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Evaluation of antibacterial immune response in *Drosophila melanogaster* and *Drosophila ananassae*

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Insects are excellent model organisms to examine the phenotypic variation of innate immunity. The innate immunity is the immediate response of invertebrates for protection against pathogens. In this study, we assessed the immune responses of *Drosophila melanogaster* and *Drosophila ananassae* against *Staphylococcus aureus* and *Escherichia coli*. It includes survival ability against infection, systemic pathogen load, and estimation of phenoloxidase activity, melanization reaction, the total number of hemocytes, quantification of fat, and the nitrite levels in adult flies and also the bacterial inhibitory effect of hemolymph. The results showed increased bacterial growth in *D. ananassae* compared to *D. melanogaster*. Due to bacterial infection, reduced fat accumulation was observed in both *D. ananassae* and *D. melanogaster*, which suggests a possible decline in physiological condition. Further, a significant elevation in phenoloxidase, nitrite levels and hemolymph reveals that bacterial cell growth was found to be inhibited due to the immune response of both *Drosophila* species. *D. ananassae* showed high sensitivity to bacterial infection, while *D. melanogaster* showed moderate sensitivity. The results have demonstrated the level of immunocompetence of laboratory stocks of *D. melanogaster* and *D. ananassae* against Gram-positive and Gram-negative bacteria.

Keywords: Antimicrobial activity, Bacterial infection, Hemolymph, Immune response, Melanization, Phenoloxidase

The innate immune system is the first line of defense that distinguishes a foreign invader, and it's an essential requirement for insects to fight against bacterial and fungal infections[1]. Many aspects of innate immunity are conserved between insects and mammals. Insects respond rapidly to microbial infection by expression of antimicrobial peptides (AMPs) to defend against invading microorganisms[2]. The fruit fly is proven as one of the premier model systems for studying the function and evolution of immune defense, which offers a unique experimental toolbox including genetic screens to identify the genetic basis of body fat storage control[3].

Drosophila has a specific antimicrobial response to various classes of microorganisms. Immune activation in *Drosophila* leads to the activation of two signal transduction cascades: the Toll and Imd pathways[4]. These pathways are induced by microbial invaders, which are dependent upon the structures and combinations of elicitors presented to the fly. The initiation of any of these pathways leads to the activation of Nuclear Factor Kappa (NFκB) transcription factors that result in the expression of various target genes, including those encoding AMP[5]. The genes encoding for antibacterial and antifungal peptides are differentially expressed after infection of microorganisms[6]. Insects can also synthesize AMPs in epithelia, which lines the outer membrane of the insect and the intestine of insects, and these are exposed first to microbial invaders. After the recognition of microorganisms by extracellular proteins, the chain reaction will be initiated and induces the production of AMPs[7].

The immune system is not only the signaling pathways that produce antimicrobials, but it is also a sum of all the biological mechanisms that protect a host from infection. Besides, disease and immunity are certainly complex processes as different microorganisms provoke different physiological responses in the fly[7]. A complex interaction between the immune response and behaviour has been described in a wide range of species[8]. Further, disease resistance can vary among the different strains of *Drosophila*. *Drosophila melanogaster* and *Drosophila ananassae* are two genetically different species that could show a distinct immune response to any particular microbe. Distribution wise both species are cosmopolitan and share the same habitat[9]. The ecological context also affects variation in the

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individual behaviour of flies[10]. For these evolutionary backgrounds, we have selected these two species to study immune response against bacteria.

Many researchers have studied the microbial infection in *Drosophila*[11,12]. Apart from these, our work was focused on to understand the variation in physiological processes such as survival, bacterial load count, locomotory activity and sleep, melanization, number of hemocytes, levels of phenoloxidase, nitrite level, and inhibition assay of *D. melanogaster* and *D. ananassae* by infecting the host with a Gram-negative (*Escherichia coli)* and Gram-positive bacteria (*Staphylococcus aureus*).

Materials and Methods

Fly stocks

Fly stocks were reared on an instant *Drosophila* diet supplemented with yeast and maintained at 25ºC. The reference fly strains *Drosophila melanogaster* (1.002) and *Drosophila ananassae* (11.001) were procured from *Drosophila* Stock Center, University of Mysore, Mysore. All stocks were cultured and maintained at room temperature (RT). Adult male and female flies aged 4-5 days old were used in all experiments.

Bacterial cultures

The following bacteria were used in all experiments: *Escherichia coli* strain (MTCC No. 723) and *Staphylococcus aureus* strains (MTCC No. 7443) obtained from MTCC, Chandigarh. All bacteria were maintained on Petri dishes containing solid agar medium. For liquid cultures, bacteria were grown in sterile tubes containing 7.5 mL of 2.5% Luria-Bertani (LB) broth and incubated for 24 h at 37°C. Cultures were pelleted down by centrifuging at 4ºC for 5 min and suspended in phosphate-buffered saline (PBS). The density of the bacterial suspension was estimated with an optical density measurement at 600 nm using a spectrophotometer[13]. For further experiments, both fly strains were infected with *S. aureus* and *E. coli* separately.

Infection and survival studies

Flies were infected by needle pricking method at the lateral side of the thorax using a 0.1 mm sterile tungsten needle dipped into the bacterial slurry[2] (Fig. 1). Fifty flies were used in each group. In control group, the infection was carried out by pricking flies with a needle dipped in PBS solution. In the treatment group, nearly 100-150 colony forming units (CFU) of bacteria were injected per fly. The pricked flies were placed into a fresh vial, laying the vial on its side until all of the flies have recovered from the anesthesia to prevent the flies from becoming stuck to the food[14]. Flies were kept at RT, and survival was monitored at 0, 3, 6, 12, 24 and 48 h intervals after infection[15]. Flies that died within the first hour of the challenge were not considered for the analysis. The experiment was carried out in triplicates.

