Investigating the Impact of Genotype, Sex, and Crotonic Acid Diet on the Survival of Drosophila melanogaster Strains Against Serratia marcescens Infections.

Abstract

Drosophila melanogaster is a vital model organism for genetic and microbiological research due to its genetic parallels with humans. Understanding how different genotypes, sexes, and environmental treatments affect survival under bacterial infection can provide insights into genetic resilience and susceptibility mechanisms. However, survival analysis has yet to explore the Daxx mutant genotype. This study explores the survival outcomes of Drosophila melanogaster, focusing on the Dahomey wildtype and Daxx mutants when subjected to Serratia marcescens infections and crotonic acid treatment. We show that male flies and the Dahomey genotype exhibit higher survival probabilities than female flies and Daxx mutants with a p-value of <0.0001.
Additionally, while crotonic acid treatment did not significantly impact overall survival rates 0.323 p-values, it delayed developmental timing in Daxx mutants. These findings suggest that sex and genotype significantly influence Drosophila's resilience to bacterial infections, with males and Dahomey flies showing more excellent resistance. The study also highlights the potential physiological effects of histone crotonylation, such as delayed development. Our results underscore the importance of considering genetic and environmental factors in studying immune responses and survival, providing a foundation for future research into the molecular mechanisms underlying these differences. Further investigations could explore the specific gene expression profiles and the broader impacts of crotonylation on Drosophila physiology.

Introduction

Drosophila melanogaster is widely used as a research model due to its genetic similarities to humans, possessing only 7,000 fewer genes (Markstein, M. 2018). The shotgun sequencing of its genome in the 2000s (Neckameyer, W.S. and Argue, K.J. 2013) has facilitated precise control of gene expression in specific cells at regulated times and levels (Pfeiffer, B.D. et al. 2010).

The Dahomey strain, a robust wildtype strain from West Africa, is often used as a control due to its health and longevity (Gubina et al., 2018). In contrast, the Daxx[NP4778] strain (Death domain-associated protein), with a mutation in the Daxx gene, exhibits altered stress responses and immune function, potentially affecting longevity and disease susceptibility (Tang, Wan, and Wu, 2015). Dahomey flies generally show higher survival rates when infected with Serratia marcescens than some mutant strains, likely due to their robust immune response (Rose et al., 2022).
Sex differences significantly influence survival outcomes in Drosophila. Typically, males show higher resilience to infections and stressors than females, possibly due to physiological, hormonal, and immune function differences (Lin et al., 2023). While both sexes in the Dahomey strain exhibit vital survival against S. marcescens (Rose et al., 2022), it remains unexplored in the Daxx[NP4778] strain.
This study investigates survival probabilities of different genotypes, sex, and treatment conditions (diet of crotonic acid) in response to Serratia marcescens infections in Drosophila

melanogaster. Understanding these dynamics is crucial for advancing genetic and microbiological research.

Drosophila's unique features, such as the easy isolation of chromosomes from larval salivary glands and the advanced genetic tools available, facilitate studying post-translational modifications (PTMs)(Koryakov and Zhimulev, 2014). PTMs, including crotonylation, a histone PTM, involve adding a crotonyl group (crotonic acid) to lysine residues on histones, impacting chromatin compaction and accessibility (Ntorla and Burgoyne, 2021). Studies using Drosophila have revealed that crotonylation marks are distributed across the genome and are associated with both active and repressed chromatin states, highlighting the complexity of PTM-mediated regulation of gene expression (Boros, 2012).

Genome-wide studies in Drosophila have mapped the distribution of crotonylation marks, indicating enrichment at promoters and enhancers of active genes, suggesting a role in transcriptional activation (Stow et al., 2022). However, crotonylation has not been further studied on different genotypes (Daxx or Dahomey) or under survival analysis of infection, in particular Serratia marcescens and their link to survival or earlier death, and the possibility of affecting the next generation with this susceptibility from the altering chromatin structure.

