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ORIGINAL RESEARCH ARTICLE

Morphological observations of haemocytes from *Agrotis ipsilon* (Lepidoptera: Noctuidae) larvae infected by *Escherichia coli*

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Abstract

Understanding the types of haemocytes involved in the immune response in insects, and the mechanism involved in determining that response, can provide a scientific reference for developing effective microbial insecticides. Therefore, the current study examined haemocytes from *Agrotis ipsilon* (Hufnagel) larvae infected with *Escherichia coli* in terms of their morphology, total counts, and relative proportions at different time points post-infection by optical microscopy technology. The results revealed six types of haemocytes (prohemocytes, plasmatocytes, granulocytes, spherule cells, oenocytoids, and cystocytes) in the haemolymph of sixth-instar larvae. Haemocyte deformation, disruption, nuclear changes, and vacuoles were recorded after infection with different dosages of *E. coli*. At each time period post-infection, the total haemocyte count was significantly higher than in the controls, peaking at 24 h post-infection and then decreasing by 48 h post-infection. The proportion of prohemocytes decreased significantly until 24 h post-infection, and then began to increase. By contrast, the proportions of plasmatocytes, granulocytes, spherule cells, oenocytoids, and cystocytes relatively increased, and peaked by 24 h post-infection, and then decreased. This revealed that strong immune response was stimulated in larva of *A. ipsilon* in a short time after infection with *E.coli*, and the results shed addition light on the cellular immune response of insects to pathogens.

Keyword *Agrotis ipsilon* (Hufnagel) · Haemocytes · Haemolymph · *Escherichia coli* · Immune response

Introduction

Insects have a complex immune system, including both cellular immunity and humoral immunity, enabling them to defend against pathogenic infections (Ardia et al. 2012; Berger and Jurčová 2012; Zdybicka-Barabas and Cytryńska 2013; Zhang and Zhang 2019). Haemocytes mainly perform the cellular immune functions, with an important role in defending against pathogen invasion. Previous studies have shown that haemocytes in the insect haemolymph can be categorized into several types, namely prohemocytes, plasmatocytes, granulocytes, cystocytes, adipohemocytes, spherule cells, and oenocytoids (Jones 1977). However, these types of haemocytes differ both among insect species and between developmental and physiological stages within the same insect species (Gillespie et al. 2000; Giannoulis et al. 2005; Beetz et al. 2008; Ruchita and Krishna 2014). Morphological studies of haemocytes can be used to investigate the cellular immune response. More than 100 species of insects have been studied in terms of their haemocytes (Ribeiro and Brehélin 2006; Strand 2008; Wu et al. 2016; Mahmood et al. 2018; Boguś et al. 2018). The primary functions of haemocytes are coagulation, phagocytosis, encapsulation, detoxification, and the storage and distribution of nutritive materials (Siddiqui and Al-Khalifa 2014). Haemocytes come into contact with pathogens via the haemolymph, whereupon they engulf the pathogen, forming nodules that are then destroyed by the immune system (Hillyer and Christensen 2002; Hillyer et al. 2003; Siddiqui and Al-Khalifa 2014). However, during the immune response, pathogens can produce toxins that damage the haemocytes, resulting in variation in the morphology, quantity and proportion of the different types of haemocytes (Wang et al. 1990; Mazet et al. 1994; Vilcinskas et al. 1997; Griesch and Vilcinskas 1998; Perveen and Ahmad 2017; Mahmood et al. 2018; Boguś et al. 2018). Such interactions can affect the efficacy of biological control approaches against pests. Therefore, studying the immune function of haemocytes, and the mechanism involved, will provide a theoretical reference for biological control of the pests. However, because the haemocytes and mechanism involved can differ among insect species

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(Wang et al. 1990; Feng et al. 2011; Ruchita and Krishna 2014), further studies on the cellular immune responses of additional insects species are necessary.

The black cutworm moth, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) is a pest of a variety of crops in many areas of the world (Gesraha and Ebeid 2021), and has become one of the most important pests on vegetables and crops throughout China (Ding et al. 2018). Several studies have investigated the biological characteristics of the pest and its control measures (Gemeno and Haynes 2000; Amin et al. 2019; Sobhy et al. 2020; Gesraha and Ebeid 2021). In the previous study, five types of haemocytes in fourth-larval instars of *A. ipsilon* were reported, changes in the haemocytes and a reduction in the total haemocyte count of *A. ipsilon* larvae after infection with insecticide dimilin and *Bacillus thuringiensis* was also observed (El-Aziz and Awad 2010). However, given that haemocyte types can vary among developmental stages of the same insect species, and under different ecological and physiological conditions (Gillespie et al. 2000), as well as in terms of infection with different pathogens (Wang et al. 1990; Feng et al. 2011), there is a need to understand the effects of pathogens on the morphology of haemocytes across the developmental stages of the Chinese population of *A. ipsilon*.

