Phage life history traits: regulation of Pseudomonas aeruginosa and evolution of phage resistance within the Drosophila melanogaster host environment

Heather Lindberg

University at Albany, State University of New York, tabbittin@gmail.com

The University at Albany community has made this article openly available. Please share how this access benefits you.

Follow this and additional works at: https://scholarsarchive.library.albany.edu/legacy-etd

Part of the Biology Commons

Recommended Citation

https://scholarsarchive.library.albany.edu/legacy-etd/930

This Dissertation is brought to you for free and open access by the The Graduate School at Scholars Archive. It has been accepted for inclusion in Legacy Theses & Dissertations (2009 - 2024) by an authorized administrator of Scholars Archive. Please see Terms of Use. For more information, please contact scholarsarchive@albany.edu.
Phage life history traits: Regulation of *Pseudomonas aeruginosa* and evolution of phage resistance within the *Drosophila melanogaster* host environment

by

Heather Lindberg

A dissertation submitted to

The University at Albany, State University of New York

in partial fulfillment of the Requirements for the

Degree of Doctor of Philosophy

College of Arts and Sciences

Biology

2013
Phage life history traits: Regulation of *Pseudomonas aeruginosa* and evolution of phage resistance within the *Drosophila melanogaster* host environment

by

Heather Lindberg

Copyright 2013
# Table of Contents

<table>
<thead>
<tr>
<th>Abstract</th>
<th>........................................................................................................</th>
<th>iv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preface</td>
<td>........................................................................................................</td>
<td>v-x</td>
</tr>
<tr>
<td><strong>Chapter 1:</strong> Bacteriophage <em>in vitro</em> fitness predicts efficacy of phage therapy in treating <em>Pseudomonas aeruginosa</em> infections in <em>Drosophila melanogaster</em></td>
<td>1-29</td>
<td></td>
</tr>
<tr>
<td><strong>Chapter 2:</strong> Population dynamics of a bacteriophage/<em>Pseudomonas aeruginosa</em> infection within <em>Drosophila melanogaster</em></td>
<td>30-54</td>
<td></td>
</tr>
<tr>
<td><strong>Chapter 3:</strong> Experimental evolution of bacteriophages alters phage life history traits and ability to treat <em>Pseudomonas aeruginosa</em> infections in <em>Drosophila melanogaster</em></td>
<td>55-82</td>
<td></td>
</tr>
<tr>
<td><strong>Chapter 4:</strong> The enemy of my enemy is my friend: <em>Pseudomonas aeruginosa</em> selected for resistance to lytic bacteriophage are less virulent to their <em>Drosophila</em> host</td>
<td>83-104</td>
<td></td>
</tr>
<tr>
<td><strong>Conclusion</strong></td>
<td>........................................................................................................</td>
<td>105-109</td>
</tr>
<tr>
<td><strong>References</strong></td>
<td>........................................................................................................</td>
<td>110-120</td>
</tr>
</tbody>
</table>
Abstract

Due to the increase in antibiotic resistant bacteria, there has been renewed interest in using bacteriophage to treat bacterial infections (phage therapy). This body of work examined different aspects related to the interaction between bacteria and phage within a host using the system of *Drosophila melanogaster* infected with *Pseudomonas aeruginosa*, which is then treated with lytic bacteriophage. Using this system four specific areas were addressed: I) How *in vitro* phage life history traits may be predictive of *in vivo* therapeutic efficacy, II) How the *in vivo* phage/bacteria dynamics differ for two different phages throughout the course of infection, III) How modification of phage life history traits alters phage therapeutic ability and IV) The variable costs of phage resistance within *P. aeruginosa*.

The results show that not only can phage therapeutic ability be predicted by phage *in vitro* fitness, but therapeutic ability can be altered and improved using simple *in vitro* serial transfer methods. Treatment with different phages shows different dynamics within *D. melanogaster* as well as bacteria having different levels of phage resistance evolving. Despite phage resistance evolving, we found phage resistance does incur a cost to the bacteria, which results in a decrease in within-host virulence.

In conclusion, addressing these questions not only illustrated the use of *D. melanogaster* as a good model system for phage therapy, but also provided insight into the complexities of the phage/bacteria interaction within a living host. In addition, beginning to understand the variable costs of phage resistance has extended the knowledge of the complexities behind phage therapy.
Since their discovery in 1915-1917, bacteriophages have been used to ameliorate bacterial infections (Summers 2005), treatments known as phage therapy. Phage therapy has been used to treat bacterial infections in animals and humans and has even had some agricultural uses (Summers 2005). The early years of phage therapy were very promising. Felix d’Herelle successfully treated bubonic plaque and dysentery patients with phage (d’Herelle 1926). Phage therapy was also successfully used to treat thousands of cholera patients in India (Sulakvelidze and Kutter 2005; Summers 2005). Given these early successes, the enthusiasm for phage therapy boomed and the applications for phage therapy spread rapidly. Soon commercial laboratories were making many different phage preparations containing many different types of phage to treat abscesses, wounds and respiratory infections (Sulakvelidze and Kutter 2005). Unfortunately, companies sometimes sold lysates which still contained bacterial debris (Sulakvelidze and Kutter 2005). This led to patients having adverse reactions and discredited the promise of phage therapy (Sulakvelidze and Kutter 2005). In addition, many companies did not understand the basic biology of phages and used methods which either decreased the phage titer in the preparations or eliminated the phage altogether, leading to treatment failure (Summers 2001).

Phage therapy was not only being discredited by the false promises of the commercial companies; many scientific studies were unable to find consistent positive results for phage therapy. In 1934, Eaton and Bayne-Jones published a review on over 100 phage studies and concluded phages were not living organisms and had no therapeutic value (Eaton 1934). This negative review of phage and their therapeutic
ability coincided with the discovery of broad spectrum antibiotics, which led to the demise of the study of phage therapy in the West (Sulakvelidze and Kutter 2005; Summers 2005; Summers 2001).

Interest in phage therapy has returned due to the increase in antibiotic resistant bacteria (Levin and Bull 2004). Two papers by Smith and Huggins helped to rekindle interest in phage therapy. They showed evidence of phage being a more effective treatment of *E.coli* infections in mice than many antibiotics such as tetracycline and ampicillin, and being as effective as multiple doses of streptomycin (1982). They also found phage treatments were capable of controlling diarrhea in calves, lambs and piglets (1983). Not only were phage treatments able to control diarrhea, they were able to easily treat calves by orally inoculating them with fecal material from calves previously treated with phage. These two well designed studies which had the proper controls and showed repeatability of results showed renewed potential for the use of phage to treat bacterial infections. Not only were phage treatments as effective as antibiotics, phage in fact were potentially the more desirable treatment due to the need for only one application (Smith and Huggins 1982). Phage were still viable within fecal material, increasing the chances of passive protection of non-treated animals within the same enclosure (Smith and Huggins 1983).

Smith and Huggins’ studies are just a few of the multitude of studies which have been done since antibiotic resistant bacteria have become a concern and interest in phage therapy has been reignited. Many researchers have isolated their own phage and used either single phage or phage cocktails to successfully treat infections caused by a variety of clinically relevant bacterial infections such *Pseudomonas aeruginosa, Staphylococcus*
aureus, and Escherichia coli (Atterbury et al. 2007b; McVay, Velasquez, and Fralick 2007; Watanabe et al. 2007; Wang et al. 2006; Chibani-Chennoufi et al. 2004; Biswas et al. 2002; Barrow, Lovell, and Berchieri 1998; Soothill 1992). In addition to isolating phages and using them to treat bacterial infections, several studies have begun to study what makes a phage therapeutically effective in order to be able to quickly identify better therapeutic quality phages for use. Mathematical models have predicted which traits would be advantageous for a therapeutically successful phage (Levin and Bull 1996; Levin and Bull 2004; Payne and Jansen 2003, 2001); however few studies have tested these predictions. One of the studies which studied the traits of a therapeutically successful E.coli phage found that in their case, the enzymatic activity on the tail of the phage was more important than particular phage life history traits (Bull, Otto, and Molineux 2012; Bull, Vimr, and Molineux 2010).

In addition to studying what makes a good therapeutic phage, several researchers are exploring the possibilities of using different model systems in order to study phage therapy. In addition to the mouse model which has been used for many years recently, insect model systems have begun to be used. Both wax moths larvae (Hall et al. 2012) and Drosophila melanogaster (Heo et al. 2009) have been used to study phage’s ability to treat Pseudomonas infections. Both invertebrate model systems are less costly to maintain thus facilitating the usage of larger sample sizes in the studies. In addition, in the D. melanogaster study (Heo et al. 2009), the flies were fed the phage, rather than being injected with the phage, illustrating that the phages can cross the gut barrier to treat a systemic bacterial infection.
With the recent advances in sequencing technology, some researchers have begun to look for therapeutically viable phages by doing full genome sequencing (Gill and Hyman 2010). By sequencing the phage genomes, phages carrying either lysogenic genes or genes encoding for toxins can be eliminated from consideration for therapeutic use (Gill and Hyman 2010). In addition to identifying phages that are unsuitable for therapeutic use, knowledge of the phage genome has allowed researchers to modify phages. Genomic data can also be used to inform which phages are suited for phage cocktails, as knowledge of the phage genome may provide insight into the bacterial receptor the phage likely adsorbs to. This knowledge aids researchers in creating phage cocktails that contain phages utilizing different receptors (Goodridge 2010). Phage cocktails thus prepared can either decrease the likelihood of bacteria becoming resistant, or greatly increasing the cost of phage resistance in bacteria (Goodridge 2010).

While knowledge of the phage genome can help to eliminate the phages that carry lysogenic and toxin genes, genomics cannot provide details about the phage growth dynamics, an important aspect of phage therapy (Loc-Carrillo and Abedon 2011; Abedon and Thomas-Abedon 2010). A phage may have all of the important genomic characteristics, but if the phage is unable to proliferate well on the bacterial host, it will not be a therapeutically viable option. Understanding the phage growth dynamics and being able to identify therapeutically viable phages based upon their growth dynamics prior to full genomic characterization will decrease the costs associated with identifying therapeutically viable phages (Loc-Carrillo and Abedon 2011).

My research examined different aspects related to the interaction between bacteria and phage within a host using the system of Drosophila melanogaster

viii
infected with *Pseudomonas aeruginosa*, which is then treated with lytic bacteriophage. Using this system I addressed four specific areas: I) How *in vitro* phage life history traits may be predictive of *in vivo* therapeutic efficacy, II) How the *in vivo* phage/bacteria dynamics differ for two different phages throughout the course of infection, III) How experimental evolution in structured and non-structured environments alters phage life history traits and therapeutic ability and IV) The variable costs of phage resistance within *P. aeruginosa*. Addressing these questions not only illustrated the use of *D. melanogaster* as a model system to study phage therapy, but also provided insight into the complexities of the phage/bacteria interaction within a living host.

My experimental model system was chosen based upon how well the model system fit the goals of my research such as the ability to use a large sample size; as well as based upon previous work done by other researchers. The lytic bacteriophages have been isolated from various water samples similar to other studies (Atterbury et al. 2007b; Watanabe et al. 2007; Wang et al. 2006; Chibani-Chennoufi et al. 2004; Biswas et al. 2002; Soothill 1992; Smith and Huggins 1982, 1983). Lytic phage are easily isolated from water samples and because aqueous environments may be exposed to larger throughput of suitable hosts than soil environments, the chances of isolating lytic bacteriophage instead of lysogenic bacteriophages are increased in water samples (Wilcox and Fuhrman 1994).

*P. aeruginosa* is a ubiquitous bacterium found in a variety of environments, and has been identified as a pathogen within both plants and animals in addition to being an opportunistic human pathogen (Sadikot et al. 2005; Prithiviraj et al. 2005; D'Argenio et
al. 2001a; Stover et al. 2000; Rahme et al. 1995). In humans, it is the main cause of morbidity and mortality in cystic fibrosis patients as well as the cause for many other nosocomial infections (Lyczak, Cannon, and Pier 2002; Oliver et al. 2000; Hsueh et al. 1998; Aloush et al. 2006). *P. aeruginosa* has both its genetics and molecular pathways very well characterized (Mahajan-Miklos et al. 1999; Darzins and Russell 1997; Chapon-Hervé et al. 1997; Friedman and Kolter 2004; Whitchurch et al. 2005; Ventre et al. 2006; Mougous et al. 2006; Mattick 2002). The *P. aeruginosa* strain PAO1 has been fully sequenced (Stover et al. 2000) and a comprehensive transposon library of PAO1 is available through the University of Washington (Jacobs et al. 2003).

Studying the interactions of *P. aeruginosa* and its phage within a *D. melanogaster* system allows for a more realistic testing environment than liquid culture. Within *D. melanogaster, P. aeruginosa* not only has to interact with its phage, it also has to interact with the spatial structure of the fly, as well as the fly’s innate immune system. *D. melanogaster* has previously been used as a model system to study *P. aeruginosa* infections, their virulence factors (Apidianakis and Rahme 2009; Apidianakis et al. 2005; D'Argenio et al. 2001a; Erickson et al. 2004; Chugani et al. 2001; Fauvarque et al. 2002) and how those virulence factors interacted with the innate immune system (Lau et al. 2003)
Chapter 1: Bacteriophage in vitro fitness predicts efficacy of phage therapy in treating Pseudomonas aeruginosa infections in Drosophila melanogaster

Authors: Heather Lindberg, Kurt A. McKean, Ing-Nang Wang

Institution: State University of New York, University at Albany.

*All data presented in this chapter was collected by Heather Lindberg
Abstract

Since the discovery of bacteriophage in the 1910s, researchers have been intrigued by the potential of using bacteriophage to treat bacterial infections and have actively pursued the possibility. However, many phage therapy studies simply isolated new phages and immediately tested them without full characterization of phage traits, which could have potentially informed their understanding of the therapeutic efficacy of the phage. Characterizing phage traits may facilitate finding more therapeutically effective phages. In this study, we isolated and characterized the life history traits of six phages and assessed their ability to treat a *Pseudomonas aeruginosa* infection of *Drosophila melanogaster*. We found all six phages were able to significantly increase survival time of infected *D. melanogaster*. Phage *in vitro* fitness was positively correlated with therapeutic efficacy. However, despite being highly varied, we found no single life history trait predictive of therapeutic efficacy. This study also introduces *D. melanogaster* as a model system in which to test the therapeutic efficacy of phages. In addition, this study provides insight into the difficult process of selecting therapeutically valuable phages.
Introduction

Shortly after the discovery of bacteriophages (phages), they were used as a treatment for bacterial infections. In its infancy, phage therapy was seen as a plausible antibacterial treatment and was pursued vigorously (Summers 2005). In most of the initial studies, phages were isolated and immediately used to treat infections, often without understanding their basic biology or details of their interaction with bacteria. This lack of understanding led to poor or inconsistent results (Summers 2005), which, when coupled with the discovery of broad spectrum antibiotics, eventually resulted in declining interest in phage therapy in Western medicine in the 1940’s (Summers 2005).

Renewed interest in phage therapy emerged in the 1980s, and was pursued using a more scientifically rigorous approach. Smith and Huggins were among the first to document the *in vivo* interaction of phage and bacteria over the course of treatment, first using mice (Smith and Huggins 1982) and later calves, lambs and piglets (Smith and Huggins 1983). Despite this attention to detail regarding how phage and bacteria interacted within a host, Smith and Huggins only minimally characterized the phages to determine if they required the K1 antigen of *Escherichia coli* for infection. They did not characterize the phage traits which can be obtained from the one step growth curve which has been around since the 1940s (Delbruck 1940). Even now, of the many studies that test phages’ ability to treat bacterial infections (Sulakvelidze and Barrow 2005; Sulakvelidze and Kutter 2005; Saussereau and Debarbieux 2012; Hall et al. 2012; Bull, Otto, and Molineux 2012; Bull, Vimr, and Molineux 2010; Wang et al. 2006; Alemayehu et al. 2012; Tiwari et al. 2011; Prasad et al. 2011; Heo et al. 2009), only a few characterize phage life history traits (Bull, Otto, and Molineux 2012; Bull, Vimr, and Molineux 2010;
Wang et al. 2006) and only one has attempted to correlate life history traits to therapeutic efficacy (Bull, Otto, and Molineux 2012).