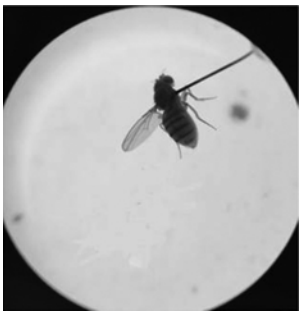

Fig. 1 — Stereo-binocular microscopic view of a fly during needle pricking method of infection. Here, the dorsolateral thorax region was pinched with a 0.1 mm tungsten needle.

Behavioural assays

The sleeping behaviour of flies was assessed by visual observation of the locomotor activity of flies as per protocol with certain modifications[16]. The locomotor behaviour was observed as the number of flies active in the 45 s period immediately after the disturbance. The treated flies were placed in empty plastic vials. After a 20 min rest period, the flies were tapped down and the number of flies being able to climb 3 cm in 30 s was recorded. The experiment was repeated three times at 1 min intervals. All observations were made in constant light (8-11 a.m.) and temperature (26ºC) to eliminate the influence of the circadian clock on behaviour and infection. Climbing percent (%) was calculated using the formula $1/2[(n_{tot} + n_{top} - n_{bot})/n_{tot}] \times 100$. i.e., mean of the numbers of flies at the top (n_{top}) and the bottom (n_{bot}), expressed as percentages of the total number of flies (n_{tot})[17].

Estimation of bacterial load

The total number of bacterial CFU in the fly was estimated by crushing twenty flies at 0, 3, 6, 12, 24, and 48 h post-bacterial infection. The infected flies were homogenized in LB broth, serially diluted[18] and spread onto LB agar plates and incubated at 37ºC for 24 h. A control group of flies injected with PBS alone was used to verify that the infection was not contaminated with any other bacterial species[8]. The experiment was carried out in triplicates.

Melanization reaction

The melanization reaction is considered as an important facet of the insect host defense. Individual flies were observed for melanization on the pricked region in a stereo-binocular microscope at 24 and 48 h post-infection in both infected and normal flies[19].

Hemocytes count

Adult flies were injected with the bacteria and estimated for hemocytes level at 0, 3, 6, 12, 24, and 48 h post-infection. A 10 µL of hemolymph was isolated from 30-40 anesthetized flies by centrifuging the flies at 1500 ×g for 6 min at 4ºC. The obtained hemolymph was immediately added to 100 µL ice-cold Ringer solution. The hemocytes were finally collected by centrifugation and counted using a Neubauer's hemocytometer[20].

Fat quantification

The flies were infected with bacteria and total fat was estimated at 0, 3, 6, 12, 24 and 48 h post-infection. In brief, flies were homogenized in 0.05 % Tween 20 (Sigma) and heated at 70ºC for 5 min. The homogenate was centrifuged at 10,000 xg[21] and the supernatant obtained was mixed with a Thermo infinity trig solution (Himedia), followed by incubation at 37ºC for 5 min. The absorbance corresponding to the concentration of total fat in the sample was measured at 570 nm on a Multiskan-Ex ELISA reader[22]. The results were expressed in percent of fat produced in treated flies relative to control flies.

Estimation of nitrite levels

Nitrite level was estimated in flies at 0, 3, 6, 12, 24 and 48 h post-bacterial infection. Flies were homogenized in buffer (0.1 M PBS; pH 7.0), followed by centrifugation at 10,000 xg for 10 min/4ºC[21]. The supernatant was mixed in a 1:1 proportion with Griess reagent (Sigma) and incubated at RT for 15 min. The optical density was measured at 595 nm using the Multiskan-Ex ELISA reader[18].

Phenoloxidase activity

The phenoloxidase (PO) activity was determined in hemolymph from flies at 0, 3, 6, 12, 24 and 48 h post-infection by microplate method. Thirty flies of each strain were infected with bacteria and PBS separately and hemolymph was extracted. It was diluted using 10 mM PBS and centrifuged at 1500 ×g for 5 min. A 40 µL of supernatant was obtained and mixed with 160 µL L-DOPA (Himedia) in 96-well plates. A linear increase in absorbance was measured at 490 nm over 30 min [23]. The experiment was carried out in triplicates.

Resazurin based antibacterial activity

The antibacterial activity was carried out in fly's hemolymph obtained at 24 h post-infection by the resazurin microtitre plate method with certain modifications[24]. A 5 µL of hemolymph (*S. aureus* or *E. coli*) was pipetted into each well except control well. A 5 µl of PBS and Ciprofloxacin was added to negative and positive control wells respectively. Then, 50 µL of nutrient broth was added. A 5 µL of the resazurin indicator solution was added. Finally, 10 µL of bacterial suspension (*S. aureus* or *E. coli*) was added to each well having a concentration of 5×10^5 CFU/mL. The plates were prepared in triplicate and incubated at 37ºC for 24 h. The absorbance was measured at 600 nm using the Multiskan-Ex ELISA reader.

