# An investigation on fruit fly mortality and the carrier state in disease transmission with the bacterium *Serratia marcescens*.

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FIG.1. *Test vial*

## ABSTRACT

**This study focused on the bacterium *Serratia marcescens*; specifically to assay its effect on fruit fly mortality, its ability to use the fruit fly as a living reservoir, and its transmissibility between fruit flies. It was hypothesized that this insect pathogen would kill susceptible fruit flies leaving those more resistant in a carrier state capable of transmitting the bacteria to uninfected flies. One unique property of the bacterium is its ability to produce a distinct red pigment that can be used to trace its presence. Experimental methods involved infecting fruit flies with the bacterium by allowing them to ingest the bacterium mixed with their food supply. More susceptible flies developed an infection and died. Fruit flies that survived were tested for the presence of *S. marcescens* in and on their bodies. Survivors were then successfully used to transmit the bacterium to fresh uninfected flies. This study is important because: [1] it represents an animal model for studying innate immunity to infectious agents (*S. marcescens* is capable of causing human infections) and [2] it provides a relatively safe, inexpensive classroom exercise to demonstrate the significance of the carrier state in transmission of infectious disease.**

## INTRODUCTION

The bacterial species *Serratia marcescens*, from the family Enterobacteriaceae, is a facultative anaerobic gram negative bacillus that is a significant opportunistic human pathogen in nosocomial and post-operative infection. About 250,000 nosocomial infections occur yearly, and in 64% of cases *S. marcescens* was present (3). *S. marcescens* causes wound infections, septicemia, conjunctivitis, endocarditis, bacteriuria, meningitis, and respiratory tract infections (7,12,13). The bacterium is often spread through reservoirs of hospital workers hands, aqueous solutions, and medical instruments (6,7). Some adults have *Serratia* in their alimentary canal and it is especially dangerous in immunocompromised patients and the elderly (1, 2, 10).

In nature, it contaminates various areas such as soil, starchy foods, petri dishes, and water reservoirs. *S. marcescens* commonly infects insects because it's found in their environment and food. Mortality is not the result of mere presence in the alimentary canal of insects, but rather upon admission to the hemocoel where it leads to colonization and death (9). In a cotton bollworm study, larvae ingested *S. marcescens*, which resulted in 66.3% mortality of the sample, slowed growth, and led to the penetration of the hemolymph to displace their gut flora (11).

*S. marcescens* has been shown to be spread by asymptomatic hosts, referred to as a carrier state. The most famous carrier of an infectious disease in history was Mary Mallon, better known as "Typhoid Mary", who was a vector for spreading typhoid fever to 33 people (5). This infamous case is a good example of why disinfection procedures should be strictly adhered to, and also of the dangers of carrier states.

## HYPOTHESES AND OBJECTIVES

We have hypothesized the following concerning the interaction of the fruit fly *Drosophila melanogaster* and *S. marcescens:*
[1] This insect pathogen will cause the mortality of fruit flies that are susceptible to infection.
[2] The more resistant fruit flies will survive and will become;
[3] Possible carriers of disease that will be able to transmit the infection when exposed to new uninfected fruit flies

The fruit fly immune system is comparable to humans, therefore conclusions relevant to our immune system can be reached. This study could be a useful exercise to emphasize the importance of the carrier state in disease transmission.

## MATERIALS AND METHODS

A simple method to infect the flies is to feed bacteria to them directly. This leads to not only infection but also displacement of their natural bacterial flora, and mortality. This technique has been established in previous studies using *S. marcescens* (4, 8).

**1. Fly Infection by Feeding: Mortality Experiments 1 (Survivorship Days 0-7)**
Each experiment lasted 17 days, and consisted of four stages., Sterilized glass vials containing 1.5% agar with 2% sucrose were used as a food source for the flies. They were stoppered with porous foam stoppers. The bacteria used to contaminate the vials were *Serratia marcescens* cultures grown overnight on tryptic soy agar (TSA). Test vials were intentionally contaminated using a sterile cotton swab that had touched the middle of a colony of bacteria, and then rubbed across the surface of the sucrose agar in the test vial. Each vial contained approximately fifteen mature *Drosophila melanogaster* flightlesst fruit flies that were 4-7 days old. Vials were maintained at a temperature of 22C and were exposed to approximately 8 hours of darkness and 16 hours of light. Vials were examined once daily for one week to quantify mortality. Results were compared to a control containing no bacteria and 15 flies. Vials were prepared in triplicate. The mortality counts were conducted by visual examination through the glass vial to avoid contamination.

