5-2023

Impacts of bacterial evolution on host lethality in drosophila

Andrew Preston
University of Louisville

Evolution of *Pseudomonas aeruginosa* Virulence Using *Drosophila melanogaster* as a Model Organism

By

Andrew Preston

Submitted in partial fulfillment of the requirements for graduation *summa cum laude*

and

for graduation with Honors from the Department of Biology

University of Louisville

May 2023

Abstract

Evolution is the process by which species change their genetic traits, such as the pathogenicity of bacteria, over time in response to changes in their environment. Although the genetic mechanisms underlying many evolutionary processes have been revealed, it is still not well understood how opportunistic pathogens, such as *Pseudomonas aeruginosa*, become virulent. The overall goal of this thesis is to test the Coincidental Evolution Hypothesis, which proposes that the virulence of opportunistic pathogens evolves coincidentally as a by-product of their interaction with their natural predators. I hypothesized that the virulence of ancestral *Pseudomonas aeruginosa* changes over time if it co-evolves with its natural predator, the amoeba. Specifically, I predicted that evolved Pseudomonas aeruginosa becomes more virulent to survive against the amoeba. To test this hypothesis, I infected *Drosophila*, the fruit fly, as an alternative host to humans with ancestral and evolved *Pseudomonas aeruginosa*. Survival analysis showed that the evolved strain of *Pseudomonas aeruginosa* was more virulent than the ancestral strain. This provides insights into how opportunistic pathogens might evolve and could eventually be used in pharmaceutical research to combat bacterial antibiotic resistance.

Keywords. Evolution, *Pseudomonas aeruginosa, Drosophila melanogaster,* Coincidental Evolution hypothesis, survival, virulence, infection

Introduction

Evolution is a natural process of all organic life that can be defined as the change in characteristics of organisms over the span of both generations and time (Ashraf, 2016). This process results in the development of new genes, phenotypes, convergence of species, divergence of species, etc. Overall, this process is driven by natural selection, or the preferred selection for one phenotype over another. In a sense, natural selection "edits" the population to what is best suited in that current environment. With respect to bacteria, this domain has one of the fastest evolution rates due to their ability to replicate in a very short amount of time compared to other species. This factor alone has made bacterial infection an ongoing and increasing problem within due to human population growth as more strains of multidrug-resistant types have become prevalent (Aslam et al., 2018). More specifically, over two million individuals globally each year have contracted antibiotic-resistant strains, with approximately 23,000 of those individuals dying due to it (CDC, 2021). Yet, mechanisms underlying how non-threatening opportunistic bacteria evolve to become pathogenic over time and how pathogenic bacteria become resistant to antibiotics are still poorly understood.

Two main hypotheses that have been proposed as to how these pathogens evolve are the Within Host Selection hypothesis and the Coincidental Evolution hypothesis. The Within Host Selection hypothesis suggests that pathological microbes colonize other organisms first, and then natural selection subsequently drives them to have higher a virulence (Farrah Bashey, 2015) (Figure 1). With this hypothesis, this is to mean that the virulence factors are due to directly evolving within the host rather than from another host. The other hypothesis, or the Coincidental Evolution hypothesis, suggests that virulence is a coincidental byproduct that evolved coincidentally because it is linked to other traits that increase fitness in survival or reproduction

of bacteria (Adiba et al., 2012) (Figure 1). For example, the relationship between *P. aeruginosa* and amoeba is complex. This relationship is usually with the bacteria trying to limit the amoeba from phagocytosing and eating the bacteria. By doing so, the bacteria and amoeba will evolve with each other in order to try and "outcompete" the other. Eventually, it has been seen experimentally that the coevolution of the two will reach an attenuated standpoint (Leong et al., 2022). This evolution within *P. aeruginosa* and its virulence factors, however, could be virulent towards other species and was thus a coincidental circumstance.

