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# Hemocytes: Central drivers of antimicrobial peptide expression and immune proteins in both cellular and humoral responses of *Galleria mellonella*

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## Abstract

Insects have an effective innate immune system that includes both cellular and humoral responses for defense against pathogens. Antimicrobial peptides like gallerimycin and galiomycin, as well as immune proteins like hemolin, are the important effectors of the humoral immune response in *Galleria mellonella* L. (Lepidoptera:Pyralidae). Encapsulation, on the contrary, is one of the important cellular immune responses. This study investigated the tissue-specific expression of an immune effector in *G. mellonella* larvae after injection with *Candida albicans* (C.P. Robin) (Ascomycota: Debaryomycetaceae) and silica beads. The gene expression of gallerimycin, galiomycin, and hemolin was examined in total larvae, hemocytes, and fat bodies at 4 and 24 h following injection. Our findings indicate that hemocytes serve as the main site for AMP synthesis, especially after bead injection, implying a more effective immune recognition mechanism relative to pathogen injection. Furthermore, we detected higher hemolin expression in hemocytes than fat tissue, indicating its role in hemocyte-mediated immune responses. Encapsulation rates were also evaluated in bead-injected larvae. At 4 h post-injection, most beads were weakly encapsulated, whereas by 24 h, the majority were strongly encapsulated, reflecting a time-dependent maturation of the immune response. These results show that *G. mellonella* has a unique immune system, with hemocytes playing a key role in regulating AMP production and immune responses during infection. This study provides deeper insights into the molecular and cellular mechanisms of insect immunity, positioning *G. mellonella* as a valuable model for studying host–pathogen interactions.

**KEYWORDS**
*Candida albicans*, early defense system, encapsulation, fat body, galiomycin, gallerimycin, gene expression, greater wax moth, hemolin, silica beads

## INTRODUCTION

Insects have developed a complex immune system to protect themselves from pathogens, such as bacteria, fungi, viruses, and even larger parasites, like nematodes and parasitoid eggs. Upon the penetration of foreign particles through the physical barriers, the innate cellular responses (e.g., phagocytosis, nodule formation, and encapsulation) and humoral responses (e.g., immunological peptides, such as AMPs, immune proteins, and lectins) are triggered, leading to the rapid removal of foreign entities (Eleftherianos, Heryanto, et al., 2021; Strand, 2008). These immune responses are directly or indirectly mediated by circulating hemocytes.

The greater wax moth (*Galleria mellonella* L. [Lepidoptrea: Pyralidae]) is an excellent model organism for studying immunological responses to invaders or microbial infections due to its small injectable size, short life span (7–8 weeks), ease of handling and maintenance without specialized laboratory equipment, lack of ethical approval requirements, survival temperature range (25–37°C), etc. (Binder et al., 2016; Desalermos et al., 2012; Ménard et al., 2021; Pereira et al., 2018; Ramarao et al., 2012; Wojda, 2017). *Galleria mellonella* contains various types of hemocytes, such as prohemocytes, spherulocytes, oenocytoids, plasmocytes, and granulocytes (Lavine & Strand, 2002; Ribeiro & Brehélin, 2006; Senior & Titball, 2020; Wu et al., 2016). Prohemocytes may be differentiated into other hemocyte types (Yamashita & Iwabuchi, 2001). Spherulocytes, which transport cuticle components, and oenocytoids, which participate in the melanization process, are grouped as non-adherent hemocytes (Jiravanichpaisal et al., 2006; Ribeiro & Brehélin, 2006; Strand, 2008). The majority of circulating hemocytes are granulocytes and plasmatocytes, have the ability to adhere to diverse surfaces, including non-self particles, and are involved in cellular immune response (e.g., encapsulation) (Schmit et al., 1977; Lavine & Strand, 2002; Jiravanichpaisal et al., 2006; Hillyer, 2016).

Encapsulation is a cellular immune response used by insects to deal with large invaders, such as parasite eggs, larvae, or foreign bodies that cannot be phagocytosed by individual hemocytes (Dubovskiy et al., 2016). Hemocytes, granulocytes, and plasmatocytes, aggregate around the invader, forming multiple layers, and secrete a layer of melanin through the melanization process, leading to the formation of a physical barrier. Investigations into encapsulation mechanisms demonstrated that granulocytes experience self-lysis within the first 5 min of recognition, and plasmatocytes gather around the foreign body within 20 min, leading to a gradually thickening of the capsule layers (Pech & Strand, 1996; Schmit et al., 1977). The cellular layer around the capsule functions as a benchmark for evaluating the magnitude of the encapsulation reaction (Richards & Dani, 2008). Studies examining the encapsulation response of *G. mellonella* commonly utilize silica beads ranging from 40 to 120 μm, which generate a similar response (Altuntaş et al., 2012; Er et al., 2010; Kaya, 2023; Kaya et al., 2021; Kaya et al., 2022; Wiesner & Götz, 1993).