**Experiment 1 (Survivorship Days 8-14)**
At the end of week one for Stage 2, 2 surviving flies were allowed to walk across TSA medium for 1.5 minutes. Plates were then incubated at 35C, and then examined for red trails which indicated a possible external carrier. Results were documented by photography.. The living control flies were expected to leave white or no bacterial trials indicative of the absence of *S. marcescens.* During Stage 3, the surviving possible carriers were transferred to vials containing uninfected flies and no bacteria. Each trial contained 1 possible carrier from a vial and 15 uninfected flies. A control vial with no bacteria, 15 uninfected flies and 1 noncarrier fly was tested simultaneously. Mortality counts were recorded for 1 week.

**Fly Infection by Feeding: Sub-lethality Experiments**
Stage 4 consisted of fly sacrifice and testing for bacterial presence. The flies were sacrificed by washing them in a petri dish containing 70% ethanol to kill external bacteria, crushed with the swab tip, and streaked onto each TSA plate to see if *Serratia marcescens* had colonized their GI tract. Then plates were examined for red colonies indicative of *S. marcescens* and photographed. Experiments were repeated to attain enough data for adequate statistical analysis.

**2. Starvation, Endotoxin/Food Supplementation, and Sucrose Concentration Variable Testing Experiment 2**
We also tested for the effect of different concentrations of sucrose in the media ( 0% vs 2%), as well as no bacteria vs live bacteria vs dead bacteria. The experiments with no bacteria and 2% sucrose **(N2)** were the same as the normal control vial to examine longevity. Live bacteria with 2% sucrose **(L2)** was our normal test vial. Dead bacteria with 2% sucrose **(D2)** was done to determine whether dead bacteria might free something (e.g. lipopolysaccharide) toxic or alternatively might serve as an additional food source for the flies.. No bacteria with 0% sucrose **(N0)** was a starvation control. Live bacteria with 0% sucrose **(L0)** was to see if the presence of bacteria compounded the effect of starvation. Dead bacteria with 0% sucrose **(D0)** was to check if the presence of dead bacteria compounded the starvation effects or alternatively provided a food source to counter the starvation effect. To answer the question of whether bacteria were utilizing sugar in the media and causing mortality of the fruit flies by starvation, we also tested bacteria stored in a 2% sucrose vial for 2 day before adding the flies **(2L2)**. If bacteria used the sugar, results should be similar to L0, if they did not it should be similar to L2.

**Dead Bacteria Procedure (D)**
The method to obtain dead bacteria was to introduce bacteria to a dense 5-10 ml saline suspension and heat them at 80C in a H2O bath for 20 minutes. Then we dipped the swab into saline 1-2 times and swabbed the agar surface of the vial.

**3. The main statistical evaluation of the data** was by Kaplan-Meier survival curves made with the Cox Proportional Hazards model which is a non-parametric method commonly used in the medical field. This method is used when considering mortality versus time due to different variables. R commander statistical survival analysis software (from R-Project.org) was used to produce graphs. In this study, the change in mortality of fruit flies due to presence of *S. marcescens* was examined as well as the effect of different variables.

## RESULTS & DISCUSSION

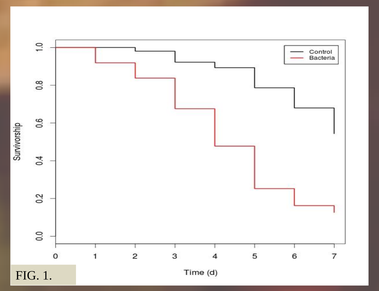



FIG. 1.

