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Histological Observation and Expression Patterns of antimicrobial peptides during Fungal Infection in *Musca domestica* (Diptera: Muscidae) Larvae

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ABSTRACT

Housefly, *Musca domestica*, has a complicated immune system. However, its underlying operating mechanism remains elusive. *Candida albicans* is a major pathogen affecting humans by causing deep infection fungous disease, but it is non-symbiotic in houseflies. To investigate the *C. albicans* infection process in housefly, the changes in morphological and histological and expression patterns of antimicrobial peptide were monitored to indicate the insect's response to fungal infection. The results showed that scattered brown spots were comprising melanized encapsulation and encapsulated fungal cells were initially observed at the inner side of larvae's body wall 3 h post-infection (PI). Between 6 and 36 h PI, the whole body of larvae was densely covered with the brown spots, which then gradually disappeared. The majority had disappeared at 48 h PI. Some fungi colonized in the gaps between the body wall and the muscle layer, as well as among muscle fibers of the muscle layer at 12 h PI and hyphal was observed at 18 h PI. These fungi colonized distribution changed from a continuous line to scattered spots at 24 h PI and virtually disappeared at 48 h. The results of quantitative PCR analysis revealed that in coordination with the variation during the infection, the expression levels of four antimicrobial peptides were up-regulated. In conclusion, *C. albicans* infection in *M. domestica* larvae involved the following stages: injection, infection, immune response and elimination of the pathogen. The rapid response of antimicrobial peptides, melanized encapsulation and agglutination played a vital role against the pathogenic invasion.

Key words: *Musca domestica*, larvae, *Candida albicans*, infection, innate immunity

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INTRODUCTION

Insects lack the adaptive immune responses typical of vertebrates; thus, they are heavily dependent on their innate immune system to guard against pathogen invasion (Hoffmann 2003). Vector insects, with their susceptibility to human pathogens, which can carry a great variety of pathogens and cause many diseases, hence lead to serious damages to human health, and their immune response mechanism is highly homologous to the mammalian innate immune response (Cirimotich et al. 2011; Stokes et al. 2015). In what way the insect innate immune system responds to human pathogens has become a recent focus of research. Many studies have investigated the host–pathogen interaction between insects and pathogen; for example, the interaction between the insects *Galleria mellonella*, *Drosophila melanogaster*, silkworm and *Tribolium castaneum* and the pathogens *Candida albicans*, *Cryptococcus neoformans*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae* (Altincicek et al. 2008; Fuchs et al. 2010; Chamilos et al. 2011; Wang et al. 2013). Recent research indicates that the insect immune system can be activated by the alien pathogens. When the insects are infected by the invading pathogens, the pattern recognition proteins/receptor (PRPs) in insects would firstly recognize and combine with pathogen-associated molecular pattern (PAMPs) in the pathogens, and then initiate the activation and regulation of a series of innate immune response (Ohta et al. 2006). On the one hand, the hemolymph cell served as the major cellular immune response triggering cytophagocytosis and melanization tubercle formation (Lavine and Strand 2002; Lu 2008; Wu et al. 2015). On the other hand, immune effector molecules such as antimicrobial peptides (AMPs) and lysozymes synthesized and secreted by fat body cells (i.e. similar to mammals' liver), hemocytes and other cells trigger humoral immune responses (Lemaitre and Hoffmann 2007). The innate immune response in insects is divided into humoral immunity and cellular immunity. They function together to kill and eliminate the pathogens via phagocytosis, nodulation, encapsulation, coagulation, and melanization.

Houseflies are present worldwide and considered to be an important medical insect which can carry and transmit over 100 human pathogens and zoonotic agents, but they rarely to be infected (Scott et al. 2014). It has been demonstrated that housefly larvae were prone to induce the generation of AMPs such as attacin, cecropin, defensin and diptericin by means of heat shock, ultraviolet exposure and needle piercing (Wang et al. 2006; Liang et al. 2006; Dang et al. 2010; Liu et al. 2011). Once the adult housefly is infected with *Beauveria bassiana*, hemocyte density changes and fungi number in the hemolymph are synchronized (Mishra et al. 2015). The published genome sequences analysis of housefly indicated that compared with Drosophila melanogaster, the genome contains a rich resource of shared and novel protein coding genes, a significantly higher amount of repetitive elements, and substantial increases in copy number and diversity of both the recognition and effector components of the immune system, including a large number of genes encoding PAMPs, immune-related signalling pathways, immune effector molecules and antimicrobial peptides (Scott et al. 2014). Moreover, many specific peptides with strong antibacterial effects are now being discovered in houseflies, including MAF-1, Muscin and MDAP-2 (Fu et al. 2009, 2015; Pei et al. 2014; Yang et al. 2015). These results indicate that houseflies are gifted with a complicated innate immune system that has likely adapted to the complex living environment, particularly to the pathogens these houseflies carry and transmit. However, the mechanisms underlying the operation of this unique innate immune system remains elusive, and the interactions between houseflies and pathogens require further studies.

C. albicans is an opportunistic fungus that can cause deep fungal infections and severely harm human health (Miceli et al. 2011). However, it is an exogenous and non-symbiotc fungus in a housefly (Phoku et al. 2014), furthermore, there was no direct evidence that *Candida albicans* is a natural pathogen of *M. domestica*. In our preliminary study, all the larvae which injected with 210 nl PBS buffer were able to complete their life cycle, and in the meanwhile, the larvae injected with an equal volume of the fugal suspension (i.e. approximately 2×10^4 CFU of *C. albicans*) were controlled with the ratio of approximately 50%. On the basis of predecessors study, it provides a good opportunity to investigate the interrelation between an exogenous fungus and the innate immune system of a housefly during the period of infection and recovery. Therefore, we sought to describe the

infection process and broaden the understanding of the insect's immune response to fungal infections.

MATERIAL AND METHODS

House fly rearing

Houseflies were bred in the Modern Pathogenic Biology Laboratory, Guizhou Medical University (Guiyang, China) (Fu et al. 2009). Their larvae were raised on an artificial diet comprising bran and water and routinely reared at 26–28°C with 70–80% relative humidity and a photoperiod of 12 h: 12 (Light: Dark) h for up to the third-instar larval stage.

C. albicans culturing

The *Candida albicans* (ATCC10231) used in infection experiments was stored at our laboratory. *C. albicans* was cultivated in Sabouraud dextrose agar (SDA) and its cell suspension was prepared by inoculating a single colony in Sabouraud dextrose broth (SDB) at 37°C for 12 h with agitation (Kelly and Kavanagh 2011). Fungus entering the logarithmic growth phase was selected for infection experiments. *C. albicans* cells solution were centrifuged at 4000 g/min and washed and re-suspended in phosphate buffered saline (1 × PBS 0.01M, Solarbio, China) and standardized to 1×10^8 CFU/ml.