Recent reviews regarding the future of phage therapy have highlighted the importance of phage choice in therapeutic success. Phages must be chosen based not only upon their stability and ability to be maintained in a laboratory setting (Gill and Hyman 2010), but they must also be identified as virulent and have phage growth characteristics suitable for therapeutic purposes, such as the phage’s ability to adsorb to the host and replicate efficiently (Loc-Carrillo and Abedon 2011; Abedon and Thomas-Abedon 2010). These recent reviews also discuss the importance of genomic characterization of these phages to identify genes which encode toxins or indicate lysogeny, however, the phage’s ability to adsorb and replicate within the bacterial host is generally not able to be identified by genomic characterization (Loc-Carrillo and Abedon 2011; Gill and Hyman 2010). Characterizing phage life history traits prior to rigorous genomic characterization may aid in narrowing the number of phages being subject to more stringent genomic characterization to only those which have already show therapeutic potential in vivo. This will decrease the cost and time spent on the verification of therapeutic quality phages.

Current, prior to any genomic characterization, studies often isolated several phages to test in vivo in pursuit of a therapeutically successful phage. This shotgun approach seems inefficient, increasing both the time and funds required to identify therapeutically effective phages. The ability to predict phage therapeutic efficacy through in vitro characterization rather than in vivo testing would not only aid in the search for therapeutic quality phages, but may also facilitate the targeting of traits for future genetic manipulation of therapeutic phage.
Here we evaluate whether phage life history traits and in vitro fitness estimates are predictive of therapeutic efficacy in vivo. Our model system involves the phage treatment of a systemic, lethal Pseudomonas aeruginosa infection of Drosophila melanogaster. P. aeruginosa is a ubiquitous Gram-negative bacterium associated with many human medical conditions, such as nosocomial infections (Emori and Gaynes 1993; Suarez et al. 2011) and infection of burn wounds (McManus et al. 1985), as well as being a major cause of morbidity and mortality in cystic fibrosis patients (Lyczak, Cannon, and Pier 2002). D. melanogaster has previously been used as a model system to study P. aeruginosa infections (Ye, Chenoweth, and McGraw 2009; D'Argenio et al. 2001a; Mulcahy et al. 2011; Apidianakis and Rahme 2009), including one study evaluating the efficacy of phage therapy in treating infection (Heo et al. 2009). In a previous study, Heo et al. evaluated the efficacy of two Caudovirales phage strains, MPK1 and MPK6 in treating P. aeruginosa infections in mice and in D. melanogaster (Heo et al. 2009). While there were only two phages tested, there was broad agreement in that the phages were capable of treating infections in both systems. In that study only a very cursory evaluation of the phage was presented; a major goal of the present study was to evaluate how characteristics of the phage-bacteria interaction tested in vitro may predict the efficacy of therapy in vivo.

**Materials and Methods**

**Fly Host**

The D. melanogaster used in the experiments were 4-6 day old females from a laboratory population established in 2008 with flies caught at Indian Ladder farms in
Voorheesville, NY. This population is maintained as a large outbred population kept at 25°C with a 12:12 light cycle. Adult females used in infection assays were collected from low larval density vials established by placing 10 males and 10 females in a vial for 24 hours.

**Bacteria**

The bacteria used are the laboratory strain of *P. aeruginosa*, MPAO1, obtained from the University of Washington Genome center (Jacobs et al. 2003) and PA4525 also obtained from University of Washington Genome center. PA4525 is a pilA transposon mutant of MPAO1 obtained from the University of Washington Genome center transposon mutant library (Jacobs et al. 2003), which lacks type IV pili (a common phage receptor). The *P. aeruginosa* clinical isolate, PA14 (O'Toole and Kolter 1998) was also used as an enrichment culture for the isolation of phages.

**Isolation of Phage**

Phages capable of infecting *P. aeruginosa* MPAO1 were isolated from a wastewater treatment plant in Menands, NY and water from the Passiac River (sampled in NJ). For isolation, a 25 ml culture of log-phase MPAO1 in Luria-Bertani (LB) broth was inoculated with 100 μl of one of the water samples and incubated for 24 hours in a 30 °C shaking incubator. After 24 hrs, the culture was centrifuged at 14,400 rpm for 10 min at room temperature, and the supernatant passed through a 2 μm filter to remove any remaining bacterial debris. Approximately 100 μl of the phage suspension was mixed with 100 μl of log-phase MPAO1 grown at 30 °C This mixture was allowed to pre-adsorb at room temperature for 5 minutes, then mixed with 3 ml of molten top agar (2.5 g
tryptone, 2 g NaCl, 2 g agar per 250 ml H2O) held at 45°C and poured onto an LB plate. The plate was incubated overnight at 30°C. The following day, a single phage plaque was picked off the plate and suspended in 100 μl of distilled water. This suspension was then vortexed briefly, re-plated as described above and incubated for 24 hours. After 24 hours, a single plaque was picked and added to 25 ml of a growing culture of MPAO1 shaking at 30°C for 24 hours. After 24 hours, the culture was centrifuged, filtered and stored at 4 °C as stock.

HWPB-1, HWPB-2, and HWNPB-2 were isolated from sewer water sampled from a waste water treatment plant in Menands, New York, while HWPB-3 and HWNPB-1 were isolated from a sample from the Passiac River in New Jersey. All phages used MPAO1 as the enrichment host. Phage HWNPB-3 was also isolated using the waste water treatment plant sample from Menands, NY using P. aeruginosa isolate PA14 as the enrichment culture. Phage HWNPB-3 has the ability to plaque and proliferate on both MPAO1 and PA14 while the other five phages are unable to plaque on PA14.

To determine if any of the isolated phages utilize the type IV pilus to infect P. aeruginosa, the six isolates were tested for their ability to plaque on PA4525. Phages unable to form plaques on PA4525 likely utilize the type IV pilus for adsorption and were classified as HWPB (Heather Wilson Pilin Binding) phage. Phages able to adsorb onto PA4525 and plaque did not utilize the type IV pili for entry into the cell and were classified as HWNPB (Heather Wilson Non Pilin Binding) phage.

**Phage purification, DNA isolation and sequencing**
10 ml of 10⁹ phage ml⁻¹ phage particles were collected, purified and DNA isolated as described in Lee and Clark (1997). The purified DNA was resuspended in distilled water and cut by various restriction enzymes according to manufacturer’s protocols for sixteen hours. HWPB-1 and HWPB-3 were cut using SmaI (Thermo Scientific), HWPB-2 was cut with StyI (Fermentas), HWPB-1 and HWPB-3 were cut with EcoRV (Fermentas), and HWPB-2 was cut with Hind III (Fermentas). Restriction digests were visualized on a 1% agarose gel, and bands under 4Kb in length were excised from the gel and purified (QIAquick gel extraction kit: Qiagen). HWPB-1 and HWPB-3 fragments were then ligated to a pSMART vector (Clonesmart Blunt Cloning Kit: Lucigen). The ligated product was then transformed into XL1-B cells (Stratagene) and screened for insertion by plating cells on LB agar plates containing kanamycin (40 ug/ml). Colonies from the kanamycin plates were then checked for insert by PCR.

HWPB-2 fragments cut with StyI were blunted using T4 polymerase (Fermentas) and then purified (QIAquick PCR purification kit: Qiagen). After purification, the fragments were ligated into pSMART vectors (Clonesmart Blunt Cloning Kit: Lucigen) and transformed as described above. HWPB-1 and HWPB-3 and HWPB-2 fragments were ligated with T4 DNA Ligase (Fermentas) to pUC19 cut with SmaI or Hind III respectively. The ligated product was then transformed into XL1-Blue cells (Stratagene) and screened for insertion using the blue-white screening method by plating cells on LB agar plates containing isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).
Colonies from either the kanamycin antibiotic plates or white colonies from the blue-white screening were checked for the presence of insert by PCR. Recombinants, which showed the presence of insert, were selected for plasmid purification. Plasmids were purified (QIAprep miniprep spin kit; Qiagen) and used as templates for DNA sequencing. DNA sequencing was performed at the DNA Analysis Facility on Science Hill at Yale University. Homologous sequences were identified by using the BLAST tool from National Center for Biotechnology Information (NCBI). Overall, at least 4 kb for each phage was compared to Genebank in order to make a preliminary identification. Primers used for cloning and sequencing of using the Clonesmart system were SL1: 5' - CAGTCCAGTTACGCTGGAGTC - 3' and SR2: 5' - GGTCAGGTATGATTTAAATGGTCAGT - 3'. Primers used for cloning and sequencing using pUC19 were forward: 5'-AAGGGGGATGTGCTGCAAGG - 3' and reverse: 5'-CGGCTCGTATGTGGTGTGGTGGAAGGGAATGGGTCAGT-3'.

**Adsorption rate estimation**

Methods described in Wang et al. (2008) were adapted to estimate phage adsorption rates. In brief, a 25 ml culture of MPAO1 was grown to log-phase in a 30°C water bath. Phage at a concentration of $1 \times 10^4$ pfu ml$^{-1}$ was mixed with log-phase MPAO1 at $1.1 \times 10^7$ cfu ml$^{-1}$ in a total culture volume of 5 ml. Samples (0.5 ml) were taken every five minutes for 20 minutes and filtered through an AcroPrep 96 filter plate (Pall, Ann Arbor MI). The filtrate was plated on MPAO1 to assess the number of free phage. The adsorption rates were obtained by fitting the data to the model of $\ln(P_{20}/P_0) = -\alpha ft$, where $P_0$ and $P_{20}$ are the phage concentrations at time zero and 20 min respectively, $\alpha$ is the adsorption rate constant and $\beta$ is the initial cell concentration. We assumed a
constant bacterial cell concentration throughout the assay as there was only a negligible increase in bacterial cell count throughout the 20-min assay. Adsorption rates are based on three replicates for each phage.

**One step growth curves**

Methods described by Wang (2006) were adapted for use to estimate the lysis time and burst size of each of the six phages. In brief, a 25 ml culture of MPAO1 was grown to log-phase in a 30°C water bath. Phage at a concentration of 2.3-3.5 × 10^7 pfu ml\(^{-1}\) was mixed with 1.10 × 10^8 cells ml\(^{-1}\) MPAO1 for 20 minutes at 25°C then diluted 10^4 fold in LB broth to a total culture volume of 10 ml. Every 10 minutes, for up to 180 minutes, 0.4 ml aliquots were drawn and filtered through a 2 µm filter. The filtrate was plated for plaque assay on MPAO1 to determine phage numbers. Lysis times and burst sizes are based on three replicates for each phage.

**In vitro fitness assay**

Phage *in vitro* growth, was assayed using methods adapted from Wang (2006). In brief, ~ 4 × 10^4 pfu ml\(^{-1}\) phages were combined with log-phase MPAO1 (~1 × 10^8 cfu ml\(^{-1}\)) in 3 ml LB broth. The mixture was incubated at 30°C and agitated in a tissue culture roller drum (New Brunswick Scientific, Edison NJ) at setting 7. Fitness was calculated as \(\omega = \ln[P_3/P_0]/3\), where \(P_0\) and \(P_3\) are free phage concentrations at times 0 and 3 hours respectively. The fitness of each phage was estimated in three independent replicates.

**Therapeutic efficacy of phage in treatment of *P. aeruginosa* infected *D. melanogaster***
The therapeutic efficacy of the phages was assessed by comparing the survival of phage-treated *D. melanogaster* to untreated flies following experimental infection with *P. aeruginosa* MPAO1. To establish bacterial infections for each of three independent replicates, 50 female *D. melanogaster* were injected by piercing the thorax with a 0.1 mm diameter minutien pin (Fine Science Tools, Foster City, CA) that had been dipped in a liquid culture of MPAO1 diluted to OD$_{600}$ = 0.097 ±0.011, resulting in an initial inoculation of ~10$^3$ cfu per fly. Six hours post bacterial infection, flies were injected using a Nanoject injector (Drummond Scientific) with 50.6 nl of either sterile LB broth or filtered phage lysate. Initial phage concentrations were ~10$^4$ pfu per fly, corresponding to an estimated multiplicity of infection (MOI) of 10. After phage treatment, flies were checked for survival every six hours for 72 hours. Those flies surviving more than 72 hours were right-censored for analysis using a Kaplan-Meier survival analysis. The LT$_{50}$ for each treatment group was calculated as the time at which fifty percent of the population had died. Three replicates of the experiment were performed for each phage.

**Data Analyses**

All statistics were performed using the JMP 10 statistical software package. The survival assay was performed using a Kaplan-Meier survival analysis. The three replicates of the assay were not significantly different, and therefore were pooled prior to analysis. Pairwise comparisons to assess the differences among the survival times were done using a Cox proportional hazards model. The differences among the adsorption rates, lysis times, burst sizes and *in vitro* fitness were assessed using a non-parametric Kruskal-Wallis test. The correlations were performed using a bivariant analysis. In
instances where there were multiple comparisons done within one data set, the comparisons were corrected for using a Bonferroni correction (Rice 1989).

Results

Phage identification

Of the six environmentally isolated phages, partial genomic sequencing revealed the phages belong to three different phage families. HWPB-1 and HWPB-3, isolated from waste water and river water respectively, belong to the family Siphoviridae, HWPB-2, which was isolated from waste water, belongs to the family Podoviridae and HWNPB-1, HWNPB-2 and HWNPB-3, all belong to the family Myoviridae. HWNPB-1 was isolated from river water which HWNPB-2 and HWNPB-3 were both isolated from waste water. HWPB-1 and HWPB-3 show high sequence similarity to M6, YuA and MP1412 phages (Ceyssens et al. 2008; Bae et al. 2012). HWPB-2 shows high sequence similarity to \( \phi \)KMV and members of the \( \phi \)KMV subgroup, \( \phi \)KF77, LUZ19 LKD16, PT2, PT5 and \( \nu \)B Pae Tbilis M32 (Lavigne et al. 2003; Ceyssens et al. 2006). All three non-pilin binding phages show high sequence similarity to PB-1 and PB-1 like phages (F8, KPP12, SN, LMA2, JG024, NH-4, 14-1), which utilize the lipopolysaccharide for entry into the bacterial cell (Jarrell and Kropinski 1977; Garbe et al. 2010).

Phage life history traits

We found significant variation among the six phages in their life history traits. The six phages have significantly different adsorption rates (Kruskal-Wallis \( \chi^2 = 15.1520 \) df = 5 p = 0.0097) however, there is no significant difference in adsorption rates among phages utilizing the same receptor for entry into the cell (Among non-pilin binding:...
Kruskal-Wallis $\chi^2 = 4.355$ df = 2 p = 0.1133)(Among pilin binding: Kruskal-Wallis $\chi^2 = 5.4222$ df = 2 p = 0.0665), but there are significant differences in adsorption rates between pilin binding and non-pilin binding phages (Kruskal-Wallis $\chi^2 = 12.1657$ df = 1 p = 0.0005) (Figure 1). The non-pilin binding phages have an average adsorption rate of $1.57 \times 10^{-9}$ cell$^{-1}$ mL$^{-1}$ min$^{-1}$ while the pilin binding phage have a higher average adsorption rate of $1.36 \times 10^{-8}$ cell$^{-1}$ mL$^{-1}$ min$^{-1}$.

Analysis of one-step growth curves (Figure 2) further showed significant variation in lysis times (Kruskal-Wallis $\chi^2 = 16.7302$ df = 5 p = 0.0050) and burst sizes (Kruskal-Wallis $\chi^2 = 12.9532$ df = 5 p = 0.0238). Lysis times range from 30 to 130 minutes while burst sizes range from 35 to 558 phages per infected cell (Table 2). When comparing among phage types, we saw no groupings of lysis times or burst sizes associated with receptor type.

**Phage in vitro fitness**

The *in vitro* fitness of the phages range from 4.83 to 0.32 hr$^{-1}$. Overall, there was a significant difference in *in vitro* fitness among the phage (Kruskal-Wallis $\chi^2 = 16.5789$ df = 5 p = 0.0054) with each phage’s *in vitro* fitness being significantly different from the others, even after Bonferroni corrections to account for multiple comparisons (Figure 3). There was not however, a significant difference between the fitness of pilin-binding verses non-pilin binding phages (Kruskal-Wallis $\chi^2 = 0.0476$ df = 1 p = 0.8273).