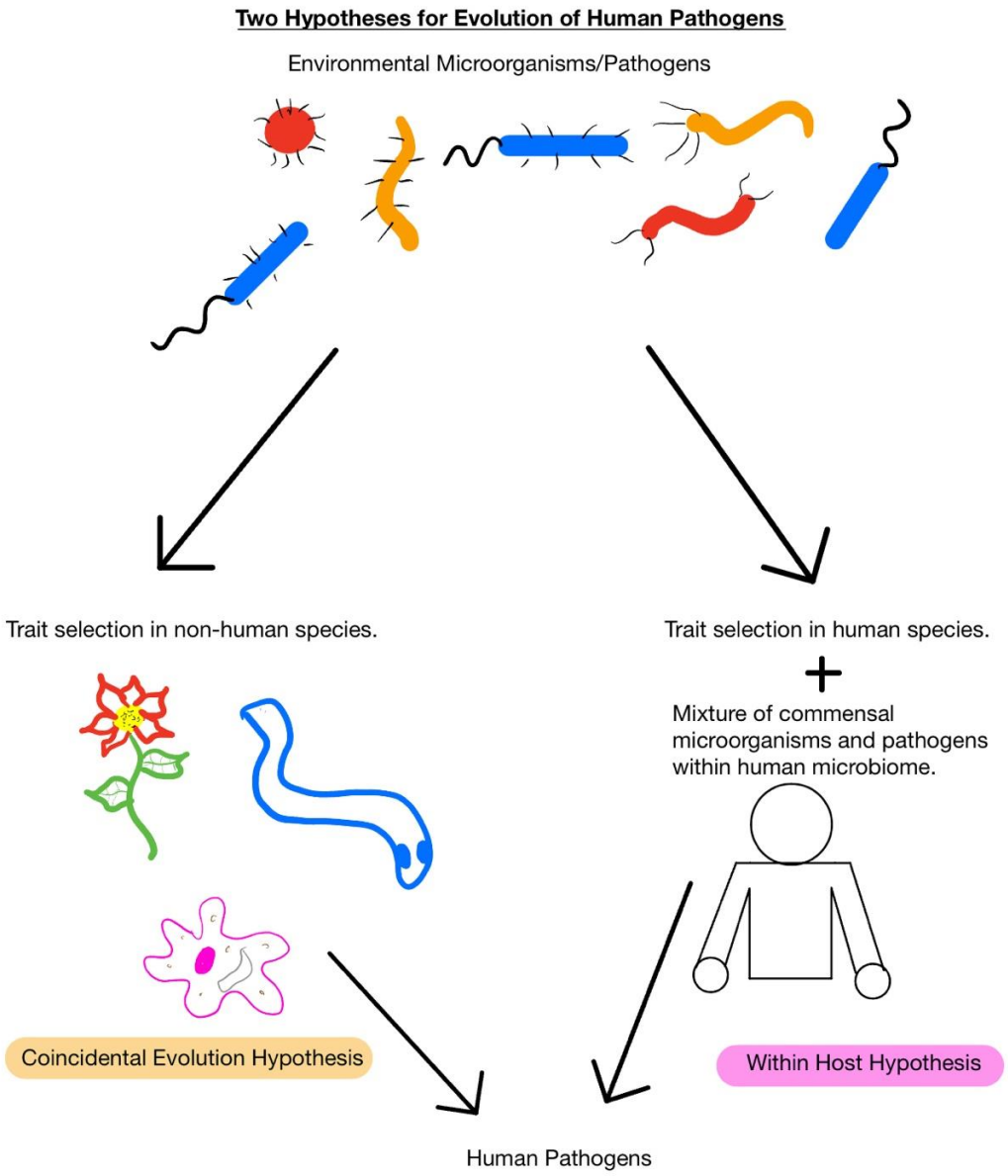

Figure 1: Comparison of the Coincidental Evolution hypothesis and the Within Host Hypothesis

Of the two hypotheses proposed, the focus of this experiment was with the Coincidental Evolution hypothesis. This is due to identifying changes in virulence within a host outside of the original one. The hypotheses proposed within this experiment were:

1. Does evolution of opportunistic pathogens, specifically *Pseudomonas aeruginosa*, impact overall virulence when in pathogenic-capable environments?

2. Do opportunistic pathogens, specifically *Pseudomonas aeruginosa,* tend to evolve via the Coincidental Evolution hypothesis modality?

To test these hypotheses, two different strains of *Pseudomonas aeruginosa* were used, SRP17-047 Ancestral and SRP17-047 Naïve (both were originally isolated and maintained by and at Dr. Yoder-Himes' laboratory) The SRP17-047 Ancestral strain (or the pre-evolved strain) was derived from a bathroom sink drain (a place where *Pseudomonas aeruginosa* is commonly found) and maintained in a non-pathogenic opportunity medium whereas the Naïve SRP17-047 (or the evolved strain) was co-cultured with *A. castellanii*, an amoeba that is a natural predator of *P. aeruginosa* for approximately 6 months (Table 3). Now with a direct host, the reason for virulent-related evolution of the Naïve strain might result in an overall change in virulence when compared to the Ancestral strain. These strains were then used to infect iso31 *Drosophila melanogaster* flies (a wild-type fly strain) via a pricking method. Once infected, the flies were then monitored, and the amount of alive and dead flies were tracked at specific intervals for up to a 48-hour period. The data was then statistically measured using Kaplan-Meier estimation.

With respect to the Coincidental Evolution hypothesis, the naïve bacterial strain would theoretically have a higher virulence in comparison to the ancestral strain. This would be due to the evolutionary relationship between *P. aeruginosa* and *A. castellani* coincidentally creating virulence factors that impact the second host, *D.* melanogaster.

Materials and Methods

1. General Fly Husbandry

Table 1

Fly Type	Source	Stock Number
iso31/w1118	Bloomington *Drosophila* Stock Center (https://bdsc.indiana.edu/)	5905

Table 1. Iso31 fly strain general information.

The strain of *Drosophila* used in this experiment was iso31, a wild type (Table 1). Flies were fed a strict Nutri-Fly® diet (Flystuff, Genesee Scientific, Sand Diego, CA), a cornmeal diet that is also used by the Bloomington *Drosophila* Stock Center (the sourcing location of this strain). Flies used were always in a 12 Light: 12 Dark cycle and kept at 25 degrees Celsius.

2. Fly Expansion Protocol

For each experiment, iso31 flies were allowed to mate and lay eggs in the fly media vials (not pre-sexed so that both males and females are within the same vial) for five-day periods before being removed from the vials. The previous vials were then kept, as they had larvae at this point. This process was consistently repeated throughout the span of this experiment in order to keep a steady supply of iso31 flies for both experimentation and continuation of the expansions.

3. Fly Nutrient Food Protocol

Table 2

Nutri-Fly® Ingredients

Ingredient	**Brand Name**	**Catalogue Number**
Nutri-Fly® Bloomington Formulation	Flystuff	66-121

Propionic Acid	Avantor J.T.Baker	02-003-884
Nipagen/Tegosept	Apex^{TM}	20-259
Ethanol	Milipore	818760
Parafilm	amcor	26121-91

Table 2. All major ingredients and products used to create the fly nutrient food.