In this study, we investigated the haemocytes of *A. ipsilon* in terms of their morphology, total number and relative proportion of count numbers in *A. ipsilon* larvae at different time points following infection with *Escherichia coli.* The results will be useful to further understanding of the types of haemocytes involved in the immune response and the mechanisms involved in their immune functions, providing a scientific reference for the development of more effective microbial insecticides.

Materials and Methods

Insects

Larvae of *A. ipsilon* were collected from Leshan tobacco-planting areas around Zunyi in Guizhou province, China in March 2015. The larvae were reared in the laboratory at 25 ± 1℃ and 70 ± 7 relative humidity (RH) under a 14:10 (L:D) hours photoperiod until the third instar stage. Thereafter, they were reared individually to avoid cannibalism. Larvae were fed on Chinese cabbage leaves, *Brassica pekinensis*. Four generations of larvae were reared, and the sixth-instar larvae from each generation were used in the study.

Preparation of bacterial suspension

Escherichia coli was purchased from Shanghai Luwei Technology company, China, and cultivated on Luria–Bertani (LB) medium at 37 °C. After centrifugation at 2, 000 rpm for 10 min, the bacteria were collected and diluted with normal saline to give six concentrations: 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 cells/mL. Concentrations of bacteria were determined using a hemocytometer under the optical microscope. First, the number of bacteria in each small square of hemocytometer was measured, and then converted into the number of bacteria in each milliliter of bacterial solution. So, the bacteria number in 1 mL of bacterial suspension = the average number of bacteria in each square (n) × coefficient (k) × dilution ratio of bacterial suspension (d).

Morphological observations on haemocytes of larvae

Ten sixth-instar larvae of *A. ipsilon* were collected and washed with sterile water. The abdomen of each larva was punctured with a dissecting needle, and a small amount of haemolymph was extracted and placed on a microscope slide with a pipette, forming a blood film. Three drops of Wright's dye was added to the blood film, followed, 2 min later, by three drops of Giemsa stain and a phosphate buffered solution (PBS, pH7.2). The slides were left for 30 min, washed with tap water, and allowed to dry at room temperature. Each slide was then observed under a light microscope. The haemocytes observed were classified according to the classification standard suggested by Jones, who identified the commonest seven types of haemocytes in insects haemolymph, namely prohemocytes, plasmatocytes, granulocytes, cystocytes, adipohemocytes, spherule cells, and oenocytoids (1977).

Variations in morphology and counts of haemocytes infected by *E. coli*

To study the variation in morphology and counts of haemocytes from larvae infected by *E. coli*, 3 μL of a bacterial suspension was injected into the abdomen of larvae using a microinjector. The larvae were then transferred to an artificial climate box and fed on Chinese cabbage leaves. Haemolymph of larvae was collected at 6, 12, 24, 48 h post-injection. The total haemocyte counts (THCs) in each sample of haemolymph was determined using a hemocytometer filled with anticoagulant buffer (Leonard et al. 1985). The haemolymph samples were each placed on a microscope slide, and dyed using three drops of Wright's-Giemsa stain. Each slide was then examined under a light microscope(640×) and haemocyte counts and variation in haemocyte morphology were recorded. Photographs of haemocytes were captured with a digital camera (Canon, EOS-200D). To accurately measure the counts of haemocytes, the haemolymph was thoroughly

mixed with the anticoagulant buffer to disperse the haemocytes. For each treatment,45 larvae / time point / dosage were used, and three replicates were conducted. A control experiment was also run using larvae treated with normal saline.

Statistical analysis

All data were analyzed by SPSS version 11.5 software (SPSS Inc., Chicago, IL, USA). Comparisons between the mean of groups at different treatment times and bacterial dosages were analyzed using one-way analysis of variance (ANOVA) and two-way analysis of variance (ANOVA), where the differences among means were compared with the Tukey's multiple comparison method at $P<0.05$ level that the test results are false, reject.

Results

Morphology of larval haemocytes

Six types of haemocytes were found in the haemolymph of *A. ipsilon* sixth-instar larvae: prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherule cells (SPs), oenocytoids (OEs), and cystocytes (CYs). The total haemocyte count was 18,897.33 indiv./mL in the haemolymph, prohemocytes were the most abundant haemocytes with the count of 8,999.45 indiv./mL, followed by plasmatocytes (2,758.66 indiv./mL), granulocytes (2,346.71indiv./mL), spherule cells (2,122.05 indiv./mL) and cystocytes (1,514.26 indiv./mL), oenocytoids were the least abundant (1,156.20 indiv./mL) (Fig. 1A), the difference among them reached significant level ($df_1=6$, $df_2=13$, $F=1703.82$, $P<0.0001$). Each cell type in the total haemocyte count accounted for 47.62%, 14.60%, 12.42%, 11.23%, 8.01% and 6.12% respectively (Fig. 1B). Healthy haemocytes had well-developed cell membranes and nuclei.