Antimicrobial peptides (AMPs) and opsonins, on the contrary, are part of the humoral immune response in insects. In response to microbial infections, these proteins are rapidly synthesized and secreted into the hemolymph. AMPs and opsonins are particularly effective against bacteria and fungi, functioning by disrupting microbial membranes, inhibiting cell wall synthesis, or interfering with metabolic processes (Bulet et al., 1999; Eleftherianos, Zhang, et al., 2021; Mylonakis et al., 2016; Zhou et al., 2024). They provide a rapid and effective means of neutralizing infections that are too small to trigger encapsulation, such as bacterial or fungal pathogens. AMPs can be classified based on their amino acid contents (e.g., cysteine, glycine, and proline) and secondary structures, such as α-helical, β-sheets, or the mixture of these structures. The structure and function of insect AMPs were thoroughly examined in reviews (reviewed in Zhou et al., 2024; Eleftherianos, Zhang, et al., 2021). Insect AMPs are mainly produced in the fat body, hemocytes, salivary glands, and reproductive and digestive tracts, exhibiting varying quantities in both infected and uninfected larvae (Tsai et al., 2016). Studies conducted with *G. mellonella* discovered 20 potential AMPs which include gallerimycin and galiomycin, cysteine-rich cationic peptides (Brown et al., 2009; Cytryńska et al., 2007; Kim et al., 2004; Lee et al., 2004; Vogel et al., 2011). Gallerimycin and galiomycin are induced during fungal and bacterial infections (e.g., *C. albicans* and *Bacillus thuringiensis*) and also physical stress and temperature (Bergin et al., 2006; Trevijano-Contador & Zaragoza, 2018; Sheehan & Kavanagh, 2018; Taszłow et al., 2017; Mowlds et al., 2008; Mowlds & Kavanagh, 2008.).

Hemolin, which is a member of the immunoglobulin family, acts as a pattern recognition receptor (PRR) in insects and is induced by fungal, bacterial, and viral infections (Browne et al., 2015; Genç et al., 2024; Shaik & Sehnal, 2009; Subhagan et al., 2024; Terenius, 2008; Yu & Kanost, 2002). Hemolin recognizes fungal β-1,3-glucan, mycobacterial lipoglycans, bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA), which results in promoting the aggregation of hemocytes and phagocytosis (Asai et al., 2021; Eleftherianos et al., 2007; Jung et al., 2019; Mishra et al., 2011; Yu & Kanost, 2002). Hemolin gene expression was detected in the fat body, hemocytes, Malpighian tubes, midgut, nervous cord, and the silk glands of insects (Ladendorff & Kanost, 1991; Shaik & Sehnal, 2009).

While cellular and humoral responses are distinct immune mechanisms, they are closely linked in orchestrating a comprehensive defense strategy against various types of pathogens. So, this study investigates the cellular immune response by assessing the encapsulation ratio and the humoral immune response by quantifying the gene expressions of AMPs (e.g., gallerimycin and galiomycin), as well as immune receptors like hemolin, in response to two distinct immune challenges: the microbial pathogen *C. albicans* and beads that simulate the size of parasite eggs. Gene expressions were assessed in whole larvae, fat bodies, and hemocytes using RT-qPCR at two pivotal time intervals: 4 and 24 h post-injection.

# MATERIALS AND METHODS

## *Candida albicans* growth

*Candida albicans* (C.P. Robin) (Ascomycota: Debaryomycetaceae) cells were grown in YPD medium (1% yeast extract [wt/vol]; 2% peptone [wt/vol]; 2% glucose [wt/vol]) at a constant temperature (30°C) and constant shaking at 250 rpm up to the stationary phase. Then, 100 μL of saturated cultures were inoculated into fresh YPD medium and incubated under the same conditions up to the logarithmic phase ($OD_{600}$: 0.6–0.7). At this stage, a cell suspension comprising about $2 \times 10^5$ cells was centrifuged and washed with sterile distilled water. Yeast cells ($2 \times 10^5$ cells/larva) suspended in 5 μL of distilled water were used for injection (Vertyporokh & Wojda, 2020).

## Insect rearing, injection and sampling

Samples of *G. mellonella* were obtained from larvae that reached the last stage (0.20 ± 0.02 g) after being reared in a controlled laboratory setting from eggs taken from two adults. Larvae were reared and kept in the same circumstances (at 65 ± 5% relative humidity and 30 ± 1°C temperature in darkness). A total of 180 larvae were used for each condition in both bead and pathogen injections, encompassing the whole larvae, hemocytes, and fat tissue, for 4 and 24 h ($n = 180$).

The larvae selected for the experiment underwent first surface sterilizing. Sephadex A-25 chromatography beads with an average of 10–15 beads in 10 μL PBS solution (pH = 7) after staining with 1% Coomassie Brilliant Blue-G dye (Merck KGaA, Darmstadt, Germany) were used for injection. Fifteen larvae for each group were injected with either chromatography beads (10–15 beads/larvae) using a 22-gauge microsyringe (50 μL, Hamilton, Reno, NV, USA) or pathogen ($2 \times 10^5$ cells/larva) using a 26-gauge microsyringe (25 μL, Hamilton,) through the top right proleg of the larvae. Larvae were maintained under the same conditions, and sampling (whole larvae, hemocytes, and fat bodies) was done 4 and 24 h after infection.

At the end of the waiting period, the larvae were segregated into two groups, and the first group of larvae, which received no treatment, was stored at −80°C after being frozen in liquid nitrogen. The second group of larvae was used for collecting both hemocytes and fat tissue. The hemolymph from all the larvae was collected in a 2-mL microcentrifuge tube, including 100 μL of anticoagulant (0.098 M NaOH, 0.186 M NaCl, 0.017 M $Na_2$EDTA, and 0.041 M Citric Acid pH: 4.5[Merck KGaA, Darmstadt, Germany]). The collected hemolymph was centrifuged at 10000 rpm for 5 min (IKA, Staufen, Germany). The supernatant was discarded, and the pellet containing hemocytes was frozen in liquid nitrogen and thereafter kept at −80°C. The larvae whose hemolymph was collected were dissected, and the fat tissue was transferred to a 2-mL microcentrifuge tube containing 300 μL of anticoagulant. The collected fat tissue was thoroughly homogenized and frozen in liquid nitrogen, then stored at −80°C. Fat tissue and hemocytes were extracted from the same larvae.