This study aims to enhance understanding of survival outcomes in specific genotypes under controlled conditions. We hypothesise significant survival differences between sexes, with males showing greater resilience to S. marcescens. Additionally, the Dahomey genotype is expected to have higher survival rates than the Daxx genotype under the same conditions, and specific environmental treatments (e.g., P.ent. vs vehicle) will significantly impact survival probabilities. Furthermore, interactions between genotype, sex, and diet of crotonic acid are anticipated to yield significant variations. For example, Daxx and a diet of crotonic acid will delay egg laying and later development of hatching a day later than the Daxx or Dahomey Drosophila eating regular food or Dahomey on the crotonic acid diet, either impacting survival outcomes, influencing susceptibility to infection.

Methods

Drosophila culturing: Cage setup

Two separate mesh cages attached to large Petri dishes were created to provide a habitat for both genotypes (Dahomey/wildtype and Daxx/mutant) to be comfortable in the microenvironment, especially for a female to lay their eggs. The bottom of the Petri dishes consisted of apple juice plates, offering nutrients and sugar essential for mating and egg laying.

Preparation of Apple Juice Plates

The following materials were gathered to prepare apple juice plates for approximately 2 90mm Petri plates: agar, sucrose or dextrose, Nipagin (methylparaben) or Tegosept (butylparaben), ethanol, and apple juice. A hob was turned on to its highest setting. In a plastic jug, 1.5g of agar was mixed with 50ml of distilled water and microwaved on maximum power for less than 10 minutes. Simultaneously, 1.2g of sucrose or dextrose was mixed with 25ml of distilled water and 25ml of apple juice in a small glass container and heated on the

hob. The hot agar solution was added and boiled for 1 minute. The mixture was cooled quickly under running water. For Nipagin, 0.2g was dissolved in 1.5 ml ethanol (partial dissolution was acceptable). When the solution cooled to approximately 70°C, 1.5ml of ethanol with 0.2g dissolved Nipagin or 1.5ml of Tegosept 10% in ethanol was added. The plates were stored at 4°C. Once firmed, attach apple plates to the created cages. Then, add Dahomey into one cage and Daxx into the other and leave for 24 hours.

Experimental Setup for crotonic acid and control

Next, four tubes for each parameter were prepared: Dahomey (wildtype) containing crotonic acid, Dahomey with regular food and water, Daxx with crotonic acid, and Daxx with regular food and water. Overall, 12 tubes were prepared for each parameter.

Preparation of Tubes

To prepare fly food for a total volume of 15L, the following steps were taken:
A whole kettle of dH2O was boiled. In a bucket, 2.00L of cold dH2O, 1.15kg of maise flour, 1.15kg of dextrose, and 0.50kg of yeast powder were mixed. For the 15L preparation, 100g of agar was mixed with 1L of cold dH2O in the stock pot. Boiling dH2O from the kettle (12.50L) was added to the pot. When the pot reached 80-90°C, the bucket ingredients (maise flour, dextrose, yeast powder, and cold dH2O). The ingredients were heated to 95°C for approximately 30 minutes, maintained at that temperature for 20 minutes, and stirred well. The hob was turned off, and approximately 1L of frozen dH2O was used to cool the mixture to 75°C. Preservatives were then prepared: 46.50ml of Tegosept (10% w/v in ethanol) and 52.5ml of propionic acid. These preservatives were added to the mixture at 75°C and mixed for a few minutes. Food was dispensed at 10mL per tube.
The tubes were then split into equal groups of 12, and 50mM of crotonic acid was added to half. The remaining half was controlled with added water. All tubes were labelled correctly.

Egg harvest

Thirty eggs were harvested from the cage with an apple agar plate and placed in each labelled tube prepared before. Immediately After the flies were hatched, they were separated into males and females using anaesthetics and separated into separate labelled tubes to produce 15 tubes for each male and female.