The nutrient food described was made and used for the entire duration of the experiment with the flies (Table 2). To make one batch of nutrient food, one liter of water was put into the cooker and set to 100 degrees Celsius to boil. While waiting for the water to boil, 262.5 grams of the Bloomington nutrient food was weighed out and then dissolved into 500 milliliters of water (not from the cooker). In addition to this, 15 milliliters of propionic acid was measured out and parafilmed until needed. Lastly, 4.5 grams of nipagen (a 0.3% food preservative) was dissolved into 22.5 ml of absolute ethanol. Once the water from the cooker reached a boil, the nutrient food mixed with the water was then added to the hot water. The cooker was then covered and allowed to boil once again for twenty minutes and stirring. After the twenty minutes had elapsed, the mixture was allowed to cool down to approximately 80 degrees Celsius before the nipagin and propionic acid were added. This was stirred at 60 degrees Celsius for approximately two minutes. While cooling, two trays were set up and 100 vials were placed each. One tray was then placed under the food dispenser. To the food dispenser, approximately 700-750 milliliters of the food was placed into the top. Once filled, the gate was opened, and the food was dispensed evenly into the 100 vials. This process was repeated with the other tray of vials. Once the vials were fully cooled, cotton balls were then added to the top. Each tray was then labeled accordingly (Nutrient food + 1% propionic acid + 0.3% nipagin) and then placed into the fridge

for use throughout the week. Trays of food were kept in the fridge for no more than 7-10 days in order to reduce the amount of dried food vials being used in the experiment.

4. General Bacteria Sourcing

Table 3

Bacterial Strain	**Laboratory Sourcing**	**Original Sourcing**
SRP17-047 Ancestral	Dr. Yoder-Himes' Laboratory	Bathroom sink drain
SRP17-047 Naive	Dr. Yoder-Himes' Laboratory	Bathroom sink drain*

Table 3. Bacterial strains used in the experiment. *SRP17-047 Naïve bacteria were originally sourced from the SRP17-047 Ancestral line**

Bacteria used in the experiment were directly sourced from Dr. Yoder-Himes' Laboratory (Table 3). The entirety of this procedure was conducted and done by Rhiannon Cecil of said laboratory. In brief, the SRP17047 Ancestral line was isolated directly from a bathroom sink drain. Before receiving the bacteria, a pre-evolved ancestor line was cultured in 8 mL of HL5 media in 6-well tissue culture plates. The media was removed and replaced with 8 mL of fresh 1% HL5 every two to three days. Once every seven days, the cells were scraped from the bottom of the 6-well culture plates with a cell scraper. The cells were then centrifuged and diluted (1:50) back into fresh media.

To generate the fully evolved (24-week-old evolved lines), replicates of the pre-evolved ancestor were inoculated into 6-well tissue culture plates. The bacteria were then placed in a medium with *A. castellanii* (an amoeba that *P. aeruginosa* can host) that was replenished with the amoebae every seven days over the 24-week period. To separate the bacteria cells from the

amoebae, each sample was centrifuged at 600g for five minutes (this pelleted the amoebae, but not the bacteria). The supernatant was then removed and centrifuged at 5,850 rpm for 15 minutes in order to pellet the bacteria. A portion of the bacteria were cryopreserved every one to two weeks in order to generate a fossil record. Pyocin profiles were tested with each strain each week using colony PCR in order to ensure no cross-contamination occurring.

5. Plate Culturing Protocol

When bacteria were not suspended in a liquid broth media directly prior to infection, they were matured on agar plates. Prior to starting the plate culturing, all plates were dated and labeled with the corresponding bacterial strain with a marker. Gloves were used for the entirety of this protocol and sterilized frequently with ethanol. With a gas flame, the inoculation loop was heated until the loop visibly changed to a red color. After this was achieved, the loop was allowed to cool for approximately thirty seconds. After the thirty seconds had elapsed, the loop was dipped into the agar of a previous culture of bacteria. The loop was then used to take a colony of the culture. With this colony, it was streaked across one quadrant of the new plate in a zig-zag pattern and ensuring that the loop does not go over the same place twice. This process was repeated for the remaining three quadrants. Once all four quadrants were looped with the cultures, the agar plate was then lidded and flipped upside-down so that the agar was on the top. The plates were then allowed to incubate for 24 hours in a 25 degrees Celsius incubator before being parafilmed and stored for use. Cultures used in the experiment were no older than two weeks before this protocol was repeated.

6. LB Broth Protocol

Table 4

Ingredient	**Brand Name**	**Catalogue Number**
LB Broth, Lennox	Difco™	240230
Ethanol	Milipore	818760
Parafilm	amcor	26121-91

Table 4. All major ingredients and products to make the LB broth.

The LB broth used in this experiment was for use as a food source for the bacteria cultures while creating the liquid cultures (Table 4). For this, 400mL of water was used and poured into the cooker. The cooker was then set to 80 degrees Celsius with stirring. Approximately 8 grams of Difco LB Broth, Lennox was weighed out and then added to the water. The cooker was then lidded and stirred for 15 minutes. After 15 minutes had allotted, the broth was then put into a sterile 500mL glass bottle with a rubber lip and allowed to cool. Once cooled, the bottle was capped. The bottle was then labeled with the date and stored in the fridge for up to two weeks.

7. LB Agar Protocol

Table 5

Ingredient	**Brand Name**	**Catalogue Number**
LB Agar, Miller (Luria-Bertani)	Difco™	244520
Ethanol	Milipore	818760
Parafilm	amcor	26121-91

Table 5. All major ingredients and products used to create the LB Agar.

The LB agar was a food source for the bacterial cultures while plated (Table 5). To the cooker that was sterilized with ethanol prior, 400mL of water was added and preheated to 100 degrees Celsius with stirring. 16g of LB agar powder was weighed out. Once the water was preheated, the LB agar powder was added to the heated water and covered. Once covered, the solution was heated back to 100 degrees Celsius with stirring for an additional 15 minutes. The lid was removed, and the solution was allowed to cool for approximately two to three minutes before being transferred to a total of 12 agar plates. Once evenly distributed, the plates were allowed to cool for 30 minutes, uncovered. After the plates were cooled and set, they were then covered and inverted to prevent condensation from occurring on the agar. The plates were then allowed to sit for two days before being parafilmed and then refrigerated for up to two weeks.

