessed from Ensembl Metazoa (https://metazoa.ensembl.org/biomart/martview/). After that, a custom annotation file was generated to assign associated GO terms to corresponding genes using Python3 in PyCharm. Each DEG cluster was analyzed using Fisher's exact test for ontologies corresponding to biological processes (BP), cellular components (CC), and molecular functions (MF). The gene ontologies for each DEG gene cluster were determined using the topGO package (Alexa and Rahnenfuhrer, 2023) to determine associated processes affected by wounding or infection. In addition, upregulated and downregulated DEGs in response to wounding and infection were separately subjected to GO enrichment analysis to determine the physiological processes affected by wounding and infection, respectively. nodeSize of 2 and 5 were used to prune GO hierarchy from terms with less than 2 and 5 annotated genes for cluster analysis and treatment, respectively. The top 25 GO terms (topNodes = 25) contributing to BP, CC, and MF for each gene cluster were summarized. A process was deemed to be significantly affected (either upregulated or downregulated) at $P < 0.05$ (Fisher's exact test), and the processes involved by wounding or infection were visualized in bubble plots using the ggplot2 package. The enrichment factor (ratio of DEGs attributed to a specific process compared to the total number of genes attributed to that process) was used to visualize the most affected processes.

Expression heatmaps of the immunity-related pathways

The $\log_2$-fold change in DEGs associated with specific immunological pathways or pathogen-recognition molecules, metabolic processes, and antimicrobial peptides for wounding and infection were plotted using ggplot2. Their homologs were identified in *H. illucens* using BLAST search against the RefSeq genome of *H. illucens* (GenBank assembly accession: GCA_905115235.1). An expression heatmap indicating the scaled count of all genes belonging to these pathways has been summarized in the Supplementary Materials. Additionally, an expression heatmap containing scaled counts of Imd and Toll pathway genes was plotted to determine their temporal expression.

3 Results

A total of 45 high-quality cDNA libraries were obtained with an average read depth of 19.2 million reads (average GC% = 38.5; Supplementary Table S1). Of 15,712 genes in the reference genome, we observed 15,196 genes in our dataset. Genes with counts ≤20 were filtered out to prevent false positive results during downstream analysis, resulting in 11,900 genes. The gene counts were normalized to the respective library size before further analysis.

Sample clustering indicates time- and treatment-specific effects of wounding and infection

Principal component analysis (PCA) of the top 500 genes with the highest row variance reveals treatment- and time-specific sample clustering (Figure 1). PC1 and PC2 explain 75% and 11% of the variation in gene expression between samples, respectively. At 2 h, samples from larvae subjected to PBS- or bacteria injection clustered closely, indicating that both treatments generated a similar response in BSF larvae early on (Figure 1). However, samples from the three treatments clustered separately at 6 h, indicating a divergence in transcriptomic response to bacteria- and PBS-treatment. At the last sampling time (13 h), samples from untreated and PBS-treated larvae clustered together, whereas those from bacteria-injected larvae clustered separately (Figure 1).

Genes contributing to sample variation along PC1 and PC2 are involved in immunity and developmental processes in multiple insect species, such as *cecropin*, *cuticle protein*, and *seminal metalloprotease 1-like*. The top loadings of the PC2 axis (Figure 1), i.e. genes contributing most to sample distribution along the PC2 axis, are interesting because this axis separates the three treatments. Of the top 15 genes contributing to positive sample loadings along the PC2 axis, thirteen were cecropin-encoding genes (Supplementary Figure S1), suggesting their potential importance as an effector molecule against bacterial infection with *P. protegens* Pf-5.

BSFL transcriptomic response to wounding and infection

Wounding and infection elicited significant changes in gene transcription in BSF larvae, including upregulation and downregulation of genes (Figure 2). The list of differentially expressed genes generated from wounding and infection along with their log2 fold expression at each time point is presented in Supplementary Tables S2 and S3, respectively. The number of upregulated DEGs generated by wounding decreased over time, while infection increased the number of upregulated DEGs over time (Figure 2). The expression of DEGs from both wounding and infection was affected by time and treatment, separating the DEGs into 10 distinct gene clusters (Figure 3).

Response to wounding

Wounding resulted in the upregulation of 206 genes. After 2 h, 113 genes were upregulated in response to wounding, of which 51 genes were still upregulated at 6 h (Figure 2). The number of upregulated genes post-wounding was reduced considerably at 13 h, as only 59 genes were significantly upregulated, of which 24 genes were already upregulated at the previous time point, i.e. at 6 h. A total of 19 genes (including attacin-A, lysozyme, two genes encoding for beta-1,3-glucan-binding protein, peptidoglycan-recognition protein SA, two defensin genes, cecropin (LOC119656619) and multiple uncharacterized genes; Figure 2) were consistently upregulated across all time points in response to wounding. Genes such as *Relish*, *cecropin*, *defensin*, and *attacin* were significantly upregulated at 2 h post-wounding, but their expression level reduced over time, and they were no longer differentially expressed at 13 h post-wounding.

Wounding resulted in significant downregulation of 21 and 5 genes at 2 h and 6 h, respectively (Figure 2). Downregulated genes in response to wounding included *cytochrome P450* and *pancreatic triacylglycerol lipase*, which are involved in metabolic processes (Figure 4B). No genes were downregulated in response to wounding at 13 h post-injection.

Response to infection

Infection resulted in significant upregulation and downregulation of 1,014 and 884 genes, respectively, in BSF larvae (Figure 2). Only 6 genes were upregulated at 2 h in infected larvae, while no gene was downregulated at 2 h in the infected larvae (Figure 2). At 6 h, the number

