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RESEARCH ARTICLE

AVOIDANCE BEHAVIOR BY WINGLESS DROSOPHILA MELANOGASTER (DIPTERA: DROSOPHILIDAE) OF FOOD SOURCES INFECTED WITH LIPOPOLYSACCHARIDES

Sydney C. Kehrmann, Brianna Niebrugge and *Marianne Robertson

Millikin University, United States

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***Corresponding Author:**
Marianne Robertson

ABSTRACT

Individuals conserve energy through pathogen avoidance by preventing costly immune responses. Lipopolysaccharides (LPS) are gram-negative bacterial endotoxins that initiate an immune response without causing infection. We tested adult male and female wingless *Drosophila melanogaster* using lipopolysaccharide concentrations of 0.5 mg/ml and 1.0 mg/ml. Control flies were not pre-exposed to lipopolysaccharides, while experimental flies were exposed to a food source containing lipopolysaccharides. We placed an individual fly into an arena that contained an uninfected food source (no LPS) and an infected food source (LPS). For each control (n = 30) and experimental (n = 30) fly, we recorded how many times the fly landed on each food source, and the duration of time spent on each food source. There were no significant differences in number of visits or time spent on the infected food source by control males or control females at 0.5 mg/ml LPS. There were no significant differences in number of visits or time spent on the infected food source by experimental males or experimental females at 0.5 mg/ml LPS. There were no significant differences in number of visits or time spent on the infected food source by control males or control females at 1.0 mg/ml LPS. Experimental males pre-exposed to 1.0 mg/ml LPS spent significantly less time on the infected food source and had significantly less visits to any food source. Experimental females had no significant differences in number of visits or time spent on the infected food source at 1.0 mg/ml LPS. There was a significantly smaller proportion of active flies following exposure to the 1.0 mg/ml concentration of lipopolysaccharides in both males and females. These differential results between male and female flies demonstrate sex-specific behaviors following exposure to an endotoxin.

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INTRODUCTION

Gram-negative bacteria have a thin outer membrane composed of peptidoglycan and an endotoxin, known as lipopolysaccharides (LPS). LPS functions as a permeability barrier against host chemical defenses (El-Garawani et al., 2020). *Drosophila melanogaster* can taste LPS and avoid egg laying in LPS infected areas (Soldano et al., 2016). LPS can be detected by the immune system as a dangerous bacterium. Flies clean themselves after contact with LPS to potentially avoid further infection. Furthermore, LPS induced a bitter sensation in the throat and mouth of the flies when ingested (Soldano et al., 2016). *Drosophila melanogaster* experience high rates of exposure to gram-negative bacterial strains in their native environment (Istas et al., 2019). Immune response production is costly and initiating an efficient immune response to all gram-negative pathogenic provocation would be ultimately ineffective (Vale and Jardine, 2017). Hosts that can reduce the chances of infection may prevent deleterious effects as well as elude the energetic costs of an immune response. Therefore, the first non-immunological defense known to occur in a broad range of host taxa is pathogen avoidance (Vale and Jardine, 2017). *Drosophila melanogaster* is an ideal species to study the interaction between behavior and infection because it is one of the most developed models for behavioral ecology, genetics, and host-pathogen interactions (Vale and Jardine, 2017).

Drosophila melanogaster is an ideal species to study the interaction between behavior and infection because it is one of the most developed models for behavioral ecology, genetics, and host-pathogen interactions (Vale and Jardine, 2017). A virulent pathogen infection acted as an unconditioned stimulus that fruit flies associated with cues and caused them to decrease the number of times they approached an infected food source (Babin et al., 2014). Animals must weigh the benefits of nutritional value of the food to the costs of post-ingestion effects and behave accordingly (Kobler et al., 2020). Flies with no synaptic output of the mushroom body, apart of the brain involved in memory, lacked the ability to differentiate between an infected food source and an uninfected food source. Flies with synaptic output of the mushroom body avoided an infected food source for several hours. These findings suggest that pathogen avoidance is an adaptive behavior in *D. melanogaster* (Kobler et al., 2020).