**Therapeutic efficacy of phage**
Adult female *D. melanogaster* were injected with \( \sim 10^3 \) *P. aeruginosa* and six hours later either injected with between \( \sim 2.40 \times 10^4 \) and \( 1.20 \times 10^5 \) phage or sterile LB broth. *P. aeruginosa* infection of this size is 100% fatal in untreated flies within 24-30 hours at 25°C (Figure 4A). At the time of phage treatment, bacterial numbers had increased to \( \sim 10^4 \) bacteria, giving a multiplicity of infection (MOI) of \( \sim 10 \). The survival data for the phage treatments are shown in Figure 4A. Overall, treatment with phage increased the survival of the flies (Kaplan-Meier survival analysis \( \chi^2 = 353.11 \) df=6 \( p<0.001 \))(Figure 5). There was significant among-phage variation in treatment efficacy (Proportional Hazards model \( \chi^2 = 99.07 \) df=5 \( p<0.001 \)) (Figure 4, Table 1): the LT_{50} (time until which 50% of the flies had died) of untreated flies was 22.11 ± 0.38 hours, while those for phage-treated flies ranged from 27.21 ± 0.5 hours for the least efficacious phage treatment to 42.86 ± 0.35 hours for the most (Figure 4B, Table 1). In general, the pilin binding phages were significantly more effective at treating the bacterial infection (PB vs NPB: Kaplan-Meier survival analysis \( \chi^2 = 49.006 \) df=1 \( p<0.0001 \)). Even if the most effective phage, *i.e.* HWPB-2, is removed from the analysis, the pilin binding phages still significantly increase the survival time of *D. melanogaster* when compared to the non-pilin binding phages (Kaplan-Meier survival analysis \( \chi^2 = 4.47 \) df=1 \( p=0.0345 \)). In pairwise comparisons, three distinct groups emerged, with HWPB-2 and HWNPB-1 each being significantly different from all other phages in their treatment efficacies (Kaplan-Meier survival analysis \( p<0.0002 \)), while the other four phages clustered together and were not significantly different from each other in their therapeutic efficacy (Kaplan-Meier survival analysis \( p<0.05 \)) (Figure 4B).

**Correlations between phage traits and therapeutic efficacy of phage**
One of the major potential benefits of phage therapy is the vast number and diversity of phages present in nature. One of the big downfalls of phage therapy currently is the method of testing for therapeutic quality phages, which is in vivo testing. Being able to correlate easily assessed in vitro phage life history traits to in vivo therapeutic ability would not only increase productivity in finding new therapeutic quality phages, but also decrease the time and cost associated with finding these phages. Overall, none of the individual life history traits: adsorption rate ($r^2=0.2409 \ F_{(1,4)}=2.5594 \ p=0.3228$), lysis time ($r^2=0.3901 \ F_{(1,4)}=2.5594 \ p=0.1849$) or burst size ($r^2=0.1418 \ F_{(1,4)}=0.6610 \ p=0.4618$) were significantly correlated with the in vivo therapeutic efficacy of the phages (Figure 5). Phage in vitro fitness, however, was significantly positively correlated with therapeutic efficacy ($r^2=0.909 \ F_{(1,4)}=40.0 \ p=0.0032$)(Figure 6). In general, the faster the phage is able to grow in vitro, the better able the phage is in combating the bacterial infection within D. melanogaster. This positive correlation remains significant even if the most effective phage (HWPB-2) is removed from the correlation analysis ($r^2=0.664 \ F_{(1,4)}=7.93 \ p=0.048$).

**Discussion**

In recent years, phage therapy has re-emerged as an alternative treatment of bacterial infection, mainly due to the rise of antibiotic resistant bacteria (Bull et al. 2002). The success or failure of phage therapy will depend on a detailed understanding of phage/bacteria interaction within the host organism. As a first step, it is important to determine if there are phage characteristics, which can be identified in vitro, that may be able to predict in vivo efficacy. Previous phage therapy studies have only partially characterized the phages used, by assessing either morphological or genomic
characteristics (Heo et al. 2009), the phage’s ability to lyse a culture (Smith and Huggins 1982, 1983; McVay, Velasquez, and Fralick 2007; Soothill 1992; Watanabe et al. 2007) or studying the phage pharmokinetics (Atterbury et al. 2007a; Payne and Jansen 2003; Levin and Bull 1996; Bull et al. 2002). However, the life history traits of the phages are rarely characterized (Wang et al. 2006) and even more rarely are the phages selected based upon specific life history traits (Bull, Vimr, and Molineux 2010). Furthermore, the development of an alternative model system in which to test phage therapy may be desirable given the significant costs associated with large scale experiments needed to evaluate alternative hypotheses concerning what drives variation in treatment efficacy. The goal of the present study was to 1) evaluate the potential of D. melanogaster as a system in which to study phage therapy and 2) evaluate if life history traits characterized in six environmentally isolated phages can be used to predict the efficacy if phage therapy in P. aeruginosa infected D. melanogaster.

**Isolated phages vary greatly in life history traits as well as in vitro fitness.** The vast difference in the phage life history traits illustrates the importance of characterizing at least some of the life history traits prior to testing their ability to control a bacterial infection. As a group, the non-pilin binding phages had lower adsorption rates than the pilin binding phage, although that may simply be due to the relative number of available receptors; P. aeruginosa only has a few type IV pili (Weiss 1971; Skerker and Berg 2001) while the lipopolysaccharide layer may allow for more potential adsorption points for the phages. In addition to having different adsorption rates, the phages also greatly varied in their lysis times and burst sizes. The variation seen among the life history traits for these phages is not all that surprising given the different families they belong to. The phage
with the longest lysis time and smallest burst size is HWPB-3, which is a *Siphoviridae* phage with sequence similarity to M6, YuA (Ceyssens et al. 2008) and MP1412 (Bae et al. 2012). The genomes for all three phages carry markers of lysogeny, however at least with YuA, a stable lysogen has never been isolated (Ceyssens et al. 2008). Under the conditions we used for this study, neither HWPB-3, nor HWPB-1 (another *Siphoviridae* phage also similar to M6, YuA and MP1412) exhibited any lyosogenic traits such as turbid plaque or lysogenic bacterial colonies in the center of the phage plaque. HWPB-2 is a *Podoviridae* phage with sequence similarity to \( \phi \)KMV (Lavigne et al. 2003) and \( \phi \)KMV-like phages (Ceyssens et al. 2006). \( \phi \)KMV and the \( \phi \)KMV-like phages are highly virulent phages that are T7-like (Ceyssens et al. 2006). \( \phi \)KMV has a lysis time of approximately 15-20 minutes and a burst size of 25-30 phages per cell when tested at 37°C (Lavigne et al. 2003). HWPB-2 has a slightly longer lysis time of 30 minutes, which may be due to being tested at 30°C rather than 37°C, and a larger burst size of 182 phages per cell.

All of the non-pilin binding phages are *Myoviridae* phages with sequence similarity to lytic broad host range phages PB-1 and PB-1-like phages (Ceyssens et al. 2009; Garbe et al. 2010) which use lipopolysaccaride for adsorption. The broad host range of these phages may explain the relatively low burst size compared to the lysis times of these phages. Being broad host range phages may mean the phages are more inefficient at producing progeny as they are not as specialized to a single *P. aeruginosa* strain (Jensen et al. 1998; Bull 2006).

Phage fitness was determined *in vitro* as the increase in phage numbers in three hours of growth, a time frame which allows for multiple infection cycles (Wang 2006).
There was tremendous variation in the in vitro fitness of the phages, which is unsurprising given the differences in life history traits. While all phages significantly differed from each other, HWPB-2 had a very high fitness of almost 5 hr\(^{-1}\) while the other phages all have fitness less than 3 hr\(^{-1}\). Having a fitness of 5hr\(^{-1}\), while high in this study, is not necessarily very high overall, given that T7 phage has a much higher fitness of 35.6 hr\(^{-1}\), (Heineman and Bull 2007). Having an in vitro fitness of approximately 2 hr\(^{-1}\) is very similar to results seen in λ phage, where fitness of isogenic λ strains differing in only lysis timing ranged from 1.89 hr\(^{-1}\) to 2.83 hr\(^{-1}\) (Wang 2006). This may suggest that even though the six phages have significantly different in vitro fitness, HWPB-2 may be particularly rare, with a fitness that is almost double that of any of the other phages isolated in this study, or λ phage, despite having a much lower fitness than T7 phage.

**Drosophila melanogaster is a useful model system to test the therapeutic efficacy of phage.** The majority of phage therapy studies have been done in the mouse model, which has several limitations including the potentially high monetary cost of maintaining enough test animals to have adequate sample sizes or to test multiple phages. Insects, such as wax moth larvae (Hall et al. 2012) and *D. melanogaster* (Heo et al. 2009), are easy to work with, cost less to maintain and allow for much higher sample sizes, and may therefore potentially be important alternative model systems in which to test the effectiveness of phage therapy. Extrapolation of results from such systems is complicated by important differences between insects and mammals, such as dramatically different body plans, circulation and the lack of antibody-mediated acquired immunity (Hoffmann and Reichhart 2002; Leclerc and Reichhart 2004). However, the presence of innate immune function in insects does ensure that phage therapy trials are carried out in
a living host where the contribution of host immune function, in combination with the phage, acts to limit bacterial growth. There is the added advantage of an incredible array of genetic resources available in the *D. melanogaster* model system. Immune function mutants are available that could be used to better understand the role host immune function plays in the efficacy of therapy.

We found that by testing phages for their ability to treat *P. aeruginosa* infections within *D. melanogaster*, we are able to discriminate among the phages based upon their therapeutic ability. While all six phages were able to significantly increase the survival time of *D. melanogaster* to some degree, only HWPB-2 was able to nearly double the survival time of the flies (Figure 4). In addition to almost doubling the survival time of the flies, there was a 7% survival rate of flies (11 flies) that lived past 72 hours compared to between zero and two flies surviving past 72 hours for the other five phages. The significant difference in survival time, as well as the much larger percentage of flies surviving infection suggests that of the phages isolated, HWPB-2 is the most effective therapeutic phage, and is a good candidate phage for mammalian model testing.

Mathematical models have predicted that phage life history traits such as short lysis times or high burst sizes may enhance the therapeutic efficacy of phage (Levin and Bull 2004; Levin and Bull 1996). Despite these predictions, we did not find any single life history trait predictive of therapeutic efficacy in treating the *P. aeruginosa* infection in *D. melanogaster* (Figure 5). However, we did find a positive correlation between the *in vitro* fitness of the phages and their therapeutic efficacy. These results are in agreement with predictions from several theoretical models of phage therapy (Payne and Jansen 2003; Levin and Bull 1996; Payne and Jansen 2001; Levin and Bull 2004).
Despite these results following theoretical predictions of what traits make a phage therapeutically successful, our results are slightly different from results found by the Bull et al. studies (Bull, Vimr, and Molineux 2010; Bull, Otto, and Molineux 2012). Using E. coli and two phages, a K1-dependent and a K1-independent phage, they found the endosailasidase activity of the K1-dependent phage’s tailspike was responsible for the therapeutic efficacy of the phage in treating a mouse infected with E. coli (Bull, Vimr, and Molineux 2010), rather than the fitness of the phage in broth (Bull et al. 2002), although the fitness in serum was found to be predictive of therapeutic efficacy (Bull et al. 2002; Bull, Vimr, and Molineux 2010). The increase in fitness for the K1-dependent phage was attributed to their ability to produce endosailasidase. In contrast, within our P. aeruginosa phage system, we were able to find a predictive relationship between in vitro fitness in broth culture and therapeutic efficacy in D. melanogaster. Both our study and previous studies (Bull, Vimr, and Molineux 2010; Bull et al. 2002) were able to use in vitro fitness as a way to predict in vivo therapeutic efficacy; although the predictive relationship between in vitro fitness and therapeutic efficacy were found in different media.

Given the variation with the results regarding the predictability of phage therapeutic efficacy within two different model systems, the relationships between different phage traits and in vivo efficacy may be specific to either the host bacterial species being used or the in vivo system where the therapy is being tested. Fitness assays may need to be performed in more than one media in order to attain good predictive results of therapeutic efficacy, depending on the system being utilized. Regardless, in addition to selecting phages to test for therapeutic ability based upon which their ability
to produce non turbid plaques and completely lyse a bacterial culture, suggesting a
virulent lytic phage rather than a lysogenic phage, selecting phages based upon high *in vitro* fitness will help to increase the efficiency in selecting high quality therapeutic phages. Despite having several different criteria that can be used in selecting for different phages, in the future, the most effective means of getting a high quality therapeutic phage may be to manipulate a therapeutically successful phage to increase its effectiveness. This technique has already shown promise with Merril *et al.* (1996) selecting for phages that had the ability to circulate for longer periods within the mouse, and as a result, had a higher efficiency in clearing the bacterial infection.
Figure 1: Phage concentration declines of the six phages. Decreases in phage numbers are plotted over time. Non-pilin binding phages (broken black) overall adsorb faster than pilin binding phages (black). The adsorption rates for the six phages are significantly different (Kruskal-Wallis $\chi^2=15.152$ df=5 $p=0.0097$), and the non-pilin binding phages have significantly faster adsorption rates compared to pilin binding phages (Kruskal-Wallis $\chi^2=12.1657$ df=1 $p=0.0005$).
Figure 2: One-step growth curve. Increases in phage concentrations for both pilin binding phages (solid black) and non-pilin binding phages (broken black) are plotted against time after 20 minute pre-adsorption. There are significant differences in both lysis time (Kruskal-Wallis $\chi^2=16.7302$ df=5 $p=0.0050$), and burst size (Kruskal-Wallis $\chi^2=12.9532$ df=1 $p=0.0238$) among the six phages, however there are no significant differences in lysis time or burst size between non-pilin binding and pilin binding phages.
Figure 3: In vitro fitness of phages. In vitro fitness was assessed using a three hour fitness assay in LB broth at 30°C. Fitness was calculated as $\omega = \ln[P_3/P_0]/3$, where $P_0$ and $P_3$ are free phage concentrations at times 0 and 3 hours respectively. There was significant variation among the six phages (Kruskal-Wallis $\chi^2=16.5789$ df=5 $p=0.0054$). The fitness of each of the six phages was significantly different from each other, even after correcting for multiple comparisons using a Bonferroni correction ($p<0.05$).
Figure 4: Survival data for *D. melanogaster* treated with either phage or LB broth. All six phage were able to increase the median time of death in *D. melanogaster*. *D. melanogaster* were injected with *Pseudomonas aeruginosa* and then six hours post infection, given a 50.6 nl injection of phage suspended in LB broth or LB broth alone as the control. Each treatment had a sample size of 50 females, and flies were checked every 6 hours for death for 72 hours. **A)** Full survival curves for all six phages compared to the control (untreated) flies. LB Control (Thick black broken line) HWPB-1 (Thin black line) HWPB-2 (black dotted line) HWPB-3 (black dashed line) HWNPB-1 (Grey line) HWNPB-2 (Grey dotted line) HWNPB-3
The LT_{50} of the survival assays. The LT_{50} is calculated as the time at which fifty percent of flies in that treatment are dead. The assay was repeated three times. Overall the addition of phage significantly increased the survival of the flies (Kaplan-Meier survival analysis $\chi^2=353.11$ df=6 $p<0.001$), however there were significant differences among the phages’ therapeutic abilities (Proportional Hazards model $\chi^2=99.07$ df=5 $p<0.001$). In pairwise comparisons, after corrections for multiple comparisons, HWPB-2 and HWNPB-1 were both significantly different compared to the all other phage treatments (Kaplan-Meier survival analysis $p<0.0002$), while HWPB-1, HWPB-3, HWNPB-2 and HWNPB-3 were not significantly different from each other.