## Encapsulation

To compare the results from gene expression experiments, encapsulation responses were assessed using Sephadex A-25 chromatography beads. Therefore, beads were injected into untreated larvae, and after 4 and 24 h, these beads were collected through dissection under a stereomicroscope (Leica EZ4, Wetzlar, Germany) and placed on a slide with a drop of PBS. The slide was sealed, and the cellular layer surrounding the beads was examined using a phase-contrast microscope (Olympus BX51, Tokyo, Japan). The beads were categorized as none, weak, or strong based on the criteria provided by Richards and Dani (2008), and the encapsulation response was assessed. Fifteen larvae were utilized for each sampling time.

## Real-time quantitative PCR

The measurement of gene expression was conducted using larvae, hemocyte, and fat body from each group: naive, null injection, $dH_2O$ injection, *C. albicans* injection, and bead injection. A total of 45 larvae were employed for each group to provide triplicate samples. After 4 and 24 h injection, the five larvae and separated fat bodies of the five larvae were immediately ground in liquid nitrogen. The hemolymphs of the same larvae were carefully collected, and hemocytes were obtained following centrifugation. The larvae, fat body, and hemocytes were then stored at −80°C until the RNA was isolated. The total RNA from samples of fat bodies and hemocytes was extracted using the RNA purification kit (Thermo Fisher Scientific Baltics, UAB-Vilnius/Lithuania, K0732). In order to remove any DNA contained in the homogenate, the samples were further treated with DNase (Thermo Scientific, EN0525). The Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Vantaa, Finland) was used to evaluate the RNA yield. Subsequently, the first-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622), following the guidelines provided by the manufacturer. The cDNA synthesis was performed using RNA amounts adjusted to obtain a final cDNA concentration of 500 ng per sample. Following the manufacturer's instructions, real-time quantitative PCR (RT-qPCR) was conducted using a PikoReal™ Real-Time PCR System (Thermo Scientific) with the RealQ Plus 2x Master Mix Green Without ROX (Ampliqon, Odense, Denmark A323402). The primer sequences for gallerimycin, galiomycin, hemolin, and housekeeping (β-actin) genes used in this study were as follows (5′–3′): Gallerimycin F: *GAAGTCTACAGAATCACACGA* and R: *ATCGAAGACATTGACATCCA*; Galiomycin F: *CGTTTCGTCACCGCAAAATG* and R: *GCCGCAATGACCACC TTTAT*; Hemolin F: *ATCACTGTTGGCCCTGATGG* and R: *CCGTGAGGGAGTCGATGAAG*; β-actin F: *CCCTGTGCTGC TCACCGA* and R: *ACAGTGTGGGTGACCCCGTC*. The gallerimycin, hemolin, and β-actin primers were employed in accordance with the methodology outlined previously (Lange et al., 2018; Genç et al., 2024). The galiomycin primer sequences were determined in this study using the NCBI primer-BLAST tool with the *G. mellonella* nucleotide database. The specificity of primers was checked against the *G. mellonella* genome and confirmed by agarose gel electrophoresis. Each qPCR reaction was carried out at a final volume of 20 µL, containing 20 ng of cDNA, 5 µL of 2x SYBR Green, and 10 pmol/µL of each primer. The RT-qPCR protocol consisted of the following conditions: 95°C for 15 min, then 30 cycles (95°C for 30 s, 52°C for 30 s, and 72°C for 30 s). The RT-qPCR protocol consisted of the following conditions: 95°C for 15 min, then 30 cycles (95°C for 30 s, 52°C for 30 s, and 72°C for 30 s). Each reaction was performed in triplicate, subsequently calculating the average threshold cycle (Ct) for each sample. The mRNA quantity has been normalized to the β-actin gene. The comparative CT (-ΔΔCt) method was employed to calculate fold differences for each gene with respect to the control (naive) (Livak & Schmittgen, 2001).

# RESULTS

## Gallerimycin expression after *Candida albicans* and bead injection

The expression of the gallerimycin gene in the larvae, hemocyte, and fat body of *G. mellonella* following injection with *C. albicans* is presented in Figure 1A. In larvae, minimal gene expression was seen at 4 h (16-fold) compared with 24 h (103-fold) relative to the control (naive larvae FD = 1.0), which indicates a delayed immune response in the larvae. Similarly, the expression of gallerimycin in hemocytes is significantly lower at 4 h (11-fold) compared with 24 h (680-fold) following the injection of the pathogen. The massive increase in gene expression at 24 h suggests that hemocytes develop a robust defensive response against *C. albicans* over time. However, gallerimycin expression in the fat body was relatively higher at 4 h (108-fold) than at 24 h (23-fold). The elevated gene expression in the fat body compared with larvae and hemocytes after 4 h indicates an early immune response in the fat body. The declining gene expression after 24 h indicated that the immune activity in the fat tissue diminishes after an initial response.