Infection experiments

Each experimental group comprised 200 randomly chosen third-instar larvae of appropriate weight (26 ± 0.5mg). The infected group (IC group) larvae were injected with 210 nl suspension, i.e. approximately 2×10^4 CFU of *C. albicans* at the 10th segment of segmental venter of larvae using the Auto-Nanoliter Injector (Nanoject II Nanoliter Injector, Drummond Scientific Co., Broomall, USA) under a stereomicroscope (SMZ25, Nikon, Japan) (Khalil et al. 2015). After injection, these larvae were raised on sterilized bran and water. At 3, 6, 12, 18, 24, 36 and 48 h post-infection (PI), the larvae were collected. Untreated larvae were also collected (blank control group, C group), and larvae injected with an equal volume of PBS buffer were used as the negative control group (PBS group).

Morphological and histological observations

Larvae were collected at the indicated time points. Morphology was observed using a stereoscopic microscope (SMZ25, Nikon, Japan). After embedding in Tissue-Tek optimal cutting temperature compound (OCT, Sakura Finetek, Netherlands), the larvae were transferred to a microtome cryostat (MEV3.01, SLEE, Germany). Histological sections of 10 μm were removed and subjected to periodic acid Schiff (PAS) staining (Okada et al. 2013), observed and photographed using a microscope (Eclipse Ci-S, Nikon, Japan) and *NIS-Elements System* (NIS-Elements, Nikon Instruments Inc., USA).

qPCR analyses of AMP genes expression

Total RNA was isolated from 6-8 larvae using TRIzol® Reagent (TRIzol® Reagent, Invitrogen, USA) strictly following the manufacturer's protocol. The concentration and purity level of samples was detected by NanoDrop (ND1000, Thermo Scientific, USA) and agarose (1%) gel electrophoresis. Then, 1 μg of total RNA was reverse-transcribed in 10 μl reaction volumes using PrimeScript RT (PrimeScriptTM RT reagent Kit with gDNA Eraser, Takara Bio, Japan) with random hexamer and oligo dT primers. After synthesis, cDNA was diluted 1: 10 and used in quantitative polymerase chain reaction (qPCR) analysis. Each reaction was performed using the SYBR Premix (SYBR Premix Ex TaqTM II, Takara Bio, Japan), and reactions were performed using the real-time PCR system (ABI PROSM 7300, Applied Biosystems, USA). Reaction mixtures were incubated for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 34 s at 60°C. Primers for attacin, cecropin, defensin, diptericin and reference (rps18) genes were selected on the basis of previous studies on housefly immune responses (Wang et al. 2009; Fleming et al. 2014). The primers were designed using Primer 5.0 software (Primer PREMIER Version 5.00, PREMIER Biosoft International 2000). The primers used for qPCR are listed in Table 1. For relative expression analysis, CT values for calibrator conditions (i.e. larvae untreated at each indicated time points, C group) and treatment groups (i.e. PBS and IC groups) were calculated using the $2^{-\Delta\Delta CT}$ method (Pfaffl et al. 2002). Log_2-fold change in the expression ratios of the target genes attacin, cecropin, defensin and diptericin were compared with the calibrator condition using the reference gene rps18. Statistical significance difference in the IC and PBS groups were analyzed by multiple *t*-test using the Holm-Sidak method (PASW Statistics 18, SPSS Inc. 2010). All data was

analysed to determine differences between groups ($P < 0.05$).

Table 1 Primer sequences for qPCR

Gene	Access no.	Sense Sequence (5′-3′)	Antisense Sequence (5′-3′)
rps18	KC424479.1	GCGTGACGATTTGGAACGCTTGA	TTCTTGGATACACCGACAGTGCGA
attacin	DQ062744.1	CGAATTCATGTTCTCTAAATC	CTCTAGATTAAAAATCATGACTT
cecropin	ES608437.1	GGACAAAGTGAAGCTGGATGGTTG	GCTGGGCCACACCAATAGTTTGA
defensin	ES608345.1	AAATTTCGTCCATGGAGCTGACGC	ACCGCTCAACAAATCGCAAGTAGC
diptericin	ES608652.1	AGTGCAACATTTGTGGTTGCCGAC	GCCATAACCTGCTGTGGCATCA

RESULTS

Morphological observations

Morphological observations revealed no changes under the epidermis of the larvae in the C and PBS groups (Fig. 1A, Fig. 1B). However, under the body wall of the larvae in the IC group, many scattered brown spots were observed PI. At 3 h PI these spots were scattered and distributed at the inner side of larvae's body wall (Fig. 1C), and at 6 h PI, the spots had substantially increased in both number and size (Fig. 1D). At 12, 18 and 24 h PI, the entire body was densely covered by brown spots of various sizes (Figs. 1E, 1F and 1G). At 36 h PI, the quantity of these spots had significantly decreased and their colour had faded (Fig. 1H). Majority of the spots had disappeared at 48 h PI (Fig. 1I).

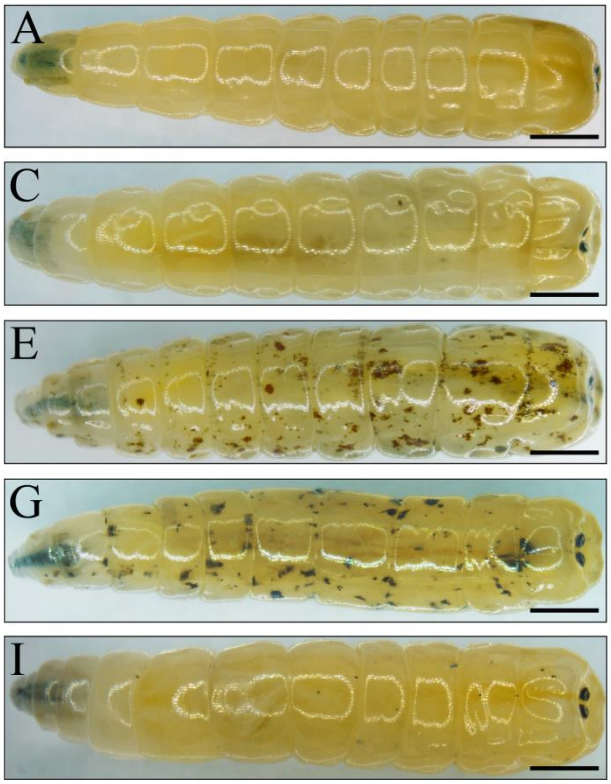

Figure 1 Morphology observation of housefly larvae after *Candida albicans* injection. **A** Blank control group: untreated larvae. **B** PBS negative control: larvae were injected with 210 nl PBS buffer. **C-I** Infection group: the larvae were injected with 210 nl suspension of *C. albicans* (2×10^4 CFU). **C** 3 h post infection (PI), **D** 6 h PI, **E** 12 h PI, **F** 18 h PI, **G** 24 h PI, **H** 36 h PI and **I** 48 h PI. The arrow indicated the brown spots under the body wall of the larvae. (A–I) Scale Bar = 1.0 mm.