Statistical analysis

Data were presented as mean ± SD and statistical variations were determined using Graph-pad Prism 8.0. For data analysis, means were compared using repeated measures two-way analysis of variance (ANOVA) with Dunnett's Post-Hoc test or an unpaired two-tailed t-test (bacterial load) or Brown-Forsythe ANOVA (Hemocytes). A comparison between survival curves (percent death) was conducted using a log-rank (Mantel-Cox) test. Values were considered significant when $P < 0.05$.

Results

The rate of survival was higher in *D. melanogaster*

The rate of survival was varied after infection with *S. aureus* and showed 70 and 59% at 48 h time point in *D. melanogaster* and *D. ananassae*, respectively. After infection with *E. coli*, the survival rate was decreased by up to 74 and 59% at 48 h in *D. melanogaster* and *D. ananassae.* The *D. melanogaster* showed relatively high resistance to both bacterial infections. Most flies survived after aseptic control infection, indicating that flies succumbed to the infection and not to the injury (Fig. 2 A & B).

Infection promotes sleep and changes in locomotory behaviour

Fly sleep behaviour was assessed by visual observations. Based on this, an increase in sleep was observed in *D. melanogaster* and *D. ananassae* flies infected with *S. aureus* and *E. coli* compared to the PBS group.

The fly locomotor behaviour was also changed after bacterial infection, and the climbing of the flies was observed. The flies were not able to climb upwards for every tapping timepoint and instead, they lied at the bottom of the vial. A significant decrease in climbing behaviour was seen starting from 6 h and up to 48 h post- *S. aureus* infection in both fly strains. However, post-*E. coli* infection, the climbing behaviour has significantly decreased only after 12 h and decreased until 48 h in *D. melanogaster* and *D. ananassae* flies (Fig. 3).

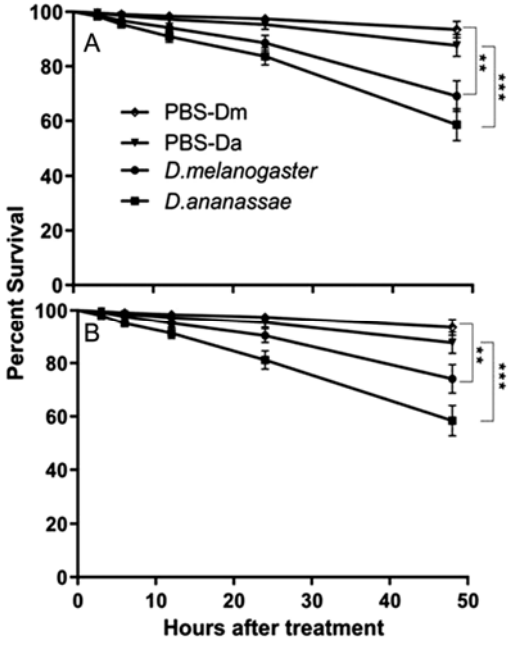

Fig. 2 — The rate of mortality after the injection of *D. melanogaster* and *D. ananassae* with PBS (control), *S. aureus* (A) and *E. coli* (B). The number of flies survived after 0, 3, 6, 12, 24 and 48 h of infection was indicated in percentage. *D. melanogaster* has shown more resistance to infection from both bacteria with the highest survival rate. [Data were presented as mean ± SD from three independent experiments. Significance as compared to PBS group. ***P <0.01*, **** P <0.001*]

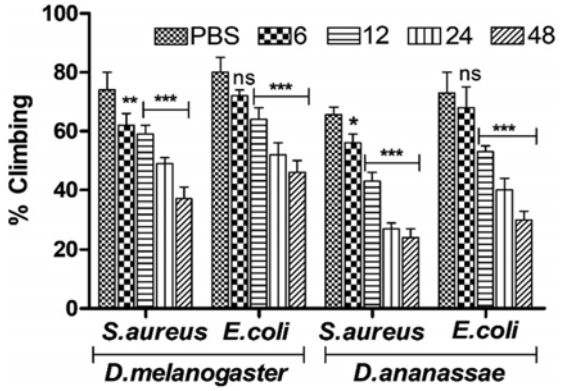

Fig. 3 — The Locomotory activity was studied based on percent climbed by *D. melanogaster* and *D. ananassae* after infection with *S. aureus* and *E. coli*. The *D. melanogaster* showed more climbing activity than *D. ananassae* in both infections. [Data were presented as mean ± SD from three independent experiments. Significance as compared to PBS group. ***P <0.01*, **** P <0.001*]

Bacterial load in infected flies

The bacterial load was not observed after 24 h post-infection in both *D. melanogaster* and *D. ananassae* flies. A significant increase in CFU was observed until 12 h post- *S. aureus* infection in both fly strains, after which no significant CFU was observed until the study period. In *E. coli* infected flies, CFU has significantly increased till 12 h post-infection in *D. melanogaster*. Though the bacterial load was significantly higher at 24 h post-infection in *D. ananassae*, the highest level was observed only at 12 h post-infection, with further reduction observed till the time points studied (Fig. 4 A & B).