Previous research examined pathogen avoidance in a wide variety of harmful bacteria, fungi, and viruses. Babin *et al*. (2014) exposed fruit flies to the intestinal pathogen *Pseudomonas entomophila*. Pereira and Detrain (2020) used an entomopathogenic fungus, *Metarhizium brunneum*, to examine pathogen avoidance in ants. Vale and Jardine (2017) used *Drosophila* C Virus (DCV) to determine whether fruit flies exhibited pathogen avoidance behavior of an infected food source. Previous studies focused on the direct effect of a pathogen in

the avoidance of infection, but little is known about which component of a gram-negative cell institutes this avoidance. Therefore, we looked at the direct effect of a bacterial endotoxin, LPS, on the avoidance of an infected food source in wingless *D. melanogaster.* We hypothesized that unexposed control flies would not demonstrate avoidance of an LPS infected food source, whereas pre-exposed experimental flies would demonstrate avoidance through decreased number of visits to and time spent on the infected food source. Male *D. melanogaster* avoid eating LPS infected food, and we hypothesize that this behavior would be apparent in female conspecifics as well (Soldano et al., 2016)

METHODS

Avoidance of LPS at 0.5 mg/ml: We obtained living, vestigial, wingless *D. melanogaster* cultures from Josh's Frogs, and we ordered LPS derived from *Escherichia coli* from Santa Cruz Biotechnology. We retrieved adult flies from the cultures and placed them into four 122 mL polypropylene vials containing 40g of Formula 4-24® Instant *Drosophila* Blue Medium mixed with 40 mL of distilled water. We randomly selected two of these cultures to further extract *D. melanogaster* by numbering the vials from one to four and using a random number generator. We examined flies from these cultures under a stereo microscope to determine their sex based on presence or absence of sex combs. Sex combs are male-specific and are used to grasp females during courtship (Massey et al., 2019). We extracted the flies which were separated into 12 different vials containing 5 flies each per sex. We then numbered the vials from 1 through 12 and used a number generator to randomly select 6 vials to be the control group and 6 vials to be the experimental group for each sex. Our control group consisted of 30 fruit flies that were not pre-exposed to LPS. Our experimental group consisted of 30 fruit flies that were pre-exposed to LPS before the trials.

The control flies had a food source of 10 g of Formula 4-24® Instant *Drosophila* Medium and 50 mL of water in the vials, with no previous exposure to LPS. We exposed each experimental fly to an LPS solution composed of 20 mL of water, 0.8 g of sucrose, and 10 mg of LPS for 48 h before the trial (Istas et al., 2019; Lushchak et al., 2013). We kept both solutions in a refrigerator at 2.7 ℃ consistently throughout the duration of the research. We exposed the experimental flies to the LPS solution with a piece of Whatman® qualitative filter paper (Grade 1), 9.0 cm in diameter. We fully soaked the filter paper with the LPS solution and placed it into the experimental fly vial for 48 hours preceding the trial. We repeated this method for all experimental trials.

We placed 2 petri dishes (10 cm dia x 1.5 cm H), which contained 3 g of Formula 4-24®Instant *Drosophila* Medium, 15 mL of water and a piece of fully soaked filter paper, one with one with 4% sucrose solution (control) and one with LPS solution (experimental), into a transparent plastic arena (35.6 cm L x 20.3 cm W x 12.4 cm H) separated by a length of 8.0 cm. We then placed an individual fly into the arena with the two food sources. We covered the arena with a mesh lid. We observed five flies simultaneously for 2 hours in their own individual arenas according to the methods of Babin *et al*. (2014). We recorded the number of visits to each food source as well as the duration of time spent on that food source for each visit. We repeated these methods for all experimental and control flies. After trial completion, flies were preserved in ethanol.

We analyzed the raw data using SPSS 25.0 (IBM), and we considered $P < 0.05$ statistically significant. Further, we used a student's t-test to analyze the total number of visits to each separate food source with the group as the independent variable and total number of visits as the dependent variable. We also used a student's t-test to analyze the total amount of time spent on each separate food source with the group as the independent variable and total amount of time as the dependent variable.

Avoidance of LPS at 1.0 mg/ml: The same methods were repeated using an LPS concentration of 1.0 mg/ml to replicate the methods of Soldano *et al.* (2016). The control flies (n = 30) were not exposed to LPS. Experimental flies (n = 30) were pre-exposed to 1.0 mg/ml LPS solution, composed of 20 mL of water, 0.8 g of sucrose, and 20 mg of LPS (Istas et al., 2019; Lushchak et al., 2013; Soldano et al., 2016). The housing of the flies, trials, and data collection remained the same as conducted in the 0.5 mg/ml LPS trials.