Histological observations

We performed histological studies to better observe *C. albicans* infection process in the housefly. Periodic Acid Schiff (PAS) staining could clearly distinguish larvae tissue and fungi; tissue was stained light or dark blue, whereas *C. albicans* was stained purple red. There were no differences between the larvae in the C and PBS groups (Figs. 2A and 3A, Figs. 2B and 3B). We observed a certain amount of *C. albicans* cells distributed in the gaps between the body wall and the muscle layer, as well as among muscle fibers of the muscle layer of infected larvae in the IC group. At 3 and 6 h PI, considerable aggregation of *C. albicans* cells (yeast forms) distributed in these gaps (Figs. 2C and 3C, Figs. 2D and 3D). At 12 h PI, fungal cells were observed colonization in a linear arrangement in these gaps between the body wall and the muscle layer and among muscle fibers of the muscle layer. The growth of the fungus and its filamentation led to thses tissues invasion, some of them were observed with short hyphae (Figs. 2E and 3E). At 18 h PI, the distribution of fungal changed from a continuous line to scattered spots, and a significant number of *C. albicans* with hyphae and yeast forms were observed (Figs. 2F and 3F). At 24 h PI, majority of the *C. albicans* had hyphae (Figs. 2G and 3G). At 36 h PI, the number of *C. albicans* had significantly decreased; furthermore, *C. albicans* with hyphal forms were sparsely dotted in the muscle tissue (Figs. 2H and 3H) and had virtually disappeared at 48 h PI (Figs. 2I and 3I).

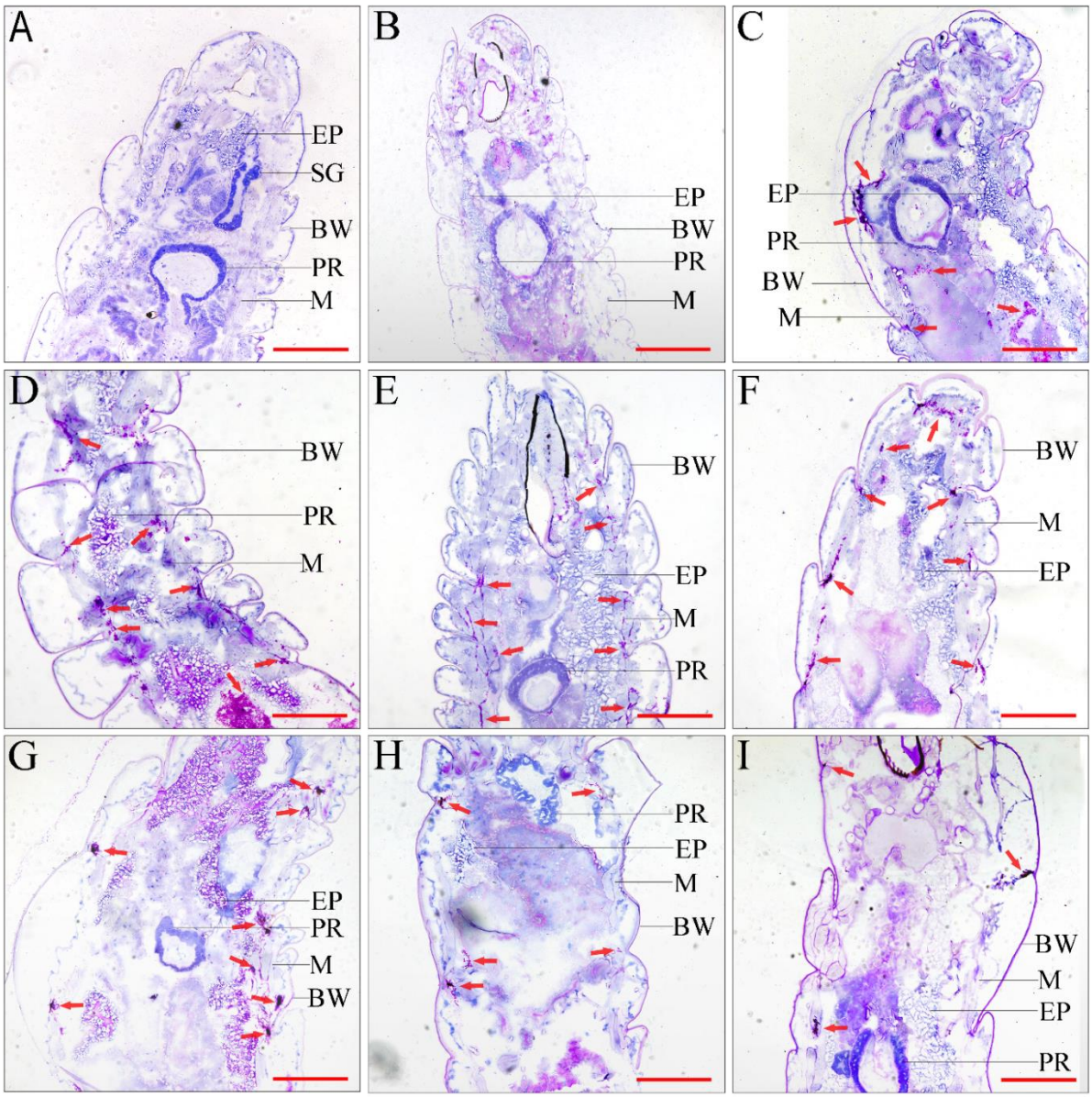

Figure 2 Histopathological identification of *C. albicans* in housefly larvae (PAS stain, ×40). **A** Blank control group: untreated larvae. **B** PBS negative control: larvae were injected with equal volume PBS buffer. **C-I** Infection group: larvae were injected with *C. albicans* larvae. **C** 3 h post-infection (PI), **D** 6 h PI, **E** 12 h PI, **F** 18 h PI, **G** 24 h PI, **H** 36 h PI and **I** 48 h PI. Proventriculus (PR), salivary glands (SG), body wall (BW), epiploon (EP), muscle (M), arrows indicate *C. albicans*. *Bar* = 500 µm