The gallerimycin expression in the larva, hemocyte, and fat body after injection of bead is given in Figure 1B. The expression of gallerimycin in larvae at 4 h (460-fold) was greater than that at 24 h (293-fold) following bead injection. After 4 h of injection, gallerimycin expression was comparable in hemocytes (255-fold) and fat body (278-fold); however, the gene expression significantly increased at 24 h in both hemocytes (5125-fold) and fat body (510-fold). The gene expression in fat body increased slightly, though the overall levels were much lower compared with hemocytes. This suggests the fat body mounts a delayed, but relatively weaker, immune response compared with hemocytes. Overall, it was noted that gallerimycin expression levels measured in larvae, hemocytes, and fat body following bead injection were significantly greater than those recorded after pathogen injection, at 4 and 24 h periods.

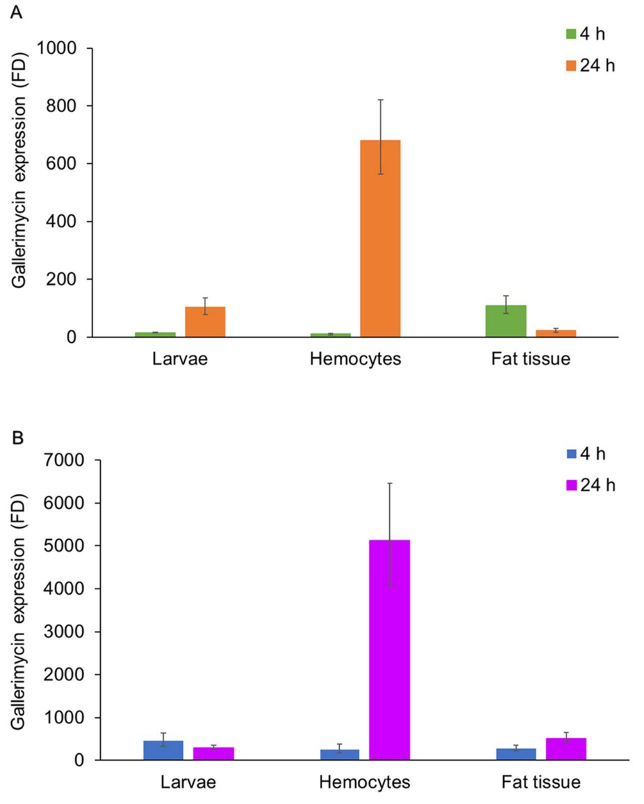


**FIGURE 1** Gallerimycin expression in whole larvae, hemocytes, and adipose tissue of *Galleria mellonella* following injection with (A) *Candida albicans* and (B) silica beads. Error bars represent asymmetric SD: For *Candida albicans* (A) [+1.13/−1.06 (Larvae 4 h); +3.12/−2.42 (hemocytes 4 h); +34.51/−26.18 (Fat tissue 4 h); (+32.62/−24.78 (Larvae 24 h); +141.34/−117.02 (hemocytes 24 h); +6.97/−5.37 (Fat tissue 24 h)]. For bead injection (B) [+178.17/−128.46 (Larvae 4 h); +123.75/−83.30 (hemocytes 4 h); +66.98/−53.98 (Fat tissue 4 h); (+61.93/−51.13 (Larvae 24 h); +1337.83/−1060.89 (hemocytes 24 h); +141.95/−111.03 (Fat tissue 24 h)].

## Galiomycin expression after *Candida albicans* and bead injection

In larva, hemocyte, and fat body, the galiomycin expression after 4 and 24 h injection of *C. albicans* was given in Figure 2A. After 4 h, the expression of galiomycin in larvae was similar to the control but increased eightfold after 24 h post-pathogen injection. The galiomycin expression was parallel in hemocytes (16-fold) and fat tissue (14-fold) after 4 h post-pathogen injection. Conversely, gene expression in hemocytes increased up to 88-fold, whereas it diminished in fat cells, falling below the control level. Hemocytes responded immediately to the fungal infection and continued to increase over time. Also, the immune response of fat tissue to pathogens may be more critical during the early phase of infection. While gallerimycin and galiomycin expression patterns were similar among larvae, hemocytes, and fat body after *C. albicans* injection, the expression levels were markedly different.

The galiomycin expression in larva, hemocytes, and fat body following bead injection is presented in Figure 2B. The