Prohemocytes

Prophemocytes were identified as small haemocytes (10.25 ~ 12.13 μm × 7.34 ~ 8.81 μm) in the haemolymph of *A. ipsilon* sixth-instar larva. They were circular or ovate in shape with a clear outline, a nucleus in the center of cell, and a high nuclear:cytoplasmic ratio. The cytoplasm was relatively homogeneous, without any obvious granular materials (Fig. 2A).

Plasmatocytes

Plasmatocyte, also called protogonocytes, occurred in different shapes and sizes (16.13~17.65μm × 8.87~10.29μm). There was a single nucleus in the center of the cell, accounting for half of the overall volume of the cell; the cytoplasm was relatively homogeneous, without any granules (Fig. 2B).

Granulocytes

Granulocytes were common in the haemolymph of *A. ipsilon* sixth-instar larva, and were circular, ovate, or irregular in shape with different sizes (19.23 ~ 29.41 μm × 8.85 ~ 14.71 μm) (Fig. 2C). The nucleus was circular or ovate, and centrally located. There were heterogeneous lysosome-like materials present in the cytoplasm, enabling these cells to be distinguished from plasmatocytes.

Spherule cells

Spherule cells were circular haemocytes of medium-large size, in the range of 13.53 ~ 16.67 μm × 9.86 ~ 14.17 μm, and contained many pearl-like inclusions forming a circle within the cells (Fig. 2D). It was difficult to locate the nucleus.

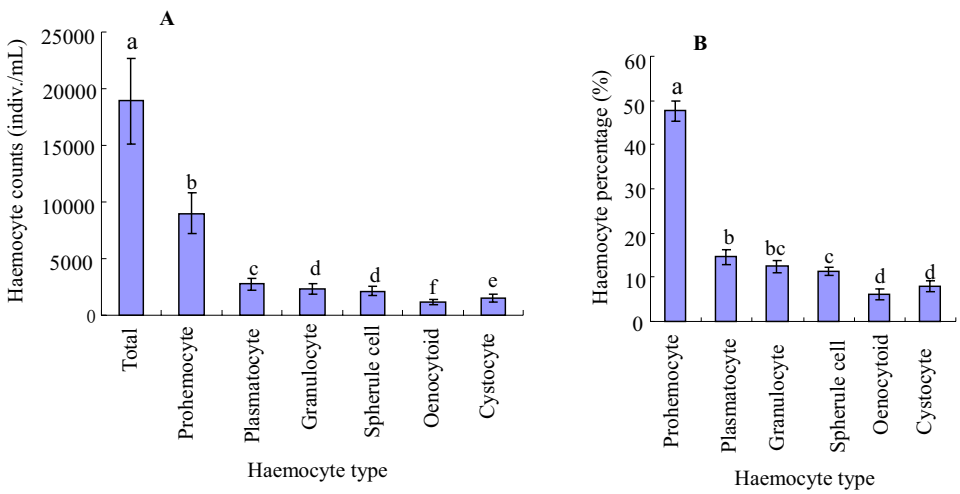

Fig. 1 Haemocyte counts and percentages of *A. ipsilon* larva. **A**:Haemocyte counts; **B**:Haemocyte percentages. The letters on the column are the results of Tukey's multi comparison, the different lowercase letters(a,b,c,d,e,f) represent statistically significant differences in cell counts or percentage among different haemocyte type at $P<0.05$ level. The error bars represent the standard error (SE)