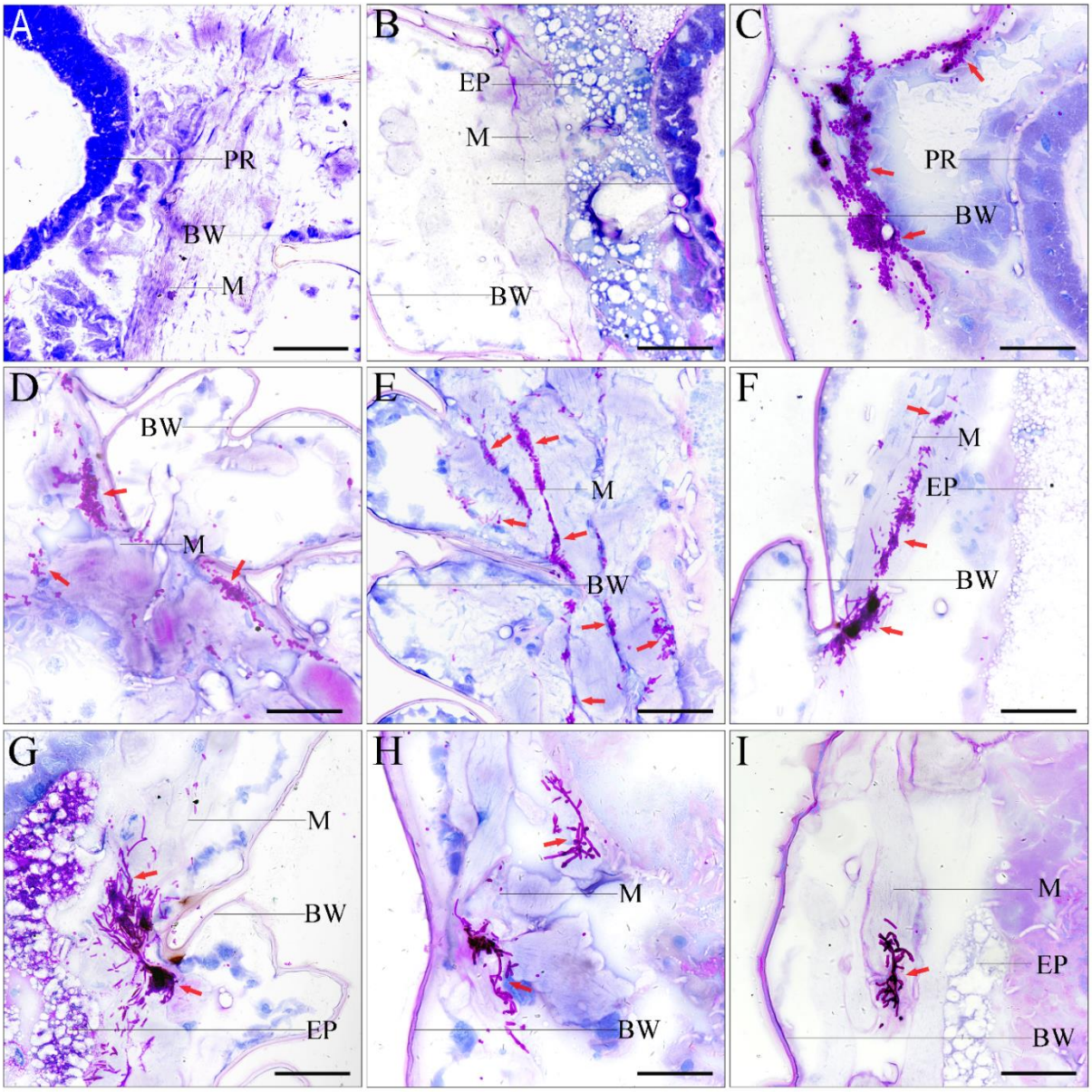

Figure 3 Histopathological identification of *C. albicans* in housefly larvae (PAS stain, ×200). **A** Blank control group: untreated larvae. **B** PBS negative control: larvae were injected with equal volume PBS buffer. **C-I** Infection group: larvae were injected with *C. albicans* larvae. **C** 3 h post-infection (PI), **D** 6 h PI, **E** 12 h PI, **F** 18 h PI, **G** 24 h PI, **H** 36 h PI and **I** 48 h PI. Proventriculus (PR), body wall (BW), epiploon (EP), muscle (M), arrows indicate *C. albicans*. *Bar* = 100 μm

In all infected larvae, many scattered brown spots were observed. These spots were dark in colour because of the accumulation of melanin. Another plane of histological sections revealed that these spots were comprising melanized encapsulation and encapsulated agglutination of fungal cells, spread among the coelom, muscle tissue and the inner side of the body wall (Fig. 4). Between 3 and 12 h PI, we observed a considerable number of *C. albicans* with yeast forms aggregated around the melanized encapsulation (Figs. 4A, 4B and 4C), the yeast and hyphal forms were both present between 18 and 24 h (Figs. 4D and 4E). *C. albicans* majority existed as hyphal forms at 36 h and 48 h (Figs. 4F and 4G), and some empty capsules without fungal cellss were found at 48 h PI (Figs. 4H and 4I).