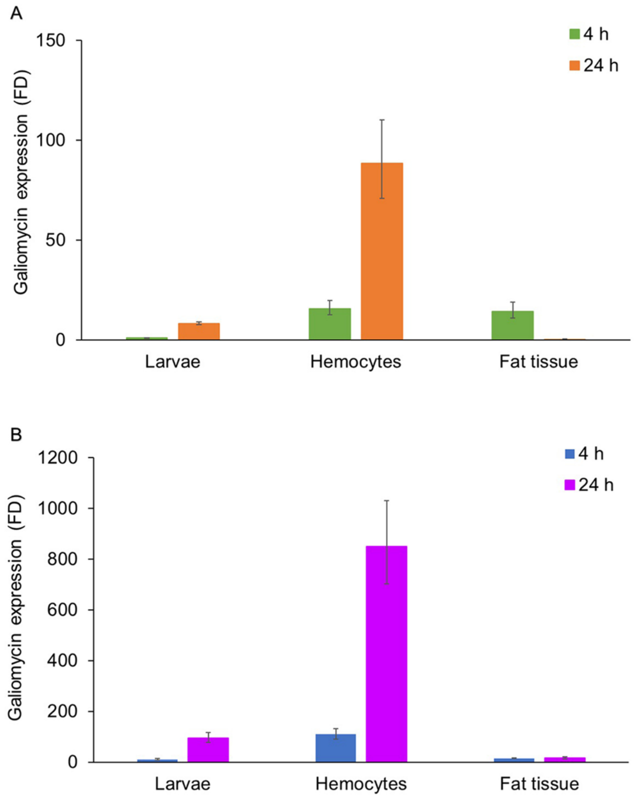


**FIGURE 2** Galiomycin expression in the whole larvae, hemocytes, and adipose tissue of *Galleria mellonella* following injection with (A) *Candida albicans* and (B) silica beads. Error bars represent asymmetric SD: For *Candida albicans* (A) [+0.08/–0.08 (Larvae 4 h); +4.04/–3.22 (hemocytes 4 h); +4.57/–3.47 (Fat tissue 4 h); (+0.73/–0.67 (Larvae 24 h); +21.68/–17.41 (hemocytes 24 h); +0.12/–0.09 (Fat tissue 24 h)]. For bead injection (B) [+3.22/–2.44 (Larvae 4 h); +22.62/–18.77 (hemocytes 4 h); +1.78/–1.60 (Fat tissue 4 h); (+21.87/–17.78 (Larvae 24 h); +180.35/–148.82 (hemocytes 24 h); +4.20/–3.34 (Fat tissue 24 h)].

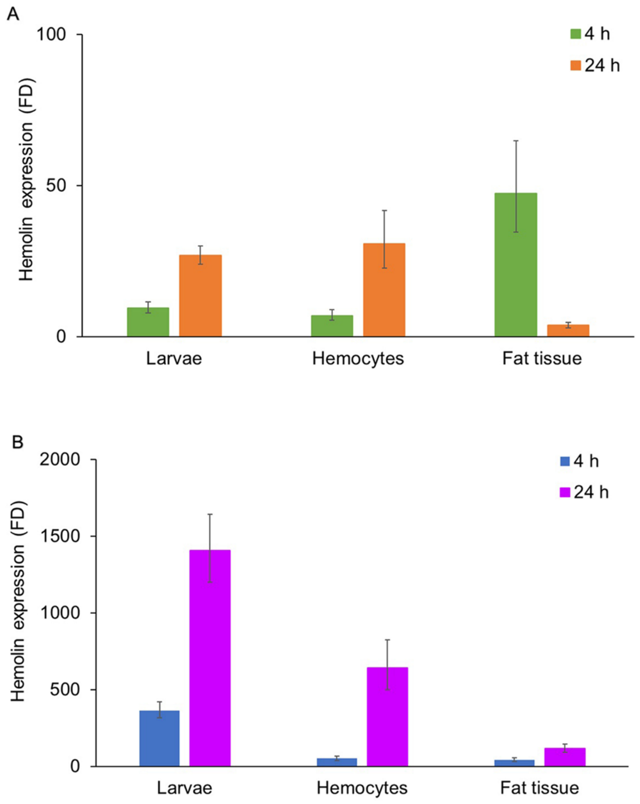


**FIGURE 3** Hemolin expression in the whole larvae, hemocytes, and adipose tissue of *Galleria mellonella* following injection with (A) *Candida albicans* and (B) silica beads. Error bars represent asymmetric SD: For *Candida albicans* (A) (+2.05/–1.69 [Larvae 4 h]; +1.85/–1.47 [hemocytes 4 h]; +17.45/–12.75 [Fat tissue 4 h]; +3.21/–2.86 [Larvae 24 h]; +11.01/–8.11 [hemocytes 24 h]; +1.01/–0.79 [Fat tissue 24 h]). For bead injection (B) (+55.67/–48.29 [Larvae 4 h]; +14.40/–11.21 [hemocytes 4 h]; +14.62/–10.85 [Fat tissue 4 h]; +238.80/–204.12 [Larvae 24 h]; +182.02/–141.82 [hemocytes 24 h]; +28.89/–23.10 [Fat tissue 24 h]).

gene expression in larvae at 4 h (10-fold) was lower than that at 24 h (95-fold) after bead injection, which indicates the gradual increase in galiomycin expression in larvae. Following bead ejection, gallerimycin expression in larvae decreased, but galiomycin expression increased, suggesting that the expression of AMPs occurs sequentially and gallerimycin serves as the primary response. Hemocytes showed a massive increase in gene expression both at 4 h (110-fold) and 24 h (851-fold) following bead injection. The progressive increase in galiomycin expression in larvae and hemocytes indicated a strong and long-lasting immune response, even to non-pathogenic foreign particles. Following the bead injection, galiomycin expression in the fat body was higher than control after 4 h (15.07-fold) and maintained its level at 24 h.

## Hemolin expression after *C. Albicans* and bead injections

The expression of the hemolin gene in the larva, hemocyte, and fat body of *G. mellonella* following injection with *C. albicans* was given in Figure 3A. The expression of hemolin in larvae following 4 h post-injection (10-fold) was lower than 24 h (27-fold), suggesting a delayed response. Hemocytes showed extremely low gene expression at 4 h (sevenfold), which eventually reached the same level as larvae at 24 h. The hemolin expression in the fat body was significantly increased in comparison with larval and hemocyte levels (47-fold) at 4 h, but it subsequently decreased to fourfold at 24 h.