Melanin production does occur after bacterial infection

A dark spot was observed at the region of pricking, indicating the deposition of melanin at the injection

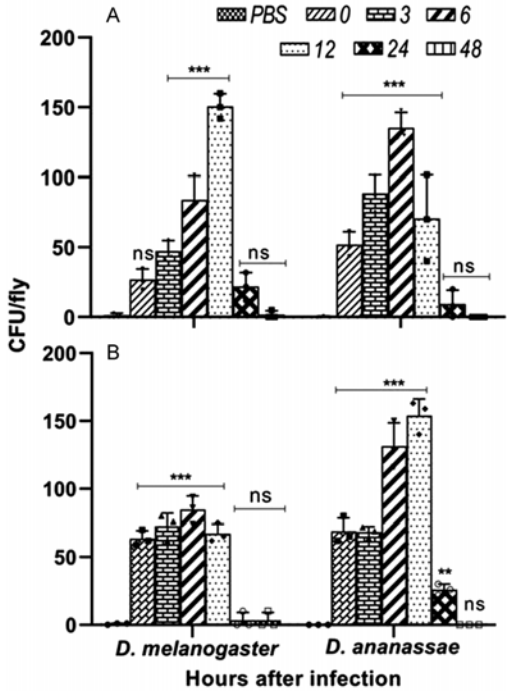

Fig. 4 — Bacterial load (CFU) in *D. melanogaster* and *D. ananassae* after infection with *S. aureus* (A) and *E. coli* (B). The number of recoverable bacteria as represented in CFU/fly was counted at 0, 3, 6, 12, 24, and 48 h after infection. The CFU was lower in *D. melanogaster* as compared to *D. anannassae*. [Data were presented as mean ± SD from three independent experiments. Significance as compared to PBS group. ***P < 0.01*, **** P < 0.001*]

site in both D. melanogaster and D. ananassae (Fig. 5 A & B). However, there was no melanization spot observed in PBS treated flies. Higher melanization spots were observed in 24 h post-infection *D. melanogaster* and D. ananassae flies than in 48 h infected flies.

Hemocytes as a cellular immune mediator

The number of hemocytes was significantly higher until the 48 h time point studied in bacterial infected *Drosophila* spp., when compared to PBS treated flies. In *S. aureus* infected flies, a significant increase in the number of hemocytes was observed in all the time points, the highest level was only found at 12 h post-infection in both the fly strains.

However, In *E. coli* infected flies, no significant difference was found at 0 h in comparison with PBS treated flies. A significant difference in hemocytes was found till 48 h with the maximum level being observed at 6 h in *D. melanogaster* and 12 h in *D. ananassae* (Fig. 6 A & B).

Fat deposition reduces post-bacterial infection

A gradual decrease in fat content was observed in both *D. melanogaster* and *D. ananassae*. The total fat present in *D. melanogaster* and *D. ananassae* was less when compared to PBS treated flies. In *S. aureus* infected flies, there was no significant relative fat content observed till 24 and 6 h in *D. melanogaster* and *D. ananassae*, respectively. However, the fat level was reduced at 48 h in both the fly strains. In *E. coli* infected flies, no significant differences was observed till 24 h in *D. melanogaster* and *D. ananassae*. Simultaneously, a significant reduction in the fat deposition was observed at 48 h post-infection in both the fly strains (Fig. 7 A & B).

Nitrite levels promote increased immune activity

For PBS infections, lower nitrite levels were observed in both *D. melanogaster* and *D. ananassae*. In *S. aureus*

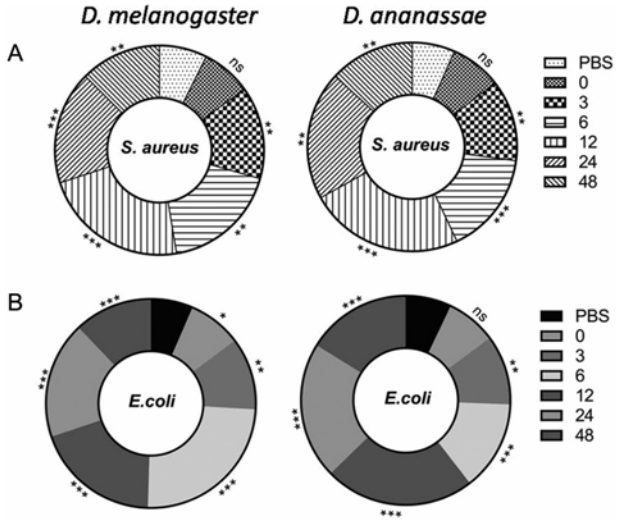

Fig. 6 — Number of hemocytes recovered were determined as per microliter of hemolymph from the *D. melanogaster* and *D. ananassae* after infection with PBS (control), *S. aureus* (A) and *E. coli* (B) at 0, 3, 6, 12, 24, and 48 h after infection. Hemocyte level was unaffected at 0 h but maintained at an increased level until further time points studied. [Data were presented as mean ± SD from three independent experiments. Significance as compared to PBS group. **P <0.05*, ***P <0.01*, **** P <0.001*]