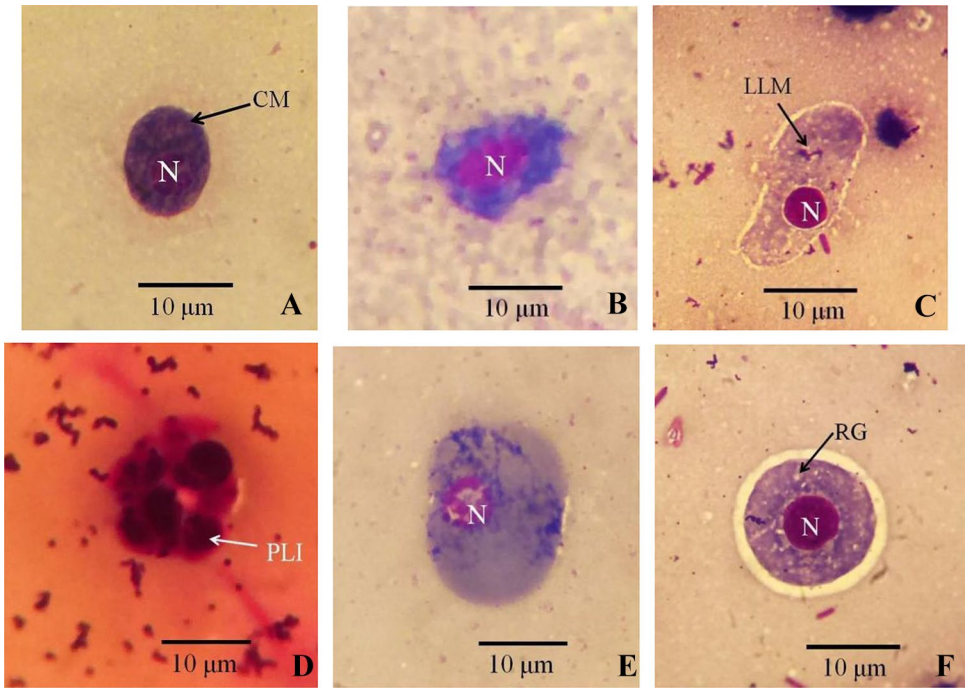

Fig. 2 Haemocytes isolated from the haemolymph of sixth-instars of *A. ipsilon* larva. **A**: Prophemocytes (640×); **B**: Plasmatocyte (640×); **C**: Granulocytes (640×); **D**: Spherule cells (640×); **E**: Oenocytoids (640×); **F**: Cystocytes (640×). N, nucleus; LLM, lysosome-like materials; PLI, pearl-like inclusions; RG,refractive granules

Oenocytoids

Oenocytoids were irregular in shape, with a small, circular nucleus. The cell sizes were 17.68 ~ 20.39 µm × 12.92 ~ 15.8 3 µm. The cytoplasm was dense and homogeneous (Fig. 2E).

Cystocytes

Cystocytes were usually medium-sized haemocyte (14.32 ~ 25.11 µm × 13.33 ~ 23.53 µm), with a circular or oval shape. The outline was relative smooth, and the cytoplasm contained some refractive granules of different sizes (Fig. 2F).

Morphological variations in haemocytes from *A. ipsilon* larvae infected by *E. coli*

The infection of *A. ipsilon* by *E.coli* induced haemocytes to adhere to each other to form aggregates (Fig. 3A), of which most of the haemocytes were plasmatocytes and granulocytes. Six hours after infection, some haemocytes showed considerable structural changes. For example, plasmatocytes lost their smooth outline and complete profile, developing a pleated or distorted outer membrane. The nucleus appeared displaced to one side of the cells with presence of vacuoles of various sizes (Fig. 3B). The granulocytes appeared deformed with many vacuoles in the cytoplasm (Fig. 3C). The membranes of cystocytes became uneven, and ruptured in some cells, which resulted in the out-flowing of cytoplasm content including nucleus (Fig. 3D).

Variation in total haemocytes counts in *A. ipsilon* larva infected by *E. coli*

Infection of sixth-instar larva of *A. ipsilon* with different dosages of *E. coli* led to significant increases in the number of THCs after 6, 12, 24, and 48 h post-infection (Fig. 4), with the number of THCs increasing significantly with increasing bacterial dosage ($df_1 = 6$, $df_2 = 21$, $F = 34.945$, $P < 0.0001$). The number of THCs in each bacterial dosage group peaked at 24 h post-infection (Fig. 4). Overall, infection of *A.*

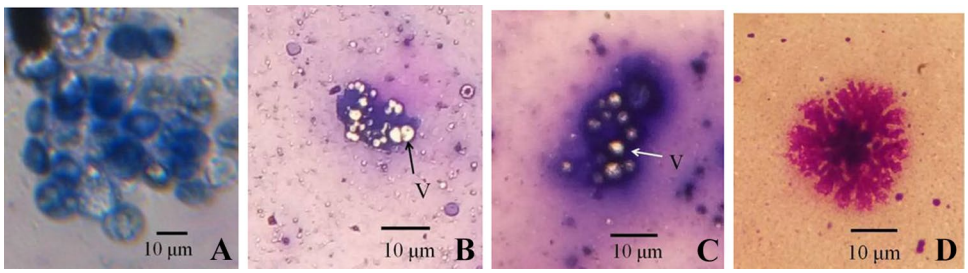

Fig. 3 Morphology variation in larval haemocytes of *A. ipsilon* infected with *E. col*. **A**: Aggregation of haemocytes (100×); **B**: Plasmatocytes (640×); **C**: Granulocytes (640×); **D**: Cystocytes (400×). V, vacuoles