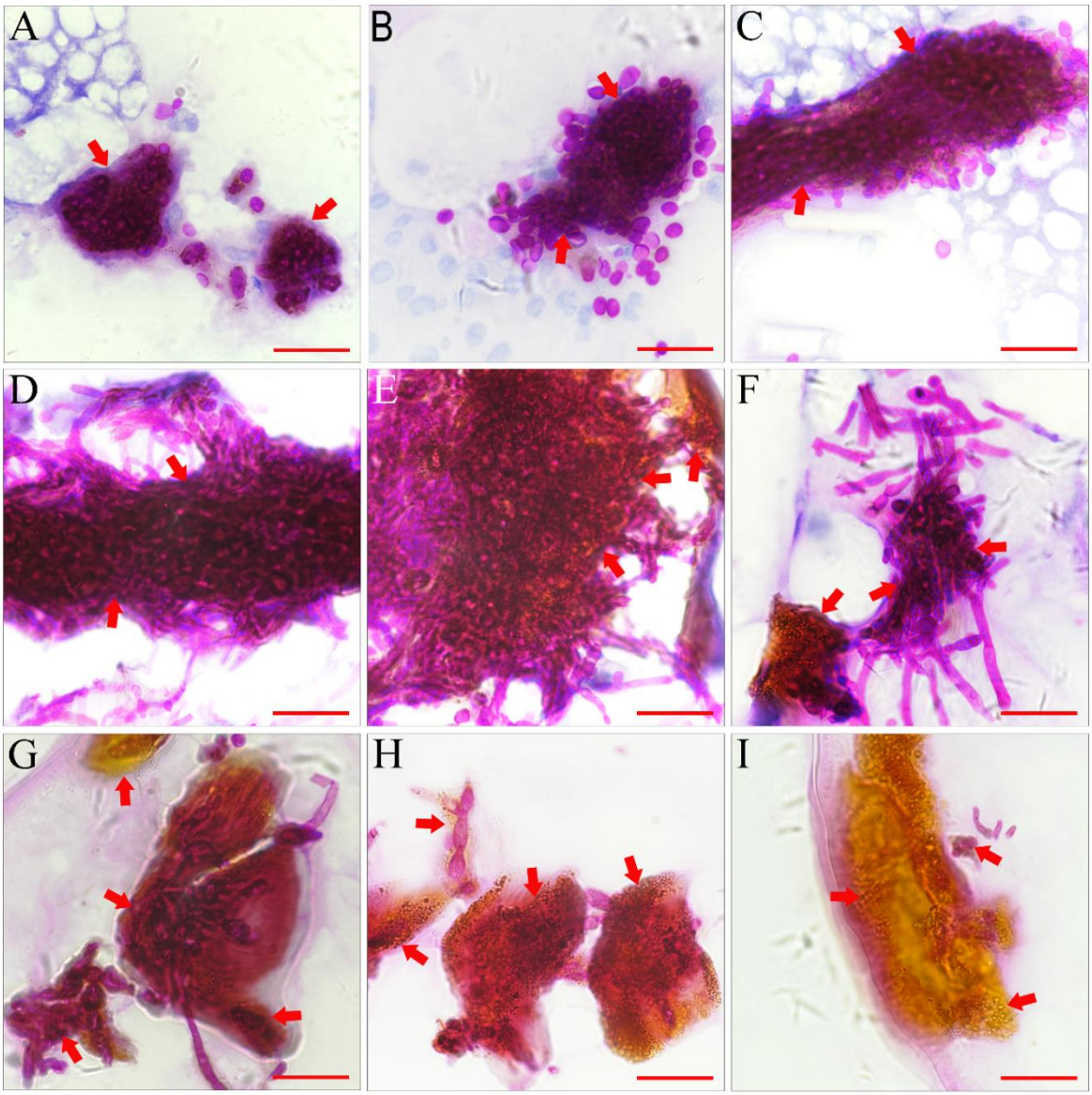

Figure 4 Histopathological identification of melanized encapsulations. **A** 3 h post-infection (PI), melanized encapsulations encysted yeast from fungal cells. **B** 6 h PI similar to 3 h PI. **C** At 12 h PI, melanized encapsulations encysted yeast from fungal cells. **D** At 18 h PI, melanized encapsulations encysted yeast and hyphae from fungal cells. **E** 24 h PI similar to 18 h PI. **F** At 36 h PI, melanized encapsulations encysted hyphae from fungal cells. **G** At 48 h PI, melanized encapsulations encysted hyphae from fungal cells. **H** At 48 h PI, melanized encapsulations encysted limited amount of hyphae from fungal cells. **I** At 48 h PI, empty encapsulations. Arrows indicate melanized encapsulations. *Bar* = 50 μm

qPCR analyses of AMP genes expression.

qPCR was used to assay the temporal patterns of mRNA from attacin, cecropin, defensin and diptericin at respectively challenged 3rd-instar larva at 3, 6, 12, 18, 24, 36 and 48 h after *C. albicans* infection. Comparing with the PBS group, the results revealed that in coordination with the variation during the infection process, the expression levels of four antimicrobial peptides were up-regulated, but their expression patterns were different from each other (Fig. 5). Attacin, cecropin and defensin were gradually increased at 3 h PI, but diptericin was not clearly up-regulated ($P > 0.05$). Between 6 and 24 h PI, all four AMPs were significantly up-regulated ($P < 0.05$, in all cases). Expression level of attacin, cecropin peaked at 12 h PI, denfensin peaked at 3 h PI and diptericin peaked at 24 h PI. And then from 36 to 48 h PI, expression level of attacin, defensin and diptericin sharply decreased ($P > 0.05$, in all cases), and cecropin

expression level slowly decreased ($P < 0.05$, in all cases).

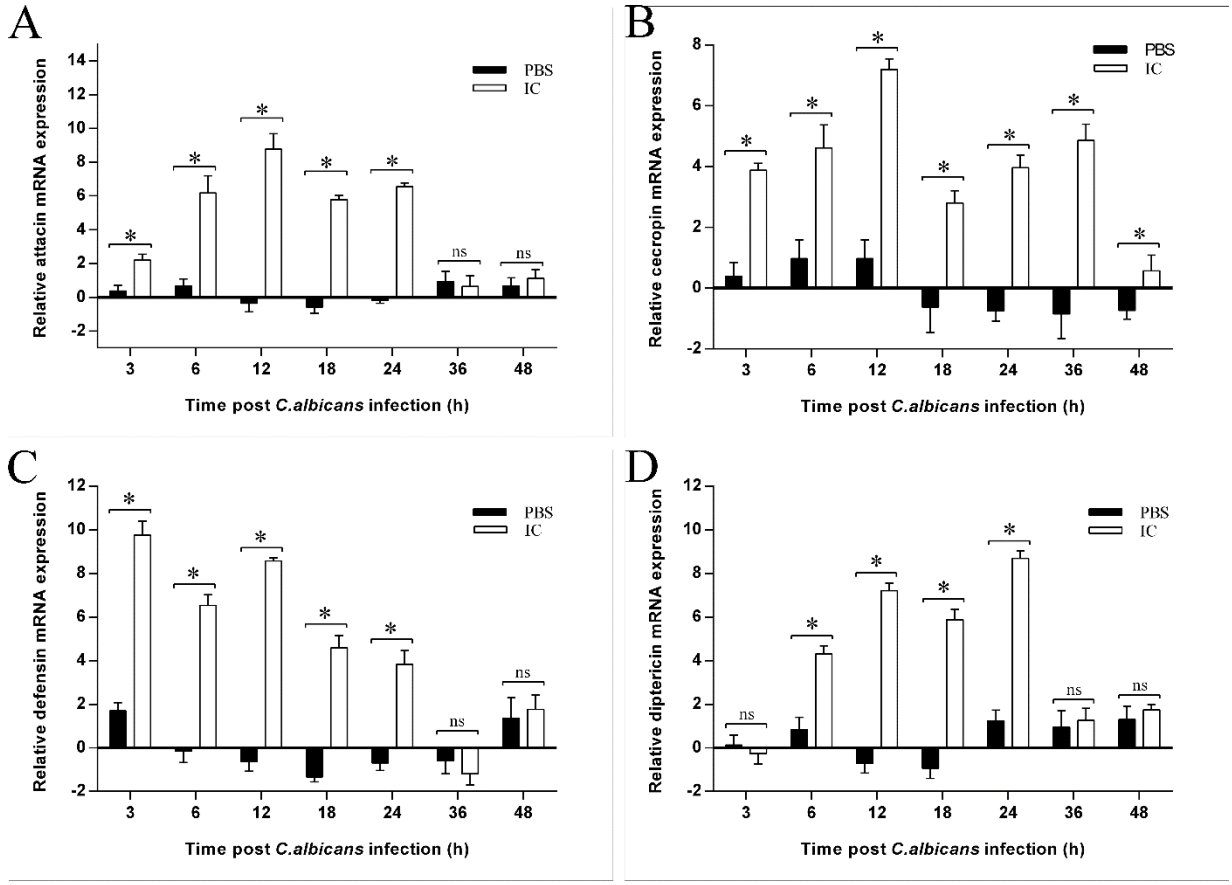

Figure 5 AMP genes expression pattern in housefly larvae with *C. albicans* infection. In the IC group, the larvae were injected with 210 nl suspension of *C. albicans* (2×10^4 CFU). In the PBS group, the larvae were injected with equal volume PBS buffer. The C group was untreated. Collecting samples ($n = 4$ larvae per time point) at 3, 6, 12, 18, 24, 36 and 48 h post-infection. The C group was set as calibrator conditions, compared with the PBS and IC groups. For relative expression analysis, CT values for calibrator conditions (i.e. larvae untreated at each indicated time points, C group) and treatment groups (i.e. PBS and IC groups) were calculated using the $2^{-\Delta\Delta CT}$ method. Log_2-fold change in expression ratios of the target genes attacin, cecropin, defensin and diptericin was compared with the calibrator conditions using the reference gene rps18. **A** attacin, **B** cecropin, **C** defensin, **D** diptericin. Data was presented as mean ± SE. Significance levels: * $P < 0.05$; ns, not significant