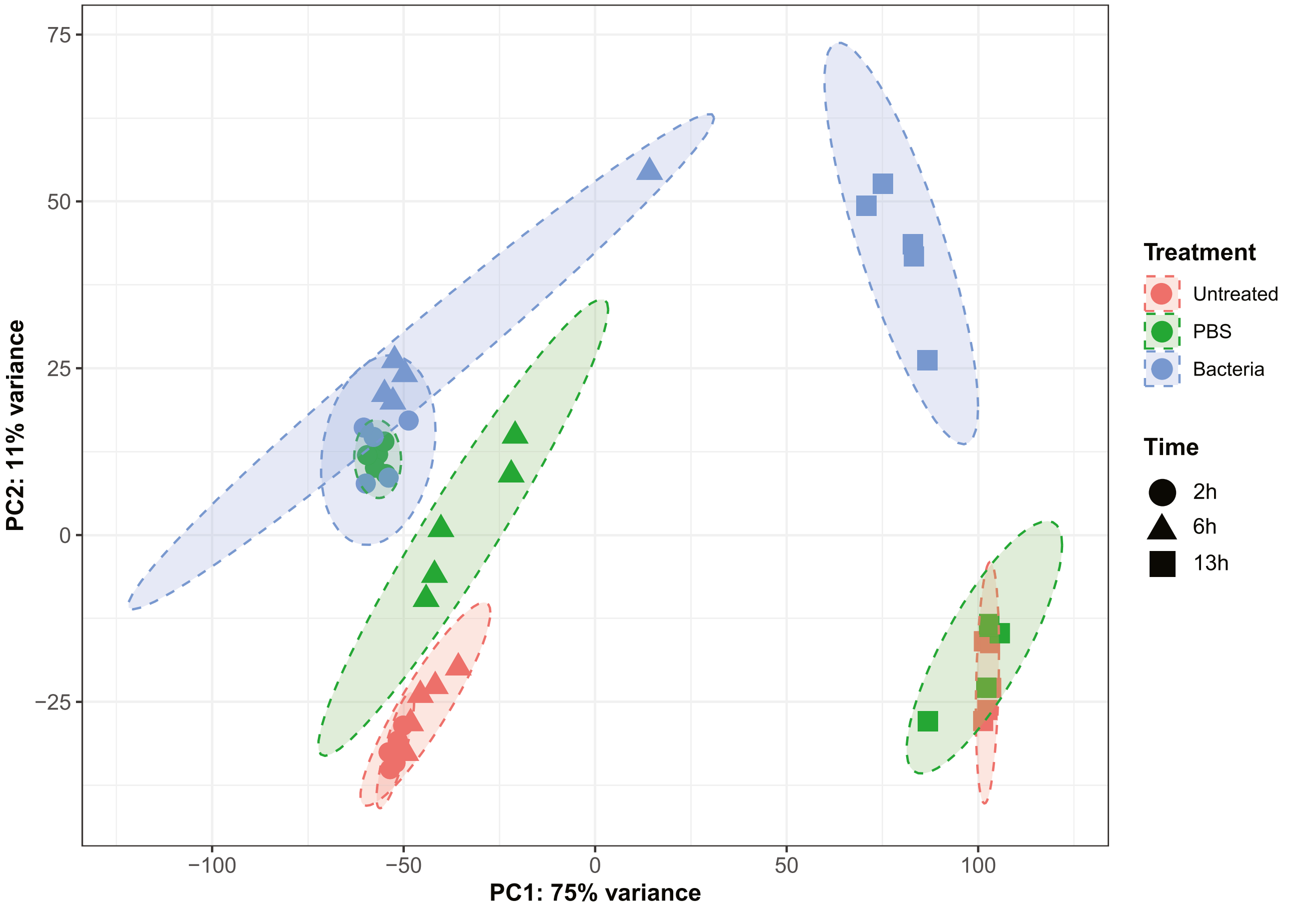

FIGURE 1 Principal Component Analysis (PCA) of the top 500 genes with the highest row variance across all treatment x time combinations. Five biological replicates (of two pooled larvae each) are represented for each treatment at a particular time. The elliptical line around samples for respective treatment x time combination clusters represents the 95% confidence level.

of upregulated and downregulated genes in response to infection was 347 and 341, respectively. The upregulated DEGs in infected larvae at 6 h included genes encoding for Relish, multiple PGRPs, and AMPs, including numerous copies of *attacin*, *cecropin*, and *defensin* genes (Figure 4A-C). *Relish* was upregulated across all time points in infected larvae. Genes related to metabolism, such as *lipase* and 29 cytochrome-encoding genes, were downregulated in infected BSF larvae at 6 h.

The number of upregulated DEGs in infected BSF larvae increased to 818 genes at 13 h. Of these 818 genes, 156 were upregulated at both 6 h and 13 h, indicating their consistent role in response to pathogen infection. From the 156 consistently upregulated genes, several genes are associated with immunity-related functions such as cecropin (23 genes), defensin (5 genes), heat shock 70 (2 genes), melanization protease 1, and serine protease (3 genes), while 38 other genes are characterized for having potential immunological functions (Figure 2).

Infection resulted in a significant downregulation of 341 and 678 genes at 6 h and 13 h, respectively (Figure 2). Genes associated with metabolic- and developmental processes were downregulated in infected BSFL at 6 h and continued to stay downregulated at 13 h: this includes general odorant-binding protein 99a/b (14 genes), cytochrome P450 family (13 genes), glycine N-methyltransferase (2 genes), and 87 uncharacterized genes (Figure 2). Thirty-two out of 137 genes downregulated in infected larvae at both 6 h and 13 h are uncharacterized.

DEG clusters

To gain insight into the temporal pattern of DEGs in the response to wounding and infection, DEGs were grouped (using hierarchical clustering, with complete linkage) into 10 different clusters (Supplementary Figure S2) based on expression patterns over time in response to treatments (Figure 3). Clusters 1, 3, 9, and 10 were composed primarily of genes upregulated by bacterial infection, while DEGs in clusters 2, 4, and 8 were significantly downregulated in response to infection (Figure 3).

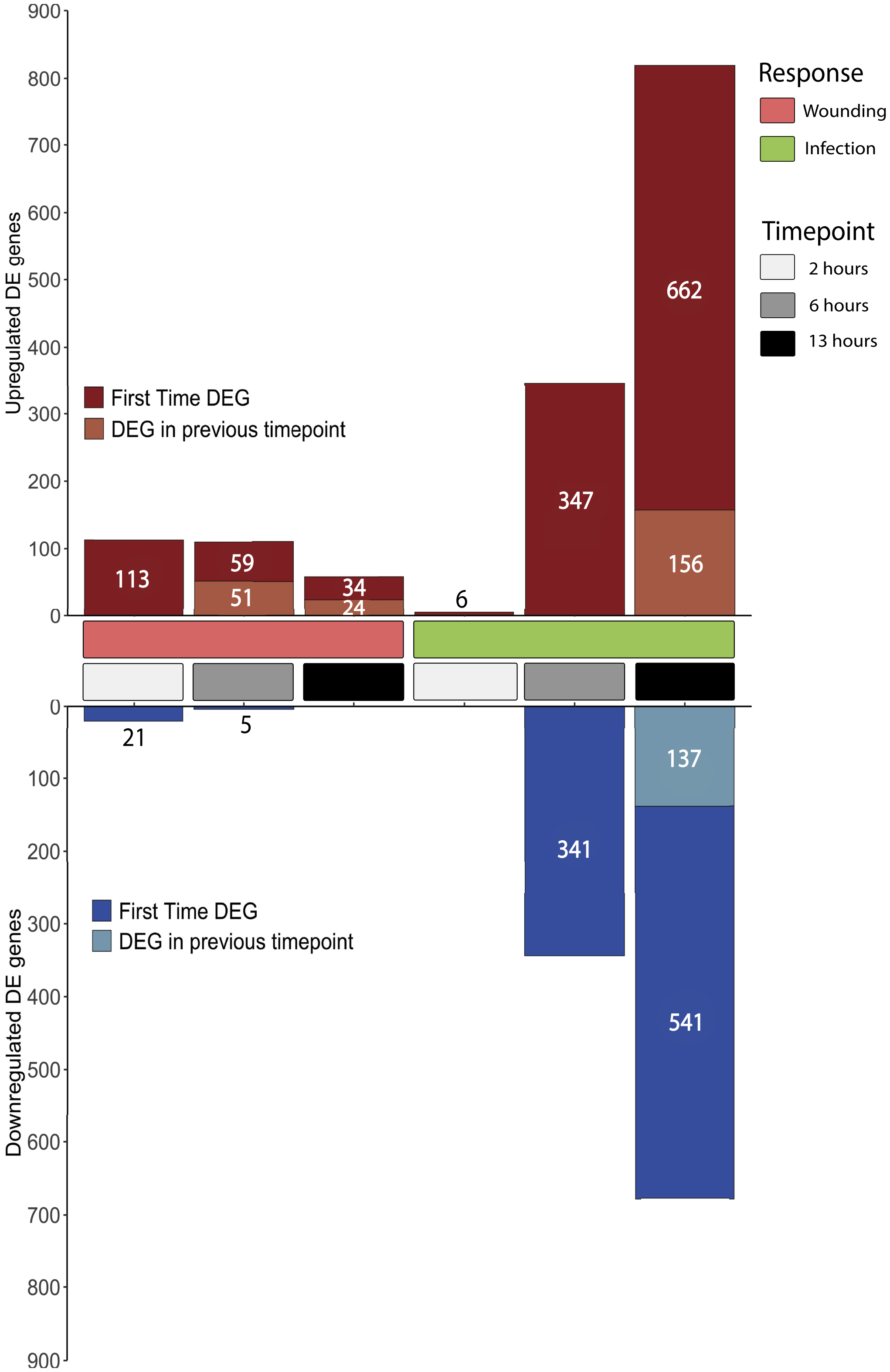

FIGURE 2 Stacked bar plot indicating the number of DEGs in five-day-old *H. illucens* larvae in response to either wounding (comparison of treatments: PBS versus Untreated) or bacterial infection (comparison of treatments: Bacteria versus PBS) (indicated as pink or green, respectively, along the x-axis) at three sampling time points (2 h, 6 h, and 13 h since treatment), indicated along the x-axis as white, grey and black blocks, respectively). Genes were identified as differentially expressed genes (DEGs) when FDR < 0.001 and $\log_2$FC ≥ 2 ('upregulated') or $\log_2$FC ≤ −2 ('downregulated'). Upregulated DEGs are indicated in dark red (unique to a specific time point) or red (DEG commonly expressed at the previous time point) color. Downregulated DEGs are shown in blue (DEGs unique to a particular time point) or light blue (DEGs overlapping with the previous time point). The number of DEGs is shown within the respective column.