8. Liquid Culture Protocol

For the infection of the flies, a liquid culture was made. During the entire process of making the liquid culture, gloves were always worn with frequent ethanol sterilizations. Using the LB broth previously described, a total of 2000 microliters of the broth was placed into sterilized test tubes using a micropipette and sterilized micropipette tips. The cap of the test tube was always opened and closed using the pinky finger to reduce potential contamination. Once each test tube was filled with a total of 2 milliliters, the tubes were allowed to come to room temperature. After reaching room temperature, the culturing process was allowed to begin. All desired plated cultures were brought and the parafilm was removed from each one. With a Bunsen burner, the inoculation loop was heated until it turned red and then removed from heat to cool for approximately 30 seconds (with emphasis on preventing the loop from touching anything). After the loop was completely cooled, a few colonies from the plate were then

removed and then lightly swirled into the LB broth test tube. The test tube was then capped and labeled with a marker on the side with the strain, date, and time. These steps were repeated until all desired LB broth test tubes were completed. The test tubes were then incubated on the shaker for approximately 16-18 hours and then allowed to rest for approximately 12 hours so that a pellet would form. After use in the infection protocol, each tube was filled with bleach and ethanol before being disposed of in the biohazard bin.

9. Infection Protocol

Infection protocol starts approximately 24 hours before the actual infection. Young flies of approximately 72 hours cohorts were put under carbon dioxide anesthesia, sexed, and then put into new vials (with each vial ranging from approximately 10-20 flies). The vials were then placed onto their sides, or horizontally, for the first hour in order to prevent the anesthetized flies from becoming stuck within the food. After this hour allotted, the vials were then placed in their traditional vertical position and placed into the 25 degrees Celsius incubator for approximately 24 hours. This time was to allow the flies to have ample time for recovering from the carbon dioxide before being put under carbon dioxide for the true infection. Approximately 1mL of each liquid culture of bacteria were then micropipetted into sanitized microcentrifuge tubes for better accessibility. Another centrifuge tube was filled with ethanol. Several rounds of pilot infection trials indicated a higher variation in survival in female flies presumably because their mating and reproduction (egg laying) status is known to significantly affect physiology and immune response (Short et al., 2012).

Therefore, for the entirety of the actual infection experiment with ancestral and evolved *P. aeruginosa*, only male flies were used. To reduce the total exposure of carbon dioxide with the flies, only one vial of flies was anesthetized at a time.

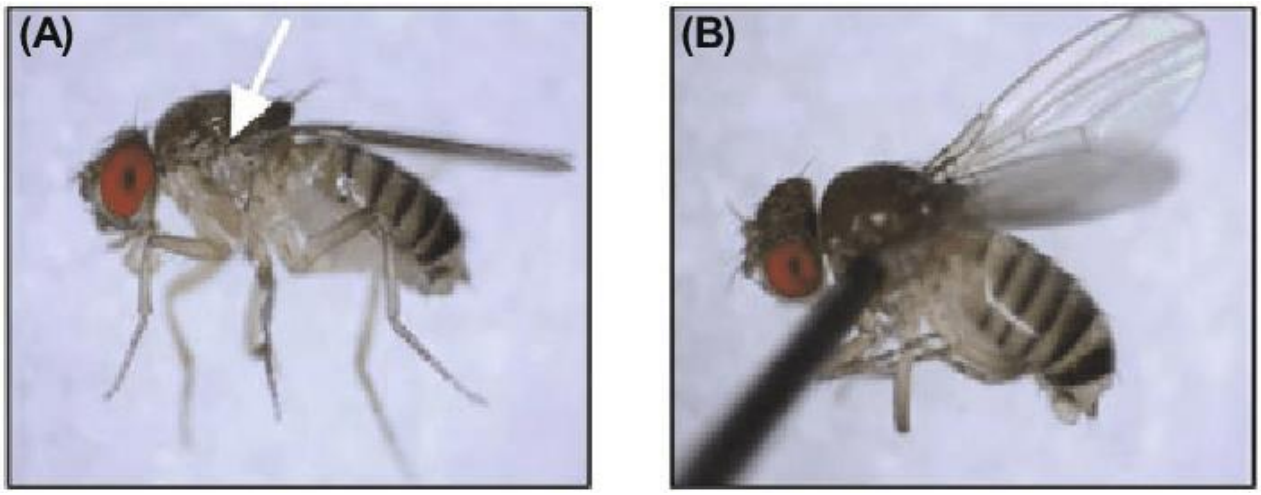

Figure 2. Needle pricking using D. melanogaster. The flies are softly pricked (less than 0.2 mm deep) with a needle that has been dipped into the bacterial pellet. The dorsolateral thorax is punctured with the tip of the needle. (A) The pricking position (designated by arrow). (B) The side view upon pricking. Image and caption adapted from Young-Joon Lee et al., 2018.

The control flies used during the experiment were mock infected by first dipping the infection needle into the ethanol, stabbing a fly (Figure 2), and then dipped back into the ethanol. Enough time was allotted before each prick so that the ethanol on the needle evaporated and no longer visible under the microscope. For a given bacterial strain, a needle would be dipped into the ethanol and allowed to evaporate. After evaporation, the needle would then be dipped into the liquid culture and looked under a microscope to ensure that there was liquid on the needle. If so, the fly would then be pricked (Figure 2). After pricking, the needle would be dipped into ethanol and the process would be repeated until all desired flies were pricked. Ten flies of the same condition would then be grouped into one vial. With each condition and trial, at least 25 flies were used. Vials were placed horizontally for about an hour before being placed vertically

to prevent flies from getting stuck to the food. Each vial was dated and appropriately and the approximate time the condition was pricked. Afterwards, the vials were then placed into the 25 degrees Celsius incubator. Vials were checked 10 hours post-pricking to count for the number of flies alive and dead in each vial, and then approximately every two hours until 24-28 hours had elapsed. After 24-28 hours, vials were then checked every 6 hours until a total of 48 hours had elapsed if necessary.