ipsilon with different dosages of *E. coli* (1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 cells/mL.) increased the number of THCs by 82.46%, 97.64%, 111.29%, 126.35%, 139.27%, and 155.39% as compared to the control after 24 h post-infection, respectively. After 48 h post-infection, the number of THCs declined, although the differences among the different treatment time remained significant ($df_1=3$, $df_2=20$, $F=2.498$, $P<0.0001$). Two-way analysis of variance showed that the interaction of dosages of *E. coli* and treatment time had a significant influence on the number of THCs in sixth-instar larva of *A. ipsilon* ($df=15$, $F=7.835$, $P<0.0001$).

Proportions of haemocyte types of *A. ipsilon* larva infected by *E. coli*

Variations in the relative proportion of the six haemocyte types in *A. ipsilon* sixth instar larva were observed at different time points post-infection with *E. coli* (Fig. 5). The percentage in number of prohemocyte decreased significantly relative to the control with increasing bacterial dosage ($df_1=6$, $df_2=21$, $F=7.586$, $P<0.0001$) and time post infection up until 24 h post-infection ($df_1=3$, $df_2=20$, $F=14.317$, $P<0.0001$) (Fig. 5A), which decreased 22.78%, 26.98%, 29.10%, 31.06%, 33.15%, and 35.10% than control, separately. Whereupon it began to increase.

The percentages in number of plasmatocyte ($df_1=6$, $df_2=14$, $F=4.80$, $P=0.007$), granulocyte ($df_1=6$, $df_2=14$, $F=4.162$, $P=0.013$), spherule cell ($df_1=6$, $df_2=14$, $F=2.518$, $P=0.072$), oenocytoid ($df_1=6$, $df_2=14$, $F=3.242$, $P=0.033$), and cystocyte ($df_1=6$, $df_2=14$, $F=5.343$, $P=0.005$) all increased relative to the control at 6 h post-infection, and with increasing bacterial dosages, peaking at 24 h post-infection (Fig. 5B–F).

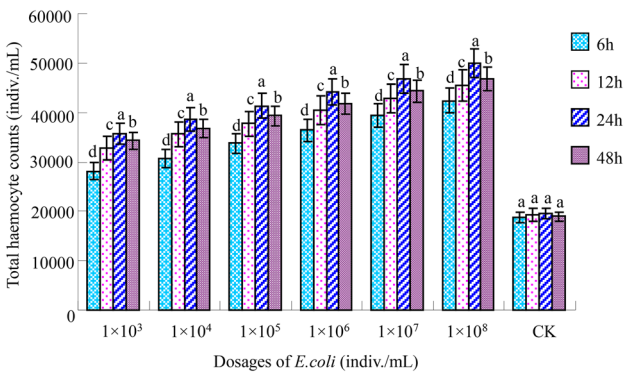

Fig. 4 Total haemocyte counts of *A. ipsilon* larva infected by *E. coli*. The letters on the column are the results of Tukey's multi comparison between different time points at the same dosage, the different lowercase letters(a,b,c,d) represent statistically significant differences in total cell counts among different time points post-infection at $P<0.05$ level. CK is the control treatment, the error bars represent the standard error (SE)

For each dosage, the percentage in number of plasmatocyte increased 5.39%, 6.21%, 6.85%, 7.44%, 8.05%, and 8.44%, separately, granulocyte increased 4.70%, 5.52%, 6.11%, 6.83%, 7.49%, and 7.87%, spherule cell increased 3.90%, 4.72%, 5.04%, 5.20%, 5.51%, and 5.65%, oenocytoid increased 4.01%, 4.56%, 5.04%, 5.18%, 5.50%, and 5.64%, and cystocyte increased 4.78%, 5.97%, 6.06%, 6.41%, 6.60%, and 7.50%. The difference between different treatment times were not significant in plasmatocyte ($df_1=3$, $df_2=20$, $F=12.987$, $P=0.955$), granulocyte ($df_1=3$, $df_2=20$, $F=12.35$, $P=0.995$), spherule cell ($df_1=3$, $df_2=20$, $F=13.926$, $P=0.509$), oenocytoid ($df_1=3$, $df_2=20$, $F=14.159$, $P=0.306$), and cystocyte ($df_1=3$, $df_2=20$, $F=17.978$, $P=0.59$). Thereafter, the percentages in number of the five haemocytes had begun to decrease by 48 h post-infection.