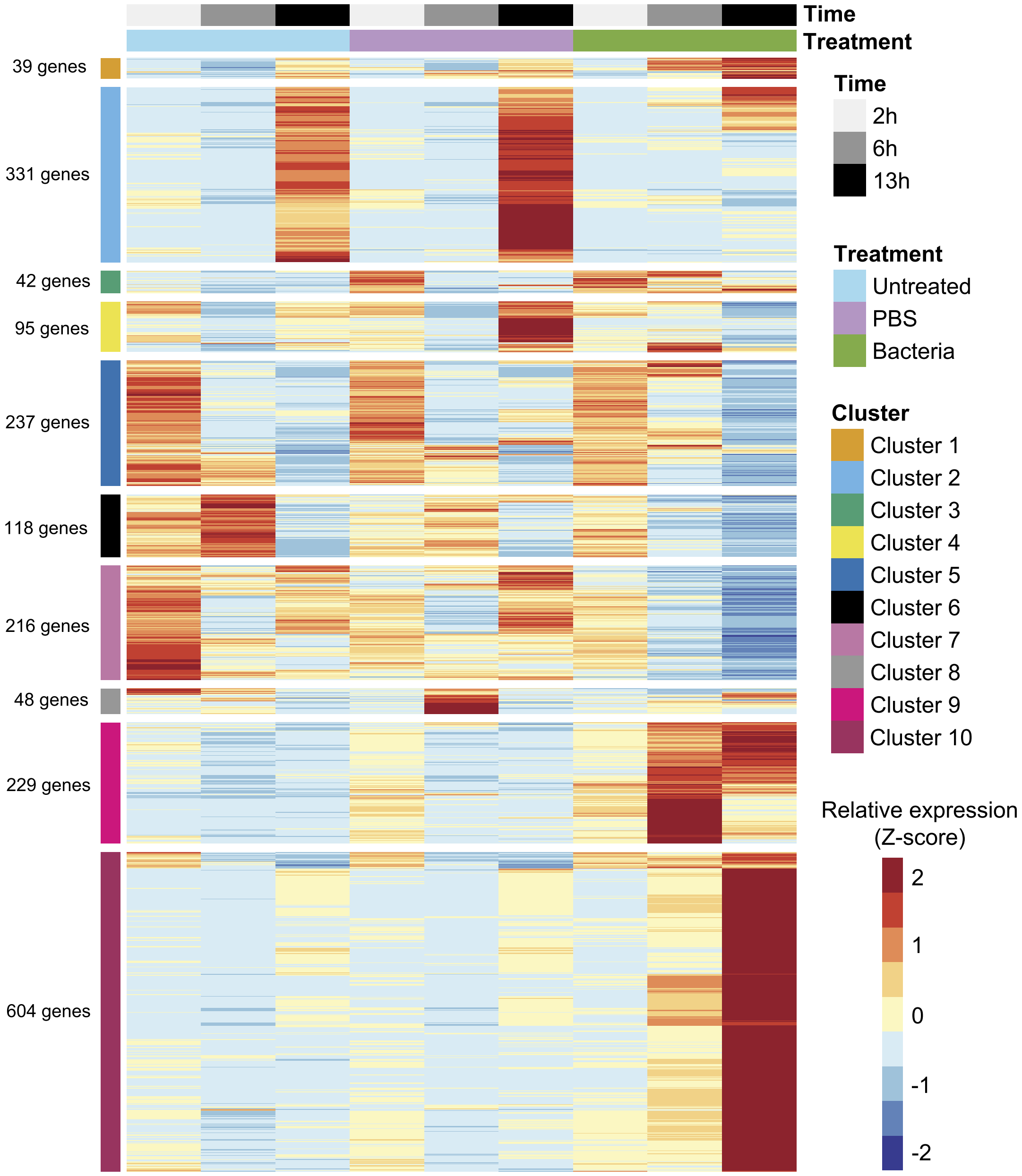

FIGURE 3 Cluster analysis of 1959 differentially expressed genes (DEGs) in response to wounding or infection in the larvae of *H. illucens*. Genes were considered DEGs if their false discovery rate was < 0.001 and $|\log_2 FC| \geq 2$. The genes were divided into 10 distinct clusters based on the outcome of hierarchical clustering with complete linkage. Log_2 (normalized counts per million) counts were centered and scaled by row (yielding a row z-score). Each row represents a scaled gene count for a specific DEG, ranging from 2 (red indicates upregulation) to −2 (blue indicates down-regulation).

Gene clusters upregulated in response to infection (i.e. clusters 1, 3, 9, and 10) are primarily involved in cellular response to stress, humoral immune response, antimicrobial response, and DNA repair. Genes in cluster 1 (comprised of 39 genes) were upregulated in bacteria-treated larvae at 6 h and 13 h; they include *ABC transporter G family member 23*, *lysozyme*, *pancreatic triacylglycerol lipase*, and *pupal cuticle protein Edg-78E*. The genes within cluster 1 are involved in various biological processes, such as cellular response to stimulus, DNA repair, cellular response to stress, and post-replication repair. Genes within cluster 3 and cluster 9 were significantly upregulated by wounding and infection at 2 h; however, their expression remained upregulated in response to infection while decreasing over time in response to wounding. Clusters 3 and 9 were composed of 42 and 229 genes, respectively, including various genes such as *serine protease-7*, *cecropin peptide 1*, *defensin-A*, *CD109 antigen*, *lysozyme*, *NF-kappa-B inhibitor cactus*, *peptidoglycan-recognition protein SA*. The genes within clusters 3 and 9 are involved in humoral immune response, antimicrobial