We analyzed data using SPSS 25.0 (IBM), and we considered $P < 0.05$ statistically significant. Due to unequally distributed data, we changed our data analysis from using a parametric student's t-test to using a non-parametric Wilcoxon signed ranked test and a z-scoretest. We used a Wilcoxon signed rank test to analyze the total number of visits to each separate food source with group type (control or experimental) as the independent variable and total number of visits as the dependent variable. We also used a Wilcoxon signed rank test to analyze the total amount of time spent on each separate food source with group as the independent variable and total amount of time as the dependent variable. The z-score test was used to analyze the proportion of active flies with group as the independent variable and number of visits to any food source type as the dependent variable.

RESULTS

Avoidance of LPS at 0.5 mg/ml: We found no significant difference between control and experimental female flies in the total number of visits to the uninfected food source (Fig. 1; $P = 0.262$). We found no significant difference in the total number of visits to the infected food source between control and experimental treatments (Fig. 1; $P = 0.865$). We found no significant difference between control and experimental female flies regarding the total amount of time spent on the uninfected food source (Fig.1; $P = 0.123$). We also found no significant difference in the total amount of time spent on the LPS infected food source between control and experimental treatments (Fig. 1; $P = 0.304$).

We found no significant difference between control and experimental male flies in the total number of visits to the uninfected food source (Fig. 2; $P = 0.196$). We found no significant difference in the total number of visits to the infected food source between control and experimental treatments (Fig. 2; $P = 0.402$). We found no significant difference between control and experimental male flies regarding the total amount of time spent on the uninfected food source (Fig. 2; $P = 0.275$). We also found no significant difference in the total amount of time spent on the LPS infected food source between control and experimental treatments (Fig. 2; $P = 0.277$).

Avoidance of LPS at 1.0 mg/ml: There were no significant differences of number of visits to ($Z = -0.813$, $P = 0.416$) or time spent ($Z = -0.097$, $P = 0.922$) between food sources by control female flies (Fig. 3). There were no significant differences of number of visits to ($Z = -1.027$, $P = 0.304$) or time spent ($Z = -.402$, $P = 0.687$) between food sources and experimental female flies (Fig. 3). There was no significant difference in the total proportion of female flies visiting any food source based on previous exposure (Fig. 4; $z = 0.3139$, $P = 0.757$).

There were no significant differences of number of visits to ($Z = -.108$, $P = 0.457$) or time spent ($Z = -0.241$, $P = 0.809$) between food sources and control male flies (Fig. 5). Experimental male flies previously exposed to LPS visited the infected food sources significantly less often ($Z = -1.611$, $P = 0.053$) and spent significantly less time on the infected food source ($Z = -2.442$, $P = 0.015$) (Fig. 5). There was a significant difference in the total proportion of male flies visiting any food source based on previous exposure ($z = 2.234$, $P = 0.025$), demonstrating male flies previously exposed to LPS have a decrease in activity (Fig. 6).

Comparison of avoidance at differing LPS concentrations: There was a significantly smaller proportion of active flies following exposure to the higher concentration (1.0 mg/ml) of LPS in both females (Fig. 7; $p < 0.00001$) and males (Fig. 7;$p < 0.00001$).