Two-way analysis of variance showed that the interaction of dosages of *E. coli* and treatment time had no significant influence on the percentage in number of prohemocyte ($df=15$, $F=1.048$, $P=0.427$), plasmatocyte ($df=15$, $F=0.014$, $P=1.00$), granulocyte ($df=15$, $F=0.012$, $P=1.00$), spherule cell ($df=15$, $F=0.087$, $P=1.00$), oenocytoid ($df=15$, $F=0.112$, $P=1.00$), and cystocyte ($df=15$, $F=0.149$, $P=1.00$) in sixth-instar larva of *A. ipsilon*.

Discussion

Haemocytes are the main cells involved in vital physiological activities in insects, for example, prohemocytes are putative stem cells that involved in division of haemocytes; plasmatocytes are the main capsule formation cells that involved in forming of capsule (Jones 1977; Schmidt et al. 2001; Lavine and Strand 2002; Hillyer et al. 2003; Castillo et al. 2006). Studies shows that granulocytes are the professional phagocytes that involved in phagocytizing the pathogens, and they also participated in nodule formation and envelopment (Jones 1977; Schmidt et al. 2001; Lavine and Strand 2002; Hillyer et al. 2003; Castillo et al. 2006). In addition, spherule cells are potentially a source of cuticular components that involved in secretions and storage; oenocytoids are a source of phenoloxidases, and coagulocytes are involved in clotting (Jones 1977; Schmidt et al. 2001; Lavine and Strand 2002; Hillyer et al. 2003; Castillo et al. 2006). However, the proportions and types of haemocytes vary among insect species. Our results revealed six types of haemocytes in the haemolymph of sixth instar larva of *A. ipsilon* (prohemocytes, plasmatocytes, granulocytes, spherule cells, oenocytoids, and cystocytes). Prohemocytes are by far the most abundant cell type, followed by plasmatocytes and granulocytes, and oenocytoids comprise the least proportion of total haemocyte population. These haemocyte types have already been described in diverse species, including Lepidoptera, Orthoptera, Diptera, Blattaria, Coleoptera,