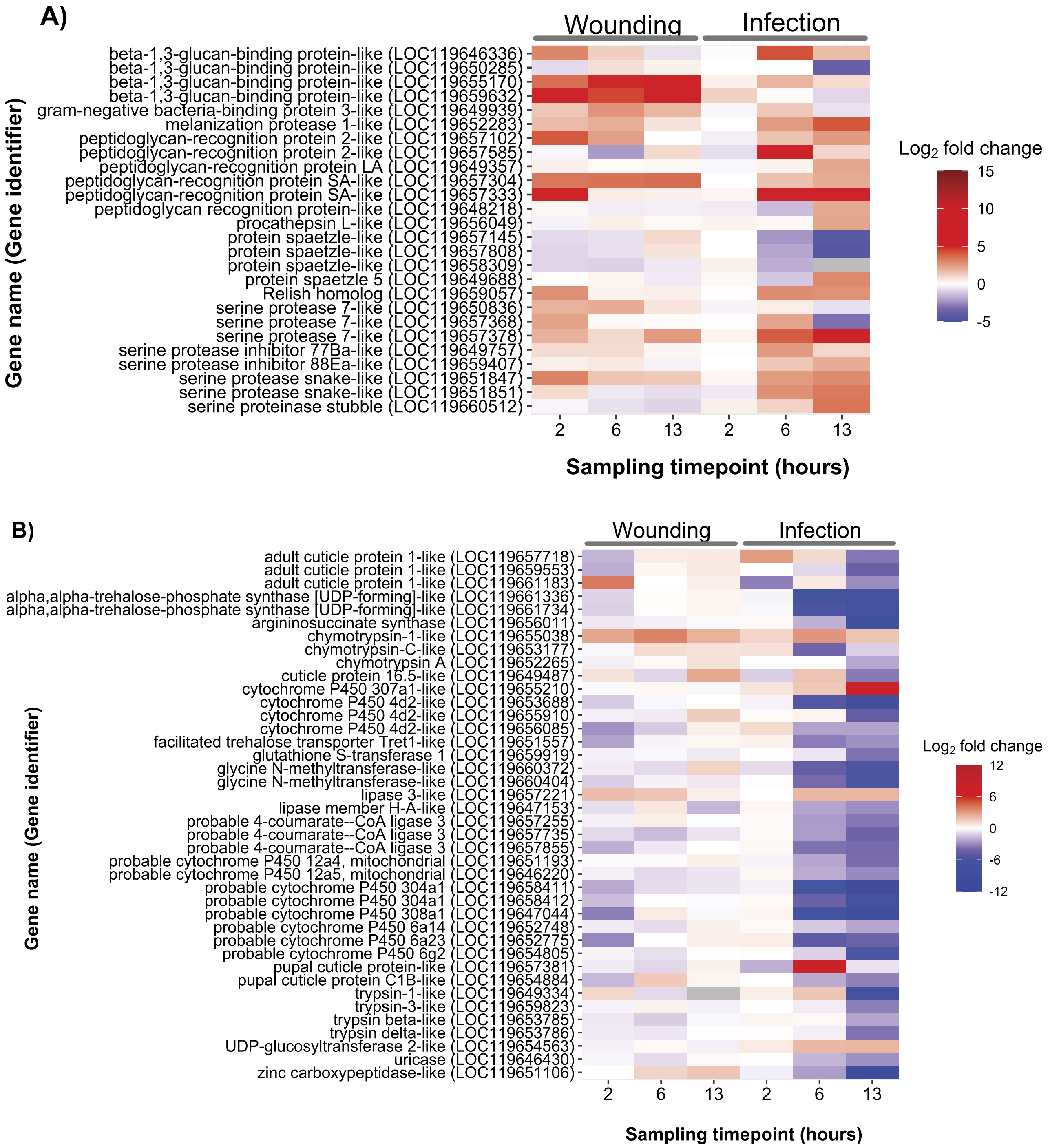

FIGURE 4 Fold-change relative to control in the expression of (A) genes involved in pathogen recognition, (B) growth and metabolism-related genes, and (C) genes encoding effector molecules such as antimicrobial peptides (AMPs) in response to wounding (comparison of treatments: PBS versus Untreated) and infection (comparison of treatments: Bacteria versus PBS) at three sampling time points (2 h, 6 h, and 13 h since treatment) in 5-day-old *H. illucens* larvae. The degree of upregulation is described in the respective legend panels. Red indicates upregulation, and blue indicates downregulation for respective genes. The name and associated gene IDs are represented at the left side.

humoral response, defense response to bacterium, and immune response. Forty-nine genes within cluster 9 are uncharacterized.

Almost one-third of all DEGs (604 from 1,959 DEGs) were grouped in cluster 10. Genes in cluster 10 contribute to biological processes associated with DNA repair, cellular response to DNA damage stimulus, cellular response to stress, and RNA biosynthetic process. Genes in this cluster encode for, e.g. caspase Dronc, cytochrome P450 6A1, cytochrome P450 6a8, DNA

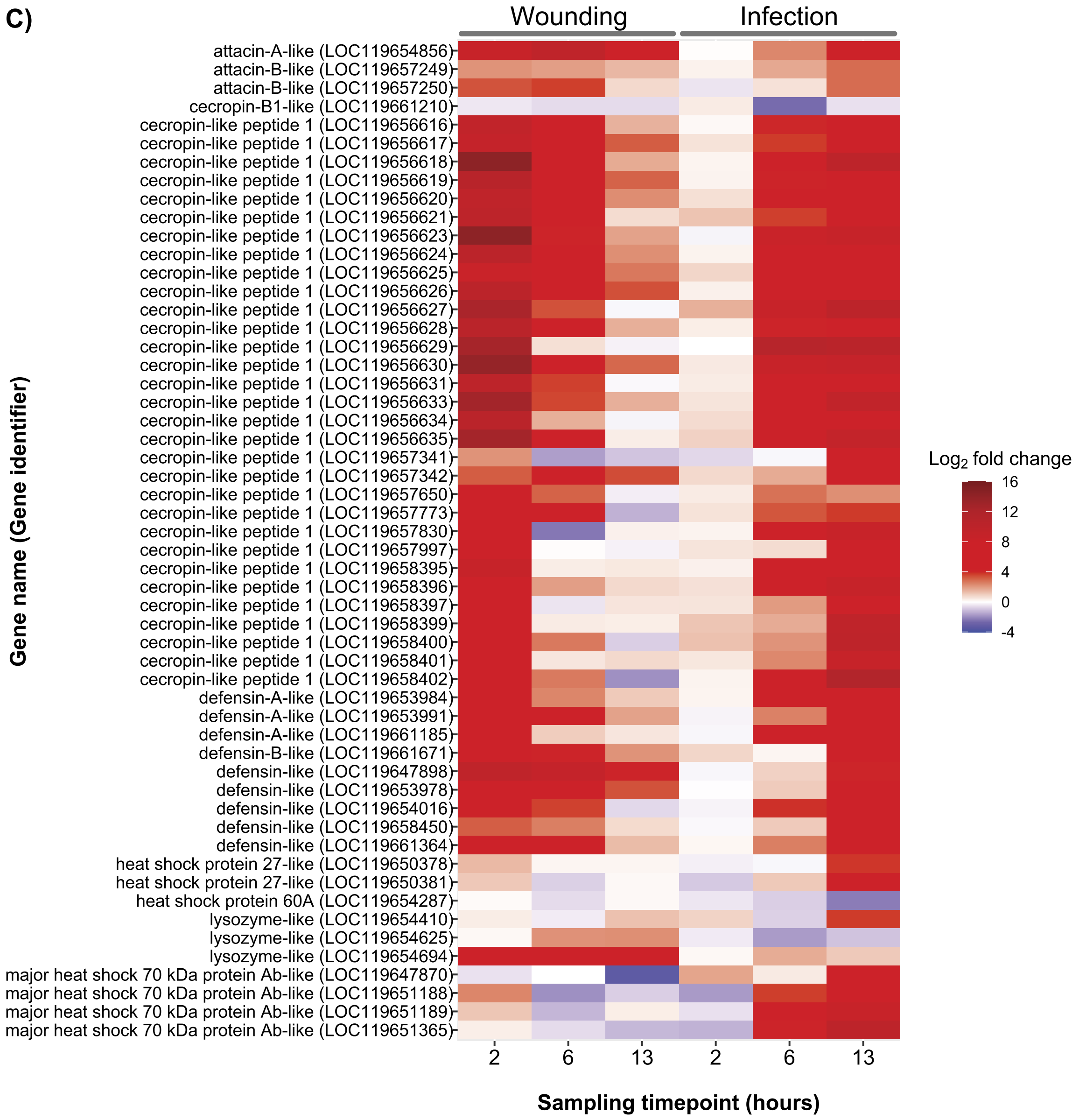

FIGURE 4 (Continued.)

repair protein RAD50, heat shock protein 27, major heat shock 70 kDa protein Ab, and melanization protease 1. Cluster 10 contains 158 uncharacterized genes that are upregulated post-bacterial infection.

Gene clusters 2, 4, 5, 6, 7, and 8 contain DEGs downregulated in infected larvae, primarily linked to metabolic and growth processes (Figure 3). Cluster 2 contains 331 genes, such as *cytochrome P450 6a2*, *lipase-3*, *carboxypeptidase-B*, *chymotrypsin-A*, *trypsin-beta*, and *peroxidase*. Downregulated DEGs in cluster 2 are involved in carbohydrate metabolic processes, lipid metabolic processes, and primary metabolic processes. In clusters 4 and 5, 95 and 237 genes were significantly downregulated in infected larvae at 13 h post-infection. Genes in cluster 4 include *trypsin-1*, *adult cuticle protein 1*, *cuticle protein 16.5*, and *Gram-negative bacteria-binding protein 3*, which are associated with carbohydrate (chitin) metabolic process and proteolysis. Cluster 5 genes include *lipase 3*, *heat shock protein 27*, *cuticle protein 12.5*, *cytochrome P450 6a13*, *argininosuccinate synthase*, and *trypsin-3*, which are associated with response to toxic substances, cellular detoxification, and lipase activity.