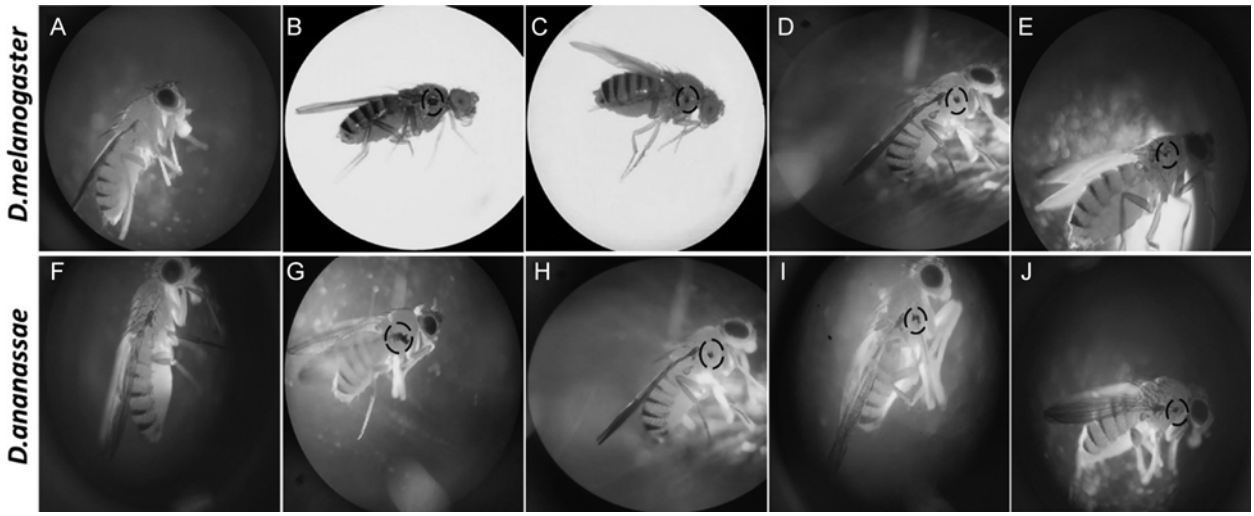

Fig. 5 — Level of melanization observed in *S.aureus* and *E. coli* infected *D. melanogaster* & *D. ananassae* flies in PBS group (A & F), 24 h group (B, G & D, I) and 48 h post-infection group flies (C, H & E, J). Melanin production is higher at 24 h but reduced after 48 h of infection. Also, the melanin production is lower in *D. ananassae* flies when compared to *D. melanogaster* flies.

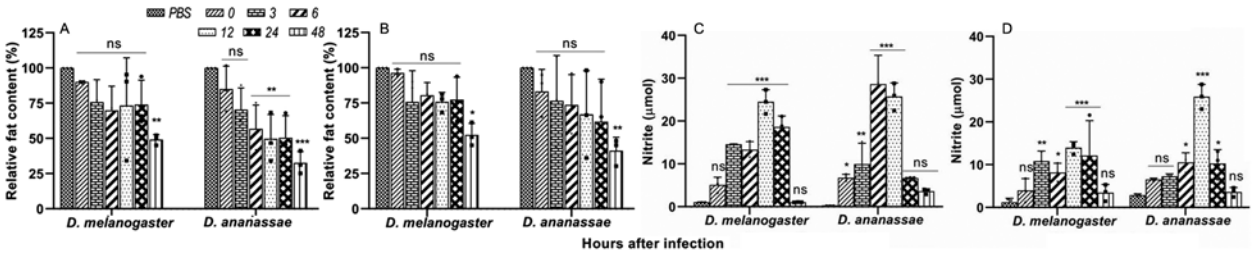

Fig. 7 — Relative percentage of total fat (A & B); and Nitrite levels (C & D) in the *D. melanogaster* and *D. ananassae* estimated at 0, 3, 6, 12, 24, and 48 h after infection with PBS (control), *S. aureus* and *E. coli*. The fat deposition has significantly reduced mostly after 48 h post-infection except in *E. coli* treated *D. ananassae* flies where the significant reduction was observed from 6 h post-infection. The nitrite levels were significantly higher until 24 h in all groups except the *S. aureus* infected *D. ananassae* group in which nitrite level was reduced 12 h post-infection. [Data were presented as mean ± SD from three independent experiments. Significance as compared to PBS group. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$]

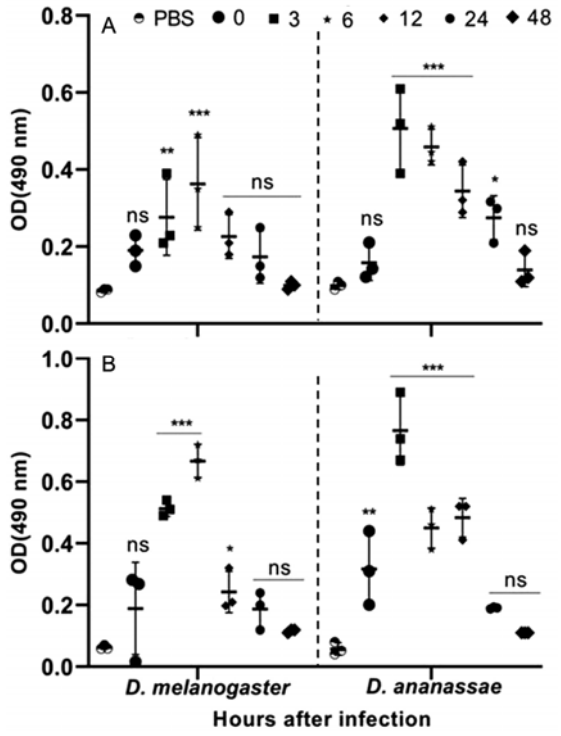

Fig. 8 — Phenoloxidase activity has significantly varied in the *D. melanogaster* and *D. ananassae* after infection with *S. aureus* (A) and *E. coli* (B). The PO activity in fly hemolymph samples was measured at 0, 3, 6, 12, 24 and 48 h after infection. [Data were presented as mean ± SD from three independent experiments. Significance as compared to PBS group. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$]

infected flies, a significant increase in nitrite level was observed till 24 h post-infection in *D. melanogaster* and 12 h in *D. ananassae*, after which no significant change in nitrite level was observed (Fig. 7C).

