International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 27-07-2021, Accepted: 12-08-2021, Published: 28-08-2021
Volume 6, Issue 4, 2021, Page No. 237-241

Negative geotaxis in *Drosophila* species during priming and challenge infections of *E. coli* and *S. aureus*

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Abstract
There is lot of emerging evidence on the association between locomotor behavior and bacterial infections in *Drosophila spp*. However, the role of immune priming in the regulation of locomotor behavior has been limited. The negative geotaxis was explored here as a parameter to study the locomotor behavior during challenge infections of *E. coli* and *S. aureus* in *D. melanogaster* and *D. ananassae*. The *Drosophila spp.* were initially immune primed with infectious dose and in another set, flies were primed with heat-killed bacteria and challenged with subsequent infection by a homologous bacteria at live lethal dose. The number of flies moving against gravity above 3 cm from the bottom of the vial was recorded. The results showed that infection has reduced the geotaxis movement irrespective of bacterial and fly species used. The *D. melanogaster* showed relatively improved geotaxis movement after immune priming or challenge infections suggesting that it is more tolerant to infection than *D. ananassae*. Further, *S. aureus* infected flies had reduced climbing movement than *E. coli* infected flies. Importantly, in challenge *E. coli* infections, the geotaxis movement was significantly higher in immune primed flies than non-primed live lethal dose injected flies. This could be possibly prefixed to the production of putative cecropin A-like peptide in these *E. coli* immune-primed flies. Finally, our report identified the bacteria and fly species-specific variation in geotaxis behavior in challenge infected *D. melanogaster* and *D. ananassae* flies. Also, a better climbing movement was observed in *E.coli* than *S. aureus*-infected flies. Further studies are needed to confirm the role of antimicrobial proteins in varied negative geotaxis movement.

Keywords: *drosophila melanogaster, drosophila ananassae,* negative geotaxis, priming, challenge infection

Introduction
The activation of the innate immune response by the injection of dead microbes or a sublethal dose of live microbes which is referred to as immunological priming [13]. In insects, this priming could protect a secondary infection with a live lethal dose of homologous bacteria. The production of antimicrobial proteins (AMPs) is a common feature during priming and is known to play a role in the insect's locomotor behaviour [2]. For example, inoculation of honeybees with lipopolysaccharide (LPS) is known to alter the defensin gene expression which in turn alters its behavior after challenge infection [20].

Drosophila melanogaster acts as a suitable model to understand the impact of immune priming on locomotor behavior. The negative geotaxis is one behavioral aspect that is similar between *Drosophila* and humans and can be used to assess locomotor function [4]. Negative geotaxis represents the fly's ability to climb vertically from the bottom of the vial, where the climbing behavior is scored by keeping track of the flies visually for the distance climbed in a fixed time [9]. The *D. ananassae* is a cosmopolitan, domestic species and has a completed genome sequence. Like *D. melanogaster*, *D. ananassae* is frequently found in association with humans [25]. This species has distinct features in its genetic behavior and is of common occurrence in India [28].

Recent studies have showed that many insects develop a strong and specific immune response such as the production of AMP and hemocytes after a subsequent challenge to immune primed insects [23, 7, 8, 26]. Further, it is well known that the nervous system and immune system mediate in a bidirectional manner [13]. So, there is still so much to understand about the impact of immune mediators generated during immune priming or challenge studies on an insect's nervous system and hence on its behavior or locomotor movements. It is also interesting to know the role of antimicrobial peptides which provides both local and systemic immune response on locomotory behavior. In our unpublished data, we have observed the production of putative cecropin A-like peptide in E. *coli* but not *S. aureus* infected *D. melanogaster* and *D. ananassae* flies. However, the impact of subsequent infection after priming on *Drosophila* locomotor behavior is little known. Hence, in continuation to our earlier studies, the effect of challenge infection on immune primed *D. melanogaster* and *D. ananassae* flies on the negative geotaxis movement was assessed.

Materials and Methods
1. Fly stocks
The fly species were procured from *Drosophila* Stock Center, University of Mysore, and Mysore. *D. melanogaster* and *D. ananassae* (1.002 & 11.001) flies were cultured on an instant *Drosophila* diet supplemented with yeast and maintained at 25°C, 12:12 h light/dark conditions. 5 days post-eclosion male and female flies were collected and transferred to a vial containing fresh media before the experiments [10, 17]. For all experiments, 4-5 days old adult flies were used.

2. Bacterial species

Escherichia coli (MTCC 723) and *Staphylococcus aureus* (MTCC 7443) species were procured from MTCC, Chandigarh. All bacterial cultures were maintained on the Nutrient Agar medium. Bacteria were grown in sterile tubes containing 5 mL of Nutrient Broth and incubated for 24 h at 37°C before use.