<table>
<thead>
<tr>
<th>Treatment vs Control</th>
<th>Flies Censored</th>
<th>LT_{50}(hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>22.11 ±0.38</td>
</tr>
<tr>
<td>HWPB-1</td>
<td>0</td>
<td>32.08 ±0.88</td>
</tr>
<tr>
<td>HWPB-2</td>
<td>11</td>
<td>42.86 ±0.35</td>
</tr>
<tr>
<td>HWPB-3</td>
<td>0</td>
<td>30.68 ±0.86</td>
</tr>
<tr>
<td>HWNPB-1</td>
<td>1</td>
<td>27.21 ± 0.6</td>
</tr>
<tr>
<td>HWNPB-2</td>
<td>2</td>
<td>31.98 ±0.77</td>
</tr>
<tr>
<td>HWNPB-3</td>
<td>0</td>
<td>30.86 ±0.86</td>
</tr>
</tbody>
</table>

Table 1: LT_{50} and pairwise comparisons with the control for phage treatments (Kaplan-Meier Survival Analysis).
Figure 5: Correlations of phage life history traits and therapeutic efficacy. None of the assessed life history traits: A) adsorption rate ($r^2=0.2409$ $F_{(1,4)}=1.269$ $p=0.3228$), B) lysis time ($r^2=0.3901$ $F_{(1,4)}=2.5594$ $p=0.1849$) and C) burst size ($r^2=0.1418$ $F_{(1,4)}=0.6610$ $p=0.4618$) were correlated with the therapeutic ability of the phage in treating a *P. aeruginosa* infection within *D. melanogaster*. 
Figure 6: Correlation between in vitro phage fitness and LT$_{50}$ of flies. The in vitro fitness of the phages is significantly correlated to their therapeutic efficacy within D. melanogaster ($r^2=0.909$ $F_{(1,4)}=40.0$ $p<0.0032$).
<table>
<thead>
<tr>
<th>Family</th>
<th>Adsorption Rate (cells$^{-1}$ mL$^{-1}$ min$^{-1}$)</th>
<th>Lysis Time (min)</th>
<th>Burst Size (pfu/cell)</th>
<th>Fitness (hrs$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HWPB-1</td>
<td>$1.11E-09 \pm 2.59E-10$</td>
<td>$123.33 \pm 2.87$</td>
<td>$391.8 \pm 127.05$</td>
<td>$1.53 \pm 0.02$</td>
</tr>
<tr>
<td>HWPB-2</td>
<td>$2.84E-09 \pm 2.43E-10$</td>
<td>$30 \pm 0.0$</td>
<td>$182.1 \pm 10.85$</td>
<td>$4.84 \pm 0.03$</td>
</tr>
<tr>
<td>HWPB-3</td>
<td>$7.62E-10 \pm 1.29E-10$</td>
<td>$130 \pm 0.0$</td>
<td>$35.2 \pm 8.62$</td>
<td>$0.72 \pm 0.05$</td>
</tr>
<tr>
<td>HWPB-1</td>
<td>$1.94E-08 \pm 3.49E-09$</td>
<td>$100 \pm 0.0$</td>
<td>$558.8 \pm 21.3$</td>
<td>$0.33 \pm 0.02$</td>
</tr>
<tr>
<td>HWPB-2</td>
<td>$1.51E-08 \pm 5.05E-09$</td>
<td>$60 \pm 0.0$</td>
<td>$245.1 \pm 31.93$</td>
<td>$2.40 \pm 0.05$</td>
</tr>
<tr>
<td>HWPB-3</td>
<td>$6.33E-09 \pm 1.53E-09$</td>
<td>$66.67 \pm 2.89$</td>
<td>$115.4 \pm 24.56$</td>
<td>$1.92 \pm 0.08$</td>
</tr>
</tbody>
</table>

Table 2: Phage life history traits. Values are averages of three replicates per phage per assay.
Chapter 2: Population dynamics of a bacteriophage/Pseudomonas aeruginosa infection within Drosophila melanogaster

Authors: Heather Lindberg, Kurt A. McKeen, Ing-Nang Wang

Institution: State University of New York, University at Albany.

*All research presented in this chapter was done by Heather Lindberg
Abstract

Phage therapy, the use of lytic bacteriophage (phage) to treat bacterial infection, has seen renewed interest since the 1980’s. Much research has been done to validate different phage’s ability to treat infections, as well as testing if phage can be manipulated to increase therapeutic efficacy. During this resurgence of interest in phage therapy, the dynamics of how bacteria and phage interact within the host organism has been neglected. The dynamics has generally been neglected due to the difficulty of the assays required. Using the model system of *Drosophila melanogaster, Pseudomonas aeruginosa*, and lytic phage, we have broken open the black box of the host organism in order to observe the interactions between bacteria and phage. We found flies treated with two different phages maintain similar bacterial titers despite having very different survival rates, and flies die with lower bacterial titers than expected. In addition to assessing dynamics we also tested for bacterial resistance to the phage within the fly, which is a potential pitfall of phage therapy. We found very few phage resistant bacteria within the flies, and when present, resistant bacteria were never the major population. Our results show the importance of understanding the dynamics of infection and treatment, as many of the assumptions of what was occurring during infection, such as bacterial population crashes and flies dying when bacterial populations escape phage control, were not seen within our system.
Introduction

Phage therapy has been around since the early 1920's when Felix d'Herrelle used bacteriophage to treat bubonic plague patients (Summers 2005). After falling out of favor in the 1940’s, in the West due to inconsistent results and the rise of antibiotics, the field has received renewed interest (Smith and Huggins 1982, 1983; Bull et al. 2002; Levin and Bull 1996; Levin and Bull 2004; Bull, Otto, and Molineux 2012; Bull, Vimr, and Molineux 2010; Merril et al. 1996; Atterbury et al. 2007b; McVay, Velasquez, and Fralick 2007, Watanabe et al. 2007). One of the various reasons for phage therapy falling from favor and one of the main cornerstones of the renewed research involves trying to pinpoint the qualities of good therapeutic quality phages. This question has been addressed from many sides. Some have genetically altered phages to not lyse in order to reduce the bacterial debris, which can be detrimental to the host (Hagens et al. 2004). Others have serially passed phages within hosts to select for phages which can circulate within the host for longer periods of time (Merril et al. 1996), or tried to pinpoint what set of phage traits makes a better therapeutic phage (Bull, Vimr, and Molineux 2010; Bull, Otto, and Molineux 2012). Despite the great deal of attention on manipulating how the phage interacts with the bacteria, very few have studied how the phage and bacteria interact within the host over the course of infection.

There have been many mathematical models created in order to predict how phage and bacteria interact, both in vitro and in vivo (Cairns et al. 2009; Payne and Jansen 2003, 2001; Levin and Bull 2004; Levin and Bull 1996; Chao, Levin, and Stewart 1977). Unfortunately many of the experimental studies which tested the models’ assumptions, found the models failed to predict either the dynamics or the end results,
mostly due to the complexities of attempting to correctly model a tri-trophic interaction (Levin and Bull 1996; Antia, Levin, and May 1994). Of those that have (Smith and Huggins 1982; Wang et al. 2006; Levin and Bull 1996; Levin and Bull 2004), various results were attained, and no real consensus was reached as to how phages were increasing the life expectancies of the model organism, or what occurred within the model organisms that died during the course of treatment.

Not only are the dynamics of what is occurring over the course of phage treatment difficult to determine, it is also extremely difficult to pinpoint the true cause of mortality when the host organism succumbs to the infection. Generally, it is assumed the host is dying when the bacteria exceeds a certain level, but it is generally not known if the host is dying due to phage susceptible or phage resistant bacteria. The assumption of the host organism dying due to an overload of bacteria may not be correct in many cases. There is evidence of hosts dying due to other complications caused by infection such as a buildup of toxins or due to the bodies over reaction to bacteria debris, causing the hosts immune system to attack self rather than the infection (Lepper et al. 2002). In order to truly understand what is causing the death of the host organism, the dynamics of the infection and subsequent treatment must be followed in not only surviving hosts, but the state of the dynamics within the host at the time of death must also be investigated.

Using *Drosophila melanogaster* as our model organism, we focused on how *Pseudomonas aeruginosa* and phage interacted over the course of infection, as well as pinpointing the cause of death in flies which succumb to infection. We document the dynamics of two phages which have very different life history traits and different therapeutic efficacies in an attempt to tease apart the differences between these two
phages and their interaction with *P. aeruginosa* within *D. melanogaster*, and how those differences are affecting therapeutic ability.

**Materials and Methods**

**Flies, bacteria, and phages**

The *D. melanogaster* flies used in the experiments were 5-7 days old females from a laboratory population established in 2008 with flies caught at Indian Ladder farms in Voorheesville, New York. This population is maintained as a large outbred population kept at 25°C with a 12:12 light-dark cycle. The *Pseudomonas aeruginosa* strain PAO1, obtained from the University of Washington *P. aeruginosa* library (Jacobs et al. 2003) as strain MPAO1, was used for fly infection.

Phages HWPB-2 and HWNPB-3, which have been previously described (Chapter 1), were used for infecting *P. aeruginosa*. HWPB-2 belongs to the *Podoviridae* and depends on the type IV pili of PAO1 for infection. The estimated adsorption rate is $2.84 \times 10^{-9}$ cells$^{-1}$ml$^{-1}$min$^{-1}$, with a lysis time of 30 minutes and a burst size of 182 phages per cell. HWNPB-3 belongs to the *Myoviridae* and does not depend on the type IV pili for infection. It has an estimated adsorption rate of $6.33 \times 10^{-9}$ cells$^{-1}$ml$^{-1}$ min$^{-1}$, with a lysis time of 70 minutes and a burst size of 115 phages per cell. These estimates of life history traits are from chapter 1.

**Growth of *P. aeruginosa* MPAO1 in *D. melanogaster***

To estimate the growth rate of *P. aeruginosa* MPAO1 in *D. melanogaster*, we infected 100 female flies with ~$10^3$ MPAO1 cells, using needlestick injection to the
Thorax. Ten living flies were sampled every 6 hours throughout the course of infection. Each fly was individually homogenized in 500 µl LB broth and immediately plated using a spiral plater (Autoplate 4000, Spiral Biotech) to assess bacterial load. It should be noted that at 24 hours post infection (hpi), only two flies were still alive. Both were homogenized for bacterial load determination (see Figure 1).

**Bacteriophage decay rate in *D. melanogaster***

To estimate how quickly the phage may decay within *D. melanogaster* in the absence of a bacterial host, 60 female *D. melanogaster* were injected using a Nanoject injector (Drummond Scientific) with 50.6 nl of phage lysate, containing ~10⁹ - 10¹⁰ phages. Six living flies were sampled every 24 hours for 10 days. Each individual fly was homogenized in 500 µl LB broth, the homogenate filtered through a 0.2 µm filter, and filtrate plated to determine phage titer.

**Population dynamics of phage and bacteria in *D. melanogaster***

The same procedures and conditions used for determining bacteria growth and phage decay were used for co-injecting bacteria and phages in *D. melanogaster*. Approximately 10³ bacteria were injected into individual female *D. melanogaster* flies, followed by injection of ~10⁴ phages 6 hours later. Six live flies were sampled every 6 hours post bacterial infection for up to 60 hours. Sampled flies were homogenized individually in 500 µl LB. After adjusting the homogenate volume to 1 ml, each homogenate was then filtered through a 25 mm, 0.2 µm filter to separate the phage from the bacteria, using a filter holder assembly and removable filter paper (Pall Corp., Ann Arbor, Michigan). The phage filtrate was stored in 4°C until phage titers were
determined. The filter paper was removed from the holder assembly and vortexed in 1 ml LB to re-suspend the retained bacteria. The bacteria concentration was then determined by using the spiral plater and Qcount Colony Counter (see above). After the bacterial suspension was plated, the remaining bacterial lysate was suspended in 15% glycerol and stored at -80°C. The experiment was conducted twice.

To obtain a more fine-grained view during the period when the host mortality is the highest (between 30 to 35 hours post infection), flies were sampled hourly to determine the bacterial and phage titers in both living and dead flies. In this particular study, two flies were sampled, one living and one dead. After each sampling, other dead flies were removed from the vials. This study was repeated three times.

Data analyses

All statistical analyses were performed using the JMP 10 statistical software. The growth of MPAO1 in *D. melanogaster* was estimated using linear regression. Survival was assessed by utilizing a Kaplan-Meier survival analysis. The three replicates of each assay were pooled prior to the assay being run. Differences between intra-host population dynamics were assessed using a parametric t’ test discussed in Sokal and Rolf (Box 13.3) (Sokal and Rohlf 1981). The differences in phage and bacteria within dead flies were assessed using a standard ANOVA to compare between phage types.

Results

Infection of *D. melanogaster* by *P. aeruginosa* MPAO1
To establish the baseline of infection in *D. melanogaster*, we infected female flies with *P. aeruginosa* MPAO1 using needlestick injection. As shown in Figure 1, the bacteria grew exponentially within *D. melanogaster* during the first 24 hours, with an estimated growth rate of 0.394 hr\(^{-1}\) or a doubling time of 1.76 hours. Corresponding to the increasing within-host bacteria titer is the reduced probability of survival experienced by the fly hosts (Figure 2). More than 90% of the infected flies would have died by 24 hours post infection (hpi), with an estimated LT\(_{50}\) (time at which 50% of the infected individuals died; mean survival time) of 22.11 ± 0.38 hours. As expected, the highest time-specific mortality rates are found at later time points. For example, during periods of 18 – 24 and 24 – 30 hpi, the time-specific mortality rates are 0.89 and 0.91, respectively. That is, 89% of the flies that survived the first 18 hours of infection would have died during 18 – 24 hpi. In fact, for a total of 144 flies at the beginning of infection, no fly was able to survive past 36 hours post infection. It is clear that, in the absence intervention, infection by *P. aeruginosa* MP AO1 is fatal to *D. melanogaster*.

**Treatment of infected *D. melanogaster* with *P. aeruginosa*-specific phages**

To investigate the effects of phage treatment on the survivability of *P. aeruginosa* MP AO1 infected *D. melanogaster*, we separately introduced two different phages, HWPB-2 and HWNPB-3, 6 hours after bacterial infection. As shown in Figure 2, when used as therapeutic agents, both HWPB-2 and HWNPB-3 significantly increase the LT\(_{50}\) of *D. melanogaster* when compared to the sham-injected control flies (Kaplan-Meier survival analysis: HWNPB-3 vs. Control: \(\chi^2 = 27.9146, df = 1, p < 0.001\); HWPB-2 vs. Control: \(\chi^2 = 75.5472, df = 1, p < 0.001\)). Although both phages are effective in prolonging the survival time of infected *D. melanogaster*, flies treated with HWPB-2
survived significantly longer than those treated with HWPB-3 (Kaplan-Meier survival analysis: HWPB-2 vs. HWPB-3: $\chi^2 = 61.5403$, df = 1, $p < 0.001$), with the estimated LT$_{50}$ of 42.86 ±0.35 hours and 30.86 ±0.86 hours for HWPB-2 and HWPB-3, respectively. In fact, out of the 150 infected flies treated by HWPB-3, only one was able to survive to 66 hpi, and none to 72 hpi; while for the 152 infected flies treated with HWPB-2, 10 were able to survive to 72 hpi. Evidently, phage intervention is effective in controlling bacterial infection, thus resulting in extending the longevity of infected hosts.

**Intra-host population dynamics of bacteria and phages**

An intuitive explanation for the difference in phage treatment efficacy is that the more efficacious phage is able to achieve lower (or completely eliminate) bacterial load by maintaining a higher phage titer in the infected flies. To elucidate the population dynamics during the course of infection, we conducted a separate experiment to track the bacteria and phage populations in infected flies at the same time points as those shown in Figure 2.

As shown in Figure 3, both phage titers increased exponentially with time, with HWPB-2 reaching a plateau of $\sim 10^8$ phages/fly 18 hours post phage introduction (24 hours post bacterial infection), while for HWPB-3 the maximum titer reached is $\sim 10^7$ phages/fly 24 hours post phage introduction (30 hours post bacterial infection). The estimated growth rates for HWPB-2 and HWPB-3 are 0.541 hr$^{-1}$ (6 – 24 hpi) and 0.301 hr$^{-1}$ (6 – 30 hpi), respectively. Clearly, under a similar condition, HWPB-2 was able to achieve a higher phage titer in a shorter time period when compared to HWPB-3.
During the assay period, despite the presence of phages, both bacteria populations also increased exponentially for the first 18 to 24 hours, with the HWPB-3-treated bacteria population reaching a higher titer (~3 × 10^5 bacteria/fly) than the HWPB-2-treated population (~5 × 10^4 bacteria/fly). The estimated growth rates during this period in the HWPB-2- and HWPB-3-treated flies are 0.146 hr\(^{-1}\) (6 – 24 hpi) and 0.421 hr\(^{-1}\) (6 – 18 hpi), respectively. Interestingly, 24 hours post phage introduction (30 hpi), both populations crashed to their lowest levels (although the titers are still significantly different; Kruskal-Wallis test: adj. \(H = 9.095\), df = 1, \(p = 0.0026\)). Afterward, both bacteria populations resumed their exponential growth. However, for the HWPB-3-treated flies, the experiment ended when all flies died at either 42 hours (replicate 1) or 36 hours (replicate 2) post infection. For the HWPB-2-treated flies, the bacteria titer was able to reach its highest level at 54 hpi before the entire population crashed again at 60 hpi.