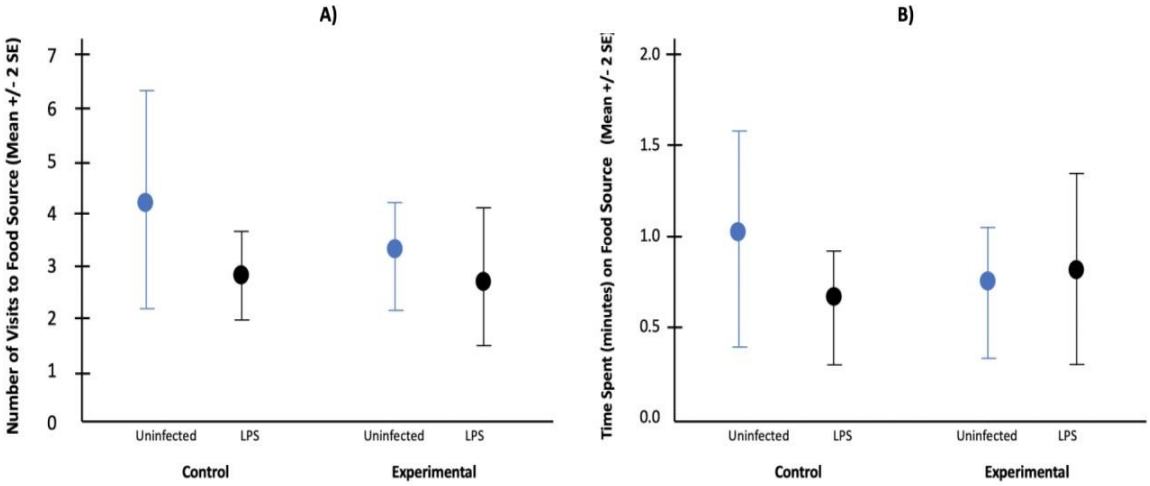

Figure 1. A) Number of visits by female *Drosophila melanogaster* to uninfected and infected food sources based on previous lipopolysaccharides exposure. B) Time spent by female *Drosophila melanogaster* on uninfected and infected food sources based on previous lipopolysaccharides exposure. Control flies were not previously exposed to lipopolysaccharides while experimental flies were previously exposed to 0.5 mg/ml lipopolysaccharide

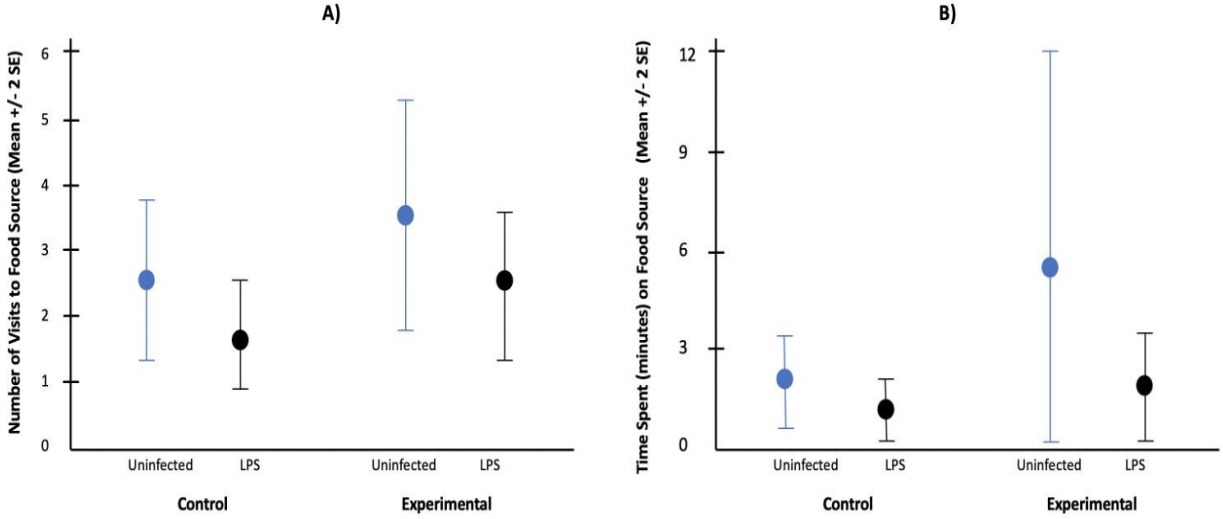

Figure 2. A) Number of visits by male *Drosophila melanogaster* to uninfected and infected food sources based on previous lipopolysaccharides exposure. B) Time spent by male *Drosophila melanogaster* on uninfected and infected food sources based on previous lipopolysaccharides exposure. Control flies were not previously exposed to lipopolysaccharide while experimental flies were previously exposed to 0.5 mg/ml lipopolysaccharide