3. Immune priming and challenge infection

Ten sets of flies (n=60) categorized into three groups were used. In the first group, four sets of flies were pricked with PBS (Phosphate buffered saline) and bacteria at 24 and 48 h. An optical density of 1 (1.0 OD_{600}=1 x 10^7 CFU) was used as an infectious dose. In the second group, six sets of flies were pricked with PBS, Live lethal (LL) and HK+LL (Heat killed+ Live lethal) at 24 and 48 h. For the preparation of heat-killed (HK) bacteria, culture with 0.5 $O.D_{600}$ was autoclaved at 121ºC for 20 min [5]. For the HK+LL set of flies, initially, flies were primed with HK bacteria and 24 h later, LL (1.5 O.D) bacteria was pricked as a challenge dose [22]. All the infection was done on anesthetized flies at the lateral side of the thorax using a tungsten needle. The flies were kept at room temperature (RT) by placing them in fresh vials until all flies were recovered from the anesthesia.

4. Negative geotaxis method

The mobility of flies from each treatment group was recorded using a negative geotaxis climbing assay [9, 16]. Flies (n=60) injected with either bacteria or PBS were placed in the media vial and gently tapped to the bottom. Each vial was vertically divided into six 1 cm tall segments and labeled in the order of ascending height. The vials were then tapped on a lab bench as a startle stimulus [18]. The flies were allowed to climb freely for 4 sec, after which the number of flies that crossed 3 cm from the bottom of the vial was recorded [12]. Each group was assayed thrice at 1 min interval and averaged. The climbing % was calculated using the formula 1/2[(ntot + ntop - nbot)/ntot] X 100. i.e., mean of the numbers of flies at the top (ntop) and the bottom (nbot), expressed as percentages of the total number of flies (ntot).

5. Statistical analysis

The data were analyzed by performing two-way ANOVA for analyzing climbing activity with Bonferroni posthoc test using Graph pad Prism software 5.0. All the values were represented as mean ± SD. Values were considered significance when *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Results

1. Priming with *S. aureus* and *E. coli* declines the climbing ability in *Drosophila spp.*

The climbing ability against gravity was impaired in both fly species infected with either *S. aureus* or *E. coli* when compared to control ($P < 0.001$). However, the geotaxis activity was varied among the fly strains and the bacterial species studied. The mobility of *D. melanogaster* and *D. ananassae* was relatively higher at 24 h than at 48 h post-infection. In the case of *D. ananassae*, only fewer fkies showed negative geotaxis mobility (Fig. 1 and 2). In *S. aureus*-infected flies, *D. melanogaster* showed only a 22 % climbing movement at 24 h which slightly improved after 48 h post-infection (29 %). In *D. ananassae* flies, climbing movement was even more reduced at 24 h post-infection (17 % flies) with a small increase after 48 h post-infection (20 %).

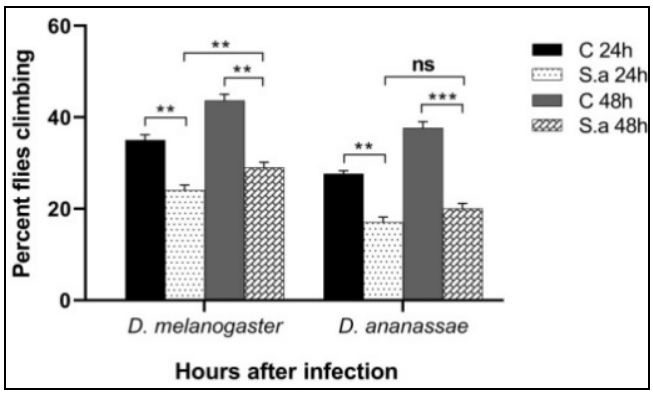

Fig 1: Negative geotaxis movement observed as percent climbing in *D. melanogaster* and *D. ananassae* flies after infection with *S. aureus* (S. a). The *D. ananassae* has shown a significant decline in climbing compared to *D. melanogaster* which had slightly recovered after 48 h post-infection. Significance was shown as compared to control. ns - Non-significant, **$P < 0.01$ and ***$P < 0.001$.

The *E. coli* infected *D. melanogaster* flies showed a declined climbing movement at 24 h (36 %) with gradual recovery after 48 h post-infection (41 %). Similarly, *D. ananassae* flies had 28 % climbing movement at 24 h post-infection with an increased 35 % climbing movement at 48 h post-infection. Overall, among the bacterial species tested, the percent climbing was relatively and significantly better in *E. coli* than *S. aureus* infected *D. melanogaster* and *D. ananassae* flies.