DISCUSSION

Insects lack the adaptive immune responses typical of vertebrates; thus, they are heavily dependent on their innate immune system to defend against pathogenic invasion. Knowledge on how the insect innate immune response fights infection may aid the understanding of the first step in the human host–pathogen interaction (Wang et al. 2013; Singh et al. 2014; Wu et al. 2014; Khalil et al. 2015). Many models and techniques of insect infection have been successfully established, including injury, rolling and ingestion methods. The rolling method involves the infection of the epithelial cell surface and the ingestion method involves the infection of the digestive tract mucous membrane. These methods model the natural infectious process of pathogenic invasion into the epidermis and intestinal tract, which can then cause local infection that may gradually develop into systemic infection (Onfelt et al. 2001; Nayduch et al. 2013; Wu et al. 2014). The injury method mainly used needles to deliver pathogens by piercing the somatic layer or directly injects pathogens into the insect coelom to cause acute infection and rapidly activate the immune response (Matsumoto et al. 2012; Singh et al. 2014). However, the inevitable mechanical injury caused by this method may affect the experimental results. Micro-injection technology has recently been applied in *D. melanogaster*

infection to achieve controllable infection and minimize mechanical injury by a precise and quantitative inoculation (Khalil et al. 2015). In our preliminary study, the third-instar housefly larvae infection model was built using minimally invasive injection methods. The previous results have indicated that all the larvae of the PBS group were able to complete their life cycle, and in the meanwhile, the larvae of the IC group were controlled with the ratio of approximately 50%. In this study, morphological and histological investigation revealed no significant difference between larvae that were not inoculated and those that were inoculated with PBS (Figs.1 and 2). Although the qPCR results revealed a slight difference in AMP expression between the PBS and C groups, no significant differences were observed. This fluctuation may have been caused by the activation of a weak immune response by the micro wound on the larvae somatic layer caused by injection.

We observed that antibacterial peptides were synthesized in response to microorganism infestation. The housefly genome contains more antibacterial peptide genes than other insect genomes, and such genes are present in the form of a gene family in the genome (Scott et al. 2014; Tang et al. 2014). It was previously reported that in houseflies challenged by microorganisms, antibacterial peptides such as attacins, cecropins, defensins and diptericins were significantly up-regulated. These rapid changes in expression were coordinated in several genes (Wang et al. 2009; Liu et al. 2011; Fleming et al. 2014). In this study, all four antibacterial peptides (attacins, cecropins, defensins and diptericins) were simultaneously up-regulated 6–24 h PI, and their expression parrterns were consistent with those of our previous experiments and some other previouslty published manuscripts (Wang et al. 2009; Fleming et al. 2014). In *D. melanogaster* AMP expression is regulated by two signal transduction pathways: the Toll and IMD signal pathways. The Toll pathway regulates the response to gram-positive bacterial and fungal infections. The IMD pathway regulates the response to gram-negative bacterial infection (Cho et al. 2012; Kleino and Silverman 2014). Even though the Toll and IMD pathways have separate components and preferential target genes, but some antimicrobial peptide are expressed following the stimulation of one of the two aforementioned pathways (Hultmark 2003; Brennan et al. 2004, Royet et al. 2005, Tanji et al. 2005). We found that the expression of attacin, cecropin, defensin and diptericin varied 3 to 48 h PI. For instance, cecropin and defensin were up-regulated at 3 h PI, whereas attacin and diptercin were up-regulated later, and in particular, diptercin was not up-regulated until 6 h PI. The rapid response of cecropin and defensin suggested that because houseflies were infected with *C. albicans*, they may preferentially respond to the Toll pathway. Other studies have shown that the Toll and IMD pathways can be cooperative and may contribute to the activation of each other (Lemaitre and Hoffmann 2007; Tanji et al. 2007, Xiong et al. 2015). Defensin and cecropin are predominantly regulated by the Toll pathway, and attacin and diptericin are predominantly regulated by the IMD pathway during the *C. albicans* infection. Cecropin is reported to possess fungistasis activity in vitro (Hultmark 2003); therefore, the maintenance of high cecropin expression throughout infection process may have contributed to the control of infection.

Melanization is another major component of arthropod immunity that is not observed in vertebrates. Melanization involves the rapid synthesis of a black pigment, melanin, at the site of infection or injury (Cerenius et al. 2008; Tang 2009; Dudzic et al. 2015). Melanized encapsulation was reported to form at the site at which pathogens infected insects and are activated by contact between the pathogen and hemolymph cells (e.g. granular cells). Hemolymph cells then release chemotactic components to absorb plasma cells and form a multicellular sheet. The encapsulation internal layer undergoes melanization to form a capsule around the pathogen through the action of phenoloxidase (Lavine and Strand 2002; Lu 2008), and the melanization cascade activates the host humoral immune defence system, as observed in *Legionella pneumophila* infection of *G. mellonella* (Harding et al. 2012). Other research showed that Silkworm serine protease homologs (e.g. SPH1 and SPH2) and prophenoloxidase (e.g. BmPO1) were involved in melanization and encapsulation (Tokura et al. 2014). Moreover, Wang reported that lectins played a role in the coordination of hemagglutination, encapsulation and melanization in *Helicoverpa armigera* pathogen elimination (Wang et al. 2014). Housefly larvae infected with *E. coli* are reported to eliminate pathogens by activating the phenoloxidase cascade (Li et al. 2015), which enhances hemagglutination and promotes cytophagocytosis through lectin (Cao et al. 2012). In this study, we observed brown spots

under the epidermis of larvae within 3 h of infection with *C. albicans*. The brown spots were Melanized encapsulations surrounding aggregated *C. albicans* mycothallus. Throughout infection, similar structures were found, and some empty encapsulations without mycothallus were found 48 h PI. Our results suggested that encapsulation and agglutination may play an important role in the housefly innate immune response against *C. albicans*.

Housefly larvae, like some other insects, have a mixocoel, and humoral hemocyte concentrated in the last segments and scattered around tissues and organs. In this study, *C. albicans* mycothallus colonized in the gaps between the body wall and the muscle layer, as well as among muscle fibers of the muscle layer of larvae. We speculated that *C. albicans* was injected into the hemocoel at the 10th segment of the segmental venter and was quickly disseminated through the blood stream during muscle contractions to the entire body. *C. albicans* was able to persist between the inner side of the body wall and muscle tissue layers because of the low concentration of hemocytes in these spaces. In these environments, *C. albicans* was able to develop from the yeast to hyphal form (Huang 2012; Mayer et al. 2013). Our data are consistent with previous research reporting the formation of pseudomycelium by *Candida tropicalis* mycothallus when hemocyte numbers were reduced in *G. mellonella* (Mesa-Arango et al. 2013). Some reports suggested that insect blood cells were constantly regenerated and kept dynamically changing. In *Locusta migratoria*, the hemocytes and hematopoietic tissue mutually assist each other to clear invading pathogens from the circulation (Duressa et al. 2015). In housefly, hemocyte number varied during fungal infection (Mishra et al. 2015).

According to these results, we infer that because a great deal of fungal spores was injected into the coelom of housefly larvae, a fraction was transported to the gaps of tissues. Because there are fewer hemocytes in these locations than in the haemocoel, fungus which stayed in these locations was not eliminated immediately by the immune system. And then began to colonize at these locations. With the regeneration hemocytes and other immune molecule such as AMPs were transported to these gaps during blood circulation, these rest fungus were gradually cleared. This may explain why the mycothallus distribution changed from a continuous line to scattered spots and then disappeared.