Among *E. coli* infected flies, there was a gradual increase in nitrite level up to 24 h which goes on decreasing in *D. melanogaster*. In *D. ananassae*, nitrite level was higher up to 12 h. A significant nitrite

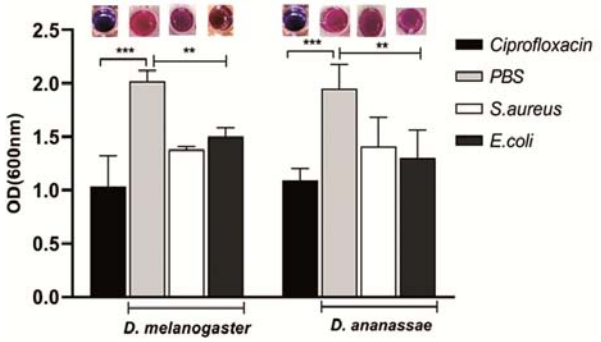

Fig. 9 — Antibacterial activity of hemolymphs isolated from *D. melanogaster* and *D. ananassae* after infection with *S. aureus* and *E. coli*. The bacterial growth was significantly inhibited by the hemolymph collected at 24 h after infection. [Data were presented as mean ± SD from three independent experiments. Significant as compared to PBS group. **$P < 0.01$, *** $P < 0.001$]

level was observed at 12 h post-infection in both the fly strains (Fig. 7D).

Phenoloxidase activity increases with a bacterial infection

The PO level was significantly increased up to 6 h in *D. melanogaster* and 24 h in *D. ananassae* infected with *S. aureus*. No further increased levels of PO was observed until 48 h post-infection. In *E. coli* infected flies, an increased PO level was observed until 12 h after which no significant PO was observed until the 48 h study period in both the fly strains (Fig. 8 A & B).

Hemolymph from infected flies reduces bacterial cell viability

The effectiveness of this modified resazurin assay has been carried out with hemolymph isolated 24 h post-infection of *D. melanogaster* and *D. ananassae*. The higher cell viability was observed in PBS when compared to treated or standard wells. The hemolymph obtained from two fly strains post-*S. aureus* and *E. coli* infection has significantly reduced bacterial growth when compared to PBS added well (Fig. 9).

Discussion

Drosophila serves as a powerful model system to study innate immunity. To expand the studies and investigation, several aspects of the humoral and cellular immune response in *D. melanogaster* and *D. ananassae* was investigated by infecting the flies with Gram-positive and Gram-negative bacterial strain.

Results of recent studies have indicated the variation between certain laboratory lines, in susceptibility to infection with bacterial pathogens[18]. Variation in fly survival was due to increased bacterial infection. The previous results reported that *Drosophila*, in response to infection with *E. coli* and *Beauveria bassiana*, the survival rate has reduced[15]. In this study, *D. ananassae* flies infected with *S. aureus* and *E. coli* showed a decreased survival rate. It indicates the susceptibility of *D. ananassae* due to the reduced ability to clear the bacteria from their body efficiently[25]. An increase in bacterial load at different time intervals led to a decrease in the survival ability of *D. ananassae* and *D. melanogaster*.

These results suggest that fatal bacterial infections are not only due to the inefficient elimination of bacteria (resistance) but also caused by a decline in metabolic fitness during infection.

For survival, sleep is essential and an evolutionarily conserved process. This concept supports that continuous sleep deprivation leads to death in flies[19]. Previous studies have shown that increased resistance to infection will occur through the enhanced activity of the NFκB transcription factor caused by increased sleep which robustly promoted survival[26]. Our observations suggest that *D. melanogaster* flies experienced more sleep and had greater resistance to the infection, which provides a link between the activation of innate immune function and higher survival rate.

The melanization reaction is the immediate immune response against invading pathogens in *Drosophila*[26,27]. Melanization has multiple roles in the immune response, including encapsulation of pathogens, clot formation, and production of cytotoxic intermediates that kill invading microorganisms[28]. Melanin prevents the loss of hemolymph and invasion of pathogens into the hemocoel[29]. This blackening reaction results from the de novo synthesis and deposition of melanin[30]. In our study, though the melanization reaction started appearing from 3 h post-infection (data not shown), the highest level was seen at 24 h post-infection in both *D. ananassae* and *D. melanogaster* which may have reduced the bacterial load. Several studies have analyzed the contribution of hemocyte-mediated melanization to *Drosophila* survival upon wounding and infection[31]. This could also represent an alarm mechanism that prepares the host in case a new pathogen breaches epithelial defences avoiding secondary infection.

Drosophila counters systemic infection through the wide-ranging action of hemocytes, which are equivalent to vertebrate blood cells[32]. Hemocytes play a key role in the survival of insect species. The number of hemocytes varies enormously in the developmental stages as well as in different physiological states of the insects[33]. In our study, the number of hemocytes started to increase at an early point and have not reduced until the study period. These results imply that *D. melanogaster* may rely mostly on the rapid activation of cellular immune defenses against bacterial infection.

To examine the physiological status of flies, we tested total fat in adult flies infected with bacteria and PBS. The body fat content of flies can vary widely and serve as a sensitive diagnostic phenotype indicating imbalances in lipometabolism. Various techniques have been used to quantify fat storage in flies[3]. In the present study, the low-fat level in infected flies shows that starvation or an overall decline in the metabolic state of the flies.