DEGs in cluster 6 (118 genes) and cluster 7 (216 genes) were significantly downregulated at 6 h and 13 h in infected BSF larvae. Cluster 6 genes include *mitotic-spindle organizing protein 1*, *cytochrome b5*, *trypsin*, *general odorant-binding protein 99a*, *general odorant-binding protein 99b*, and *hydroxylysine kinase*. The biological processes represented by genes in cluster 6 include organelle organization, cellular component organization, or biogenesis. Genes in cluster 7 are *L-xylulose reductase*, *larval serum protein 2*, *cell division cycle protein 20 homolog*, *mitochondrial fission process protein 1*, and α, *α-trehalose-phosphate synthase* [*UDP-forming*], amongst others. The genes within cluster 7 are involved in the regulation of the mitotic cell cycle and cell cycle processes.

Wounding and infection resulted in the downregulation of 48 genes in cluster 8. Downregulated genes in cluster 8 are involved in molecular functions such as heme binding, tetrapyrrole binding, and monooxygenase activity; including *carboxypeptidase B*, *chymotrypsin-2*, *cytochrome P450 6A1*, and *procathepsin L*.

Infection causes a trade-off between metabolism and immunity-related genes

Of the genes attributed to specific immunological pathways (Supplementary Figure S3), as described in Vogel *et al.* (2022), various genes from the Imd pathway are upregulated during bacterial infection (Supplementary Figure S4A), while fewer genes from the Toll pathway are upregulated (Supplementary Figure S4B). DEGs known to contribute to pathogen recognition (Figure 4A), metabolic processes (Figure 4B), and antimicrobial peptides (Figure 4C) were selected, and their relative expression (Log_2 fold change) to wounding and infection was plotted (Figure 4).

Wounding resulted in significantly upregulated expression of the genes *beta-1,3-glucan binding protein* and *peptidoglycan-recognition protein 2*, which are not significantly upregulated in infected larvae (Figure 4A). Upregulation of the gene encoding the serine protease *snake* indicates upregulation of the phenoloxidase pathway in response to wounding (Figure 4A). *PGRP-SA* (LOC119657333), *Relish*, *melanization protease-1*, and *serine protease* 7 were significantly upregulated in infected larvae (Figure 4A). *Peptidoglycan-recognition protein 2* (LOC119657585) was only upregulated in infected BSFL (Figure 4A), suggesting its role in the identification of pathogen-associated molecular patterns (PAMPs). Spaetzle proteins, associated with Toll pathway, were significantly downregulated in infected BSF larvae (Figure 4A).

Wounding and infection directly affected the expression of genes involved in growth (*adult cuticle proteins*, *cuticle protein 16*.5, and *pupal cuticle protein C1B*) and metabolism (*chymotrypsin*, *lipase*, *trypsin*, and *uricase*) (Figure 4B). Although wounding initially reduced the expression of these genes, their expression recovered at later timepoints (Figure 4B). In contrast, infection caused a sustained decrease in the expression of these genes over time (Figure 4B).

Similarly, wounding initially induced the expression of AMP-encoding genes, but this declined over time (Figure 4C). In contrast, the expression of AMP-encoding genes increased significantly in infected larvae over time (Figure 4C). The differentially expressed AMP-encoding genes include *attacin*, *cecropin*, *defensin*, and *lysozyme* (Figure 4C). Additionally, the gene encoding the enzyme tyrosine-3-monooxygenase, which catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), the initial and rate-limiting step in the melanin biosynthetic pathway, was significantly upregulated in wounded larvae only at the early timepoint while its expression was upregulated (> 1 $\log_2$-fold) in infected BSF larvae across all timepoints.

Gene ontology (GO) enrichment analysis

To gain insight into processes affected (either upregulated or downregulated) by wounding or infection, DEGs generated from wounding and infection were subjected to GO analysis to determine affected biological processes (BP), cellular components (CC), and molecular function (MF).

Wounding and infection resulted in common upregulation of genes involved in innate immune response. However, wounding results in the upregulation of genes involved in metabolic or catabolic processes, while infection results in the upregulation of genes involved in DNA repair and immune system processes (Figures 5A and 6A). DEGs downregulated in response to wounding and infection are associated with transition metal ion binding and oxidoreductase activity in BSF larvae (Figures 5B and 6B). DEGs downregulated in response to infection contribute to catalytic activity, DNA replication, and various metabolic processes (Figure 6B).

The DEGs upregulated by wounding contribute to 'response to stimulus' and 'innate immune response' (Figure 5A). Genes coding for molecular functions such as endopeptidase, lyase, and serine hydrolase activity were overexpressed. However, genes coding for molecular functions such as ion- and heme-binding processes were downregulated in response to wounding (Figure 5B).

Gene ontology (GO) analysis reveals that upregulated DEGs in infected BSF larvae are associated with biological processes involved in immune response, innate immune response, response to stress, and DNA repair processes (Figure 6A). These biological processes are carried out in cellular components located in the extra-