Bacterial Cultures and Infection

Bacterial cultures of *Serratia marcescens* were prepared by picking two colonies using a plastic loop and cultivating them overnight in L.B. broth with ampicillin in 500 ml flasks on a 180 rpm rotating incubator at 37°C. After 24 hours, the cultures were stored at 4°C. The cultures were then revived in 100 ml media on a 180 rpm rotating incubator at 37°C for 4-5 hours. O.D. was checked with a 1:10 dilution, and cultures with O.D. approximately one were transferred into two 50 ml falcon tubes, ensuring equal weight, and centrifuged at 3600 rpm, 4°C for 30 minutes. The supernatant was removed, and pellets were resuspended in 15 ml of 50 mM sucrose, followed by another centrifugation and resuspension in 2.5-4 ml of 50 mM sucrose to achieve an O.D. between 80-100. During the 30-minute centrifugation, a thermocol box with ice and a new fresh sucrose bottle were prepared, and all pipettes and

controllers were readied. Flies that had been separated by sex from 10:20 a.m. to 3:00 p.m. were starved in tubes with a filter paper disk and 100 µl of sterile distilled water and incubated at 25°C for 2 hours. Food tubes were prepared with 10 ml fly food, topped with a filter paper disk, 150 µl of bacteria for infection or 50 mM sucrose for control, and dried for 20-30 minutes. At 5:30 p.m., flies were anaesthetised, transferred into labelled tubes, and incubated at 25°C, with mortality checked thrice daily.

R Statistical Analysis

Raw data was recorded in Excel for R language graphical representation. The Data was processed in R studio Markdown, version 4.0.4 (2021-02-15) using packages:
"BiocManager" (Bioconductor Core Team, 2022)
"survival" (Therneau, 2022)
"tidyverse" (Wickham et al., 2019)
"survminer" (Kassambara et al., 2022)
"viridis" (Garnier, 2018)
"devtools" (Wickham et al., 2022)
"kableExtra" (Zhu, 2022)
"cachem" (Henry, 2022)
"ggtext" (Wilke, 2022)

The data was first cleaned: unwanted columns were removed, factors were converted to characters, missing data was handled, and columns with 'N.A.' in their names were converted to numeric. The dataset was sorted into relevant categories, reshaped from wide to long format, and columns were renamed for consistency. Kaplan-Meier survival curves were generated to visualise survival probabilities over time, and significant p-values were calculated. Then, pairwise survival difference tests and results were demonstrated through graphs. The complete R script used for the analysis is available in the appendix.

Notes
Pathogen: S. marcescens Db11 (OD~600~=100)
Starvation time for this experiment was 3-4 hours

Results

Firstly, the petro-petro statistical test yielded a significant p-value of 0.0105. While not overwhelmingly confident, this result indicates the pathogenicity of *Serratia marcescens* toward the flies.
The survival analysis of flies infected with *Serratia marcescens* strongly supports our hypotheses. It shows that male flies consistently maintained a higher survival probability of 0.95 throughout the duration than female flies, who experienced a notable decline to 0.80 around the 160-hour mark (Figure 1).