Results

Virulence Comparison

For the final trial, both bacteria strains used for this result (Figure 3) were inoculated and used at the same time. After inoculated, both strains were spun for 16 hours before allowed to rest for 12 hours. Only the pellet portion of the inoculation medium was pipetted out into sterilized tubes and used. Of the total of 120 flies used, only 20 were put under carbon dioxide at a given time. After 20 flies were pricked (Figure 2) with one of the bacteria strains, the needle was flame heated and then began using the other bacteria strain. This was repeated until all flies were pricked and infected.

Control populations and mock infections (Figure 3) were monitored for a total of 24 hours and had a 96% survival rate over the time frame. Control populations were only monitored for 24 hours due to mock infections not showing significant differences in acute mortalities between 24 hours and 72 hours (Chambers et al., 2014).The population size for the control totaled 100 flies.

JMP software was used to produce the plots (Figures 3 and 4), the summary (Table 5), the quantiles (Table 6), and overall data (Tables 8 and 9). When comparing the infection survival of the SRP17-047 Ancestral and SRP17-047 Naïve, the Ancestral infection resulted in a 30%

death rate and the Naïve infection resulted in an 83.33% death rate over the course of 48 hours (Figure 4). The death rate for the Ancestral infection was higher than the death rate for the Naïve infection until approximately 20 hours. The Ancestral infection and Naïve infection were also concluded to be statistically different (Table 7).

Figure 3

Survival Plot of Mock Infected *Drosophila*

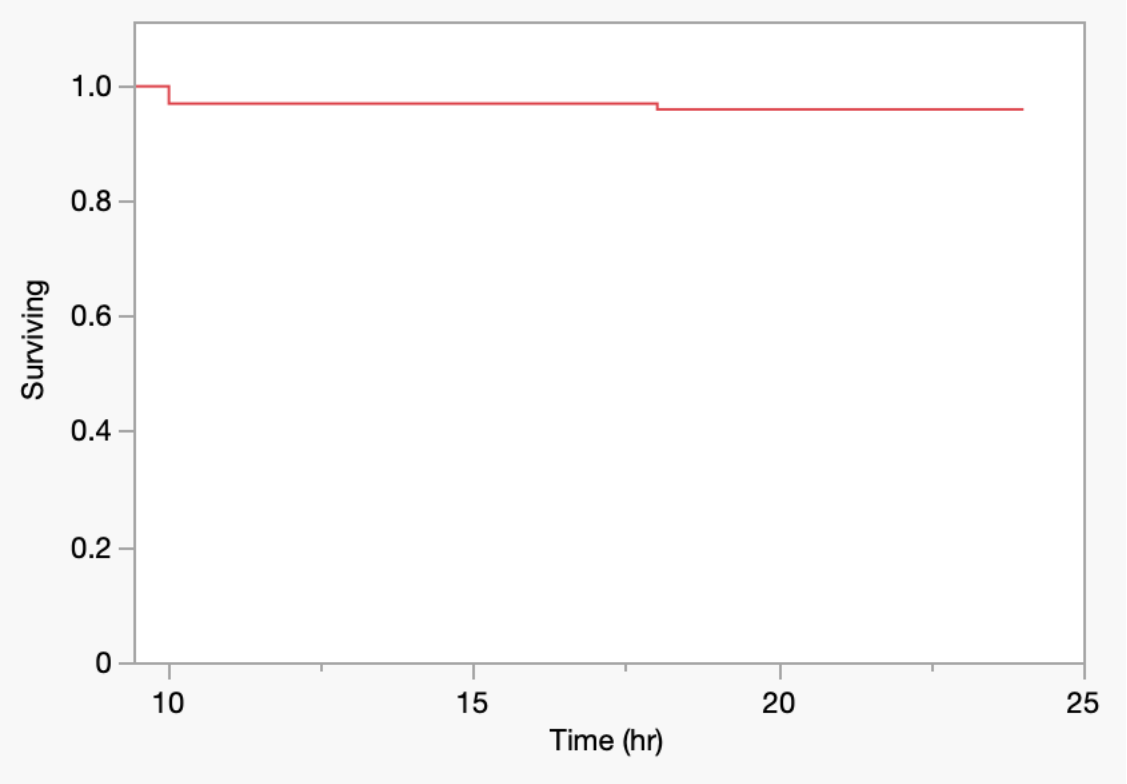

Figure 3. Death rates of flies that were pricked with no bacteria.