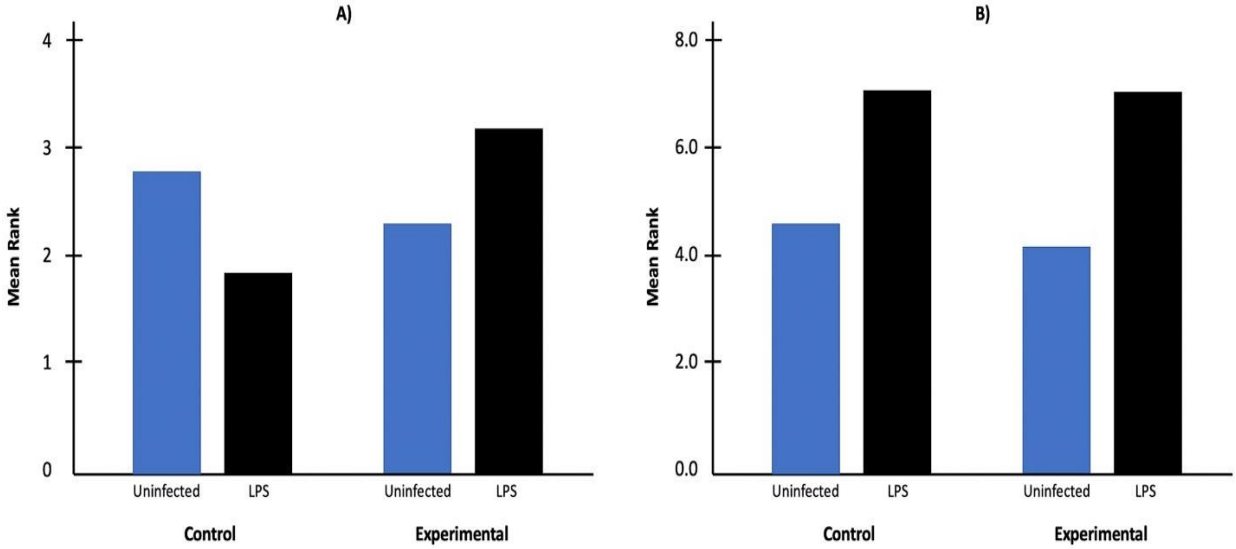

Figure 3. A) Number of visits by female *Drosophila melanogaster* to uninfected and infected food sources based on previous lipopolysaccharides exposure. B) Time spent by female *Drosophila melanogaster* on uninfected and infected food sources based on previous lipopolysaccharides exposure. Control flies were not previously exposed to lipopolysaccharides while experimental flies were previously exposed to 1.0 mg/ml lipopolysaccharide

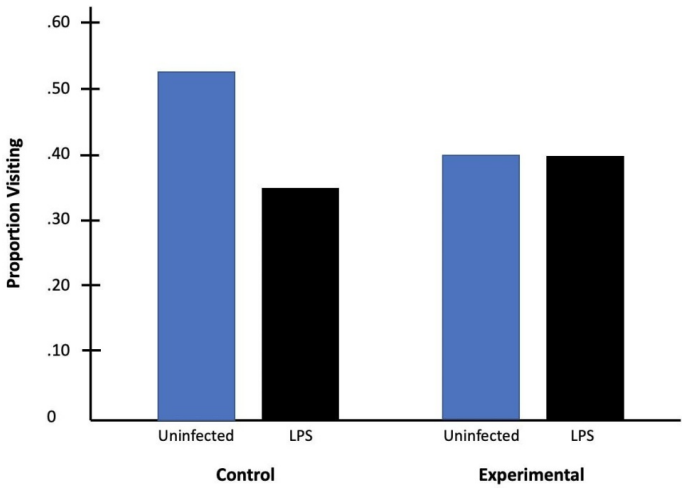

Figure 4. Proportion of female *Drosophila melanogaster* visiting uninfected and infected food sources based on previous lipopolysaccharides exposure. Control flies were not previously exposed to lipopolysaccharides while experimental flies were previously exposed to 1.0 mg/ml lipopolysaccharide