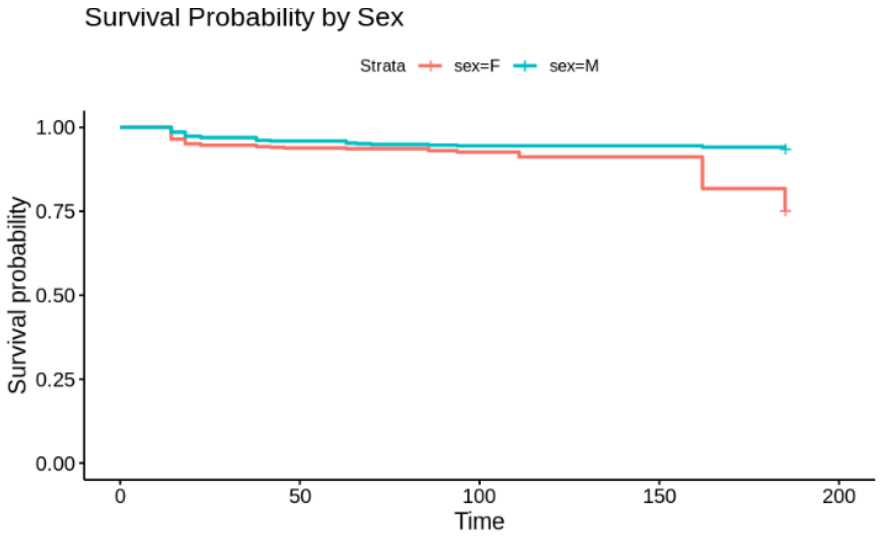

Figure 1. This Kaplan-Meier survival curve illustrates the survival probability of flies over time, categorized by sex (male and female), with the x-axis representing time in hours and the y-axis representing survival probability, ranging from 0 to 1. The dataset consists of survival times for male and female flies, comparing the survival of females (red line) and males (blue line) to assess whether sex impacts overall survival. Each step down in the survival curve represents a death event among the flies. The legend indicates the sex groups, with sex=F for females and sex=M for males.

Further analysis of only infected flies revealed significant differences in survival probabilities based on sex (figure2). Male flies consistently exhibited slightly higher survival probabilities of 0.96 throughout the timeframe than female flies, with a survival rate of 0.87 at 160 hours. The survival probabilities for only non-infected flies differed by sex (figure 3), with male flies having higher survival rates of 0.97 consistently than female flies, with a first initial decline of 0.85 at 110 hours and then a more considerable decline of 0.75 in 160 hours, further supporting our hypotheses.

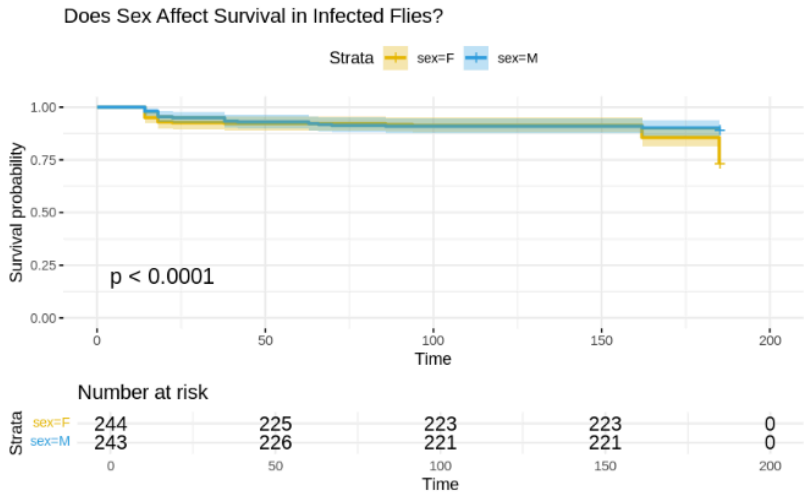

Figure 2. This Kaplan-Meier survival curve illustrates the survival probability of infected flies over time, categorized by sex (male and female), with the x-axis representing time in hours and the y-axis representing survival probability, ranging from 0 to 1. The dataset consists of survival times for male and female flies infected with a pathogen, comparing the survival of females (red line) and males (blue line) to assess whether sex impacts survival under infection. Each step down in the survival curve represents a death event among the flies. The legend indicates the sex groups, with sex=F for females and sex=M for males.