Figure 4

Survival Plot of Ancestral and Naïve infected *Drosophila*

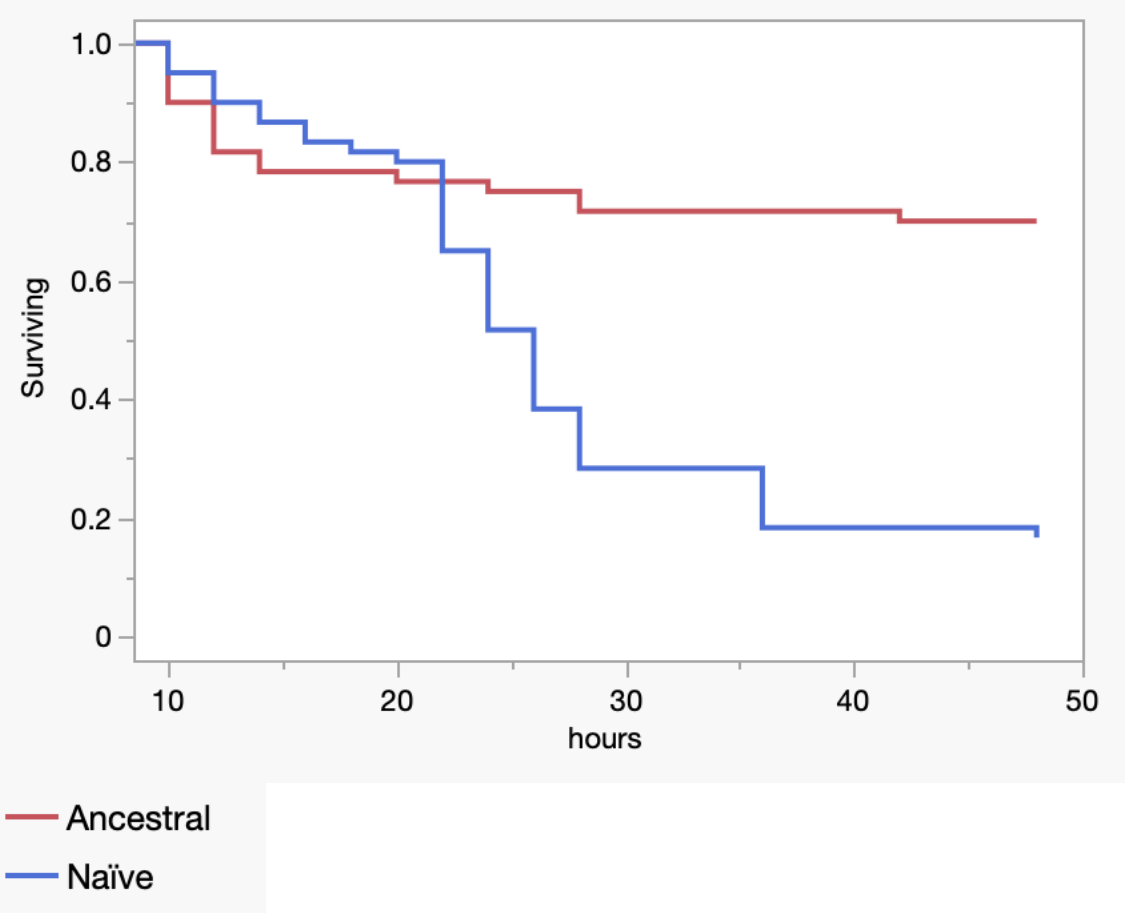

Figure 3. A comparison between the death rates of flies infected by both the Ancestral and Naïve bacterial lines over a period of 48 hours.

Table 5
Summary

Group	Number failed	Number censored	Mean	Std Error
Ancestral	18	42	34.2333 Biased	1.69712
Naïve	50	10	27.9	1.4918
Combined	68	52	33.1667	1.33643

Table 5. When using JMP software, number failed denotes the number of flies that have died while number censored denotes the number that survived after 48 hours.

Table 6
Quantiles

Group	Median Time	Lower 95%	Upper 95%	25% Failures	75% Failures
Ancestral	.	.	.	26	.
Naïve	26	24	28	22	36
Combined	32	26	.	22	.

Table 6. Quantiles of Ancestral, Naïve, and Combined infection of *Drosophila.* Median time represents the middle corresponding time that flies died, with both an upper and lower 95% range. 25% and 75% failures represent the amount time that it took for the infection to kill 25% and 75% of the sample, respectively. For the ancestral line, a median time (along with the upper and lower 95% ranges of the median) and 75% failure rate were unable to be achieved due to not enough deaths occurring.

Table 7
Tests Between Groups

Test	ChiSquare	DF	Prob>ChiSq
Log-Rank	26.3182	1	<.0001*
Wilcoxon	15.9276	1	<.0001*

Table 7. When data was put into both a Log-Rank test and a Wilcoxon test, both resulted in a p-value of less than 0.0001.

Table 8
Ancestral

hours	Survival	Failure	SurvStdErr	Number failed	Number censored	At Risk
0.0000	1.0000	0.0000	0.0000	0	0	60
10.0000	0.9000	0.1000	0.0387	6	0	60
12.0000	0.8167	0.1833	0.0500	5	0	54
14.0000	0.7833	0.2167	0.0532	2	0	49
20.0000	0.7667	0.2333	0.0546	1	0	47
24.0000	0.7500	0.2500	0.0559	1	0	46
28.0000	0.7167	0.2833	0.0582	2	0	45
42.0000	0.7000	0.3000	0.0592	1	0	43
48.0000	0.7000	0.3000	0.0592	0	42	42

Table 8. Direct data that was used to make the Ancestral plot in Figure 3.

Table 9
Naïve

hours	Survival	Failure	SurvStdErr	Number failed	Number censored	At Risk
0.0000	1.0000	0.0000	0.0000	0	0	60
10.0000	0.9500	0.0500	0.0281	3	0	60
12.0000	0.9000	0.1000	0.0387	3	0	57
14.0000	0.8667	0.1333	0.0439	2	0	54
16.0000	0.8333	0.1667	0.0481	2	0	52
18.0000	0.8167	0.1833	0.0500	1	0	50
20.0000	0.8000	0.2000	0.0516	1	0	49
22.0000	0.6500	0.3500	0.0616	9	0	48
24.0000	0.5167	0.4833	0.0645	8	0	39
26.0000	0.3833	0.6167	0.0628	8	0	31
28.0000	0.2833	0.7167	0.0582	6	0	23
36.0000	0.1833	0.8167	0.0500	6	0	17
48.0000	0.1667	0.8333	0.0481	1	10	11

Table 9. Direct data that was used to make the Naïve plot in Figure 3.