In larva, hemocyte, and fat body, the hemolin expression after bead injection is given in Figure 3B. The expression level of hemolin in larvae was found to be higher after 24 h (1405-fold) of bead injection compared with 4 h (364-fold). Following bead injection, the gene expression in larvae is much greater than that observed after pathogen injection at both time points. Hemocytes showed a lower expression of hemolin at 4 h (51-fold) compared with 24 h (642-fold). The expression level of hemolin in the fat body at 4 h (42-fold) was comparable to the hemocyte level and exhibited a progressive increase after 24 h (115-fold).

The expression of hemolin in larvae was elevated in both *C. albicans* and bead injections; however, the increased expression following bead injection was considerably stronger (36 times at 4 h and 52 times at 24 h) than the modest increase observed in pathogen. In both *C. albicans* and bead injections, hemolin expression exhibited a delayed elevation, reaching its zenith at 24 h. Nonetheless, its response to the bead is greater than that elicited by the pathogen. The fat body showed a unique pattern. Hemolin expression level remained the same following beads and pathogen injections at 4 h; however, the expression increased after bead injection, whereas it decreased following pathogen injection at 24 h.

## Encapsulation ratio

In the encapsulation assay, a total of 193 and 271 silica beads were retrieved from larvae at 4 and 24 h post-injection, respectively, and categorized as non-encapsulated, weakly encapsulated, and strongly encapsulated (Figure 4). After 4 h, 13.5% of the beads were classified as non-encapsulated, 62.3% as weakly encapsulated, and 24.2% as strongly encapsulated (Figure 5). After 24 h, the proportions of non-encapsulated beads (4.6%) and weakly encapsulated beads (32.2%) decreased, whereas the ratio of strongly encapsulated beads (63.2%) increased. The results indicate a distinct, time-dependent increase in the encapsulation intensity of the immune cells of *G. mellonella*. The gradual enhancement of the encapsulation response may indicate the synchronized function of hemocytes, especially following the first recognition phase.

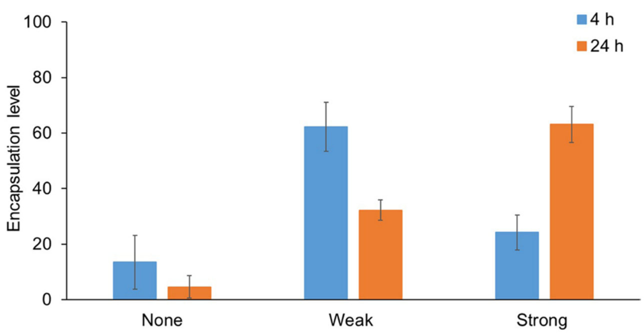


**FIGURE 4** Silica bead encapsulation ratios in *Galleria mellonella* larvae. Each bar represents the percentage of encapsulation determined in the larvae ($n = 15$). Error bars represent symmetric SD: None (±9.65 for 4 h; ±4.02 for 24 h); Weak (±8.81 for 4 h; ±3.69 for 24 h); Strong (±6.29 for 4 h; ±6.49 for 24 h).

## DISCUSSION

Insects possess an experienced innate immune system comprising both cellular and humoral systems that can function in coordination. The cellular responses include phagocytosis, nodulation, and encapsulation, mediated by hemocytes, whereas the humoral response involves AMPs, such as gallerimycin and other immunological proteins, like hemolin, which function as PRR. While the structure and antibacterial characteristics of AMPs vary among insect species, some AMPs are prevalent throughout the species. As of now, 20 distinct defense peptides have been found in the hemolymph of *G. mellonella*, with some peptides, such as gallerimycin and galiomycin, exhibiting increased levels in response to immunological challenges (Brown et al., 2008; Brown et al., 2009; Cytryńska et al., 2007; Tsai et al., 2016; Vilcinskas, 2011). The lethal dose of *C. albicans* ($2 \times 10^5$ cells/larva) injection into *G. mellonella* larvae increased the expression of gallerimycin and galiomycin genes in the fat body by 25-fold and 120-fold, respectively (Vertyporokh & Wojda, 2020). However, in another study, the gallerimycin expression (250-fold) was higher than the galiomycin expression (40-fold) in the fat body of larvae challenged with *Pseudomonas aeruginosa* (Schröter) (Andrejko et al., 2021).

In another study, AMPs expressions were determined in total larvae in response to oral administration of *Bacteroides vulgatus* Eggerth and Gagnon (1933) and *Escherichia coli* (Migula). Depending on the bacterial type, gallerimycin expression significantly increased at 4 h (approximately 1000-fold), with *E. coli* showing a more pronounced increase (Lange et al., 2018). Hemolin expression had a similar pattern, and at 4 h, it was found that hemolin expression was lower than gallerimycin expression in larvae. Previous studies determined AMP expressions either in the total larvae or in the fat body after challenged with various pathogens or compounds (Andrejko et al., 2021; Genç et al., 2024; Lange et al., 2018; Reis et al., 2023;