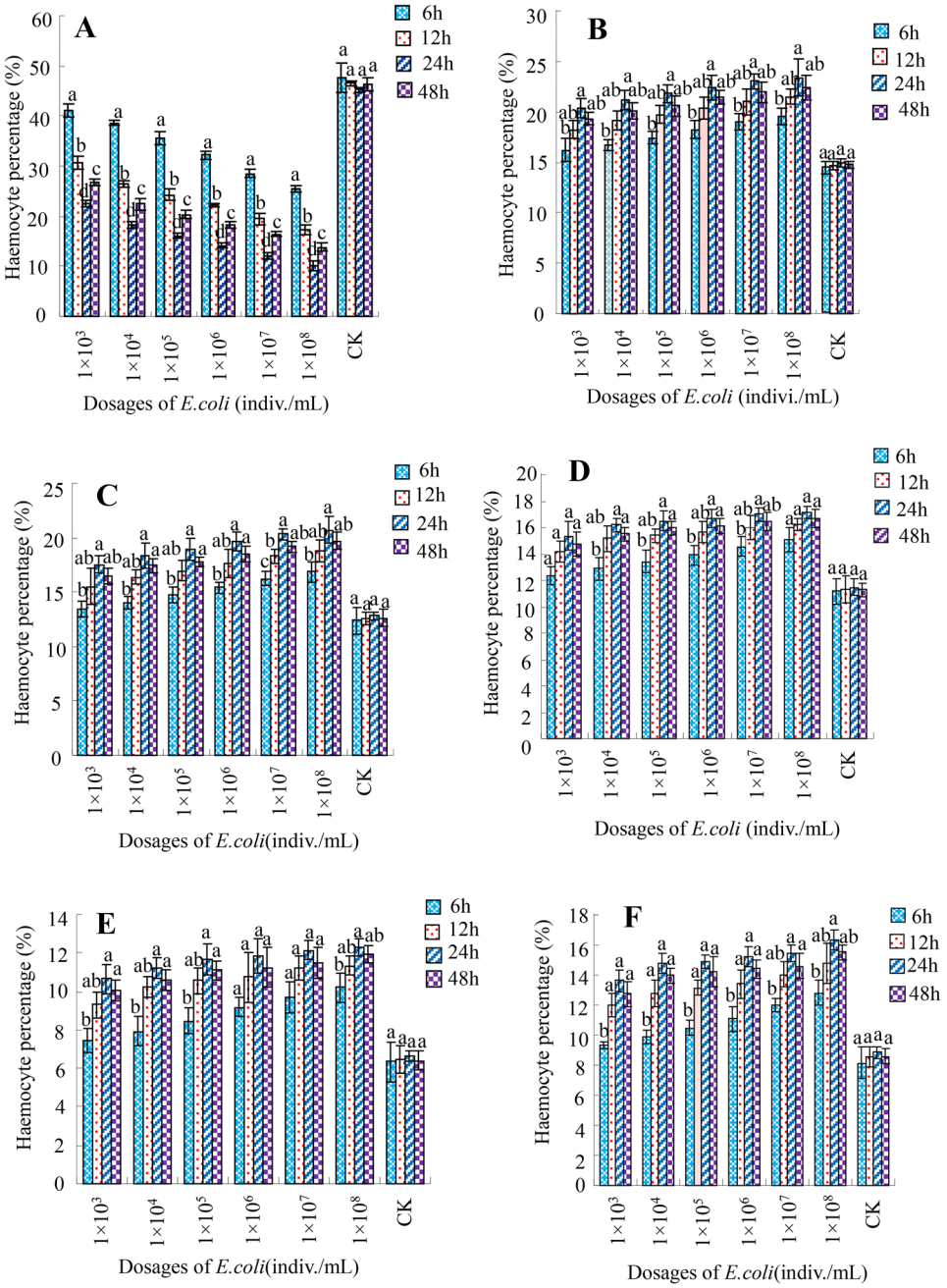

Fig. 5 Percentages of count number of haemocytes of *A. ipsilon* larva infected by *E. coli*. **A**. Percentages of prohemocyt; **B**. Percentages of plasmatocyte; **C**. Percentages of granulocyte; **D**. Percentages of spherule cell; **E**. Percentages of oenocytoid; **F**. Percentages of cystocyte. The letters on the column are the results of Tukey's multi comparison between different time points at the same dosage, the different lowercase letters(a,b,c,c,d) represent statistically significant differences in percentages of count number of haemocytes among different time points post-infection at $P < 0.05$ level. CK is the control treatment, the error bars represent the standard error (SE)

Hymenoptera, Hemiptera, and Collembola (Jones 1977; Lavine and Strand 2002; Hillyer et al. 2003; Ribeiro and Brehélin 2006; Castillo et al. 2006; Boguś et al. 2018). However, five types of haemocytes were found in the haemolymph of fourth-instar larvae of *A. ipsilon* (prohemocytes, plasmatocytes, granulocytes, spherule cells, and adipohemocytes) (El-Aziz and Awad 2010). These differences further demonstrated that haemocytes differ among developmental stages of the same insect species (Gillespie et al. 2000; Beetz et al. 2008). Furthermore, different foods eaten by the insect and different collection methods of haemocytes may also greatly affect the number and types of haemocytes obtained from the haemolymph (Castillo et al. 2006).

Cellular immunity depends on the phagocytosis, aggregation and encapsulation of haemocytes to pathogens. Plasmatocytes, granulocytes, and oenocytoids are the main haemocytes that involved in the procedure of cellular immunity (Hillyer et al. 2003). Our study shows that plasmatocytes and granulocytes of *A. ipsilon* adhere to each other to form aggregations after infection with *E.coli*. These unstructured aggregations may be encapsulated by other haemocytes. Given the role of haemocytes in the cellular immune response against pathogens, it can

be hypothesized that pathogen invasion is enhanced by changes in morphology and quantity of the haemocytes in affected insects. In our study, haemocytes from sixth-instar larva of *A. ipsilon* infected by *E. coli* underwent considerable structural changes, including deformation, membrane disruption, changes in the position of the nucleus, and occurrence of vacuoles. This showed that larva of *A. ipsilon* rapidly elicited strong immune responses against inoculated bacteria. These results are in concordance with those of El-Aziz and Awad, who reported that the infection of *A. ipsilon* with *B. thuringiensis* induced several pathological detribitons in haemocytes, the contents of the granules seem to swell giving the cells an extremely vacuolated appearance (El-Aziz and Awad 2010). And in other species, such as *Plodia interpunctella* and *Musca domestica*, many haemocytes also showed considerable structural changes after infection with bacteria, including occurrence of vacuoles (Wang et al. 1990; Yan et al. 2009; Boguś et al. 2018).