Overall, our result is a confirmation of the intuitive notion that the efficacy of phage therapy is mediated through its ability to lower the bacterial load by maintaining a high phage titer in the infected host.

**Contribution of phage-resistant bacteria to bacteria population dynamics**

Despite the presence of high phage titers, upwards of \(10^7 – 10^8\) phages per fly, bacteria persisted within the flies at a somewhat surprising titer throughout the course of infection. Even at a later time, when the majority of the fly population has succumbed to infection and died, the bacterial titers in the living flies remained high. A closer inspection of Figure 3, particularly the bacteria population in HWPB-2-treated flies,
suggests the existence of a cyclic population dynamics, possibly due to the emergence of phage-resistant strains. To test this possibility, we randomly picked 30 bacterial colonies from each of the 12 flies (six flies for two replicate experiments) at each time point during bacterial infection and phage treatment (as in Figure 2) and tested their sensitivity to corresponding phages. That is, for each phage treatment at each time point, a total of 360 random bacterial colonies were interrogated for their phage sensitivity. For the HWPB-3-treated flies, no phage-resistant colony was found during the entire course of infection. For the HWPB-2-treated flies, we did observe the presence of a very low level of phage-resistant bacteria (Figure 4), ranging from 0 to 4.44% (i.e., 48 hpi, 16 out 360). However, as manifested by the very large standard deviation associated with the mean frequency at each time point, the occurrence of phage-resistant strains is quite sporadic among the flies. Most often, no phage-resistant bacterium is found among majority of the flies at a given time point. When a resistant colony is present, it is usually found in one of the 12 flies in one of the 30 random colonies. The highest level of phage-resistant bacteria is found at 48 hpi, in which 10 out of 30 random colonies in one fly and 2 out of 30 random colonies in three flies were found to be phage-resistant.

Even though the level of phage-resistant bacteria does increase at the later stage of the infection, our result shows that the proportion of phage-resistant strains is simply too low to account for the second rise of bacteria titer, as seen in Figure 3. Therefore, it is unlikely that the emergence of phage-resistant strain(s) is the main factor in contributing to fly mortality.

**Bacteria and phage population dynamics in dead flies**
Up to this point, we have only surveyed bacteria and phage titers in living flies at time points that were 6 hours apart. There are two inevitable consequences from such an experimental design: (1) since only living flies were surveyed, we may have systematically underestimated the average lethal bacteria load and (2) even if we were to estimate the bacteria titers in dead flies, the 6 hour time span between sampling would render the estimates unreliable. To address this concern and to determine whether there are differences in bacteria and phage titers between living and dead flies during the course of infection, we conducted a separate experiment to sample one dead and one living fly every hour for at least six consecutive hours over the course of infection. In order to compare between the two phage types, flies treated with HWNPB-3 and HWPB-2 were assayed at the same time points during infection. We focused on the time period of 30-35 hpi to increase the chance of obtaining one dead and one living fly every hour (see Figure 2). Excess dead flies were removed after each sampling, so dead flies had died no more than one hour prior to sampling. From each fly, both phage and bacterial titers were assessed.

For HWNPB-3-treated flies, we found that, across time points, dead flies had higher bacteria titers than the live flies \( (F = 8.8998, p = 0.0056) \) (Figure 5), while no significant differences were found for the HWPB-2-treated flies \( (F = 1.4929, p = 0.2312) \) (Figure 6). On the other hand, HWPB-2-treated flies had significantly higher phage titers in live flies \( (F = 13.2174, p = 0.0010) \)(Figure 6), while no significant difference was found in the HWNPB-3-treated flies \( (F = 3.9110, p = 0.0566) \)(Figure 5).

Our more detailed survey of the bacteria and phage titers in infected flies during the period when the fly mortality is high showed that, on average, flies were dying with
lower bacterial levels than those seen in flies injected with only bacteria (Figure 1), and while phage reduced the instantaneous mortality rate of infected hosts, phage levels are not always indicative of fly survival.

**Stability of phage particles in fly**

One concern regarding utilizing phage to treat bacterial infections is the host’s immune reaction to the phage particles themselves. To see whether the differential efficacy between HWNPB-3 and HWPB-2 in treating *P. aeruginosa* infection is due to their differential stability in the fly, we introduced ~10^9 phages (2.50 × 10^9 phages/fly for HWPB-2 and 6.83 × 10^9 phages/fly for HWNPB-3) into individual flies in the absence of their bacterial host and followed phage decay in plaque formation for nine days by determine daily phage titers.

As shown in Figure 7, both phages decayed rapidly within a day of being introduced into the flies. For the first day, only 0.5% HWPB-2 retained the ability to form plaques, while an extremely small portion of ~0.005% HWNPB-3 did. Clearly, HWNPB-3 is much less stable than HWPB-2 in *D. melanogaster*. From the third day on, both phages stabilized at a much lower titer, with an average of ~0.02% of its initial titer for HWPB-2 and ~0.0004% for HWNPB-3. From this study, it seems that differential stability in phage particles in the *D. melanogaster* can at least partially explain the differential efficacy in prolonging the survival time between these two phages.

**Discussion**

Our results illustrate both phages are able to persist within *D. melanogaster* in the absence of *P. aeruginosa*, even though they persist at a very low level suggesting that the
*D. melanogaster* immune system does not recognize and attack the phages. Any phage lost during early infection is most likely due to the fly's immune response to the wounding as well as the difference in phage stability, rather than an immune response to the phage particles themselves, given that phage titers plateau and stabilize at a low titer approximately three days after injection through the end of the assay. The *D. melanogaster* response to *P. aeruginosa* is very different. At the dosage administered to the flies, *P. aeruginosa* is a 100% lethal infection in *D. melanogaster* when left untreated. Overall, our assay shows at least some flies are able to survive until the bacteria reach over $10^7$ cfu/ml within the fly, although by the time the infection reaches those levels, the time specific mortality rates are extremely high. In addition, the bacterial growth rate is exponential with a doubling time of 1.76 hours, suggesting that the *P. aeruginosa* overtakes the host immune system rather quickly and grows unchecked.

In regards to utilizing phage to treat the bacterial infection, HWPB-2 is the better therapeutic quality phage. HWPB-2 is able to significantly improve the survival time as compared to untreated flies or flies treated with HWNPB-3 (Figure 2). Flies treated with HWNB-3 are able to survive longer than untreated control flies, but have a much shorter survival time as compared to HWPB-2 treatment. This difference in survival is likely due the different dynamics throughout infection. HWPB-2 has a higher growth rate than HWNPB-3 and was able to proliferate much more quickly early in infection, increasing from $10^4$ pfu/ml to $10^8$ pfu/ml in approximately 12 hours where HWNPB-3 is only able to increase from $10^4$ pfu/ml to $10^7$ pfu/ml in the same amount of time (Figure 3). HWPB-2 titers plateau around $10^8$ pfu/ml 18-24 post infection and maintains those levels until very late in infection. HWNPB-3 does not appear to plateau, but that may be due to the slower
rate of amplification and the shorter duration of the sampling due to the flies dying sooner. Despite having a much shorter sampling time frame, it does appear that HWNPB-3 can reach similar levels to HWPB-2, since HWNPB-3 titers around \(10^8\) pfu/ml at hour 42, which is similar to the titers of HWPB-2 at hour 24. HWNPB-3 may have eventually exhibited a similar pattern of hitting a plateau around \(10^8\) pfu/ml as HWPB-2, had the flies survived longer. This plateau might simply be the maximum capacity of the host model system, as once phages replicate, they do not appear to be removed from the system by the host immune system.

Interestingly, throughout the course of infection/treatment, bacteria persist at a higher titer than expected given the high phage titers. Despite the high levels of phage amplification early in infection, a corresponding decrease in bacterial titers is not seen, in fact, in both treatments bacterial titers increase until about 24 hours after infection where there is a slight drop off before they rebound again and continue their exponential growth. This drop off in bacterial titers may be attributed the circadian rhythm of the flies. It has been shown that circadian rhythm can affect the regulation of immune genes (McDonald and Rosbash 2001). All flies were infected in the morning, so at 24 hours post infection, it would again be the early morning, so an up-regulation of immune genes may explain the sudden dip in bacterial titers seen at 24 hours and even slightly at 48 hours in the HWPB-2 treated flies.

Overall, bacteria being able to persist at high titers within the fly despite high phage titers may be due to several factors. The bacteria may be escaping phage detection hiding in refugia within the \(D.\ melanogaster\) body. Being physically isolated from the phages may allow for some subpopulation of bacteria to persist despite the high titers of
phage. Phage susceptible bacteria persisting in refugia within a high phage titer system have been seen before in chemostat cultures (Schrag and Mittler 1996), so it is not totally unexpected that it might occur in a host organism as well. The rise of resistant bacteria could also be responsible for the bacterial titers seen. However, in our assays we saw very low levels of bacteria resistant to HWPB-2 (Figure 4) and none resistant to HWNPB-3, making it unlikely that resistant bacteria are causing the persistence of high bacterial titers. The bacteria could potentially be forming biofilms within D. melanogaster, making them more resistant to the phage due to an exopolymetric matrix which makes it difficult for the phage to be able to contact the bacterial cells (Azeredo and Sutherland 2008). This could have allowed the bacteria to persist within the fly in the presence of high phage titers without the bacteria themselves being phage resistant.

Overall, the flies treated with HWPB-2 survived longer, which is likely due to the HWPB-2 having a faster growth rate within the fly, which in turn, keeps the bacteria at a significantly lower level that HWNPB-3. This difference likely lead to the flies treated with HWPB-2 having seen a fewer overall number of bacterial cells over the course of infection, which increased the life of the fly.

In general, the bacterial titers seen throughout this assay were much lower than expected given how quickly the bacteria reached extremely high titers within untreated flies. One reason for the disparity in bacterial numbers is that the assay was biased to only sampling living flies, so flies which succumbed to the infection may in fact have the high bacterial titers which we expected. When assaying pairs of living and dead flies, we did find dead flies had higher bacterial titers than the living flies in HWNPB-3 treated flies but saw no differences between living and dead fly titers for HWPB-2 treated flies.
Flies died with high $10^5$ cfu/ml or low $10^6$ cfu/ml bacterial titers rather than the $10^7$-$10^8$ cfu/ml titers we observed around the time of death in the untreated flies (Figure 5,6). Fly death due to decreased bacterial titers could be due to a buildup of toxins which were either released when bacterial cells were lysed by the phages, or released as exotoxins (Hagens et al. 2004; Gallagher and Manoil 2001). The effect of the toxins may have accumulated over the longer course of infection, slowly decreasing the flies’ resistance to the bacterial infection. There is also a possibility of the flies succumbing to a lower bacterial titer at any one point, as their systems may have experienced, over the course of the longer infection, a higher overall bacterial titer than the titer found in the dead flies. We also found significantly higher phage titers in the living flies than what was found in the dead flies in HWPB-2 treated flies, suggesting the possibility of the bacteria escaping phage control due to a decrease in phage titer. While this is a possibility, it is unlikely as we do not see a similar trend in HWNPB-3 treated flies, and the bacterial levels in HWPB-2 treated flies were not significantly different between the living and the dead flies.

Overall, when comparing the results of our phage dynamics assays done in *D. melanogaster* to other phage dynamics assays done in other model systems, we do find similarities. The dynamics seen within the living *D. melanogaster* are very similar to the dynamics found by Smith and Huggins (1982) and reviewed by Levin and Bull (1996) within the inoculated muscles of mice infected with *Escherichia coli* when treated with phage eight hours post bacterial infection. Within that inoculated tissue, the bacterial cell numbers are maintained around $10^8$ cfu/ml even though phage titers are maintained around $10^8$ pfu/ml as well. These high level titers of both phage and bacteria were not
maintained in any of the other mouse tissues tested. This similarity may be due to the high levels of spatial structure within the muscle tissues of the mouse. There are very likely many spatial refuges within the muscle, enabling the bacteria to evade the phages. Despite the dynamics seen within *D. melanogaster* throughout the course of infection being different than what is seen throughout the majority of tissues within the mouse model, it is not all that unexpected. The body plan of *D. melanogaster* is vastly different with the lack of a closed circulatory system and spleen which allows the mouse to eliminate bacteria in a more effective manner. The one tissue similar to the body plan of the fly, the mouse muscle, does show very similar dynamics. Given these similarities between these two tissues, understanding the bacteria/phage dynamics within *D. melanogaster* may aid in increasing effective phage therapy treatments for the treatment of abscesses and other muscle specific wounds.
Figure 1: Bacterial titers of *D. melanogaster* infected with *P. aeruginosa*. 10 living flies were assessed at the time of infection as well as every six hours post infection to attain bacterial titers. Only 2 flies were assessed at 24 hours post infection, as there were only 2 left alive at that time point.
Figure 2: Probability of survival after infection of *P. aeruginosa* PAO1. Flies treated with either HWNPB-3 (short dash) or HWPB-2 (long dash) survived longer than sham treated controls (solid line) flies (Kaplan-Meier survival analysis: HWNPB-3 vs. Control: $\chi^2 = 27.9146$, df = 1, $p < 0.001$; HWPB-2 vs. Control: $\chi^2 = 75.5472$, df = 1, $p < 0.001$). Flies treated with HWPB-2 survived significantly longer than flies treated with HWNPB-3 (Kaplan-Meier survival analysis: HWPB-2 vs. HWNPB-3: $\chi^2 = 61.5403$, df = 1, $p < 0.001$). The vertical lines within the figure are standard errors for each time point. This figure is a modification of figure 4 in chapter 1.
Figure 3: Phage and bacterial titers in living flies throughout the course of phage treatment. HWPB-2 treated flies are represented by open symbols, while HENPB-3 treated flies are represented by closed symbols. Bacterial titers are circles and phage titers are triangles. The bars at each point are standard error bars. Every time point between phages were significantly different except the one labeled “ns”. Only those bacterial time points starred were significantly different between treatments. *, p < 0.05; **, p < 0.01; ns, not significant
Figure 4: Proportion of phage-resistant bacterial colony during HWPB-2 treatment. A total of 360 colonies were tested for resistance. 30 randomly selected colonies from each of 12 flies were streaked across HWPB-2 to test for resistance.
Figure 5: Bacterial and phage titers from living and dead flies treated with HWNPB-3. One dead and one living fly were sampled every hour. The study was repeated three times. The points shown are the averages of the three replicates and the standard error. Dead flies are represented by open symbols and living flies are represented by closed symbols. Phage titers are triangles while bacterial titers are circles.
Figure 6: Bacterial and phage titers from living and dead flies treated with HWPB-2. One dead and one living fly were sampled every hour. The study was repeated three times. The points shown are the averages of the three replicates and the standard error. Dead flies are represented by open symbols and living flies are represented by closed symbols. Phage titers are triangles while bacterial titers are circles.
Figure 7: Phage decay in *D. melanogaster* in the absence of *P. aeruginosa*. 60 female flies injected with either HWPB-2 (solid) or HWNPB-3 (broken). 6 flies homogenized per day to assess phage titers.
Chapter 3: Experimental evolution of bacteriophages alters phage life history traits and ability to treat *Pseudomonas aeruginosa* infections in *Drosophila melanogaster*

Authors: **Heather Lindberg, Kurt McKean and Ing-Nang Wang**

Institution: **State University of New York, University at Albany.**

*All research presented in this chapter was done by Heather Lindberg*
Abstract

The idea of using bacteriophage (phage) to treat bacterial infection has received renewed interest, particularly due to the rise of antibiotic-resistant bacteria. Phage therapy has many benefits, including an abundance of potentially therapeutic phages specific to different clinically relevant bacteria. Being able to modify therapeutically effective phages to make them even more effective would increase the probability of a successful treatment and potentially expand the uses for phage therapy. Here we explore the possibility of utilizing well known in vitro serial transfer techniques to alter phages’ in vivo therapeutic ability. We utilize two different phages capable of treating a Pseudomonas aeruginosa infection within Drosophila melanogaster. We found that adapting phages to a more structured environment decreased therapeutic efficacy. However, adaptation to a non-structured environment increased the therapeutic efficacy, in at least one of the phage lines. Overall, we found that simple in vitro serial transfer techniques can be used to manipulate phages and increase therapeutic ability, which may lead to advances in the field of modifying phages to increase effectiveness.
Introduction

The idea of using phage to treat bacterial infections (phage therapy) achieved a resurgence of interest in the 1980’s (Summers 2005). This resurgence may have been aided by many scientific advances which made understanding the processes behind phage therapy easier, such as increased knowledge about basic phage biology and the ability to manipulate genetics. The sheer number of phages on the planet, with an estimated $10^{10}$ phage per liter of sea water or $10^7$ phage per gram of soil (Rohwer 2003; Rohwer and Edwards 2002), makes it possible for many therapeutically viable phages to be isolated, but it is a daunting task to find good therapeutic quality phages. These naturally isolated phages have not been selected for as therapeutic agents, and therefore are not optimized by a history of natural selection to be effective in treating infections. Therefore, using existing phages, there are two routes of improving therapeutic efficacy: engineering phage to have particular traits, or using experimental evolution to allow selection to optimize traits affecting efficacy.