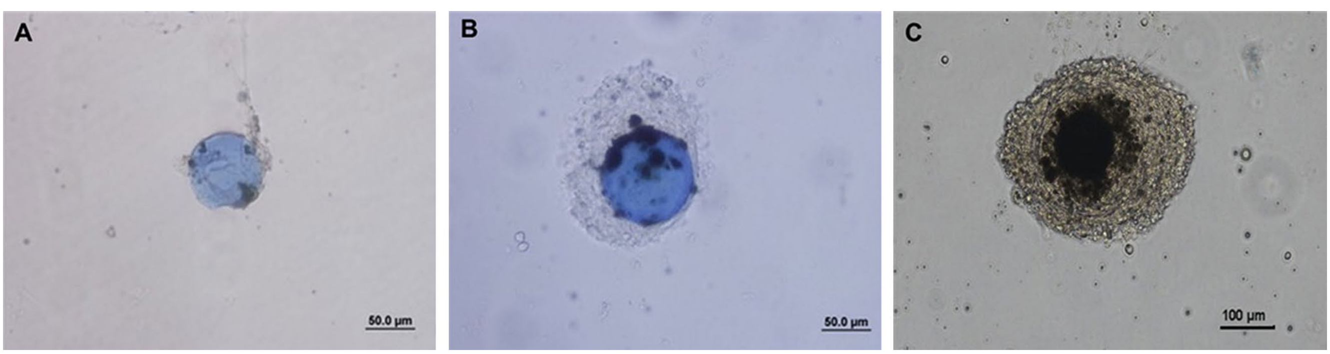


**FIGURE 5** Encapsulation levels of silica beads injected into *Galleria mellonella* larvae. (A) non-encapsulated, (B) weakly encapsulated, (C) strongly encapsulated.

Vertyporokh & Wojda, 2020). In our study, gallerimycin and galiomycin expressions were determined comparatively in total larvae, fat body, and hemocyte after injection of *C. albicans* and bead at 4 and 24 h time points. We showed that the gallerimycin expression was higher than galiomycin expression both in total larva and fat body after injection of *C. albicans* and bead, as indicated by previous studies (Andrejko et al., 2021; Asai et al., 2021; Genç et al., 2024; Lange et al., 2018). Interestingly, 24 h after pathogen and bead injections, hemocytes showed significantly higher AMP expression than fat body and total larvae. It was previously reported that in response to microbial invasion or integumentary damage, hemocytes in certain insects (e.g., *Drosophila melanogaster* (Meigen), *Bombyx mori* (L.)) produce various AMPs (e.g., cecropins, defensins, proline- and glycine-rich AMPs) and proteins (e.g., lysozymes, transferrins) (Eleftherianos, Heryanto, et al., 2021; Lemaitre & Hoffmann, 2007; Ma et al., 2019; Tattikota et al., 2020; Yadav et al., 2017; Yang et al., 2018). It has been previously noted that the cricket (*Gryllus bimaculatus* DeGeer) granulocytes play a more significant role in cellular immune responses upon encountering invading pathogens (Cho & Cho, 2019; Cho & Cho, 2024). Our study determined AMPs expression in *G. mellonella* hemocytes for the first time, and the results showed that hemocytes, not fat bodies, are the primary location of AMPs production. Consequently, hemocytes, likely granulocytes, are the principal immune cells in *G. mellonella*.

The immune response was far more pronounced to the bead than to *C. albicans*, which indicates that hemocytes are more effectively recognizing and responding to the bead, maybe due to its size, a different recognition mechanism, or longer-lasting detection of the bead as foreign. The markedly elevated response to the bead may suggest that the immune system perceives the bead as a permanent, nondegradable entity, leading to continuous immunological activity. The fat tissue showed lower levels of AMPs expression after both *C. albicans* and beads injections. Nevertheless, the bead injection resulted in a slight increase in gallerimycin expression after 24 h, whereas the expression decreased with *C. albicans*. This difference could reflect the more transient immune function of fat tissue in response to *C. albicans*, whereas the beads elicit a more sustained response. The total larva tissues have a more delayed and moderate immune response, suggesting that they contribute to the systemic immune defense but with less intensity than hemocytes. This pattern likely reflects how various tissues coordinate over time to handle the infection, with hemocytes being the major contributors to immune defense after the initial stages. Previous observations show that *G. mellonella* enhances the efficacy of its innate immune response by co-presenting several AMPs, which show increased effectiveness at lower amounts when administered concurrently (Bolouri Moghaddam et al., 2016). Our data suggest that AMP expression in fat tissue and total larvae (including other larval tissues) during the early immune response (at 4 h) was comparable to or lower than that in hemocytes, suggesting its significant role in enhancing immune response efficacy. Based on AMP expression levels in total larvae, fat bodies, and hemocytes, it can be inferred that this simultaneous AMP release occurs in various tissues at various rates and time intervals in a controlled manner.

Hemolin is an alternative indicator immune protein of cellular response pathways. It is categorized as an opsonin, facilitating recognition of pathogens and regulating hemocyte-mediated immune responses (Eleftherianos et al., 2007; Yu et al., 2002; Zhao & Kanost, 1996). Hemolin gene expression was reported in the fat body, hemocytes, midgut, and epidermis of insects, such as *Manduca sexta* Johannsen, *Plodia interpunctella* (Hübner), and *B. mori* (Aye et al., 2008; Wang et al., 1995). Previous research has documented hemolin gene expression in the fat body, silk gland, midgut, nervous system, and whole larvae of *G. mellonella* at various time intervals in response to various types of pathogens (Lange et al., 2018; Shaik & Sehnal, 2009; Sułek et al., 2024). Our work demonstrated for the first time that the hemolin gene is expressed in the hemocytes of *G. mellonella*. Hemolin expression levels and patterns in whole larvae and hemocytes were similar, indicating delayed hemolin expression, and the fat body was the site of early response following pathogen injection. After the bead injection, the hemolin gene expression pattern was similar in larvae, fat bodies, and hemocytes, but the early response site was mainly the other tissues (e.g., silk gland) in larvae rather than hemocytes and fat bodies. Previous studies have shown that the injection of bacteria increases hemolin gene expression in *G. mellonella* silk glands to a level similar to that in the fat body and midgut (Shaik & Sehnal, 2009). In our research, the expression of hemolin in the fat body was lower than that in the whole larva, which encompasses the nervous system, midgut, silk glands, fat body, and also hemocytes. Our results demonstrate that during the late response, hemolin gene expression in *G. mellonella* mostly occurs in hemocytes, as seen clearly by observations following bead injection, whereas early response expression was noted mainly in other larval parts.