Measuring nitrite levels in insects is an accurate estimation of cellular nitric oxide production. Nitric oxide (NO) is a highly reactive phagocyte derived effector molecule that acts in the nervous and immune system as a mediator in insects and vertebrates[34]. At low concentrations, NO mediates cellular and humoral immune responses[35]. Previous studies showed that NOS (Nitric oxide synthase) activity is required for a robust innate immune response to Gram-negative bacteria[2]. In our study, increased NO concentration in the fat body and hemocytes indicates that NO is produced in response to bacterial challenge. A relatively low level of NO was seen in *D. melanogaster* infected with *S. aureus*, whereas, *D. ananassae* showed a high level of NO production after infection with *E. coli*. Our indirect measurement of NO indicated an almost 5-fold increase in fat body NO after bacterial injection. It is the indication that the differential quantity of NO varies among *Drosophila* species and plays a role in the sensitivity to bacterial infection.

Crystal cells in hemocytes rupture in response to immune activation, which releases prophenoloxidase (PPO), an active form of phenoloxidase involved in the melanization cascade[36]. The phenoloxidase is the terminal enzyme, appears to play a key role in recognition and defense against bacterial infections in invertebrates[37]. Experiments estimating PO activity in both fly strains suggests that the PO is activated at different levels, which affects immune response against infections. The previous study states the important role of PPO1 and PPO2 in the survival of infection with Gram-positive bacteria and fungi, also underlining the importance of melanization in insect host defense[23].

Bacterial growth inhibition is a key indicator of an antimicrobial agent's potency. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. A blue non-fluorescent and non-toxic dye becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells[24]. The hemolymph of infected flies has inhibited the bacterial strains used, which indicated the activation of innate immune response and expression of AMPs in infected *D. melanogaster* and *D. ananassae* flies.

The hallmark of the fly host defense is explained by two main signaling pathways, Toll is activated by Gram-positive bacteria and Imd pathway (immune deficiency) activated by Gram-negative bacteria, which lead to the activation of AMPs[38]. *D. melanogaster's* innate immune response involves the inducible expression of antimicrobial peptides, which functions synergistically to fight infectious microbes[39,40]. These antimicrobial peptides are mainly identified in hemolymph and fat bodies, which are used for all biochemical investigations.

These data outline that after bacterial injection, it started to multiply in the *Drosophila* system, which promoted the upregulation in the number of hemocytes, leading further to the enhancement of PO followed by nitrite levels. This innate immune response could have guided in the clearance of bacterial load. Nonetheless, due to persistent bacterial load, fat deposition may have reduced. Each bacteria differs in their mode of infection which affects the physiology of the fly. Based on the results obtained for *S. aureus* and *E. coli* infection in both fly strains, it suggests that the fly immune response to bacterial infection can vary from one bacterium to another. However, these results can be further validated with other bacterial and fungal infection studies.

Conclusion

Overall, our study demonstrated the interspecific difference in survival rate, melanization, hemocytes, nitrite, fat level, phenoloxidase, and bacterial inhibition by hemolymph of *D. melanogaster* and *D. ananassae* species. The difference in sensitivity of *D. ananassae* in contrast to *D. melanogaster* infected with Gram-positive and Gram-negative bacteria reflects significant changes in immune responses of the two *Drosophila* species.

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Conflict of interest

The authors declare no conflict of interests.

References

1 Kim T & Kim YJ, Overview of innate immunity in *Drosophila*. *J Biochem Mol Biol*, 38 (2005) 121.
2 Foley E & O'Farrell PH, Nitric oxide contributes to induction of innate immune responses to Gram-negative bacteria in *Drosophila*. *Genes Dev*, 17 (2003) 115.
3 Hildebrandt A, Bickmeyer I & Kühnlein RP, Reliable *Drosophila* body fat quantification by a coupled colorimetric assay. *PLoS One*, (2011) 6.
4 Nishide Y, Kageyama D, Yokoi K, Jouraku A, Tanaka H & Futahashi R, Functional crosstalk across IMD and Toll pathways: Insight into the evolution of incomplete immune cascades. *Proc R Soc B Biol Sci*, (2019) 286.
5 Eleftherianos I & Schneider D, *Drosophila* immunity research on the move. *Fly* (Austin), 5 (2011) 247.
6 Lemaitre B, Reichhart JM & Hoffmann JA, *Drosophila* host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc Natl Acad Sci USA*, 94 (1997) 14614.
7 Hetru C, Troxler L & Hoffmann JA, *Drosophila melanogaster* antimicrobial defense. *J Infect Dis*, 187 (2003) 327.
8 Kuo TH, Handa A & Williams JA, Quantitative measurement of the immune response and sleep in *Drosophila*. *J Vis Exp*, (2012) 1.
9 Wallace B, Studies on intra and inter-specific competition in *Drosophila*. *Ecology*, 55 (1974) 227.
10 Matsuda M, Ng CS, Doi M, Kopp A & Tobari YN, Evolution in the *Drosophila ananassae* species subgroup. *Fly* (Austin), 3 (2009) 157.
11 Nehme NT, Liégeois S, Kele B, Giammarinaro P, Pradel E & Hoffmann JA, A model of bacterial intestinal infections in *Drosophila melanogaster*. *PLoS Pathog*, 3 (2007) 1694.
12 Khan I & Prasad NG, The aging of the immune response in *Drosophila melanogaster*. *J Gerontol - Ser A Biol Sci Med Sci*, 68 (2013) 129.
13 Ayres JS & Schneider DS, A signaling protease required for melanization in *Drosophila* affects resistance and tolerance of infections. *PLoS Biol*, (2008) 6.