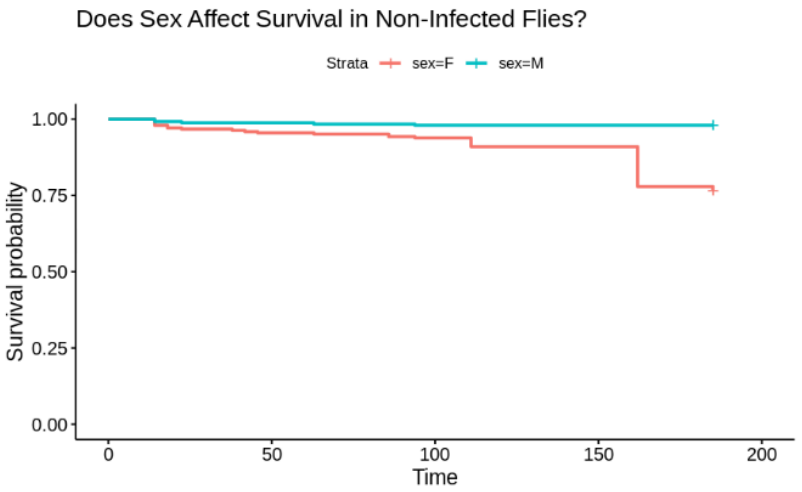

Figure 3. This Kaplan-Meier survival curve illustrates the survival probability of non-infected flies over time, categorized by sex (male and female), with the x-axis representing time in hours and the y-axis representing survival probability, ranging from 0 to 1. The dataset consists of survival times for male and female flies, comparing the survival of females (red line) and males (blue line) to assess whether sex impacts survival in the absence of infection. Each step down in the survival curve represents a death event among the flies. The legend indicates the sex groups, with sex=F for females and sex=M for males.

The analysis of different genotypes and treatments revealed significant differences in susceptibility to infection (Figure 4). The Daxx mutants without infection still had a poor survival rate of 0.60 at 160 hours, and Daxx drosophila with infection were more susceptible to *Serratia marcescens* infection than Dahomey wildtypes with a lower overall curve of 0.77 and then a lower drop at 160 than the non-infected flies at 0.70. The Dahomey survival rate of the non-infected flies started declining at 0.97 in 70 hours and again at 0.85 in 100 hours, decreasing more than the infected Dahomey's maintaining a plate of 1 throughout the experiment.

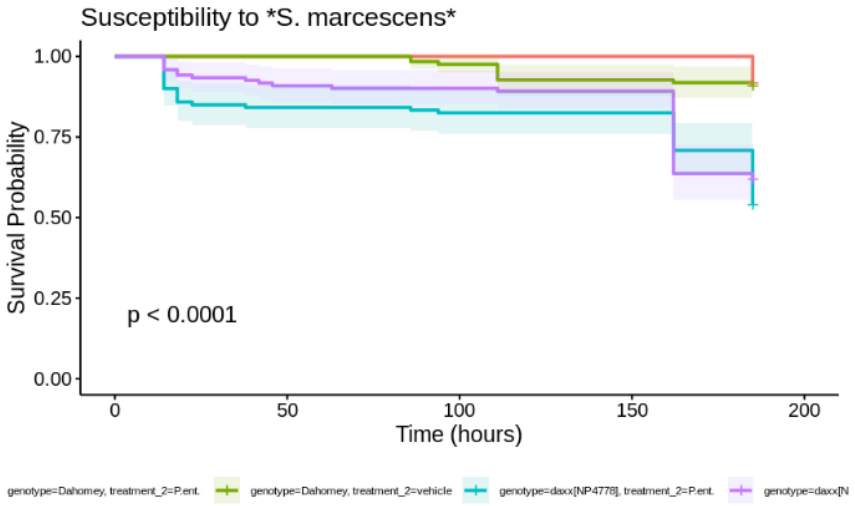

Figure 4. This Kaplan-Meier survival curve illustrates the survival probability of flies over time, categorized by genotype (Dahomey and daxx[NP4778]) and treatment (P.ent. and vehicle), with the x-axis representing time in hours and the y-axis representing survival probability, ranging from 0 to 1. The dataset compares the survival of different genotypes under various treatment conditions to assess susceptibility to S. marcescens infection. Each step down in the survival curve represents a death event among the flies. The legend differentiates between the groups: Dahomey, P.ent. (orange), Dahomey, vehicle (light green), daxx[NP4778], P.ent. (cyan), and daxx[NP4778], vehicle (purple).