Hemolin is a PRR and recognizes the pathogen associated molecular patterns (PAMPs). Hemolin attaches to hemocytes, facilitating their aggregation, recognizes specific patterns on pathogens (e.g., LPS, LTA, and β-1,3-glucan), and subsequently enhances phagocytosis to remove foreign particles (Aathmanathan et al., 2018; Asai et al., 2021; Eleftherianos et al., 2007; Jung et al., 2019). In our research, the hemolin expression level following the bead injection was elevated compared with that of pathogen injection, with the exception of fat body at 4 h, which was at a similar level to that of the pathogen injection. The bead surface lacks any structure or PAMPs recognized by the hemolin receptor, despite the presence of the β-1,3-glucan structure in *C. albicans* (Gow et al., 2017; Lenardon et al., 2020). This phenomenon can be explained by several aspects, especially those related to foreign body recognition and elimination processes. The dimensions of individual *C. albicans* cells range from 3 to 8 μm in diameter, whereas DEAE Sephadex™ A-25 chromatography beads range between 40 and 120 μm, simulating the size of an invader (e.g., parasitoid egg). When the small-sized foreign particles, PAMPs (e.g., β-1,3-glucan), were recognized by PRRs, phagocytosis was stimulated by insect hemocytes. If the number of target cells exceeds the capacity of hemocytes to phagocytize, or if the small particles form bulky clusters, the nodulation process begins. Upon recognition of large foreign invaders, including parasites, nematodes, and their analogs (e.g., silica beads), the encapsulation process was activated, leading to hemocyte (granulocytes and plasmatocytes) adhesion to the surface of foreign entities, resulting in self-destruction and release of effector immunoregulators (Dubovskiy et al., 2016; Lavine & Strand, 2002). The released immunoregulators can cause the activation of hemolin expressions as well as the expression of AMPs. It was previously reported that granules of the granulocytes may involve receptor proteins, such as PGRPs, GNBP, and βGRP (Jiang et al., 2010). The degranulation of granulocytes, which is the initial stage of encapsulation, can also result in the release of these receptor proteins present in granules into the hemolymph to trigger a humoral immune response, such as AMPs synthesis. Therefore, the gene expression levels after the bead injection are higher than the expressions after pathogen injection.

In our research, we observed a weak encapsulation rate during the initial 4 h, which was followed by a strong encapsulation rate during the subsequent 24 h, following the bead injection. In a previous study, researchers observed 9% strong encapsulation 4 h after bead injection and found that this rate increased to 65% at the end of 24 h (Kaya et al., 2022). This situation is closely related to the recognition of the beads as a foreign particle and the relatively gradual encapsulation process around them. The results demonstrated that the encapsulation rate correlated with the gene expression levels of AMPs (gallerimycin and galiomycin) and PRRs (hemolin), both of which increased 24 h post-infection. This indicates that the immune system, in opposition to the foreigner, generates an expanding response until it confines or destroys the invader. As a result, the immune system perceives the bead as a potential threat due to its size and foreign structure, triggering a broad-spectrum defense response mechanism that includes the players of both cellular (e.g., encapsulation) and humoral responses (e.g., PRRs and AMPs). The findings of this study indicated that the key player of the cellular and humoral responses is hemocytes. Therefore, we can assume that the loci of AMPs and hemolin gene expression are granulocytes, which contain granules essential for hemocyte aggregation and encapsulation.

In conclusion, our research offers new insights into the tissue-specific generation of AMPs and hemolin in *G. mellonella* larvae after immune challenges with *C. albicans* and silica beads. Hemocytes were recognized as the principal site for AMP synthesis, indicating a strong immune response. This suggests that hemocytes, presumably granulocytes, are pivotal in the immune response of *G. mellonella*, considerably enhancing early defensive systems. The delayed yet persistent immune activity in adipose tissue further confirms the orchestration of immune responses among various tissues. The results further confirm the expression of hemolin in hemocytes, underscoring its role in cellular immune processes. Collectively, our findings highlight the complex nature of the immune response in *G. mellonella*, demonstrating that it is a finely regulated process involving multiple tissues and governed by the characteristics of the immunological challenge. The synchronized engagement of various immune cells and organs ensures the effectiveness of the insect's innate immune defenses.

## AUTHOR CONTRIBUTIONS

**Serhat Kaya:** Conceptualization; investigation; project administration; funding acquisition; resources; supervision; data curation; writing – original draft; writing – review and editing; methodology. **Tülay Turgut Genç:** Conceptualization; investigation; project administration; resources; supervision; data curation; writing – original draft; writing – review and editing; methodology. **Melih Günay:** Investigation; data curation; writing – review and editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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