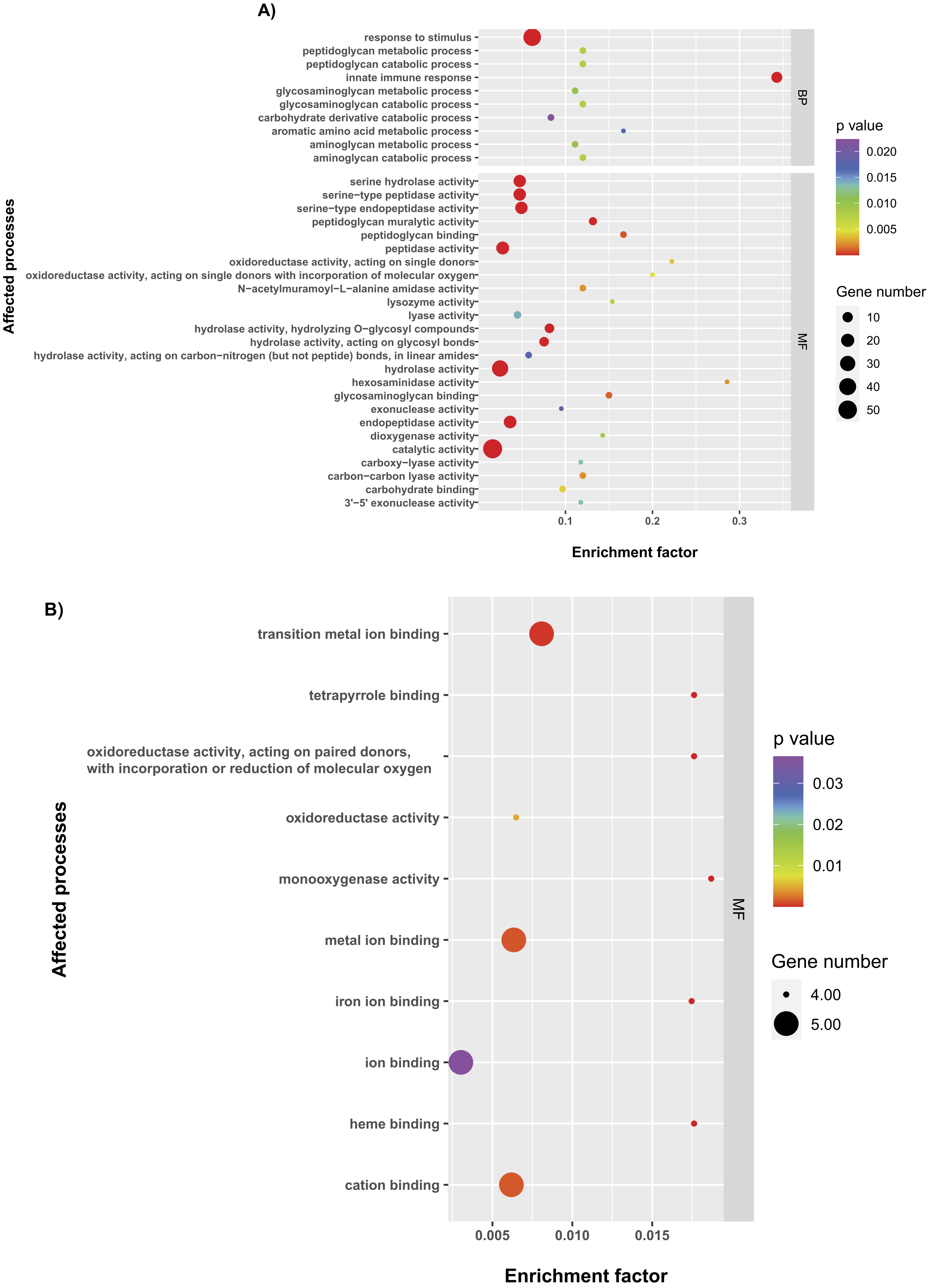

FIGURE 5 Gene ontology analysis of wounding-related DEGs to determine affected processes related to biological processes (BP) and molecular functions (MF) for (A) upregulated DEGs, and (B) downregulated-DEGs. On the right panel, BP represents a biological process (BP) or 'biological programs' performed by multiple molecular activities, cellular components (CC), and molecular function (MF) describes the molecular-level activities performed by affected gene product(s).

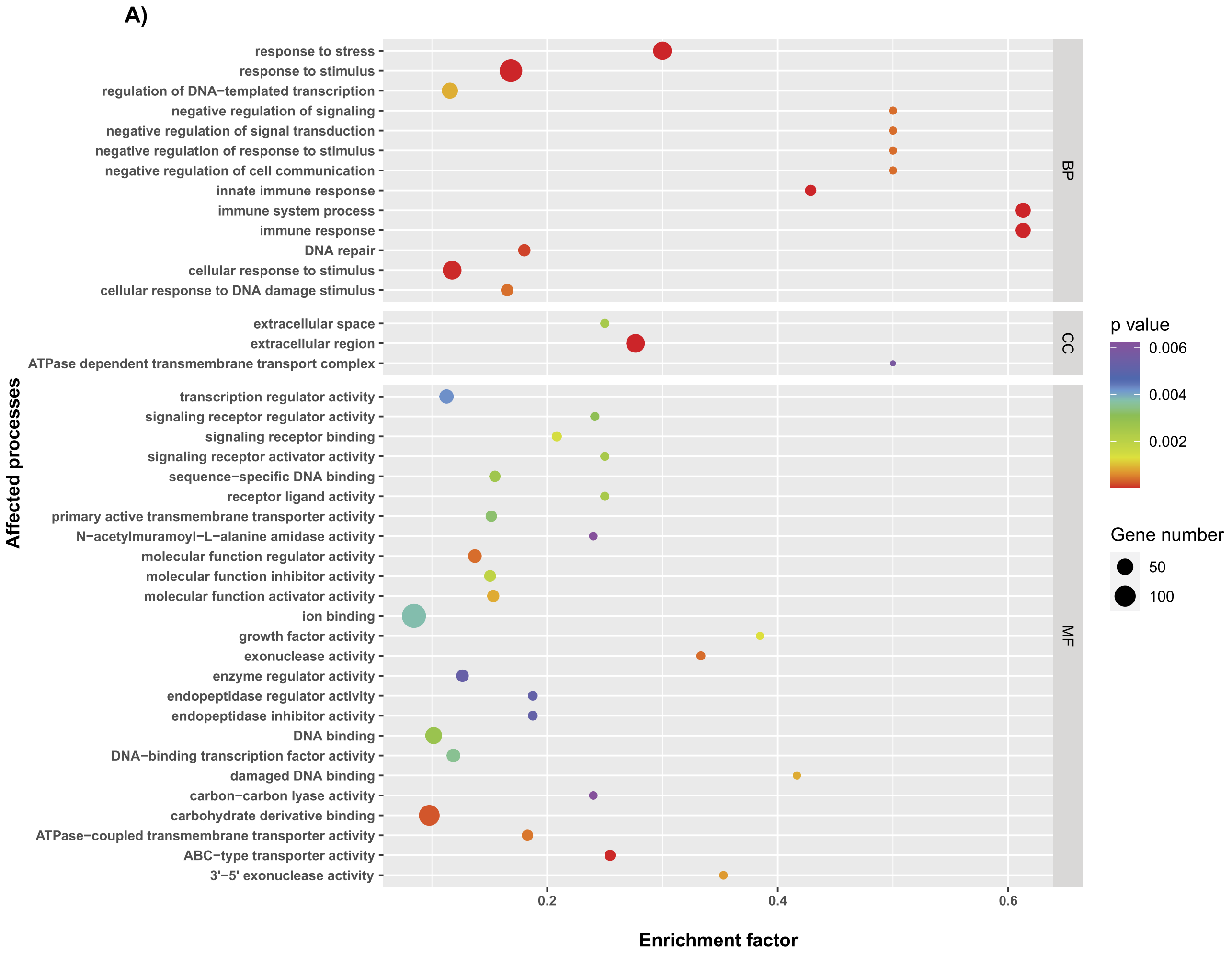

FIGURE 6 Gene ontology analysis of DEGs in response to infection to determine affected processes related to biological processes (BP), cellular components (CC), and molecular functions (MF) for (A) upregulated DEGs, and (B) downregulated DEGs. On the right panel, BP represents the biological process (BP) being performed by multiple molecular activities within cellular components (CC), in which molecular functions (MF) are being conducted by affected gene product(s).

cellular region and extracellular space (see CC in Figure 6A). Molecular functions associated with DEGs upregulated in response to infection include ABC-type transporter activity, increased receptor ligand activity, and signaling receptor regulator activity.

DEGs downregulated in response to infection are associated with molecular functions such as peptidase, lipase, and oxidoreductase activity (Figure 6B). The affected biological processes are proteolysis, lipid metabolic process, cell cycle, and carbohydrate metabolic process. The downregulated DEGs represent molecular functions and biological processes in the extracellular and chromosomal region.

4 Discussion

Knowledge of the temporal dynamics of the transcriptomic response of BSFL to pathogen infection is limited. We recorded a distinct difference in the transcriptomic response of BSFL to wounding (injection of PBS) and the injection of the Gram-negative bacterium, *P. protegens* Pf-5. The transcriptomic response to wounding and infection treatments varied over time. The early induction of immunity-related genes to wounding (i.e. in both PBS and Bacteria treatments) indicates that injury, irrespective of pathogen presence, can elicit a transcriptomic response in BSF larvae. A similar upregulation of immunity-related genes and differential modulation of immune response to wounding or infection is observed in *D. melanogaster* and *Tenebrio molitor* (Johnston and Rolff, 2013; Myllymaki *et al.*, 2014; Valanne *et al.*, 2011). The ability of insects to distinguish between wound-