The survival curves indicated lower survival probabilities for the mutants under infection, with a highly significant p-value of less than 0.0001, supporting hypothesis three.

The effect of raising larvae in crotonic acid on adult immuno-competence was assessed. The data presented in Tables 1 and 2 indicate that crotonic acid treatment did not significantly affect the survival of Dahomey wildtypes or Daxx mutants compared to their respective controls. For instance, in table 1 the p-values for comparisons between Dahomey flies treated with crotonic acid (HCr 50mM) and those not treated (vehicle) were 0.539 for both P.ent. and vehicle treatments, indicating no significant difference in survival between these conditions. Similarly, for Daxx mutants, the p-value of crotonic acid treatment (HCr 50mM) versus vehicle was 0.115, and the p-value of comparing the regular diet with P.ent. treatment to crotonic acid treatment was 0.323. These results suggest that crotonic acid treatment alone does not significantly alter survival rates.

	Dahomey, HCr 50mM, P.ent.	Dahomey, HCr 50mM, vehicle	Dahomey, Normal , P.ent.	Dahomey, Normal , vehicle	daxx[NP4778], HCr 50mM, P.ent.	daxx[NP4778], HCr 50mM, vehicle	daxx[NP477 Normal , P.ent.
Dahomey, HCr 50mM, vehicle	0.539	NA	NA	NA	NA	NA	NA
Dahomey, Normal , P.ent.	0.804	0.41	NA	NA	NA	NA	NA
Dahomey, Normal , vehicle	0.539	0.984	0.41	NA	NA	NA	NA
daxx[NP4778], HCr 50mM, P.ent.	0.984	0.539	0.821	0.539	NA	NA	NA
daxx[NP4778], HCr 50mM, vehicle	0.115	0.323	0.083	0.323	0.115	NA	NA
daxx[NP4778], Normal , P.ent.	0.079	0.016	0.115	0.016	0.083	0.003	NA
daxx[NP4778], Normal , vehicle	0.323	0.654	0.238	0.654	0.323	0.488	0.007

Table 1. This table presents pairwise p-values from survival analysis comparing groups of flies based on genotype (Dahomey and daxx[NP4778]), treatment condition (HCr 50mM, Normal), and infection status (P.ent., vehicle).

However, delayed development and hatching by a day with Daxx were noted. Crotonic acid and infection status remained dominant factors affecting survival probability, with significant differences observed between infected and non-infected groups. For instance, significant pairwise p-values from Table 1 include 0 for F, Dahomey, P.ent. vs F, Daxx, P.ent., 0.006 for M, Dahomey, vehicle vs M, Daxx, vehicle, and 0.005 for M, Daxx, P.ent. vs M, Dahomey, vehicle. Similarly, Table 2 shows significant p-values such as 0.016 for Daxx, Normal, P.ent. vs Dahomey, Normal, vehicle and 0.007 for Daxx, Normal, vehicle vs Daxx, HCr 50mM.

	F, Dahomey , P.ent.	F, Dahomey , vehicle	F, daxx[NP4778], P.ent.	F, daxx[NP4778], vehicle	M, Dahomey , P.ent.	M, Dahomey , vehicle	M, daxx[NP4778], P.ent.
F, Dahomey , vehicle	0.738	NA	NA	NA	NA	NA	NA
F, daxx[NP4778], P.ent.	0	0	NA	NA	NA	NA	NA
F, daxx[NP4778], vehicle	0	0	0.283	NA	NA	NA	NA
M, Dahomey , P.ent.	0.918	0.73	0	0	NA	NA	NA
M, Dahomey , vehicle	0.158	0.109	0	0	0.193	NA	NA
M, daxx[NP4778], P.ent.	0.127	0.193	0	0	0.114	0.005	NA
M, daxx[NP4778], vehicle	0.01	0.006	0	0	0.015	0.199	0

Table 2. This table presents pairwise p-values from survival analysis comparing different groups of flies based on sex, genotype (Dahomey and daxx[NP4778]), and treatment (P.ent. and vehicle). The groups include female and male flies under various conditions, with p-values indicating the statistical significance of survival differences between them.