One way to overcome the abundance of non-optimized phages is to modify phages which have already been identified as therapeutic agents to make them even more effective (Goodridge 2010). The ability to change phage life history traits, such as adsorption rate, lysis time, and burst size, has improved due to an increase in the understanding of basic phage biology as well as the underlying genetic components. Many studies used this ease of manipulation and increased knowledge of the phage/bacteria interaction to increase the therapeutic efficacy of phages by modifying host range or increasing phage resistance to restriction endonucleases (Goodridge 2010). Phages can also be modified by altering the mechanism by which they interact with the
bacterial host cell throughout infection. Hagens et al. (2004) genetically modified phages to kill bacterial cells but not lyse them in order to decrease the release of bacterial debris and toxins and thereby decreasing the chances of septic shock within the host organism. This reduction of bacterial debris not only aids in the reduction of toxins but also serves to decrease the amplification of the host immune reaction, decreasing the potential for septic shock.

Merril et al. (1996) took a slightly different approach in the modification of their phage. Instead of altering the phage using genetic manipulation, they used experimental evolution, passing phage through mice to increase phage within-host circulation time. This increase in circulation time corresponded to an increase in therapeutic ability within the mice. Such modification of therapeutically viable phage is not the first time phages have been used in experimental evolution studies, although it is the only one performed in vivo. In fact, the huge increase in the understanding of basic phage biology is due to phages having been used to investigate both the mechanisms of adaptation and as models for experimental evolution (Pepin and Wichman 2008; Wichman and Brown 2010; Wichman et al. 2000; Wichman, Millstein, and Bull 2005; Pepin, Samuel, and Wichman 2006; Heineman and Bull 2007). Phages make great models for experimental evolution due to their short generation times and large population sizes (Forde 2010). Not only has the field of experimental evolution been advanced by using phage models, but knowledge about basic phage biology and the phage/bacteria interaction has also been improved.

In vivo serial passage and genetic manipulation are not the only methods that have been used for the modification of phage traits. It is well know that adaptation to different in vitro environments can select for different phage life history traits and alter phage
fitness. Gallet *et al.* (2009) found that phages with lower adsorption rates were selected for in the highly structured "biofilm-like" environment, while in liquid culture serial transfer experiments, phages with faster adsorption rates, or shorter lysis times are favored by the selection process as more fit (Bull and Molineux 2008; Abedon, Herschler, and Stopar 2001; Heineman and Bull 2007; Wang 2006). *In vitro* experimental evolution has also been used to demonstrate genetic correlation among phage life history traits and that adaptation to one environment often affects fitness in different environments (Gallet, Shao, and Wang 2009; Abedon and Culler 2007; Heineman and Bull 2007).

Utilizing these different well known *in vitro* experimental evolution techniques can provide a faster and easier way to modify therapeutic quality phages and potentially make them even more effective therapeutic agents. Both Merril *et al.* (1996) and Hagens *et al.* (2004) studies, as well those studies reviewed by Goodridge (2010) show that *in vivo* experimental evolution and genetic modification of phage can increase therapeutic efficacy of the phages. However, genetic manipulation of phages requires knowledge of exactly which genes affect which phage traits, while *in vivo* serial transfers can be very time consuming and cumbersome endeavors.

The aim of our study was to adapt *Pseudomonas aeruginosa*-specific phages to two different *in vitro* environments, one structured and one unstructured, and evaluate the therapeutic efficacy of the evolved phages in treating *Drosophila melanogaster* infected with *P. aeruginosa*. Adaptation to either of these environments is known to alter phage life history traits and *in vitro* fitness, which is known to be correlated to therapeutic efficacy within *D. melanogaster*. 
Materials and Methods

Flies, bacteria, and phages

The *Drosophila melanogaster* flies used in the experiments were from a large outbred laboratory population maintained at 25°C with a 12:12 light cycle. Survival experiments were run using 4 to 6 day old adult females from low larval density vials which were established by placing 10 males and 10 females in a vial for 24 hours and then removed.

The bacterium used is the laboratory strain of *Pseudomonas aeruginosa* MPAO1 from the University of Washington transposon library (Jacobs et al. 2003).

Phages HWPB-2 and HWNPB-3 (previously described in Chapter 1) were used for all experiments. HWPB-2 is a *Podoviridae* phage likely binds to the type IV pilus of MPAO1, while HWNPB-3 is a *Myoviridae* phage that likely binds to lipopolysaccaride.

Experimental Evolution

Experimental evolution of HWPB-2 and HWNPB-3 was carried out by serial passage of replicate populations of phage infecting *P. aeruginosa* MPAO1 kept in two different environments, structured and non-structured.

For experimental evolution within a structured environment, methods described in Gallet *et al.* (2009) were modified for use. In brief, approximately 100 pfu were plated on a lawn of MPAO1 and incubated overnight at 37°C. The next day if 100 ± 25 plaques were seen on the plate, 5 ml of LB broth were poured on the plate and incubated at room temperature for 30 min. If there were either too many or too few plaques present on the plate, the previous transfer was replated. After 30 min the liquid was aspirated, collected,
and stored at 4°C. For the next transfer, the lysate from the previous transfer was diluted (approximately $10^{-5}$) so approximately 100 phages were plated. This procedure was repeated for 25 passages with six replicate populations for HWPB-2 and five for HWNPB-3. The sixth replicate population for HWNPB-3 was lost due to contamination.

For experimental evolution in a non-structured (liquid) environment, methods described in Wang (2006) were modified for use. In brief, serial passages were started by adding phages ($\sim 4 \times 10^6$ pfu ml$^{-1}$) and exponentially growing MPAO1 ($\sim 1 \times 10^8$ cfu ml$^{-1}$) to 3 ml LB broth. The mixture was incubated at 30°C and agitated in a tissue culture roller drum (New Brunswick Scientific, Edison NJ) at setting 7 for 4 hr. After 4 hr, the cultures were filtered through a 0.2um filter and the lysate stored at 4°C. Phages collected from the lysate were then used for the next passage. This procedure was repeated for 25 passages with six replicate populations for HWPB-2 and five for HWNPB-3. The sixth replicate of HWNPB-3 was lost due to contamination.

**Adsorption rate estimation**

Methods described in chapter 1 were used to estimate phage adsorption rates. In brief, a 25 ml culture of MPAO1 was grown to log-phase in a shaking 30°C water bath. Phage at a concentration of $\sim 1 \times 10^4$ pfu ml$^{-1}$ was mixed with log-phase MPAO1 at $\sim 1 \times 10^7$ cfu ml$^{-1}$ in a total culture volume of 5 ml. Samples (0.5 ml) were taken every five minutes for twenty minutes and filtered through an AcroPrep 96 filter plate (Pall, Ann Arbor MI). The filtrate was plated on MPAO1 to assess the number of free phage. The adsorption rates were obtained by fitting the data to the model of $\ln(P_0/P_{20}) = -\alpha \beta t$, where $P_0$ and $P_{20}$ are the phage concentration at time zero and 20 min respectively, $\alpha$ is the
adsorption rate constant and $\beta$ the initial cell concentration. The bacterial cell concentration was assumed to be constant throughout the assay as there was only a negligible increase in bacterial cell count throughout the 20-min assay. Adsorption rates are based on three replicates for each phage.

**One step growth curves**

Methods described in chapter 1 were used to estimate the lysis time and burst size of each phage. In brief, a 25 ml culture of MPAO1 was growth to log-phase in a 30°C water bath. Phage at a concentration of $\sim 1 \times 10^7$ pfu ml$^{-1}$ was mixed with $\sim 1 \times 10^8$ cfu ml$^{-1}$ MPAO1 for twenty minutes at 25 °C then diluted $10^4$ fold in LB broth to a total culture volume of 10 ml. Every 10 minutes, for up to 180 minutes, 0.4 ml aliquots were drawn and filtered through a 2 μm filter. The filtrate was plated for plaque assay on MPAO1 to determine phage numbers. Lysis times and burst sizes are based on three replicates for each phage.

**In vitro fitness assay**

To assess the phages' *in vitro* growth, we used methods previously described in chapter 1. In brief, phage growth was assessed by adding phages ($\sim 4 \times 10^4$ pfu ml$^{-1}$) and exponentially growing MPAO1 ($\sim 1 \times 10^8$ cfu ml$^{-1}$) to 3 ml LB broth. The mixture was incubated at 30°C and agitated in a tissue culture roller drum (New Brunswick Scientific, Edison NJ) at setting 7. Fitness was calculated as $\omega = \ln[P_3/P_0]/3$, where $P_0$ and $P_3$ are free phage concentrations at times 0 and 3 hours respectively. Phage fitness was estimated based upon three replicates.

**Therapeutic efficacy of phage in treatment of *P. aeruginosa* infected *D. melanogaster***
The therapeutic efficacy of the evolved phages was assessed by comparing the survival of *D. melanogaster* treated with evolved phages to *D. melanogaster* treated with the ancestral strain of phage following experimental infection with *P. aeruginosa* MPAO1. The method used has previously been described in chapter 1. For each replicate, 50 female *D. melanogaster* were injected by piercing the thorax with a 0.1 mm diameter minutien pin (Fine Science Tools, Foster City, CA) which had been dipped in a liquid culture of MPAO1 diluted to OD$_{600} = 0.104 \pm 0.005$, resulting in an initial inoculation of $\sim 10^3$ cfu per fly. Six hours post bacterial infection; flies were injected using a Nanoject injector (Drummond Scientific) with 50.6 nl of filtered phage lysate. Initial phage concentrations were $\sim 10^4$ pfu per fly, corresponding to an estimated MOI of 10. After phage treatment, flies were checked for survival every six hours for 72 hours. Those flies surviving more than 72 hr were right-censored for analysis using a Kaplan-Meier survival analysis. The LT$_{50}$ for each of the strains was estimated as the time at which 50% of the fly population had died.

**Effect of phage concentration on treatment of *D. melanogaster* infected with *P. aeruginosa***

The therapeutic efficacy of different concentration of ancestral phages was assessed by comparing the survival of phage-treated *D. melanogaster* to untreated flies following experimental infection with *P. aeruginosa* MPAO1. The methods used were methods previously described in chapter 1. To establish bacterial infections for each of three independent replicates, 50 female *D. melanogaster* were injected by piercing the thorax with a 0.1 mm diameter minutien pin (Fine Science Tools, Foster City, CA) that had been dipped in a liquid culture of MPAO1 diluted to OD$_{600} = 0.102 \pm 0.004$, resulting
in an initial inoculation of \( \sim 10^3 \) cfu per fly. Six hours post bacterial infection, flies were injected using a Nanoject injector (Drummond Scientific) with 50.6 nl of either sterile LB broth or filtered phage lysate suspended in LB broth. Initial phage concentrations were \( \sim 10^2, 10^3, 10^4, 10^5 \) or \( 10^6 \) pfu per fly, corresponding to an estimated MOI (multiplicity of infection) of 0.1, 1, 10, 100, and 1000, respectively. After phage treatment, flies were checked for survival every six hours for 72 hours. Those flies surviving more than 72 hours were right-censored for analysis using a Kaplan-Meier survival analysis. The LT_{50} for each treatment was estimated as the time at which 50% of the population had died.

**Data Analyses**

All statistical analyses were performed using the JMP 10 statistical software. Survival was assessed by utilizing a Kaplan-Meier survival analysis. The three replicates of each assay were pooled prior to the assay being run. Pairwise comparisons between the ancestral strain and the evolved strains as well as among evolved strains were done using the Kaplan-Meier survival analysis. Differences between ancestral and evolved life history traits and *in vitro* fitness were assessed using a non-parametric Kruskal-Wallis test was performed. Given multiple comparisons were done on each data set, corrections for multiple comparisons were done using a Bonferroni correction (Rice 1989).

**Results**

**Effect of phage concentration on therapeutic efficacy**

To test how different phage concentrations altered therapeutic efficacy; we treated infected flies with different initial phage concentrations. We found treatment with increasing concentrations of phage six hours post infection increased the efficacy of the
treatment in both HWPB-2 treated flies (Kaplan Meier survival analysis $\chi^2 = 286.64$, df = 5, $p < 0.0001$) and HWNPB-3 treated flies (Kaplan Meier survival analysis $\chi^2 = 195.10$, df = 5, $p < 0.0001$). Despite seeing overall increases in therapeutic efficacy as phage concentration increased across both phage types, there were significant differences in how therapeutic efficacy increased as phage concentration increased. While there were significant differences among treatment groups of HWPB-2 treated flies (Kaplan Meier survival analysis $\chi^2 = 19.02$, df = 4, $p = 0.0008$), in pairwise comparisons, the treatment level of MOI = 0.1 was significantly less effective than other concentrations ($p < 0.05$). All of the other treatment conditions were not significantly different from each other ($p > 0.05$) (Figure 1). There were also significant differences in treatment groups among the HWNPB-3 treated flies (Kaplan Meier survival analysis $\chi^2 = 107.73$, df = 4, $p < 0.0001$), however unlike HWPB-2, the as the phage concentration increased in HWNPB-3 treatments, the survival time of the flies also significantly increased, with each treatment being significantly better than the next lowest MOI, even after Bonferonni corrections ($p < 0.05$) (Figure 1).

**Phage life history traits**

Replicate populations of HWPB-2 and HWNPB-3 were serially passed through two different environments, a non-structured environment, *i.e.*, liquid culture, or a structured environment, *i.e.*, soft agar plates, in order to determine how *in vitro* experimental evolution can affect the therapeutic efficacy of phages *in vivo*. In addition to how experimental evolution alters therapeutic efficacy, we also tested the phages to see how adaptation altered phage life history traits. After 25 transfers in the non-structured environment, we found, as a class, both evolved HWPB-2 and HWNPB-3 showed a
significant increase in adsorption rates compared to the ancestral phages (Figure 2A and 2B) (HWPB-2: evolved vs. ancestral, Kruskal-Wallis $\chi^2 = 4.88$, df = 1, $p = 0.0270$; HWNPB-3: evolved vs. ancestral, Kruskal-Wallis $\chi^2 = 5.9227$, df = 1, $p = 0.0149$). There were no significant differences among replicate populations within either HWPB-2 or HWNPB-3 lines (HWPB-2: Kruskal-Wallis $\chi^2 = 2.59$, df = 5, $p = 0.7628$; HWNPB-3: Kruskal-Wallis $\chi^2 = 1.91$, df = 4, $p = 0.7516$).

In contrast to what was seen within the phage lines evolved in a non-structured environment, both evolved HWPB-2 and HWNPB-3 strains evolved for 25 transfers within a structured environment showed significant decreases in adsorption rate when compared to their respective ancestral strains (Figure 4A and 4B) (HWPB-2: Kruskal-Wallis $\chi^2 = 7.36$, df = 1, $p = 0.0067$; HWNPB-3: Kruskal-Wallis $\chi^2 = 7.11$, df = 1, $p = 0.0077$). There was no significant difference among the evolved HWPB-2 strains (Kruskal-Wallis $\chi^2 = 1.54$, df = 5, $p = 0.9086$), however, there was significant difference among the evolved HWNPB-3 strains (Kruskal-Wallis $\chi^2 = 10.88$, df = 4, $p = 0.0280$).