Discussion

It should be noted that the immune response of *Drosophila* is composed of both an innate immune system and a humoral immune system. When comparing the two, the innate immune system is much more dominant compared to the humoral immune system (Hoffmann, 2003). This makes *Drosophila* a great model organism for the study of innate immune function and could be used to see how conserved it is across species (especially in relation to how virulence factors evolve). With relation to the thesis, the main objective was to explore how the evolution of *Pseudomonas* under non-pathogenic and pathogenic conditions would impact virulence on *Drosophila.*

In the survival assay conducted, many more flies died and at a faster rate in the Naïve condition in comparison to the Ancestral condition by the end of the experiment. This result

indicated that co-evolved *P. aeruginosa* with amoeba for approximately six months were more virulent than the ancestral strain. Evolutionarily speaking, this could correlate to the coincidental evolution hypothesis where virulence is thought to be an adaptation to ecological niches. With these adaptations, they could then be coincidentally more virulent to other hosts, as seen in the results of this experiment.

With the data achieved in the experiment, it could be proposed that the ancestral *Pseudomonas* was in an environment with limited opportunities to be pathogenic, and thus had a larger "window" to evolve or adapt to an ecological niche. Thus, it could have allowed for a higher virulence status as a "byproduct" of occupying this new niche (Adiba et al., 2010) in the controlled *A. castellani* environment.

Limitations

While the bacteria were inoculated and span under the same conditions simultaneously, it was uncertain if the conditions had the same growth rate and created pellets of the same amount. This variable could have resulted in what appears to be a large difference in virulence or could have made the virulence look further apart. This variation can be reduced in future experiments by counting the number of bacteria prior to infection. Another limitation to this experiment was the pricking method used. With the pricking method, only one fly can be infected at a time. Due to this variable, there is a variable amount of time between each fly that could skew the data to some extent. Future experimentation with an oral method will be tested to see if it can reduce this variable time-window and increase the total sample size that can be done in a single trial.

Future Experiments

The nature of this experiment and thesis was very exploratory and has many different facets that can eventually be explored. With respect to this, there are several hypotheses that can be tested based on the data and results from this experiment:

1. Are different strains of *Drosophila* impacted differently by infection with relation to survival?
2. Are there dimorphic differences in survivability post-infection within *Drosophila?*
3. Is the level of activity changed by infection of *Drosophila* with *Pseudomonas?*
4. Does the original sourcing location of *Pseudomonas* have an impact on the evolution of its virulence?
5. Can an oral method of infection of *Drosophila* be more effective in obtaining replicable data in a shorter span of time?
6. How much does the bacterial concentration impact virulence of *Pseudomonas* and survival of *Drosophila?*

To test hypothesis one, different strains of flies will be used. Namely, Clk^{Jrk} type flies (a strain of flies created by Allada et al. (1998) that has a nonfunctioning Clk gene) are of interest due to hypothesis three. This hypothesis will be tested to see how much sleep impacts immune function, as within humans, less sleep contributes to a lowered immune system function (Garbarino et al., 2021).

To test hypothesis two, both males and females will be used of the fly strains and compared to each other. This hypothesis will be tested due to the limited information on sexual dimorphism differences in relation to infection of *Drosophila* (Belmone et al., 2019).

To test hypothesis three, use of the *Drosophila* Activity Monitor, or DAM, system will occur (Pfeiffenberger et al., 2010). With this hypothesis, fly activity can be tracked with lasers and

potentially yield more accurate survival assay data. Sleep parameters (total sleep, average bout length, sleep bout numbers, waking activity) will be obtained using the Counting Macro software (Pfeiffenberger et al., 2010). With this hypothesis, genomic correlations could be seen and how infection could impact the circadian rhythms of flies.

To test hypothesis four, implementation of the PA B80398 (also sourced from Dr. Yoder-Hime's Laboratory) *Pseudomonas* strain will be conducted. Unlike the SRP17-047 strain being sourced from a bathroom sink drain, the PA B80398 strain was directly sourced from a sputum sample from a person with cystic fibrosis.

To test hypothesis five, an oral infection method will be modified from Limmer et al, 2011, and tested. This hypothesis is due to the potential for being able to conduct much more replicable data in a shorter span of time, as well as limiting the infection time between each individual fly.

To test hypothesis six, more accurate measurements (based on concentration, not time) of the bacterial pellet will be conducted. This measurement is to decrease additional variance between samples and to more tightly regulate the variables. This measurement will be done through Dr. Yoder-Hime's laboratory analysis of samples given by Dr. Dae-Sung Hwangbo's laboratory.

Conclusions

The experiment conducted showcases that there is a virulent difference between ancestral *Pseudomonas* strains and evolved *Pseudomonas* strains. This experimental observation provides a starting place for many future experiments that can be done based off these results. With respect to the first hypothesis tested, it was concluded that different evolutionary conditions can impact virulence levels. With respect to the second hypothesis and this experiment alone, it remains inconclusive as to if opportunistic pathogens, like *Pseudomonas,* evolve more consistent

to that of the Coincidental Evolution hypothesis. This is due to the limitation of not knowing exactly how much the Ancestral strain had evolved with respect to virulence prior to collecting, as well as not knowing how many opportunities it had to act as a pathogen.

Acknowledgements

I would like to thank Dr. Dae-Sung Hwangbo for being my PI over the past two years. With all the help, insight, and guidance with experimentation, I have learned a lot that I will be carrying with me on my track to dental school. Without Dr. Hwangbo's help, I am unsure as to if I would be on the same path that I have chosen. It was due to research with him that I have found the research process very interesting and rewarding.