FIG. 1. *In Experiment 1, Fruit flies survived in the control vial (no bacteria) versus the increased mortality due to the presence of S. marcescens (test variable). Overall, bacteria increase the fruit fly risk of dying 72.8% per day relative to the control condition risk of dying. On day 7, overall populations without bacteria had a 54.5% survivorship and fruit flies treated with bacteria in their food had a 12.6% survivorship (which is the fraction of the population remaining on that day). The P value is less than .001 so the treatment did have a significant effect.*

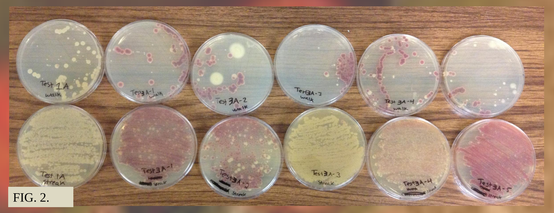


FIG. 2.

FIG. 2. *Stage 2 test flies walk for 1.5 minutes(top row) and Stage 4 GI streak (bottom row) TSA plates. All walk plates demonstrate surface contamination and 5/6 have S. marcescens as the majority. For the GI streak plates 3/6 flies have S. marcescens as the majority of their flora, where as 1/6 has about a 50:50 ratio, and 2/6 appear to be surface carriers. The bacteria on the GI streak could only have come from inside the flies, since EtOH successfully kills any surface bacteria in 1 minute.*

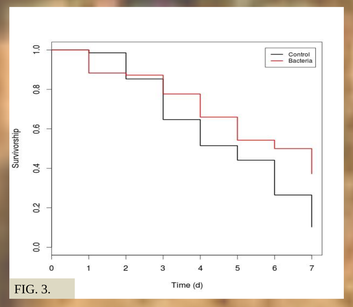



FIG. 3.

FIG. 3. *During stage 3, one surviving possible carrier was placed into a vial with 15 uninfected flies and no bacteria to test if possible carriers could transmit infection and cause increased mortality. Fruit flies were 41.2% less likely to die in vials with the flies exposed to the bacteria treatment when compared to that of the flies previously exposed to the control treatment. Bacteria reduced the odds of dying so that the overall survivorship was 37.2% by day 7 and for the control survivorship was 19.4%. The P value was .004 so the treatment did have a significant effect.*

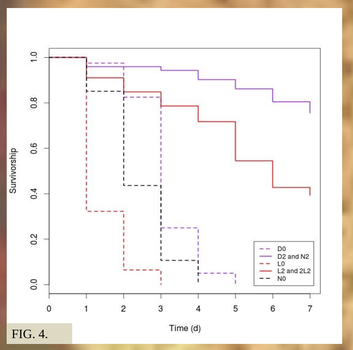



FIG. 4.

FIG. 4. *In Experiment 2, (D) is the dead bacteria treatment, (L) is live bacteria treatment, (N) is no bacteria, and 0 or 2 refers to the percentage concentration of sucrose in the media. Survivorship in 0% media, regardless of bacteria treatment (N0, L0, D0) clearly falls off earlier so that all flies are dead between Day 3 and 5 whereas in 2% media flies do survive to day 7. Therefore, flies do need to utilize sucrose as a food source in the media to survive, and the presence of dead bacteria does not supplement the food source of the fruit flies since survivorship wasn't increased in both D treatments. There does not appear to be an endotoxin effect due to the presence of dead bacteria since survivorship was increased in the D2 treatment rather than decreased due to LPS toxicity. The curves for D2 and NO2 were combined since the P value was .0509 which indicates treatments were not significantly different. There was no starvation effect when media was stored with bacteria for 2 days as compared to media with bacteria applied the day of setup, so the curves for 2L2 and L2 were combined since the difference was not significant.*

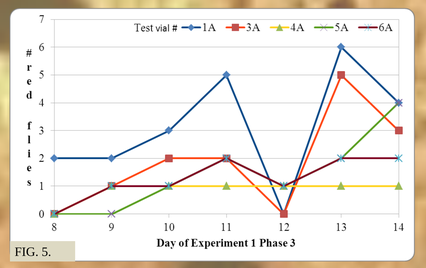



FIG. 5.