When comparing lysis times and burst sizes of the phages evolved in a non-structured environment, we found evolved HWPB-2 lines, as a class, did not have significantly different lysis times compared to the ancestral strain (Kruskal-Wallis $\chi^2 = 1.13$, df = 1, $p = 0.2888$), or variation among the replicate populations (Kruskal-Wallis $\chi^2 = 3.72$, df = 5, $p = 0.5904$). However, as a class, evolved HWPB-2 strains did have a significantly decreased burst size compared the ancestral strain (Figure 2 C) (Kruskal-Wallis $\chi^2 = 7.36$, df = 1, $p = 0.0067$) with no significant differences among the replicate populations (Kruskal-Wallis $\chi^2 = 3.13$, df = 5, $p = 0.6802$). Evolved HWNPB-3 strains showed a similar pattern, with no significant change in lysis time when compared to the
ancestral strain (Kruskal-Wallis $\chi^2 = 0.68$, df = 1, $p = 0.4096$) or difference among the replicate populations (Kruskal-Wallis $\chi^2 = 8.62$, df = 4, $p = 0.0715$). However evolved HWNPB-3 strains showed a significant increase in burst size when compared to the ancestral strain (Figure 1D) (Kruskal-Wallis $\chi^2 = 7.11$, df = 1, $p = 0.0077$), but again without any significant difference among the replicate populations (Kruskal-Wallis $\chi^2 = 1.03$, df = 4, $p = 0.9047$).

When assessing the lysis times and burst sizes of the phages evolved in the structured environment, we saw very different results compared to what was seen in the phages evolved in the non-structured environment. The HWPB-2 strains evolved in the structured environment showed no significant difference in lysis time compared to the ancestral strain (Kruskal-Wallis $\chi^2 = 0.00$, df = 1, $p = 1.000$) or among the replicate populations (Kruskal-Wallis $\chi^2 = 1.89$, df = 5, $p = 0.8643$). As a class, the evolved HWPB-2 strains showed a significant decrease in burst size compared the ancestral strain (Kruskal-Wallis $\chi^2 = 7.36$, df = 1, $p = 0.0067$), with no differences among the replicate populations (Figure 4C) (Kruskal-Wallis $\chi^2 = 5.70$, df = 5, $p = 0.3363$). The evolved HWNPB-3 strains revealed a slightly different pattern, with the evolved strains having significantly longer lysis times compared to the ancestral strain (Kruskal-Wallis $\chi^2 = 4.62$, df = 1, $p = 0.0316$), with no differences seen among the replicate populations (Kruskal-Wallis $\chi^2 = 5.59$, df = 4, $p = 0.2322$). The evolved HWNPB-3 strains, as a class, showed no significant difference in burst size compared to the ancestral strain (Kruskal-Wallis $\chi^2 = 2.95$, df = 1, $p = 0.0858$), however, there were significant differences among the evolved strains (Kruskal-Wallis $\chi^2 = 12.93$, df = 4, $p = 0.0116$) (Figure 4D).
Overall, phages which were evolved in a non-structured environment showed increased adsorption rates regardless of receptor type. In comparison, phages evolved in the structured environment showed decreased adsorption rates regardless of the receptor type. Lysis times for both phage types evolved in the non-structured environment show no difference compared to their ancestral strains. In comparison, there are differences in the lysis times for the two phage types evolved in the structured environment (Table 1-3). While the HWPB-2 phages evolved in the structured environment show no difference in lysis time compared to their ancestral strain, the HWNPB-3 lines evolved in the structured environment show a significantly longer lysis time. Despite the longer lysis times seen in the HWNPB-3 lines evolved in the structured environment, we saw no change in burst size compare to the ancestral strain. This is a different response than what is seen in the HWPB-2 strain evolved in the same environment where we actually see a decrease in burst size despite no change in the lysis time (Table 1-3). A very similar response was seen in the HWPB-2 phages evolved in the non-structured environment, where we saw a decrease in the burst size despite the lysis time not being altered. The HWNPB-3 lines evolved in the non-structured environment, on the other hand, showed an overall increase in burst size despite showing no change in the lysis time.

**In vitro fitness and therapeutic efficacy**

Experimental evolution in the non-structured liquid culture environment improved the *in vitro* fitness in the non-structured liquid environment for both evolved HWPB-2 and HWNPB-3 strains compared to their respective ancestral strains (Figure 3A and 3B) (HWPB-2: Kruskal-Wallis $\chi^2 = 7.31$, df =1, $p = 0.0067$; HWNPB-3: Kruskal-Wallis $\chi^2 = 6.49$, df =1, $p = 0.0109$). There were no significant differences in fitness among the
evolved strains for HWPB-2 (Kruskal-Wallis $\chi^2 = 1.42$, df = 5, $p = 0.922$), however there were significant differences among the evolved strains for HWPB-3 (Kruskal-Wallis $\chi^2 = 9.67$, df = 4, $p = 0.0464$). The evolved HWPB-3 strains showed significant improvement in the ability to treat a $P. aeruginosa$ infection within $D. melanogaster$ (Figure 3D) (Kaplan-Meier Survival Analysis $\chi^2 = 28.32$, df = 1, $p < 0.0001$). There were no significant differences among the evolved strains (Kaplan-Meier Survival Analysis $\chi^2 = 8.98$, df = 4, $p = 0.0616$), and in pairwise comparisons with the ancestral strain (after correction for multiple comparisons) all five evolved strains showed a significant increase in therapeutic ability. HWPB-2 evolved strains showed no significant improvement in the ability to treat a $P. aeruginosa$ infection within $D. melanogaster$ compared the ancestral strain, (Figure 3C) (Kaplan-Meier Survival Analysis $\chi^2 = 0.12$, df = 1, $p = 0.7337$) but there were significant differences among the evolved strains (Kaplan-Meier Survival Analysis $\chi^2 = 13.14$, df = 5, $p = 0.0221$). In pairwise comparisons however, none of the evolved HWPB-2 strains were significantly different from the ancestral strain.

In contrast, experimental evolution in a structured environment significantly decreased the $in vitro$ fitness in non-structured liquid media for the evolved HWPB-2 strains when compared to the ancestral strain (Kruskal-Wallis $\chi^2 = 5.34$, df = 1, $p = 0.0208$), although there was significant variation among the evolved lines (Kruskal-Wallis $\chi^2 = 15.53$, df = 5, $p = 0.0083$) (Figure 5A). In pairwise comparisons with the ancestral strain, after corrections for multiple comparisons, all strains except HWPB-2 replicate lines S4T25 and S6T25 showed a significant decrease in $in vitro$ fitness. The evolved HWPB-3 stains, as a class, did not show a significant decrease in $in vitro$
fitness compared to the ancestral strain (Kruskal-Wallis $\chi^2 = 0.28$, df = 1, $p = 0.5940$) (Figure 5B), nor was there significant difference among the evolved strains (Kruskal-Wallis $\chi^2 = 8.67$, df = 4, $p = 0.0700$). Both evolved HWPB-2 and HWNPB-3 strains showed a significant decrease in the ability to treat a *P. aeruginosa* infection within *D. melanogaster* (HWPB-2: Kaplan-Meier Survival Analysis $\chi^2 = 5.41$, df = 1, $p = 0.0200$: HWNPB-3: Kaplan-Meier Survival Analysis $\chi^2 = 6.21$, df = 1, $p < 0.0127$) (Figure 5C and 5D). The evolved HWPB-2 strains showed no significant differences among replicates (Kaplan-Meier Survival Analysis $\chi^2 = 8.85$, df = 5, $p = 0.0817$), while evolved HWNPB-3 strains showed significant difference among replicates (Kaplan-Meier Survival Analysis $\chi^2 = 14.93$, df = 4, $p = 0.0049$) and in pairwise comparisons with the ancestral strain, HWNPB-3 replicate lines S1P25 and S5P25 strains showed a significant decrease in therapeutic ability.

Overall, evolution in a structured environment decreased the *in vitro* fitness for HWPB-2, but did not alter the *in vitro* fitness for the HWNPB-3 evolved lines (Table 1). Evolution in the non-structured environment on the other hand increased the *in vitro* fitness both phage types. This increase in fitness resulted in an increase in therapeutic efficacy for HWNPB-3 phages, without showing a similar increase in therapeutic efficacy in HWPB-2 evolved lines. We see similar disparities when comparing the therapeutic efficacy and *in vitro* fitness for the phages evolved in the structured environment (Table 1). The HWPB-2 evolved lines show both a decrease in their *in vitro* fitness and therapeutic efficacy, while the HWNPB-3 lines show no decrease in their fitness, but do exhibit a decrease in their therapeutic efficacy. The differences seen in the *in vitro* fitness may translate to different effective phage concentrations *in vivo*. Given the difference
seen between the two phage types in how differences in in vitro fitness translate to therapeutic efficacy, there may be differences in how phage concentrations during phage therapy affect therapeutic efficacy and ultimately, fly survival.

**Discussion**

Using phage to treat bacterial infections is a very attractive therapeutic option. Phages are highly strain-specific, self-replicating, and have the potential to be manipulated and evolved to create more effective treatments. This study was performed in order to test whether simple in vitro serial transfer methods can be used to increase phage therapeutic efficacy in vivo.

In their ancestral states, the phages HWPB-2 and HWNPB-3 have different therapeutic efficacies within *D. melanogaster*. The therapeutic efficacy of HWNPB-3 increases as the amount of phage introduced increases, although never reaching the therapeutic efficacy of HWPB-2 (Figure 1). The therapeutic efficacy of HWPB-2 increases as the MOI increases from 0.1 to 1, but did not increase beyond that level. Given the differences seen between the therapeutic efficacies of the ancestral phage strains, we experimentally evolved both HWNPB-3 and HWPB-2 and asked if we would be able to improve the efficacy of one or both of these phages.

We found that the experimental evolution of phages in both structured and non-structured environments significantly altered phage life history traits; in vitro fitness and therapeutic efficacy (see Table 1). Furthermore, while out two different phages use different receptors for adsorption to and infection of host cells, they showed similar adaptation to the different environments. In general these results support the view that
experimental evolution in environments known to affect life history traits of critical importance in determining the efficacy of phage therapy may be a useful and cost effective means of improving therapeutic phage regardless of the phage type.

Adaptation to the structured environment decreased in vitro fitness across both phage types (Figure 5A and 5C), which may be due to the decreased adsorption rates and burst sizes found with the evolved strains (Figure 4). While we did not measure fitness in the structured environment, the observation that fitness decreased in the unstructured environment is consistent with a trade off. These results are also consistent with our previous observation that in vitro fitness predicts therapeutic efficacy in D. melanogaster (Chapter 1).

Alternatively, we saw an overall increase in in vitro fitness for both phage types when evolved in the non-structured environment (Figure 3A and 3C). Despite this similarity in fitness increase, we did observe differences in evolved changes in particular life history traits in evolved lines of HWPB-2 and HWPB-3. An increased adsorption rate and the burst size evolved in lines of HWPB-3 phage (Table 1, Figure 2B and 2D). However, in HWPB-2 the increase in adsorption rate was accompanied by a decrease in burst size (Figure 2A and 2C). Overall, despite the decrease in the burst size seen in evolved lines of HWPB-2 the increase in adsorption rate coupled with the short lysis time compensated for the loss in progeny production, leading to the increase seen in in vitro fitness.

The increases seen in fitness for the phages adapted to the non-structured environment lead to different outcomes when testing the therapeutic efficacy of the
adapted lines. We saw the expected increase in therapeutic efficacy for the non-pilin binding phages (Figure 3D) however we did not see an increase in therapeutic efficacy in the adapted pilin binding phages (Figure 3C). This result may be due to the presence of a therapeutic threshold in *D. melanogaster* rather than differences in response to experimental evolution. In general, it is assumed that the increase in therapeutic efficacy is due to the increased production of the phage, and any increase in production would result in an increase in therapeutic efficacy. However, that assumption does not hold true for HWPB-2. In the MOI study of the ancestral phages we found increasing the MOI beyond 1 did not increase therapeutic efficacy (Figure 1). Taking this into account, at least under the conditions of our current system for testing therapeutic efficacy, we would be unlikely to see any improvement in the adapted lines due to the therapeutic threshold.

The idea of a therapeutic threshold for phage therapy is not something that is solely seen in our *D. melanogaster* model system. It has also been seen by Wang *et al.* (2006), when using phage to treat mice infected with imipenem-resistant *P. aeruginosa*. This therapeutic threshold may be caused by several things, a buildup of toxins in the host system which can ultimately kill the host or the *P. aeruginosa* forming biofilm within the host, making it more difficult for phages to attack and eliminate the bacteria.

Despite only seeing an increase in therapeutic efficacy in the non-pilin binding phage adapted to a non-structured environment, this study has shown the feasibility of using simple *in vitro* serial transfer techniques to alter the therapeutic ability of phages *in vivo*. Using a much simpler and easier system of *in vitro* serial transfer, we were able to achieve a similar result to Merril *et al.* (1996), where they used *in vivo* serial transfer to increase the therapeutic efficacy of the phage. Despite not performing *in vivo* serial
transfers in *D. melanogaster* to compare to the *in vitro* results, we found that using *in vitro* serial transfer techniques has many advantages. Not only are the transfers easier and faster than *in vivo* transfers, but there are many different ways to manipulate the transfer environment to select for various traits. The idea of evolving parasites and pathogens to increase impact on host populations has precedence (Ebert 1998; Poullain et al. 2008), and several experimental evolution studies have shown that trait evolution can be predictable (Poullain et al. 2008; Gaba and Ebert 2009), allowing researchers to select their experimental evolution environment based upon the trait they want to modify. The use of serial passage to manipulate therapeutically viable phages has been done previously (Betts et al. 2013). Betts *et al.* (2013) serially passaged *P. aeruginosa* specific phages through liquid culture for six passages. Similar to our liquid transfer results, they found that serial passage of the phages increased their infectivity and evolved phages had an increased ability to reduce the bacterial population, although they were testing the phages ability to decrease the bacterial population within liquid culture rather than *in vivo*.

Overall, utilizing *in vitro* techniques to alter *in vivo* therapeutic efficacy can not only increase the effectiveness of phage therapy, but may also be used to help expand the scope of phage therapy into different settings and uses such as modifying phages for agricultural uses or for making them more effective for topical treatments.
Figure 1: Effect of MOI on therapeutic efficacy of phage therapy in *D. melanogaster*. Flies infected with *P. aeruginosa* were treated with phage treatment or LB (control) six hours post infection. Flies were checked for survival every six hours post infection for survival for up to 72 hours. Flies which survived past 72 hours were censored from analysis. Graphs show LT$_{50}$, which is the time at which fifty percent of the flies have died. There were 50 flies per treatment group and the assay was repeated three times. Grey bars show the LT$_{50}$ of flies treated with various concentrations of HWPB-2. Treatment with phage significantly improved fly survival time (Kaplan Meier survival analysis $\chi^2=286.6369$ df=5 $p<0.0001$). There are significant differences among phage treatment groups (Kaplan Meier survival analysis $\chi^2=19.0197$ df=4 $p=0.0008$). Pairwise comparisons among the phage treatment groups show treatment with and MOI of 0.1 has significantly different survival time than all other treatments, even after Bonferroni corrections. None of the other phage treatment groups were significantly different from each other. Black bars show the LT$_{50}$ of flies treated with various concentrations of HWNPB-3. Treatment with phage significantly improved fly survival time (Kaplan Meier survival analysis $\chi^2=195.1023$ df=5 $p<0.0001$). There are significant differences among phage treatment groups (Kaplan Meier survival analysis $\chi^2=107.7269$ df=4 $p<0.0001$). In pairwise comparisons, all phage treatment groups were significantly different from each other, even after Bonferroni corrections ($P<0.5$).
Figure 2: Life history traits for phages experimentally evolved in non-structured liquid environment. Ancestral strains are in broken black, replicate populations of evolved lines are in black. Adsorption rates (A and B) are shown as percent decrease in free phage numbers over a twenty-minute time period. One step growth curves are shown, after a twenty-minute pre-adsorption and the curve shown is normalized against the zero time point sampling. 