I would also like to thank Dr. Deborah Yoder-Himes and Rhiannon Cecil for their help with respect to the bacterial cultures. Without these cultures, I would have been unable to do this experiment and found a heavy interest in microbiology and pathology. It was, in part, due to this research that I will be wanting to do pathology-based research while at the University of Louisville School of Dentistry where I will also be pursuing a Master of Science in Oral Biology in conjunction with the DMD degree.

Lastly, I would like to thank Dr. Shira Rabin and Dr. Lee Thompson for taking the time to read and serve on my thesis committee. Without them, I would not have had the opportunity to get more than valuable advice and information when going forward with this thesis (as well as future theses defenses when pursuing my Master's degree)

Work Cited

Adiba, S., Nizak, C., van Baalen, M., Denamur, E., & Depaulis, F. (2010). From Grazing Resistance to Pathogenesis: The Coincidental Evolution of Virulence Factors. *PLoS ONE*, *5*(8), e11882. https://doi.org/10.1371/journal.pone.0011882

Ashraf, M. A., & Sarfraz, M. (2016). Biology and evolution of life science. *Saudi Journal of Biological Sciences*, *23*(1), S1–S5. https://doi.org/10.1016/j.sjbs.2015.11.012

Bashey, F. (2015). Within-host competitive interactions as a mechanism for the maintenance of parasite diversity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*(1675). https://doi.org/10.1098/rstb.2014.0301

Belmonte, R. L., Corbally, M.-K., Duneau, D. F., & Regan, J. C. (2020). Sexual Dimorphisms in Innate Immunity and Responses to Infection in Drosophila melanogaster. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.03075

Centers for Disease Control and Prevention. (2019, May 31). *Biggest Threats and Data*. Centers for Disease Control and Prevention. https://www.cdc.gov/drugresistance/biggest-threats.html

Chambers, M. C., Jacobson, E., Khalil, S., & Lazzaro, B. P. (2014). Thorax Injury Lowers Resistance to Infection in Drosophila melanogaster. *Infection and Immunity*, *82*(10), 4380–4389. https://doi.org/10.1128/iai.02415-14

Garbarino, S., Lanteri, P., Bragazzi, N. L., Magnavita, N., & Scoditti, E. (2021). Role of sleep deprivation in immune-related disease risk and outcomes. *Communications Biology*, *4*(1). https://doi.org/10.1038/s42003-021-02825-4

Hoffmann, J. A. (2003). The immune response of Drosophila. *Nature*, *426*(6962), 33–38. https://doi.org/10.1038/nature02021

JMP®, Version Macintosh. SAS Institute Inc., Cary, NC, 1989–2021

Khalil, S., Jacobson, E., Chambers, M. C., & Lazzaro, B. P. (2015). Systemic Bacterial Infection and Immune Defense Phenotypes in Drosophila Melanogaster. *Journal of Visualized Experiments*, *99*. https://doi.org/10.3791/52613

Leong, W., Poh, W. H., Williams, J., Lutz, C., Hoque, M. M., Poh, Y. H., Yee, B. Y. K., Chua, C., Givskov, M., Sanderson-Smith, M., Rice, S. A., & McDougald, D. (2022). Adaptation to an amoeba host leads to Pseudomonas aeruginosa isolates with attenuated virulence. *Applied and Environmental Microbiology*. https://doi.org/10.1128/aem.02322-21

Limmer, S., Haller, S., Drenkard, E., Lee, J., Yu, S., Kocks, C., Ausubel, F. M., & Ferrandon, D. (2011). *Pseudomonas aeruginosa* RhlR is required to neutralize the cellular immune

response in a *Drosophila melanogaster*oral infection model. *Proceedings of the National Academy of Sciences*, *108*(42), 17378–17383. https://doi.org/10.1073/pnas.1114907108

Medic, G., Wille, M., & Hemels, M. (2017). Short- and long-term Health Consequences of Sleep Disruption. *Nature and Science of Sleep*, *9*(9), 151–161. https://doi.org/10.2147/nss.s134864

National Institute of General Medical Sciences. (n.d.). National Institute of General Medical Sciences (NIGMS). https://nigms.nih.gov/education/fact-sheets/Pages/circadian-rhythms.aspx#:~:text=Circadian%20rhythms%20are%20physical%2C%20mental

Pfeiffenberger, C., Lear, B. C., Keegan, K. P., & Allada, R. (2010). Processing Sleep Data Created with the Drosophila Activity Monitoring (DAM) System. *Cold Spring Harbor Protocols*, *2010*(11), pdb.prot5520–pdb.prot5520. https://doi.org/10.1101/pdb.prot5520

Short, S. M., Wolfner, M. F., & Lazzaro, B. P. (2012). Female Drosophila melanogaster suffer reduced defense against infection due to seminal fluid components. *Journal of Insect Physiology*, *58*(9), 1192–1201. https://doi.org/10.1016/j.jinsphys.2012.06.002

Stone, E. F., Fulton, B. O., Ayres, J. S., Pham, L. N., Ziauddin, J., & Shirasu-Hiza, M. M. (2012). The Circadian Clock Protein Timeless Regulates Phagocytosis of Bacteria in Drosophila. *PLoS Pathogens*, *8*(1), e1002445. https://doi.org/10.1371/journal.ppat.1002445

Toda, H., Williams, J. A., Gulledge, M., & Sehgal, A. (2019). A sleep-inducing gene, nemuri, links sleep and immune function in Drosophila. *Science*, *363*(6426), 509–515. https://doi.org/10.1126/science.aat1650