The immune response should be carefully viewed as a double edged sword. Although it protected the larvae from pathogen invasion, excessive immune response could also cause self injury. Similar to the vertebrate immune system, the insect immune system is also regulated by complicated control mechanisms (Cerenius et al. 2008). A great number of immune regulators are involved in these processes, including serine proteinase inhibitors (Serpin). In *Tenebrio molitor* (Jiang et al. 2009) and *D. melanogaster* (Ligoxygakis et al. 2002; Fullaondo et al. 2011), serpins can regulate the immune response through the Toll signal pathway and prophenoloxidase-activating system. Li discovered at least 11 serpins in housefly, including a gene homologous to serpin27A of *D. melanogaster* (Li et al. 2015). In this study, at 48 h PI, the scattered brown spots gradually disappeared and empty Melanized encapsulations were observed and mycothallus colonization and AMP expression were reduced, indicating that mechanisms negatively regulating the immune response had begun to play their roles following the elimination of *C. albicans*. At 3 and 48 h PI, the expression level of 4 AMPs experienced up-regulated and then gradual recovery suggested that the balance, which provides protection for developing insects, permitting qualitatively normal inflammatory responses and protection against infection, occurred in Musca domestica as it did in vertebrates. However, the mechanisms underlying melanized encapsulation, coagulation and AMP expression during *C. albicans* infection remain to be determined. Thus, further study into fungal infection of the housefly is warranted.

CONCLUSIONS

In conclusion, we studied the morphology and histology of *C. albicans* infected *M. Domestica* larvae between 3 and 48 h PI. The infection involved a series of stages, including injection, infection, immune response and elimination of the fungal pathogen, and the housefly immune response was observed to involve the up-regulation of AMP, melanized encapsulation and agglutination.

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REFERENCES

Altincicek B, Knorr E, Vilcinskas A. Beetle immunity: identification of immune-inducible genes from the model insect *Tribolium castaneum*. *Dev Comp Immunol*. 2008; 32:585-595.

Brennan CA, Anderson KV. Drosophila: the genetics of innate immune recognition and response. *Annu Rev Immunol*. 2004; 22:457-483.

Cao XH, Zhou MH, Wang CL, Hou LH, Li YY, Chen LY. *Musca domestica* pupae lectin improves the immunomodulatory activity of macrophages by activating nuclear factor-kappa B. *J Med Food*. 2012; 15:145-151.

Cerenius L, Lee BL, Soderhall K. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol*. 2008; 29:263-271.

Chamilos G, Samonis G, Kontoyiannis, DP. *Drosophila melanogaster* as a model host for the study of microbial pathogenicity and the discovery of novel antimicrobial compounds. *Curr Pharm Design*. 2011; 17:1246-1253

Cho IH, Jeon JW, Paek SH, Kim DH, Shin HS, Ha UH, et al. Toll-like receptor-based immuno-analysis of pathogenic microorganisms. *Anal Chem*. 2012; 84:9713-9720.

Cirimotich CM, Ramirez JL, Dimopoulos G. Native microbiota shape insect vector competence for human pathogens. *Cell Host Microbe*. 2011; 10:307-310.

Dang XL, Wang YS, Huang YD, Yu XQ, Zhang WQ. Purification and characterization of an antimicrobial peptide, insect defensin, from immunized house fly (Diptera: Muscidae). *J Med Entomol*. 2010; 47:1141-1145.

Duressa TF, Vanlaer R, Huybrechts R. *Locust* cellular defense against infections: sites of pathogen clearance and hemocyte proliferation. *Dev Comp Immunol*. 2015; 48:244-253.

Dudzic JP, Kondo S, Ueda R, Bergman CM, Lemaitre B. Drosophila innate immunity: regional and functional specialization of prophenoloxidases. *BMC Biol*. 2015; 13:81.

Fu P, Wu JW, Guo G. Purification and molecular identification of an antifungal peptide from the hemolymph of *Musca domestica* (housefly). *Cell Mol Immunol*. 2009; 6:245-251. doi: 10.1038/cmi.2009.33

Fu P, Wu JW, Gao S, Guo G, Zhang Y, Liu JThe recombinant expression and activity detection of MAF-1 fusion protein. *Sci Rep-Uk*. 2015; 5:14716.

Fleming A, Kumar HV, Joyner C, Reynolds A, Nayduch D. Temporospatial fate of bacteria and immune effector expression in house flies fed GFP-*Escherichia coli* O157: H7. *Med Vet Entomol*. 2014; 28:364-371.

Fuchs BB, Eby J, Nobile CJ, El Khoury JB, Mitchell AP, Mylonakis E. Role of filamentation in *Galleria mellonella* killing by *Candida albicans*. *Microbes Infect*. 2010; 12:488-496.

Fullaondo A, Garcia-Sanchez S, Sanz-Parra A, Recio E, Lee SY, Gubb D. Spn1 regulates the GNBP3-dependent Toll signaling pathway in *Drosophila melanogaste*r. *Mol Cell Biol*. 2011; 31:2960-2972.

Harding CR, Schroeder GN, Reynolds S, Kosta A, Collins JW, Mousnier A, Frankel G. *Legionella pneumophila* pathogenesis in the *Galleria mellonella* infection model. *Infect Immun*. 2012; 80:2780-2790.

Hoffmann JA. The immune response of Drosophila. *Nature*. 2003; 426:33-38.

Huang GH. Regulation of phenotypic transitions in the fungal pathogen *Candida albicans*. *Virulence*. 2012; 3:251-261.

Hultmark D. *Drosophila* immunity: paths and patterns. *Curr Opin Immunol*.2003; 15:12-19.

Jiang R, Kim EH, Gong JH, Kwon HM, Kim CH, Ryu KH, et al. Three pairs of protease-serpin complexes cooperatively regulate the insect innate immune responses. *J Biol Chem*. 2009; 284:35652-35658.

Kelly J, Kavanagh K. Caspofungin primes the immune response of the larvae of *Galleria mellonella* and induces a non-specific antimicrobial response. *J Med Microbiol*. 2011; 60: 189-196.

Khalil S, Jacobson E, Chambers MC, Lazzaro BP. Systemic bacterial infection and immune defense phenotypes in *Drosophila melanogaster*. *Jove-J Vis Exp*. 2015; 99:e52613.

Kleino A, Silverman N. The Drosophila IMD pathway in the kactivation of the humoral immune response. *Dev Comp Immunol*. 2014; 42:25-35.

Lavine MD, Strand MR. Insect hemocytes and their role in immunity. *Insect Biochem Molec*. 2002; 32:1295-1309.

Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. *Annu Rev Immunol*. 2007; 25:697-743.

Li DX, Liang YL, Wang XW, Wang L, Qi M, Yu Y, Luan YY. Transcriptomic analysis of *Musca domestica* to reveal key genes of the Prophenoloxidase-activating system. *G3-Genes Genom Genet*. 2015; 5:1827-1841.

Liang YL, Wang JX, Zhao XF, Du XJ, Xue JF. Molecular cloning and characterization of cecropin

from the housefly (*Musca domestica*), and its expression in *Escherichia coli*. *Dev Comp Immunol*. 2006; 30:249-257.

Ligoxygakis P, Pelte N, Ji CY, Leclerc V, Duvic B, Belvin M, et al. A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*. Embo J. 2002; 21:6330-6337.