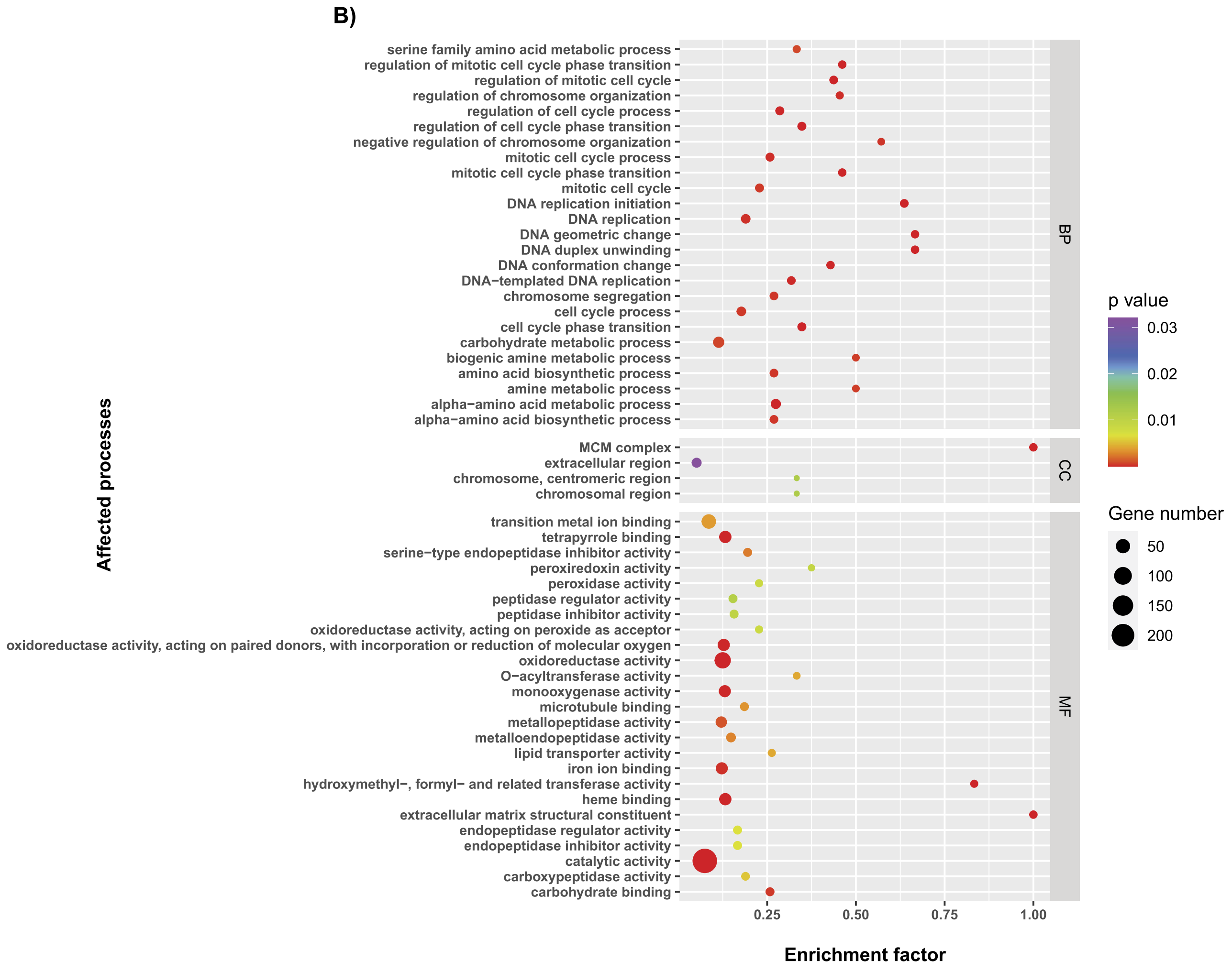

FIGURE 6 (Continued.)

ing and pathogen infection allows them to modulate their immunological responses accordingly (Johnston and Rolff, 2013). The early induction of transcriptomic response to wounding can also be gauged as a preventative measure by the larvae against pathogen proliferation.

Response to wounding

The transcriptomic evaluation indicates that wounding initially elicits a general immune response in BSFL that is similar to infection. Similarly, a common immune response to either wounding and infection has been observed in the damselfly, *Coenagrion puella*, wherein a similar set of genes involved in immune response and wound repair was initially upregulated (Johnston and Rolff, 2013). The majority of genes upregulated upon wounding encode for pathogen-recognition molecules and antimicrobial peptides that continue to stay upregulated in infected BSFL larvae, similar to those observed in *T. molitor* (Johnston *et al.*, 2014). Interestingly, genes responsible for structural processes, such as *pupal cuticle protein 36a*, *pro-resilin*, and *protein yellow*, were initially upregulated in BSFL in response to wounding. *Protein yellow* is involved in melanin production and chitin-related functions in *D. melanogaster* (Qin *et al.*, 2012; Wittkopp *et al.*, 2002), indicating an activation of a wound-repair mechanism.

Wounding of BSFL resulted in the initial upregulation of many genes involved in pathogen recognition, such as genes coding for PGRPs, GNBPs and GBPs, serine protease snake, and Relish. A similar set of genes was upregulated upon wounding in the hemocytes of *D. melanogaster* (Ramond *et al.*, 2020). Peptidoglycan-receptor proteins (PGRPs) are crucial in the initial recognition of bacterial membrane proteins by the insect host, and mediate immune pathways including Imd, Toll and PO (Aymeric *et al.*, 2010; Iatsenko *et al.*, 2016; Kurata, 2014; Neyen *et al.*, 2012; Wang *et al.*, 2019a; Wang *et al.*, 2021; Zhao *et al.*, 2018). PGRPs activate the NF-ϰB family transcription factor *Relish*, which induces AMP expression (Choe *et al.*, 2002). Infection with Gram-negative bacteria elicits the expression of

PGRP and *Relish* in *D. melanogaster* adults, generating an inflammatory response that reduced lifespan (Libert *et al*., 2006). PGRP-LB and PGRP-LC are known to function synergistically, and their activation is required to modulate a melanization response in infected insects (Takehana *et al*., 2004). Additionally, PGRPs are essential in recognizing commensal bacteria (Onuma *et al*., 2023) and in maintaining gut microbiota richness and diversity (Liang *et al*., 2023). The transfer of untreated BSF larvae to fresh substrate elicited upregulation of *PGRP-LB* and *PGRP-LC*, which play a role in pathogen detection (Iatsenko *et al*., 2016; Koyama *et al*., 2015; Wang *et al*., 2020; Yanagawa *et al*., 2017). The presence of bacteria with a DAP-type peptidoglycan is perceived by the PGRP-LC receptor in *D. melanogaster*, which induces a cleaning behavior in the insect when introduced into a substrate infested with Gram-negative bacteria (Yanagawa *et al*., 2017).

PGRP-LA and *PGRP-SA* are induced during bacterial infection in BSFL. *PGRP-SA* is reportedly activated by Gram-positive bacteria (Gottar *et al*., 2002), but we observed a significant upregulation of *PGRP-SA* in BSF larvae infected with a Gram-negative bacterium. PGRP-SA plays several roles in *D. melanogaster* and *T. molitor* by activating both the Toll pathway as well as prophenoloxidase activity, which eventually results in phagocytosis, melanin synthesis, or generation of reactive oxygen species (Dziarski and Gupta, 2006). PGRP-SA mediates melanization and AMP production in *Antheraea pernyi* caterpillars infected with Gram-negative *E. coli* and *P. aeruginosa* and in *Helicoverpa armigera* (Hübner) caterpillars infected with the Gram-negative *Enterobacter cloacae* (Xiong *et al*., 2020; Zhao *et al*., 2018). Other pathogen recognition molecules such as beta-1,3-glucan-binding protein 1 involved in the recognition of Gram-positive bacteria (Kurata, 2014; Shan *et al*., 2023) were upregulated upon wounding in BSF, but their expression was downregulated in infected individuals.

The signaling cascade initiated by PGRP upregulates genes encoding serine proteases (*snake*, *rubble*, and *Sp7*) in BSF, which are involved in the phenoloxidase pathway (Shan *et al*., 2023). Serine proteases are essential for the clearance of bacterial cells within the insect hemocoel, primarily by mediating the phenoloxidase (PO) and Toll (via Spätzle) pathways in different insect species (Kanost and Jiang, 2015). Serine proteases tightly modulate the intensity of melanization, and their activity reduces over time in the absence of a pathogen (Kanost and Jiang, 2015). Accordingly, expression of the *serine protease snake* decreases over time in response to wounding. However, we recorded genes encoding serine proteases to be consistently upregulated in infected BSFL across all time points, suggesting a function in pathogen clearance (Bae *et al*., 2021; Dudzic *et al*., 2019; Johnston *et al*., 2014; Salcedo-Porras *et al*., 2022; Shan *et al*., 2023).