The survival curves in Figure 5 illustrate that susceptibility to *Serratia marcescens* varied across different genotypes and treatments. For instance, the blue survival curve for *Ken^1^ on sucrose* shows a high survival probability, maintaining above 0.95 until the end of the observation period, with a slight decline starting around the 50-hour mark.
In contrast, the orange survival curve for *Ken^1^ on S. marcescens* demonstrates significant susceptibility, with survival probability dropping below 0.80 within the first 50 hours and continuing to decline rapidly. The green survival curve for *myoIA>Etl1^KD^ on sucrose* starts high but steadily declines to around 0.75 by 200 hours, indicating moderate susceptibility. Meanwhile, the red survival curve for *myoIA>Etl1^KD^ on S. marcescens* shows a steep decline, dropping below 0.70 within the first 50 hours and continuing to decrease, indicating high susceptibility. The significant p-values from survival analysis (Table 4) underscore these

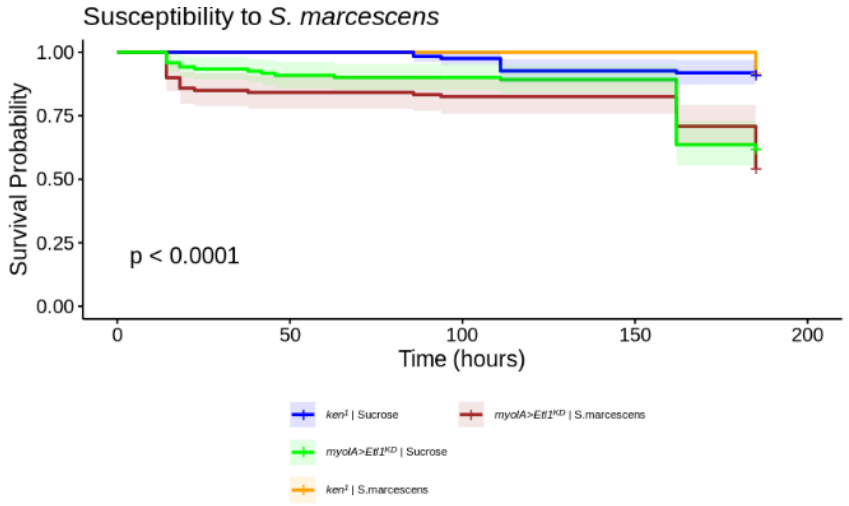

Figure 5. The plot titled "Susceptibility to S. marcescens" shows the survival probability of flies over time, categorized by genotype and treatment. The x-axis indicates time in hours, and the y-axis represents survival probability. The survival curves for four groups are displayed: ken^1^ | Sucrose (blue line), myoIA>Etl1^KD^ | Sucrose (green line), ken^1^ | S.marcescens (orange line), and myoIA>Etl1^KD^ | S.marcescens (brown line). The legend at the bottom of the plot matches the line color to each group. The plot includes a p-value of < 0.0001, indicating a significant difference in survival probability among the groups. This visualization facilitates the comparison of survival rates across different genotypes under two treatment conditions, sucrose, and S. marcescens.

interactions, with *Ken^1^ on S. marcescens* showing a p-value of 0.001, highlighting the considerable impact of infection on this genotype.

Discussion

This study aimed to explore the survival outcomes of Drosophila melanogaster genotypes under controlled conditions, focusing on sex, genotype, and crotonic acid diet in response to Serratia marcescens infections. Our results indicated significant survival differences between sexes, with males demonstrating greater resilience to S. marcescens. Additionally, the Dahomey genotype exhibited higher survival rates than the Daxx genotype under the same conditions. Specific environmental treatments, such as infection versus vehicle control, significantly impacted survival probabilities, and interactions between genotype, sex, and crotonic acid diet yielded significant variations.
Our findings support the hypothesis that male flies exhibit greater resilience to S. marcescens infection than female flies, as males consistently maintained higher survival probabilities throughout the observation period. The Kaplan-Meier survival curves and subsequent statistical analysis confirmed this, aligning with previous studies that have reported sex-based differences in immune response and stress resilience in Drosophila (Klein and Flanagan, 2016).