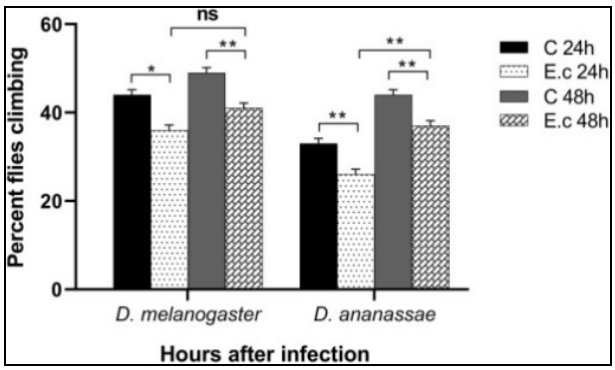

Fig 2: Negative geotaxis movement observed as percent climbing in *D. melanogaster* and *D. ananassae* flies after infection with *E. coli* (E. c). The *D. ananassae* has shown a significant decline in climbing compared to *D. melanogaster* which recovered after 48 h post-infection. The *E. coli* infected flies had better climbing activity than *S. aureus* infected flies as recorded before. Significance was shown as compared to control. ns – No significant, *$P < 0.05$, **$P < 0.01$.

2. Negative geotaxis mobility was recovered after subsequent challenge infection

The impact of secondary exposure to a homologous pathogen on the climbing ability was determined. The climbing movement was observed in *D. melanogaster and D. ananassae* flies at 24 and 48 h after secondary infection with either *S. aureus* or *E. coli*. As observed in single infection studies, the *D. melanogaster* flies showed more climbing movement than *D. ananassae*. The pricking of a live lethal dose of *S. aureus* (LL) significantly reduced the climbing movement in both *D. melanogaster* and *D.*

ananassae flies at 24 h (*D. m*=22 %, *D. a*=18 %) and 48 h post-infection (*D. m*=30 %, *D. a*=25 %). In comparision with LL infection, flies primed with heat-killed *S. aureus* and 24 h later, a subsequent infection with a live lethal dose (HK+LL) didn't show a significantly increased climbing ability after 24 h (*D. m*=27 %, *D. a*=22 %) and 48 h after reinfection (*D. m*=35 %, *D. a*=29 %). However, both the fly species climbing ability has shown to be improved after 48 h post-reinfection when compared to 24 h period.

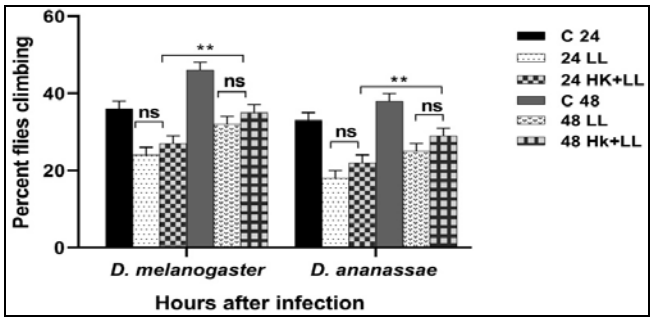

Fig 3: Negative geotaxis movement was observed after reinfection with homologous pathogen i.e., *S. aureus* in primed *D. melanogaster* and *D. ananassae* flies. The HK+LL treatment had better climbing than the LL treatment in both fly species used. Also, in *D. ananassae* climbing was declined compared to *D. melanogaster* which slightly recovered after 48 h post-infection. Significance was shown as compared to control. ns - Non-significant, $^{**}P < 0.01$.

In comparision with the PBS-treated flies, the live lethal dose of *E. coli* infection (LL) has reduced the climbing movement in both *D. melanogaster* and *D. ananassae* flies at 24 h (*D. m*=31%, *D. a*=23 %) and 48 h (*D. m*=36 %, *D. a*=30 %) after infection. In contrast with *S. aureus* infection, flies primed with heat-killed *E. coli* followed by a challenge infection with a live lethal dose (HK+LL) had improved climbing ability after 24 h (*D. m*=40%, *D. a*=31 %) and with an even more significant rise in climbing ability at 48 h period (*D. m*=47 %, *D. a*=41 %).