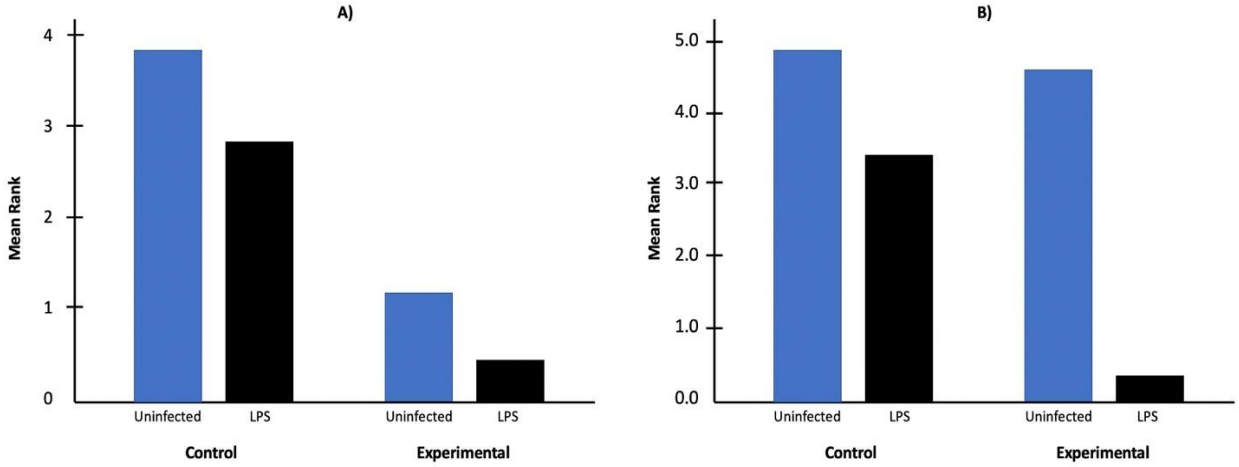

Figure 5. A) Number of visits by male *Drosophila melanogaster* to uninfected and infected food sources based on previous lipopolysaccharides exposure. B) Time spent by male *Drosophila melanogaster* on uninfected and infected food sources based on previous lipopolysaccharides exposure. Control flies were not previously exposed to lipopolysaccharides while experimental flies were previously exposed to 1.0 mg/ml lipopolysaccharide

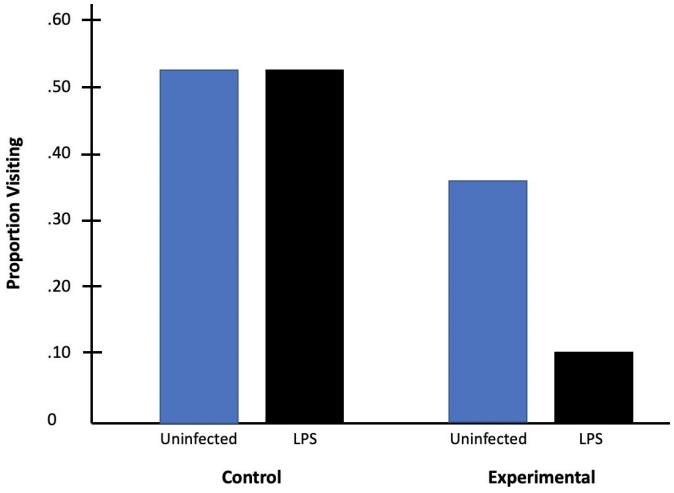

Figure 6. Proportion of male *Drosophila melanogaster* visiting uninfected and infected food sources based on previous lipopolysaccharides exposure. Control flies were not previously exposed to lipopolysaccharides while experimental flies were previously exposed to 1.0 mg/ml lipopolysaccharides.

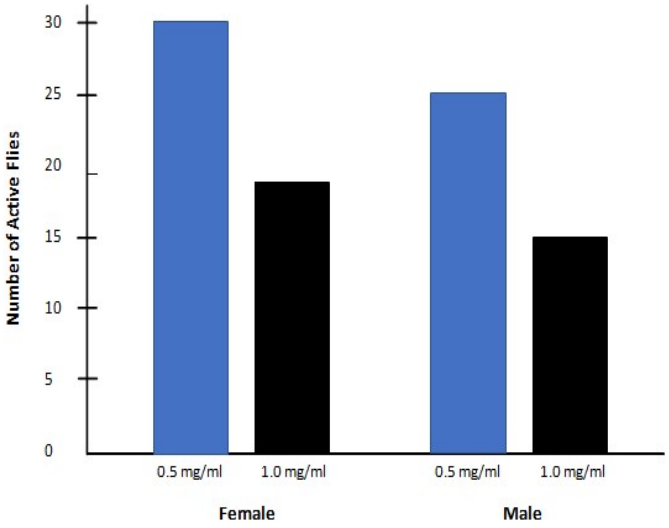

Figure 7. Proportion of active female and male *Drosophila melanogaster* after exposure to different concentrations (0.5 mg/ml or 1.0 mg/ml) of lipopolysaccharides

DISCUSSION

These data do not support our hypothesis that *D. melanogaster* will show avoidance of a food source infected with LPS after previous exposure to 0.5 mg/ml LPS. Our results were not consistent with those of previous research. Vale and Jardine (2017) found that females previously exposed to the pathogen showed a reduced preference of the food infected with the same pathogen. Soldano *et al*. (2016) found that male flies detected LPS through taste receptors and avoided eating infected food and egg laying in an LPS infected area. These studies found a trend in avoidance of a food source infected with a pathogen. Istas *et al*., (2019) found that the innate immune system is activated after exposure to LPS. The innate immune system uses increased amounts of energy to ward off infection which takes energy from other bodily processes. This ultimately resulted in a decreased appetite in *D. melanogaster* (Istas et al., 2019).