The Dahomey genotype showed significantly higher survival rates than the Daxx mutants when infected with S. marcescens. This observation aligns with the robust immune response typically seen in Dahomey flies (Leech *et al.*, 2019). The significant p-values from our survival analysis highlight the pronounced susceptibility of Daxx mutants to bacterial infections, supporting our initial hypothesis.
While the crotonic acid treatment did not significantly affect survival rates compared to controls, it did influence developmental timing, causing delayed hatching in Daxx mutants. This finding suggests that while crotonylation may not directly impact survival under infection, it could affect other physiological processes, warranting further investigation.
Several questions arise from our results that warrant future investigation. One area for future research is the specific molecular mechanisms underlying the observed differences in survival between genotypes and sexes. For example, investigating the gene expression profiles of Dahomey and Daxx flies under crotonic acid treatment and infection conditions could provide insights into the pathways involved in their immune response and stress resilience.

Additionally, the delayed development observed in Daxx mutants under crotonic acid treatment raises questions about the broader impacts of histone crotonylation on Drosophila physiology. Future studies could explore the role of crotonylation in gene regulation during development and its potential long-term effects on fitness and survival.
Our study has several limitations that should be addressed in future research. The complexity of our experimental design, which included multiple variables (sex, genotype, crotonic acid treatment, and infection status), may have introduced confounding factors that could obscure specific effects. Simplifying the experimental design with fewer variables, such as concentrating solely on genotype and infection status or sex and infection status, could provide more precise insights.

Another limitation is the use of a single bacterial strain for infection. While S. marcescens is a relevant pathogen, examining the effects of other pathogens, such as fungal or viral infections, could provide a more comprehensive understanding of the immune responses and survival strategies of different Drosophila genotypes.
Future studies could employ a more focused experimental design with fewer variables to address these limitations. For example, a study could specifically compare the survival rates of Dahomey and Daxx flies under crotonic acid treatment without the additional sex variable. Alternatively, researchers could investigate the effects of sex on survival under infection conditions without the influence of crotonic acid.

Moreover, incorporating advanced genetic tools, such as CRISPR/Cas9-mediated gene editing, could allow for more precise manipulation of specific genes involved in immune response and stress resilience. This approach could help identify the genetic basis for the observed differences in survival between genotypes and sexes.

Other studies have used similar methods to investigate Drosophila's survival and immune responses, such as those by Tower, Pomatto, and Davies. (2020) employed Kaplan-Meier survival analysis to study sex-based differences in Drosophila's response to oxidative stress. Additionally, Vieira *et al.* (2000) used survival analysis to compare the longevity of different Drosophila genotypes under various environmental conditions. These studies highlight the utility of our methodological approach and provide a framework for future research.
The findings support the hypothesis that males and Dahomey wildtype flies exhibit greater resilience to bacterial infections. However, the complexities and limitations of our experimental design suggest that future studies adopt a more focused approach to elucidate the specific molecular mechanisms underlying these observations. By addressing these questions, future research can contribute knowledge about the genetic and environmental factors influencing survival and immune responses in Drosophila and potentially other organisms.

Our results underscore the importance of considering genetic background, sex, and environmental conditions in immune response and survival studies. Future research should continue to explore these variables in more controlled and simplified experimental designs, incorporating advanced genetic tools to uncover the underlying mechanisms. Doing so can enhance our understanding of how these factors influence survival outcomes, ultimately contributing to more effective strategies for managing health and disease in model organisms and humans.

Appendixes

R markdown the entire HTML file found online at https://rpubs.com/CT27/1204779.

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