Bacterial infection has also caused a significant increase in number of THCs of sixth-instar larva of *A. ipsilon* relative to the control ($P < 0.05$), at 6, 12, and 24 h post-infection. The number of THCs increased with the treatment time extended, and reached highest at 24 h, then it began to decrease after 48 h post-infection. This revealed that strong immune response was stimulated in larva of *A. ipsilon* in a short time after infection with *E.coli*, and large numbers of haemocytes were produced and rapidly released into the haemolymph to phagocytose and encapsulate the bacteria. Similar results were obtained in other species, for example, the number of THCs in *Manduca sexta* larvae showed a marked increase after injected with *Pseudomonas aeruginosa* (Horohov and Dunn 1982). In another study, injection of *Enterobacter cloacae* also caused a sharp increase in the number of THCs of *Rhodnius prolixus* up to 7 days post-infection, followed by a decline in the number of THCs after this time (De Azambuja et al. 1991). Previous studies have showed that the development of hematopoietic organs, the proliferation of haemocytes, and the release of immobilized haemocytes all cause an increase in the number of THCs of insects (Feng et al. 2011). However, the speed of hematopoietic organs development and the proliferation of haemocytes were relatively slow, making it difficult to produce large numbers of haemocytes over a short period of time (Feng et al. 2011). Thus, we hypothesize that the immediate increase in the number of THCs in *A. ipsilon* larvae after infection with *E. coli* resulted from the rapid release of immobilized haemocytes into the haemolymph after invasion, and that the increase in the number of THCs seen at later times during the post-infection period could result from the development of hematopoietic organs and the proliferation of haemocytes. With increasing time post-infection, the number of haemocytes that be destroyed also increased, resulting in a decrease in the number of THCs (Feng et al. 2011). However, our results were in contrast to the findings of El-Aziz and Awad, who reported a significant decrease in the number of THCs of forth instar larva of *A. ipsilon* relative to the controls at 12, 24, and 48 h post-infection with *B. thuringiensis* (El-Aziz and Awad 2010). In addition, a reduction in haemocyte counts of *Trichoplusia ni* was found after exposure to *B.thuringiensis* subsp.*kurstaki* (Btk) and after injection with *E.coli* (Ericsson et al. 2009). Infection with *Conidiobolus coronatus* also caused a significant drop in the number of haemocyte types in *Galleria mellonella* (Boguś et al. 2018). These variations in the number of THCs might result, in part, from the unusual resistance of different insects to different bacterial pathogens.

After infection with *E.coli*, the relative proportion of the six haemocyte types all changed. The percentage in number of prohemocyte decreased until 24 h post-infection, and then began to increase. However, the percentages in number of plasmatocyte, granulocyte, spherule cell, oenocytoid, and cystocyte all increased, peaking at 24 h post-infection, and then decreased. The reasons may be that the prohemocyte was activated and differentiated into other haemocyte types during early post-infection periods, caused the percentage in number of prohemocyte to decrease and the percentages in number of plasmatocyte, granulocyte, spherule cell, oenocytoid, and cystocyte to increase. At later post-infection period, the plasmatocyte, granulocyte, spherule cell, oenocytoid, and cystocyte were destroyed, and the differentiation capacity of prohemocyte declined, caused the percentage in number of prohemocyte to increase and the percentages in number of other haemocyte types to decrease in the THCs. However, these results are in disagreement with those of *Musca domestica*, the percentages in number of plasmatocytes and granulocytes in the larva of *M. domestica* increased significantly at 4, 6, 8 h post-infection with *E.coli*, the percentage in number of spherule cells decreased, and the percentages in number of prohemocytes and oenocytoids did not change significantly (Yan et al. 2009). Also, bacterial injection into *M. sexta* larvae caused a significant increase in percentage in number of spherule cells and significant decrease in percentages in number of granulocytes and plasmatocytes (Horohov and Dunn 1982). No significant changes in the number of oenocytoids were detected. In addition, the infection of *Parasarcophaga surcoufi* third-instar larvae with nematode decreased the percentages in number of plasmatocytes and granulocytes at 40 h of injection (Ayaad et al. 2001). These discrepancies further highlighted that the types and counts of haemocytes revolved in the immune response differed among insect species when infected with different microorganisms.

In conclusion, after infection with *E.coli*, the morphology, quantity and proportion of the haemocytes in sixth-instar larva of *A. ipsilon* all varied, revealing a particular pattern of cellular immune response in these larvae. In the immune response procedure, plasmatocyte, granulocyte, and cystocyte showed significant morphological variation, and their percentage variations was higher than other haemocytes in haemolymph. These indicate that plasmatocyte, granulocyte, and cystocyte are the

main participants in immune response of sixth-instar larva of *A. ipsilon*. The results shed addition light on the cellular immune response of insects to pathogens. Haemocytes are not only responsible for cellular immune response, they also provide humoral immunity factors, e.g. they often release a variety of hydrolases and antioxidase by degranulation during the phagocytosis, which are involved in clearing the pathogen. Further studies on the relationship between haemocytes and humoral factors in immune response are needed.

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Declarations

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

Conflicts of interest The authors declare that they have no conflict of interest.

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