14 Siva-Jothy JA, Prakash A, Vasanthakrishnan RB, Monteith KM & Vale PF, Oral bacterial infection and shedding in *Drosophila melanogaster*. *J Vis Exp*, 135 (2018)1.

15 Taylor K & Kimbrell DA, Host immune response and differential survival of the sexes in *Drosophila*. *Fly* (Austin), (2007) 197.

16 Carbone MA, Jordan KW, Lyman RF, Harbison ST, Leips J & Morgan TJ, Phenotypic Variation and natural selection at catsup, a pleiotropic quantitative trait gene in *Drosophila*. *Curr Biol*, 16 (2006) 912.

17 Ortega-Arellano HF, Jimenez-Del-Rio M & Velez-Pardo C, Life span and locomotor activity modification by glucose and polyphenols in *Drosophila melanogaster* chronically exposed to oxidative stress-stimuli: Implications in Parkinson's disease. *Neurochem Res*, 36 (2011) 1073.

18 Eleftherianos I, More K, Spivack S, Paulin E, Khojandi A & Shukla S, Nitric oxide levels regulate the immune response of *Drosophila melanogaster* reference laboratory strains to bacterial infections. *Infect Immun*, 82 (2014) 4169.

19 Tang H, Regulation and function of the melanization reaction in *Drosophila*. *Fly* (Austin), 3 (2009) 105.

20 Ozakman Y & Eleftherianos I, TGF-β signaling interferes with the *Drosophila* innate immune and metabolic response to parasitic nematode infection. *Front Physiol*, (2019) 10.

21 Ayyaz A, Giammarinaro P, Liégeois S, Lestradet M & Ferrandon D, A negative role for MyD88 in the resistance to starvation as revealed in an intestinal infection of *Drosophila melanogaster* with the Gram-positive bacterium *Staphylococcus xylosus*. *Immunobiology*, 218 (2013) 635.

22 Tennessen JM, Barry WE, Cox J & Thummel CS, Methods for studying metabolism in *Drosophila*. *Methods*, 68 (2014) 105.

23 Dudzic JP, Kondo S, Ueda R, Bergman CM & Lemaitre B, *Drosophila* innate immunity: regional and functional specialization of prophenoloxidases. *BMC Biol*, 13 (2015) 1.

24 Sarker SD, Nahar L & Kumarasamy Y, Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. *Methods*, 42 (2007) 321.

25 Khalil S, Jacobson E, Chambers MC & Lazzaro BP, Systemic bacterial infection and immune defense phenotypes in *Drosophila melanogaster*. *J Vis Exp*, 99 (2015) 1.

26 Kuo TH & Williams JA, Acute sleep deprivation enhances post-infection sleep and promotes survival during bacterial infection in *Drosophila*. *Sleep*, 37 (2014) 5.

27 Dudzic JP, Hanson MA, Iatsenko I, Kondo S & Lemaitre B, More than black or white: Melanization and Toll share regulatory serine proteases in *Drosophila*. *Cell Rep*, 27 (2019) 1050.

28 Sheehan G, Garvey A, Croke M & Kavanagh K, Innate humoral immune defenses in mammals and insects: The same, with differences? *Virulence*, 9 (2018) 1625.

29 Bilandžija H, Laslo M, Porter ML & Fong DW, Melanization in response to wounding is ancestral in arthropods and conserved in albino cave species. *Sci Rep*, 7 (2017) 1.

30 Troha K & Buchon N, Methods for the study of innate immunity in *Drosophila melanogaster*. *Wiley Interdiscip Rev Dev Biol*, 5 (2019) 1.

31 Vlisidou I & Wood W, *Drosophila* blood cells and their role in immune responses. *FEBS J*, 282 (2015) 1368.

32 Kounatidis I & Ligoxygakis P, *Drosophila* as a model system to unravel the layers of innate immunity to infection. *Open Biol*, (2012) 2.

33 Siddiqui MI & Al-Khalifa MS, Review of haemocyte count, response to chemicals, phagocytosis, encapsulation and metamorphosis in insects. *Ital J Zool*, 81 (2014) 2.

34 Kausar S, Abbas MN, Zhao Y & Cui H, Immune strategies of silkworm, *Bombyx mori* against microbial infections. *Invertebr Surviv J*, 16 (2019) 130.

35 Sadekuzzaman M, Stanley D & Kim Y, Nitric oxide mediates insect cellular immunity via phospholipase A2 activation. *J Innate Immun*, 10 (2018) 70.

36 Salminen TS & Vale PF, *Drosophila* as a model system to investigate the effects of mitochondrial variation on innate immunity. *Front Immunol*, 11 (2020) 1.

37 Leclerc V, Pelte N, El Chamy L, Martinelli C, Ligoxygakis P & Hoffmann JA. Prophenoloxidase activation is not required for survival to microbial infections in *Drosophila*. *EMBO Rep*, 7 (2006) 231.

38 Martin M, Hiroyasu A, Guzman RM, Roberts SA & Goodman AG, Analysis of *Drosophila* STING Reveals an evolutionarily conserved antimicrobial function. *Cell Rep*, 23 (2018) 3537.

39 Tanji T, Hu X, Weber ANR & Ip YT, Toll and IMD pathways synergistically activate an innate immune response in *Drosophila melanogaster*. *Mol Cell Biol*, 27 (2007) 4578.

40 Lin SJH, Fulzele A, Cohen LB, Bennett EJ & Wasserman SA, Bombardier enables delivery of short-form bomanins in the *Drosophila* Toll Response. *Front Immunol*, 10 (2020) 1.