The concentration of LPS in the environment might not have been high enough for the fruit flies to initiate an immune response and detect the LPS. In our study, the 0.5 ml/mg LPS solution contained 20 mL of water, 0.8 g of sucrose, and 10 mg of LPS. Soldano *et al*. (2016) used a concentration of 1.0 mg/ml of LPS and found that fruit flies were able to detect and avoid LPS in different food sources. Istas *et al*. (2019) induced behavioral changes in *D. melanogaster* by tainting food with LPS, but it is unknown what concentration of LPS was present, and if any at all made it to circulating levels within the hemolymph. They suggested longer exposure times might yield different results. It is unknown how the gastrointestinal tract and the associated intrinsic bacteria in the intestines might respond during periods of LPS exposure. It is possible the innate immune system may be heightened throughout the time of exposure (Istas et al., 2019). Additionally, Soldano *et al*. (2016) starved the fruit flies for 20 hours prior to measuring avoidance. Starving the flies might cause them to be more motivated to eat. In this study the flies were not starved, which resulted in low amounts of visits and time spent on each food source.

We used a concentration of 0.5 mg/ml LPS that was low in comparison with other studies, so flies may not have been able to detect it. We conducted further research to determine whether increasing the concentration of LPS would produce different results of avoidance of an infected food source by *D. melanogaster*. We repeated our study using a higher concentration of mg/ml LPS to see if *D. melanogaster* would demonstrate decreased number of visits to andtime spent on the infected food source.

Male *D. melanogaster* demonstrated decreased number of visits to, and time spent on the LPS infected food source as well as decreased activity after previous exposure to 1.0 mg/ml concentration of LPS. These data support the hypothesis that male *D. melanogaster* show avoidance of a food source infected with LPS. These results are consistent with previous research. Male *D. melanogaster* avoid eating LPS infected food after previous exposure (Soldano et al., 2016). Additionally, males infected with the bacterium *Wolbachia* decline in total activity (Vale and Jardine, 2015). This behavior is a direct cost of infection, but these behavioral responses can be adaptive because a decline in total activity may help preserve metabolic resources that can be allotted to fighting infection (Vale and Jardine, 2015). Additionally, sleep is valuable in sustaining a vigorous immune response and health during infection (Vincent et al., 2021).

Female *D. melanogaster* demonstrated no significant differences in number of visits to or time spent on an LPS food source in addition to no significant changes in activity after previous exposure to 1.0 mg/ml concentration of LPS. These data do not support the hypothesis that female *D. melanogaster* show avoidance of a food source infected with LPS. Female fruit flies have previously shown the behavior of pathogen avoidance. Female *D. melanogaster* previously exposed to *Drosophila* C virus (DCV) showed reduced food seeking behavior when presented risk of DCV infection (Vale and Jardine, 2017). However, the difference between this study and ours is the type of pathogen used. Vale and Jardine (2017) used the viral infection of DCV whilewe used the bacterial endotoxin LPS. The only other research we found of LPS exposure and female *D. melanogaster* was that of Soldano *et al*. (2016). Soldano *et al*. (2016) found that female fruit flies avoided oviposition in a LPS infected area. However, we did not test oviposition behavior, we tested pathogen avoidance behavior. Soldano *et al*. (2016) did not have data regarding number of visits or time spent between oviposition sites. However, we wonder whether our results of no significant differences of number of visits or time spent is related to exploring different environments for egg laying.

These differential results between male and female flies demonstrate sex-specific learning behaviors following exposure to an endotoxin. One explanation for this sex-specific behavior following exposure could be due to exposure groups. Our flies were exposed to LPS in a vial containing a total of five flies. The stress of undergoing an immune response while surrounded by competition could explain the avoidance behavior demonstrated more by males than by females. In fruit flies infected with DCV, female aggregation was not affected by infection, but males aggregated further apart when infected whereas uninfected male aggregation was unaffected (Siva-Jothy and Vale, 2019). Sexual dimorphic immunity could play a role in avoidance behavior.

Our results are consistent with those of Vale and Jardine (2015) who demonstrated that males infected with *Wolbachia* declined in their total activity compared to females. However, Vale and Jardine (2015) also found DCV infection caused increased sleep in female flies but had no evident effect in males. This demonstrates a pattern of pathogen type and effect on host sex and behavior. Gram-negative bacteria infections in *D. melanogaster* yield conflicting results, showing that these infections can both increase and decrease sleep which could explain for the differences of behavior of males and females exposed to LPS (Vincent et al., 2021).