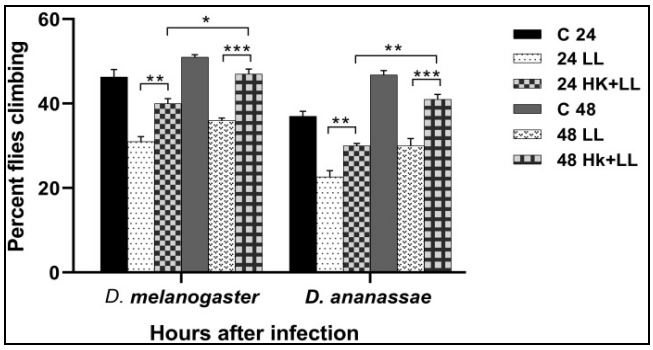

Fig 4: Negative geotaxis movement was observed after reinfection with homologous pathogen i.e., *E. coli* in primed *D. melanogaster* and *D. ananassae* flies. The HK+LL treatment had better climbing than the LL treatment in both fly species used. The *D. ananassae* climbing movement was reduced in comparision with *D. melanogaster* which slightly recovered after 48 h post-infection. Significance was shown as compared to control. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Discussion

The role of microbes including bacteria, fungi and viruses in altering the host's innate immune response and its relationship with insect behavior has been shown by many studies [15, 14]. Even the gut bacteria has been shown to regulate the locomotor activity in *Drosophila* [24]. However, it is still unclear if two different organisms could influence the host behavior distinctly. In this study, we assessed the impact of a Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria on negative geotaxis movement in two *Drosophila* species and clearly showed the altered climbing activities in fly species-specific manner. The negative impact observed in live lethal infection was certainly reduced in pre-infected flies which could be possibly due to immune priming. In insects, the protective response generated by immune priming was known to persist for up to 48 h post-priming [7]. In our study, the climbing movement was improved at 48 h post-reinfection, which could explain the possible persistence of protective response generated after primary infection by heat-killed bacteria.

In earlier studies, LPS-induced immune activation has been shown before to alter the negative geotaxis mobility in *Drosophila* only during the chronic condition [19]. The *Nora virus* and *S. pneumonia* infection decline geotaxis movement in *Drosophila* compared to control flies [21, 27]. Thus, the possible role of immune-induced molecules in providing such resistance to the infection for better climbing ability needed to be studied further. The *E. coli* infected flies had better climbing ability than flies infected with *S. aureus* which could be due to the varied immune response and AMPs produced against different bacteria [6]. In support of this, a putative cecropin A-like peptide was identified on these primed and reinfected fly species (unpublished data). Few studies have reported the role of immune-mediators in altered behavior including climbing activity. The work on *Ophiocordyceps unilateralis* has shown that the expression of AMPs along with other fungal-derived factors may bring changes in behaviour [14]. Similarly, several reports suggested that the NFκB-dependent immune response facilitates survival after septic or aseptic injuries, but with impaired sleep and climbing behavior which were the consequences of an immune-dependent injury mechanism [30, 11, 1]. The Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria contain specific cell wall components such as peptidoglycan and LPS respectively, which could cause the release of specific and differing neuroimmune signaling molecules. These in turn may affect the insect locomotory activity differently. There could be several mechanisms underlying how different bacteria could influence the *Drosophila* locomotory activity and deciphering it will vastly improve the knowledge on the host-microbe interaction and insect neurobiology.

The genetic factor has been proved as a cause for variation in tolerance to infection by different bacteria in *Drosophila spp*. A large genetic variation in locomotory behavior was found among the wild flies of *D. nasuta* and *D. immigrans* [29]. A study by Asada [3] has revealed that *D. pallidifrons* responded more actively than *D. kepulauana* to light exposure, where the analogous relationship between phylogenetic divergence and the negative geotaxis was also explained. In our study, the different genetic backgrounds between the two *Drosophila spp*. used could be the reason for better climbing ability observed in *D. melanogaster* than in *D. ananassae* flies.

Overall, our study extended the knowledge on the time and degree to which different infections modify negative geotaxis in different genetic backgrounds of *Drosophila* spp. Further studies could help us to understand the specific role of AMPs and their mechanism of action in negative geotaxis activity.

Conclusion

The results demonstrated that challenge infection of *D. melanogaster* and *D. ananassae* flies had a significantly improved climbing activity in immune primed conditions as reflected by negative geotaxis assay. The study also revealed that the *E. coli* infected flies showed better climbing activity than *S. aureus* infected *Drosophila* species.

Acknowledgments

The authors are thankful to DST, New Delhi, India for providing financial support through the INSPIRE program (Order No. DST/INSPIRE/2016/IF160813, Date: 5/10/2016).

Author's Contribution

KN developed the concept, idea and gave supervision for experimental design. MRN performed the experiments and the manuscript was drafted. KN edited the manuscript and approved it.

Conflict of Interest

"The authors do not have any conflict of interest."

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