Liu FS, Sun LL, Tang T, Wang LN. Cloning, sequence analysis and induced expression of attacin-2 gene in housefly (*Musca domestica*). *Acta Entomologica Sinica*. 2011; 54:27-33. Chinses.

Lu HS. Principles of insect immunology. Shanghai Scientific and Technical Publishers, Shanghai, China; 2008. Chinses.

Matsumoto Y, Miyazaki S, Fukunaga DH, Shimizu K, Kawamoto S, Sekimizu K. Quantitative evaluation of cryptococcal pathogenesis and antifungal drugs using a *silkworm* infection model with Cryptococcus neoformans. *J Appl Microbiol*. 2012; 112:138-146.

Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. Virulence. 2013; 4:119-128.

Mesa-Arango AC, Forastiero A, Bernal-Martinez L, Cuenca-Estrella M, Mellado E, Zaragoza O. The non-mammalian host *Galleria mellonella* can be used to study the virulence of the fungal pathogen *Candida tropicalis* and the efficacy of antifungal drugs during infection by this pathogenic yeast. *Med Mycol*. 2013; 51:461-472.

Miceli MH, Diaz JA, Lee SA. Emerging opportunistic *yeast* infections. *Lancet Infect Dis*. 2011; 11:142-151.

Mishra S, Kumar P, Malik A. The effect of *Beauveria bassiana* infection on cell mediated and humoral immune response in house fly, *Musca domestica* L. Environ Sci Pollut R. 2015; 22:15171-15178.

Nayduch D, Cho H, Joyner C. *Staphylococcus aureus* in the house fly: temporospatial fate of bacteria and expression of the antimicrobial peptide defensin. *J Med Entomol*. 2013; 50:171-178.

Okada M, Hisajima T, Ishibashi H, Miyasaka T, Abe S, Satoh T. Pathological analysis of the *Candida albicans*-infected tongue tissues of a murine oral candidiasis model in the early infection stage.[J]. *Arch Oral Biol*. 2013; 58:444-450.

Onfelt Tingvall T, Roos E, Engstrom Y. The IMD gene is required for local cecropin expression in *Drosophila* barrier epithelia. *Embo Rep*. 2001; 2:239-243.

Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res*. 2002; 30:e36.

Pei ZH, Sun XN, Tang Y, Wang K, Gao YH, Ma HX. Cloning, expression, and purification of a new antimicrobial peptide gene from *Musca domestica* larva. *Gene*. 2014; 549:41-45.

Phoku JZ, Barnard TG, Potgieter N, Dutton MF. Fungi in housefly (*Musca domestica* L.) as a disease risk indicator-A case study in South Africa. *Acta Trop*. 2014; 140:158-165.

Royet J, Reichhart JM, Hoffmann. Sensing and signaling during infection in Drosophila. *Curr Opin Immunol*. 2005; 17:11-17.

Scott JG, Warren WC, Beukeboom LW, Bopp D, Clark AG, Giers SD, et al. Genome of the house fly, *Musca domestica* L., a global vector of diseases with adaptations to a septic environment. *Genome Biol*. 2014; 15:466.

Singh S, Reese JM, Casanova-Torres AM, Goodrich-Blair H, Forst S. Microbial population dynamics in the hemolymph of *Manduca sexta* infected with *Xenorhabdus nematophila* and the entomopathogenic nematode *Steinernema carpocapsae*. *Appl Environ Microb*. 2014; 80:4277-4285.

Stokes BA, Yadav S, Shokal U, Smith LC, Eleftherianos I. Bacterial and fungal pattern recognition receptors in homologous innate signaling pathways of insects and mammals. *Frint Microbiol*. 2015; 6:19.

Tang T, Li X, Yang X, Yu X, Wang JH, Liu F, Huang DW. Transcriptional response of *Musca domestica* larvae to bacterial infection. *Plos One*. 2014; 9:e104867.

Tang HP. Regulation and function of the melanization reaction in Drosophila. *Fly*. 2009; 80:105-111.

Tanji T, Hu XD, Weber ANR, Ip YT. Toll and IMD pathways synergistically activate an innate immune response in Drosophila melanogaster. *Mol Cell Biol*. 2007; 27:4578-4588.

Tanji T, Ip YT. Regulators of the Toll and Imd pathways in the Drosophila innate immune response. *Trends Immunol*. 2005; 26:193-198.

Tokura A, Fu GS, Sakamoto M, Endo H, Tanaka S, Kikuta S, et al. Factors functioning in nodule melanization of insects and their mechanisms of accumulation in nodules. *J Insect physiol*. 2014; 60:40-49.

Wang JL, Zhang Q, Tang L, Chen L, Liu XS, Wang YF. Involvement of a pattern recognition receptor C-type lectin 7 in enhancing cellular encapsulation and melanization and melanization due to its carboxyl-terminal CRD domain in the cotton bollwork, *Helicoverpa armigera*. *Dev Comp Immunol*. 2014; 44:21-29.

Wang JX, Zhao XF, Liang YL, Li L, Zhang W, Ren Q, et al. Molecular characterization and expression of the antimicrobial peptide defensin from the housefly (*Musca domestica*). *Cell Mol Life Sci*. 2006; 63:3072-3082.

Wang Y, Jin XB, Zhu JY, Zeng AH, Chu FJ, Yang XR, et al. Expression pattern of antibacterial genes in the *Musca domestica*. *Sci China Ser C*. 2009; 52:823-830.

Wang Y, Li DD, Jiang YY, Mylonakis E. Utility of Insects for Studying Human Pathogens and Evaluating New Antimicrobial Agents. In: Vilcinskas A, editor. Yellow biotechnology I: insect biotechnologie in drug discovery and preclinical research. Springer-Verlag Berlin; 2013. pp 1-25.

Wu GQ, Li M, Liu Y, Ding Y, Yi YH. The specificity of immune priming in silkworm, *Bombyx mori*, is mediated by the phagocytic ability of granular cells. *J Insect Physiol*. 2015; 81:60-68.

Wu MH, Sugimura Y, Iwata KV, Takaya N, Takamatsu D, Kobayashi M, et al. Inhibitory effect of gut bacteria from the Japanese honey bee, *Apis cerana japonica*, against *Melissococcus plutonius*, the causal agent of European foulbrood disease. *J Insect Sci*. 2014; 14:129.

Xiong GH, Xing LS, Lin Z, Saha TT, W CS, Jiang HB et al. High throughput profiling of the cotton *bollworm Helicoverpa armigera* immunotranscriptome during the fungal and bacterial infections. *BMC Genomics*. 2015; 16:321.

Yang X, Tang T, W YL, Liu X, Cao XR, N ZH, et al. Gene cloning, expression profiling and anti-microbial assay of Muscin, a new antimicrobial peptide in the housefly (*Musca domestica*). *Acta Entomologica Sinica*. 2015; 58:617-624. Chinses.

Ohta M, Watanabe A, Mikami T, Nakajima Y, Kitaini M, Tabunoki H, et al. Mechanism by which *Bombyx mori* hemocytes recognize microorganisms: direct and indirect recognition systems for PAMPs. Dev Comp Imm

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