Apart from serine proteases, signaling molecules such as the NF-κB transcription factor Relish, are also crucial in regulating the Imd pathway in *Drosophila* (Libert *et al*., 2006; Yao *et al*., 2023). Long-term activation and production of AMPs in the absence of pathogens leads to deleterious effects in *D. melanogaster* (Libert *et al*., 2006), which are therefore tightly controlled and modulated by Relish. Relish is involved in the modulation of AMP responses in different insect species, such as *D. melanogaster*, *T. molitor*, and *M. domestica* (Asgari *et al*., 2022; Keshavarz *et al*., 2020; Libert *et al*., 2006; Yao *et al*., 2023). Wounding of BSFL results in the upregulation of *Relish*; however, its expression reduces considerably over time in the absence of pathogens. Genes encoding spätzle proteins (involved in the Toll pathway) are significantly upregulated in response to wounding but downregulated in infected BSFL except for Spz-5 which is significantly upregulated at 13 h in infected BSF. Unlike a clear distinction in the expression of the Toll and Imd pathways in *D. melanogaster*, we observe that several genes associated with the Toll pathway are initially upregulated in infected BSFL but their expression reduced over time. This is indicative of crosstalk between immunity pathways, as reported by Cho and Cho (2024) in BSFL. Collectively, our results indicate that the major immunological responses involved in BSFL response to Gram-negative bacterial infections are the phenoloxidase and Imd pathways.

Initial upregulation of immunity and repair processes upon wounding comes at the expense of downregulation of metabolic processes. However, reduced immune investments over time to wounding response increases investment towards metabolism, as the expression of genes such as *trypsin* increases significantly over time after an initial downregulation. The decreased immune response and elevated metabolic processes within a 13 h period indicate the agility and intensity of the immune response generated in BSFL, possibly to minimize negative long-term consequences on fecundity and health.

Response to infection

Infection results in a sustained induction of immunity-related processes in BSFL. Apart from sustained induction of recognition and signaling molecules, effector molecules associated with immunity-related functions were also significantly upregulated. AMP-encoding genes such as *cecropin*, *defensin*, *attacin*, *heat shock protein 27*, *heat shock protein 70*, and *lysozyme* were significantly upregulated in infected BSF larvae. These genes have also been implicated in immunity-related responses to pathogen infections in other insect species (Edosa *et al.*, 2020; Li *et al.*, 2022; Ramirez *et al.*, 2023).

Time-course experiments revealed that injection of *E. coli* lipopolysaccharide in adult *D. melanogaster* resulted in acute upregulation of several AMP genes (*attacins* and *PGRPs*) within 2 to 6 h of injection (Schlamp *et al.*, 2021), just as we observed for BSFL in this study as well.

Antimicrobial peptides are essential effector molecules secreted upon successful activation of either the Toll or the Imd pathway, and are used for clearing pathogens from the insect host (Vogel *et al.*, 2022). The BSF genome contains a wide range of AMP-encoding genes that likely enable it to survive in pathogen-rich environments (Generalovic *et al.*, 2021; Moretta *et al.*, 2020). BSF contains up to 36-37 genes encoding for cecropin (Generalovic *et al.*, 2021; Vogel *et al.*, 2022). In our study, 11 of the top 15 genes that contribute most to the separation of treatments in a multivariate analysis are cecropin-encoding genes, which indicates their important role in infection response of BSFL to an infection with a Gram-negative bacterium. Cecropin confers immunity in *D. melanogaster* adults against infection with Gram-negative bacteria like *Enterobacter cloacae* and *Providencia heimbachae* (Carboni *et al.*, 2022). Oral intake or injection of pathogen directly into the hemocoel resulted in the elicitation of multiple cecropin and defensin encoding genes in BSFL (Bruno *et al.*, 2021; Park *et al.*, 2015; Van Moll *et al.*, 2022; Vogel *et al.*, 2018; Vogel *et al.*, 2022).

Attacin represents another group of AMPs that were upregulated in infected BSFL and are known to confer immunity against infections by multiple Gram-negative bacteria in adult *D. melanogaster* (Hanson *et al.*, 2016; Poppel *et al.*, 2015; Tanji *et al.*, 2007). Infecting a triple-knockdown *attacin* mutant (*TmAtt1a*, *TmAtt1b*, and *TmAtt2*) of adult *T. molitor* with Gram-negative *Pseudomonas entomophila* resulted in increased bacterial load and reduced survival compared to control insects (Keshavarz *et al.*, 2023), indicating the importance of attacin in insect immunity. Several genes encoding heat-shock proteins 27 and 70 were upregulated in infected BSF larvae. These proteins contribute to the immune response of *Galleria mellonella* against the fungus *Conidiobolus coronatus* (Wronska and Bogus, 2020). The upregulation of heat-shock protein genes suggests their importance in the immunological response of *H. illucens* to Gram-negative bacterial infection.

In addition to AMPs, enzymes like tyrosine-3-monooxygenase that is involved in the phenoloxidase pathway was upregulated in infected BSFL. Tyrosine-3-monooxygenase catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), the initial and rate-limiting step in the biosynthesis of melanin (Gorman *et al.*, 2007). Melanin biosynthesis is essential for sclerotization of insect cuticle and healing in response to wounding (Sugumaran and Barek, 2016). The injection of PBS and the Gram-negative bacterium, *Enterobacter cloacae*, also results in an induction of tyrosine metabolism in the fat body tissue of the kissing bug, *Rhodnius prolixus* (Salcedo-Porras *et al.*, 2022).

Trade-off between immunity and metabolism

The balance in investing in metabolic and immune activities is essential for the evolutionary success of an insect (Li *et al.*, 2023). An insect's fat body is primarily responsible for energy storage, which during infection switches to the production of antimicrobial peptides to counter invading microorganisms (Dolezal *et al.*, 2019). An increased production of AMPs or humoral immunity in infected insects occurs at the expense of metabolic processes, thereby negatively affecting developmental processes (Dolezal *et al.*, 2019). Wounding of BSFL resulted in an immediate increase in the expression of AMP-encoding genes, however their expression reduced within a couple of hours. Prioritised and increased allocation of resources by BSFL to immune responses upon infection corroborates the concept of the "selfish immune system" proposed by Straub (2014), wherein the immune system is hierarchically placed above other organismal requirements during a state of infection. However, long-term immune investments incur developmental or reproductive costs. Interestingly, the actual presence of a bacterial pathogen in infected BSFL resulted in an exponential increase in the expression of AMP genes over time. An increased immune response resulted in the downregulation of metabolic activity in infected BSFL. Recently, another study recorded complementary indications for a trade-off between immunity and metabolism (Shah *et al.*, 2024b). BSF larvae that were exposed to the mycotoxin aflatoxin B_1 upregulated the transcription of genes known to be involved in aflatoxin B_1 metabolism in other insect species while downregulating genes known to be involved in the immune response (Shah *et al.*, 2024b). Thus, there is evidence for both directions of this trade-off in BSFL. Investigating the dynamics of the trade-off between immunity and metabolism will be important to understand how an insects like BSFL, that are under selection to develop rapidly in ephemeral resources, deal with pathogen exposure.

5 Conclusion

Experimental evaluation of the transcriptomic response of BSFL to an infection with a Gram-negative bacterium at a high dose of 5,000 CFU injected per larva revealed a clear transcriptomic response with little variation between samples. Although such a high level of infection is unlikely to occur in a production setup, this study offers insight into the genes and pathways that are significantly upregulated during infection in BSFL. The genes identified can be utilised to develop a health-monitoring program for BSFL in mass-rearing facilities.

Supplementary material

Supplementary material is available online at:
https://doi.org/10.6084/m9.figshare.26969074

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Author contributions

Conceptualization: P.S., SM, JvL, MD; Investigation: P.S., S.M.; Formal analysis: P.S., I.W.; Writing-original draft preparation: P.S.; Writing – review: P.S., S.M., IW, JvL, MD; Writing – editing; P.S.; Visualization: P.S., I.W.; Supervision: P.S., MD, JvL; Funding acquisition: M.D. All authors have read and approved the final manuscript.

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