There was a significantly smaller proportion of active flies following exposure to the higher concentration (1.0 mg/ml) of LPS in both males and females compared to the lower concentration (0.5 mg/ml). During the learning trials using the higher concentration of LPS, there was an abundance of inactive flies following exposure. This observation led us to analyze this observation further through statistical data analysis.

The higher concentration of LPS could have had a greater effect on *D. melanogaster* after exposure. Sickness behaviors such as lethargy (reduced activity) and somnolence (increased sleep) are frequent between most animals and might consequently be seen as general indicators of infection (Vale and Jardine, 2015). As previously stated, this behavior is a direct cost of infection, but these behavioral responses can be adaptive because a decline in total activity may help preserve metabolic resources that can be allotted to fighting infection (Vale and Jardine, 2015). Additionally, sleep is valuable in sustaining a vigorous immune response and health during infection (Vincent et al., 2021).

The results of our data could potentially be due to a trend described by Anyagaligbo *et al.* (2018) on *D. melanogaster* concerning the effects of different concentrations of LPS on heart rate. Peptidoglycan recognition proteins (PGRPs) are responsible for the recognition of LPS in insects. PGRPs activate the innate immune response. Anyagaligbo *et al*. (2018) found dose-dependent effects of LPS on direct exposure of in situ heart tubes in larval *D. melanogaster* using 1 (low), 100 (medium), and 500 (high) µg/ml LPS. LPS derived from *Serratia* marcescens increased heart rate initially in *D. melanogaster* followed by a reduction for 1 µg/ml LPS and 500 µg/ml LPS. A dose of 500 µg/ml of LPS derived from *Pseudomonas aeruginosa* caused decreased heart rate in larval *Drosophila* (Anyagaligbo et al., 2018). In both strains of LPS used, the higher concentration elicited a greater change in heart rate of larval *D. melanogaster* compared to the low and medium concentrations. This is consistent with our study since the higher concentration of LPS had a greater effect on *D.*

melanogaster, specifically in activity levels. The innate immune system is activated after exposure to LPS, and uses increased amounts of energy to ward off infection which takes energy from other bodily processes (Istas et al., 2019). Recovery from energy loss from change in heart rate and fighting infection could explain for decreased activity displayed by *D. melanogaster* following exposure to the different concentrations of LPS to preserve metabolic resources.

One possible source of error in our study could be due to the reuse of containers and food sources. Flies could have detected that there was a previous fly in the container, and that could have affected their behavior. Additionally, to obtain the sample size needed, we could not collect all the same age fruit flies, but they were all adults. Future studies could investigate the avoidance behavior of *D. melanogaster* using LPS derived from different gram-negative bacteria species such as *Proteus*, *Enterobacter* and *Klebsiella.* Future studies could investigate the threshold of LPS that induces pathogen avoidance. *D. melanogaster* could be exposed to LPS at a concentration of 0.5 mg/ml, 0.75 mg/ml, 1.0 mg/ml, 1.25 mg/ml, and 1.5 mg/ml, then tested for pathogen avoidance using the same methods. We suggest longer exposure times and starving the flies prior to the learning trials to initiate foraging behavior. We suggest recording the amount of inactivity as well as number of visits and time spent to quantify how much of the inactivity can perhaps be related to sleep. Another question that could be explored is whether exposure concentration effects lifespan.

The effect of septicemia caused by gram-negative bacteria institutes a wide variety of symptoms for patients. Studying the effects of LPS on specific parts of the body would be an interesting subject of further research. Research on the side effects of the exposure to gram-negative bacteria could produce results to potentially be imputed into the human health care system to treat septicemia caused by gram-negative bacteria in the blood system.

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CONFLICT OF INTREST: None declared.

KEY POINTS

- *D. melanogaster* do not demonstrate avoidance behavior of an LPS infected food source at 0.5 mg/ml concentration.
- Male *D. melanogaster* do demonstrate avoidance behavior of an LPS infected food source at 1.0 mg/ml concentration, whereas female *D. melanogaster* do not.
- *D. melanogaster* decrease in activity after exposure to LPS at 1.0 mg/ml concentration.
- Research on the side effects of the exposure to gram-negative bacteria could produce results to potentially be imputed into the human health care system to treat septicemia caused by gram-negative bacteria in the blood system.

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