FIG. 5. *Transmission of infection was successful as evidenced by daily counts of red flies during the course of one test run. Flies turn red after infection and death due to a unique pigment called prodigiosin produced by S. marcescens (Fig. 6). Since one possible carrier fly was placed with ~15 fruit flies in each vial, and more than one l fly consequently turned red, this implies that infection was transmitted successfully. The variation in number of red flies per day could be due to a dose dependency effect, less lethal bacteria, the point at which the infection was when the possible carrier was exposed to other flies, or some other complex interaction.*

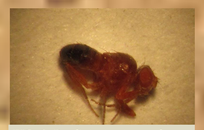
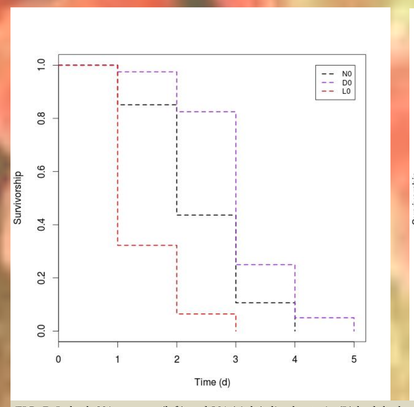


FIG. 6. *Prodigiosin gives red coloration*



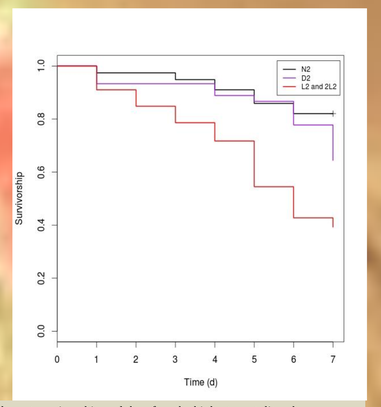




FIG. 7. *In both 0% sucrose (left) and 2% (right), live bacteria (L) had the lowest survivorship and therefore the highest mortality when compared to dead (D) and no bacteria (N) conditions, so the presence of bacteria increases mortality regardless of media. sucrose concentration.*

## CONCLUSIONS

- It was hypothesized that this insect pathogen would kill susceptible fruit flies, and this was confirmed since presence of live bacteria increased mortality regardless of sucrose percentage in the media as seen in Fig. 1.
- FIG. 5. shows a trend towards transmission of infection (although not yet statistically treatable)
- FIG. 5. seems to confirm our hypothesis that there would be a survival of some carriers that then successfully could transmit the bacteria to uninfected flies.
- Fruit flies utilize 2% sucrose medium as a food source and can survive longer (7 or more days) with it, than if maintained on 0% sucrose where they die within 3-5 days as seen in Fig 7.
- *S. marcescens* do not use the sugar in the media and starve fruit flies (Fig 4)
- There is no apparent endotoxin effect on survivorship (Fig 4)
- In a recent trial we confirmed that as suspected, fruit flies can not survive off of dead bacteria alone. We tested this by substantially increasing the amount of dead bacteria so the issue was not age. There was no endotoxin effect, and fruit flies were not using bacteria as a food source.
- We are satisfied that the bacteria were causing the increase in fruit fly mortality, not the other tested factors.
- The trend of transmission seen in FIG. 5. confirms our hypothesis, but needs further trials to have adequate sample size for statistical analysis.

## FUTURE RESEARCH

- Conduct more trials on experiments 1 and 2 to increase sample size for better statistical analysis.
- Determine if there is a dose dependency effect or if less lethal bacteria are being transmitted, since more virulent bacteria may kill off the more susceptible fruit flies first and select for bacteria that do not kill as quickly during transmission.
- Manipulate the dosage of bacteria inoculated into each vial, for example by serial dilutions
- Test different numbers of possible carriers to see at what rate mortality is increased.
- Test the effect of mixing methyl paraben, which is a mold inhibitor, into the media to see if there is increased survivorship since mold sometimes grew in the media the fruit flies were maintained on.
- Find a way to confirm carrier status or measure how many bacteria are actually carried over.

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