A) HWPB-2 adsorption rates. As a class, the evolved lines have a significantly improved adsorption rate compared to the ancestral strain (Kruskal-Wallis $\chi^2=4.88$ df=1 $p=0.0270$), and there are no significant differences among the evolved lines (Kruskal-Wallis $\chi^2=2.59$ df=5 $p=0.7628$). 

B) HWNPB-3 adsorption rates. As a class, the evolved lines had a significantly improved adsorption rate compared to the ancestral strain (Kruskal-Wallis $\chi^2=5.9227$ df=1 $p=0.0149$), and there were no significant differences among the evolved lines (Kruskal-Wallis $\chi^2=1.9137$ df=4 $p=0.7516$). 

C) HWPB-2 one step growth curves. There was no significant difference between the ancestral and evolved lines lysis times (Kruskal-Wallis $\chi^2=1.1250$ df=1 $p=0.2888$), and as a class, the evolved lines showed a significant decrease in burst size compared to the ancestral strain (Kruskal-Wallis $\chi^2=7.3636$ df=1 $p=0.0067$). There were no significant differences in burst size among the evolved lines (Kruskal-Wallis $\chi^2=3.1287$ df=5 $p=0.6802$). In pairwise comparisons with the ancestor, after corrections for multiple comparisons, all evolved strains except 3T25 have a significantly decreased burst size ($P<0.05$). 

D) HWNPB-3 one step growth curves. There was no significant difference in lysis times when comparing the ancestral and evolved lines (Kruskal-Wallis $\chi^2=0.6800$ df=1 $p=0.4096$), or among the evolved lines (Kruskal- Wallis $\chi^2=8.6154$ df=4 $p=0.0715$). There was a significant increase in burst size when comparing the ancestral and evolved lines (Kruskal-Wallis $\chi^2=7.1053$ df=1 $p=0.0077$), and there were no significant differences in burst size among the evolved lines (Kruskal-Wallis $\chi^2=1.033$ df=4 $p=0.9047$).
Experimental evolution in liquid culture significantly improved in vitro fitness of the evolved lines compared to HWPB-2 (Kruskal-Wallis $\chi^2=7.31287$ df=1 $p=0.0067$), and there were no significant differences in fitness among the evolved lines (Kruskal-Wallis $\chi^2=1.4211$ df=5 $p=0.922$). Experimental evolution in liquid culture did significantly improve the in vitro fitness of the evolved lines compared to HWNPB-3 (Kruskal-Wallis $\chi^2=6.4877$ df=1 $p=0.0109$). There were also significant differences in in vitro fitness among the evolved lines (Kruskal-Wallis $\chi^2=9.666$ df=4 $p=0.0464$). Experimental evolution in liquid culture did not improve therapeutic efficacy of the evolved lines compared to HWPB-2 (Kaplan-Meier Survival Analysis $\chi^2=0.1157$ df=1 $p=0.7337$). Among the evolved lines, there were significant differences in therapeutic efficacy (Kaplan-Meier Survival Analysis $\chi^2=13.1422$ df=5 $p=0.0221$). Experimental evolution in liquid culture significantly improved the therapeutic efficacy of the evolved lines compared to HWNPNB-3 (Kaplan-Meier Survival Analysis $\chi^2=28.3206$ df=1 $p<0.0001$), and there were no significant difference among the evolved lines (Kaplan-Meier Survival Analysis $\chi^2=8.9791$ df=4 $p=0.0616$). In pairwise comparisons, after corrections for multiple comparisons, each of the evolved strains (starred) had significantly improved therapeutic efficacy compared to the ancestral strain ($p<0.05$).
Figure 4: Life history traits for phages experimentally evolved in a structured environment. Ancestral strains are in broken black, replicate populations of evolved lines are in black. Adsorption rates (A and B) are shown as percent decrease in free phage numbers over a twenty-minute time period. One step growth curves are shown, after a twenty-minute pre-adsorption and the curve shown is normalized against the zero time point sampling.  

A) HWPB-2 adsorption rates. As a class, the evolved lines showed a significant decrease in adsorption rates compared to the ancestral strain (Kruskal-Wallis $\chi^2=7.3636$ df=1 $p=0.0067$), and there were no significant differences in adsorption among the evolved lines (Kruskal-Wallis $\chi^2=1.5380$ df=5 $p=0.9086$).  

B) HWNPB-3 adsorption rates. As a class, the evolved lines showed a significant decrease in adsorption rate when compared to the ancestral strain (Kruskal-Wallis $\chi^2=7.1126$ df=1 $p=0.0077$). There were significant differences in adsorption among the evolved lines (Kruskal-Wallis $\chi^2=10.8778$ df=4 $p=0.0280$).  

C) HWPB-2 one step growth curves. There were no significant difference between the ancestral and evolved lines lysis times (Kruskal-Wallis $\chi^2=0$ df=1 $p=1$). There was a significant decrease in burst size when comparing ancestral and evolved lines (Kruskal-Wallis $\chi^2=7.3636$ df=1 $p=0.0067$). There were no significant differences in burst size among the evolved lines (Kruskal-Wallis $\chi^2=5.7018$ df=5 $p=0.3363$).  

D) HWNPB-3 one step growth curves. Evolved lines had significantly longer lysis times compared to the ancestral line (Kruskal-Wallis $\chi^2=4.6225$ df=1 $p=0.0316$) and, there were no significant differences among evolved lines lysis times (Kruskal-Wallis $\chi^2=5.5873$ df=4 $p=0.2322$). There was no significant difference between the ancestral and the evolved lines' burst sizes (Kruskal-Wallis $\chi^2=2.9509$ df=1 $p=0.0858$). There were significant differences in burst sizes among the evolved lines (Kruskal-Wallis $\chi^2=12.9333$ df=4 $p=0.0116$).
Figure 5: **In vitro fitness and in vivo survival for phages experimentally evolved in a structured environment.** White bars represent ancestral phages, while evolved lines are in grey. 

A) Experimental evolution in a structured environment culture significantly decreased the *in vitro* fitness of the evolved lines compared to HWPB-2 (Kruskal-Wallis $\chi^2=5.3434$ df=1 p=0.0208). There were significant differences in fitness among the evolved lines (Kruskal-Wallis $\chi^2=15.5263$ df=5 p=0.0083). In pairwise comparisons with the ancestral strain, after corrections for multiple comparisons, five of the six evolved lines (starred) had significantly decreased fitness.

B) Experimental evolution in a structured environment did not alter the *in vitro* fitness of the evolved lines compared to HWNPB-3 (Kruskal-Wallis $\chi^2=0.2842$ df=1 p=0.5940). There were no significant differences in fitness among the evolved lines (Kruskal-Wallis $\chi^2=8.6667$ df=4 p=0.0700).

C) Experimental evolution in a structured environment decreased the therapeutic efficacy of the evolved lines compared to HWPB-2 (Kaplan-Meier Survival Analysis $\chi^2=5.4128$ df=1 p=0.0200). Among the evolved lines there were no significant differences (Kaplan-Meier Survival Analysis $\chi^2=8.8454$ df=5 p=0.0817).

D) Experimental evolution in a structured environment significantly decreased the therapeutic efficacy of the evolved lines compared to HWNPNB-3 (Kaplan-Meier Survival Analysis $\chi^2=6.2062$ df=1 p=0.0127). There were significant differences among the evolved lines (Kaplan-Meier Survival Analysis $\chi^2=14.9288$ df=4 p=0.0049) and in pairwise comparisons with the ancestral strain after corrections for multiple comparisons, replicate populations one and five showed a significant decrease in therapeutic efficacy compared to the ancestral line (starred) (p<0.05).
<table>
<thead>
<tr>
<th></th>
<th>Structured</th>
<th>Non-structured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HWPB-2</td>
<td>HWPB-2</td>
</tr>
<tr>
<td></td>
<td>HWNPB-3</td>
<td>HWPB-2</td>
</tr>
<tr>
<td>Adsorption rate</td>
<td>-</td>
<td>-*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Lysis time</td>
<td>No change</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No change</td>
</tr>
<tr>
<td>Burst size</td>
<td>-</td>
<td>No change*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>In vitro fitness</td>
<td>-*</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+*</td>
</tr>
<tr>
<td>Therapeutic efficacy</td>
<td>-</td>
<td>-*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No change*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1: Summary table of effects of experimental evolution. *In vitro* fitness was measured within the non-structured liquid environment. (-) means a decrease compared the ancestral strain while (+) means an increase compared to the ancestral strain. Starred boxes indicate there was within line variation seen among the experimentally evolved lines.
<table>
<thead>
<tr>
<th>Phage</th>
<th>Adsorption Rate (cells$^{-1}$ ml$^{-1}$ min$^{-1}$)</th>
<th>Burst Size (phage per burst)</th>
<th>Lysis Time (min)</th>
<th>Fitness (hrs$^{-1}$)</th>
<th>Survival (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HWNPB-3</td>
<td>-6.33E-09 ± 1.53E-09</td>
<td>115.40 ± 24.56</td>
<td>46.67 ± 2.89</td>
<td>1.21 ± 0.12</td>
<td>27.52 ± 0.55</td>
</tr>
<tr>
<td>1P25</td>
<td>-2.88E-09 ± 2.12E-10</td>
<td>512.79 ± 154.97</td>
<td>50.00 ± 0.00</td>
<td>1.85 ± 0.07</td>
<td>31.44 ± 0.69</td>
</tr>
<tr>
<td>2P25</td>
<td>-2.68E-09 ± 2.33E-10</td>
<td>436.91 ± 144.85</td>
<td>50.00 ± 0.00</td>
<td>2.08 ± 0.11</td>
<td>31.65 ± 0.57</td>
</tr>
<tr>
<td>4P25</td>
<td>-2.67E-09 ± 1.82E-10</td>
<td>538.73 ± 129.69</td>
<td>50.00 ± 0.00</td>
<td>2.41 ± 0.07</td>
<td>32.32 ± 0.70</td>
</tr>
<tr>
<td>5P25</td>
<td>-2.53E-09 ± 3.20E-10</td>
<td>510.28 ± 128.05</td>
<td>43.33 ± 2.89</td>
<td>1.71 ± 0.11</td>
<td>30.32 ± 0.72</td>
</tr>
<tr>
<td>6P25</td>
<td>-2.73E-09 ± 4.28E-10</td>
<td>589.44 ± 138.86</td>
<td>50.00 ± 0.00</td>
<td>1.64 ± 0.14</td>
<td>30.16 ± 0.61</td>
</tr>
<tr>
<td>S1P25</td>
<td>-5.23E-10 ± 4.19E-11</td>
<td>50.71 ± 4.38</td>
<td>60.00 ± 0.00</td>
<td>2.08 ± 0.02</td>
<td>28.72 ± 0.52</td>
</tr>
<tr>
<td>S2P25</td>
<td>-1.37E-09 ± 1.27E-10</td>
<td>65.93 ± 7.68</td>
<td>56.67 ± 2.89</td>
<td>2.03 ± 0.04</td>
<td>30.65 ± 0.57</td>
</tr>
<tr>
<td>S3P25</td>
<td>-9.86E-10 ± 9.09E-11</td>
<td>107.91 ± 21.71</td>
<td>56.67 ± 2.89</td>
<td>1.77 ± 0.09</td>
<td>29.44 ± 0.56</td>
</tr>
<tr>
<td>S5P25</td>
<td>-4.52E-10 ± 8.93E-11</td>
<td>4.94 ± 0.58</td>
<td>60.00 ± 5.00</td>
<td>2.03 ± 0.02</td>
<td>28.59 ± 0.56</td>
</tr>
<tr>
<td>S6P25</td>
<td>-3.96E-10 ± 4.13E-11</td>
<td>33.76 ± 4.82</td>
<td>50.00 ± 0.00</td>
<td>1.97 ± 0.01</td>
<td>30.88 ± 0.60</td>
</tr>
</tbody>
</table>

Table 2: Life history traits for ancestral HWNPB-3 and evolved replicate populations. Phages labeled 1P25-6P25 were evolved in a liquid environment, while phages labeled S1P25-S6P25 were evolved in a structured environment.
<table>
<thead>
<tr>
<th>Phage</th>
<th>Adsorption Rate (cells⁻¹ ml⁻¹ min⁻¹)</th>
<th>Burst Size (phage per burst)</th>
<th>Lysis Time (min)</th>
<th>Fitness (hrs⁻¹)</th>
<th>Survival (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HWPB-2</td>
<td>-2.84E-09 ± 2.43E-10</td>
<td>182.10 ± 10.85</td>
<td>10.00 ± 0.00</td>
<td>4.84 ± 0.03</td>
<td>42.20 ± 1.03</td>
</tr>
<tr>
<td>1T25</td>
<td>-1.20E-09 ± 1.89E-10</td>
<td>54.05 ± 17.45</td>
<td>16.67 ± 2.89</td>
<td>5.14 ± 0.03</td>
<td>42.58 ± 1.08</td>
</tr>
<tr>
<td>2T25</td>
<td>-1.76E-09 ± 3.49E-10</td>
<td>56.87 ± 8.48</td>
<td>13.33 ± 2.89</td>
<td>5.09 ± 0.04</td>
<td>40.33 ± 1.00</td>
</tr>
<tr>
<td>3T25</td>
<td>-1.73E-09 ± 3.04E-10</td>
<td>82.62 ± 17.34</td>
<td>16.67 ± 2.89</td>
<td>5.12 ± 0.04</td>
<td>44.19 ± 1.06</td>
</tr>
<tr>
<td>4T25</td>
<td>-1.59E-09 ± 3.13E-10</td>
<td>69.05 ± 7.45</td>
<td>10.00 ± 5.00</td>
<td>5.11 ± 0.02</td>
<td>40.11 ± 1.03</td>
</tr>
<tr>
<td>5T25</td>
<td>-1.78E-09 ± 5.36E-10</td>
<td>68.50 ± 12.19</td>
<td>13.33 ± 2.89</td>
<td>5.12 ± 0.02</td>
<td>40.83 ± 1.02</td>
</tr>
<tr>
<td>6T25</td>
<td>-1.55E-09 ± 3.27E-10</td>
<td>78.83 ± 8.35</td>
<td>10.00 ± 0.00</td>
<td>5.08 ± 0.03</td>
<td>42.25 ± 1.09</td>
</tr>
<tr>
<td>S1T25</td>
<td>-9.94E-11 ± 1.93E-11</td>
<td>11.98 ± 4.56</td>
<td>13.33 ± 2.89</td>
<td>4.43 ± 0.08</td>
<td>43.92 ± 1.00</td>
</tr>
<tr>
<td>S2T25</td>
<td>-1.13E-10 ± 1.27E-11</td>
<td>26.01 ± 7.13</td>
<td>10.00 ± 5.00</td>
<td>4.52 ± 0.01</td>
<td>46.75 ± 1.04</td>
</tr>
<tr>
<td>S3T25</td>
<td>-1.47E-10 ± 5.63E-11</td>
<td>19.65 ± 6.98</td>
<td>10.00 ± 5.00</td>
<td>4.59 ± 0.01</td>
<td>42.75 ± 1.10</td>
</tr>
<tr>
<td>S4T25</td>
<td>-1.31E-10 ± 7.32E-11</td>
<td>13.53 ± 3.03</td>
<td>10.00 ± 0.00</td>
<td>4.84 ± 0.04</td>
<td>43.42 ± 1.10</td>
</tr>
<tr>
<td>S5T25</td>
<td>-1.09E-10 ± 2.02E-11</td>
<td>15.14 ± 4.73</td>
<td>10.00 ± 0.00</td>
<td>4.45 ± 0.03</td>
<td>43.48 ± 0.93</td>
</tr>
<tr>
<td>S6T25</td>
<td>-2.02E-10 ± 5.65E-11</td>
<td>9.02 ± 1.43</td>
<td>6.67 ± 2.89</td>
<td>4.70 ± 0.01</td>
<td>44.88 ± 1.11</td>
</tr>
</tbody>
</table>

Table 3: Life history traits for ancestral HWPB-2 and evolved replicate populations. Phages labeled 1T25-6T25 were evolved in a liquid environment, while phages labeled S1T25-S6T25 were evolved in a structured environment.
Chapter 4: The enemy of my enemy is my friend: *Pseudomonas aeruginosa* selected for resistance to lytic bacteriophage are less virulent to their *Drosophila* host.

Authors: Heather Lindberg\(^1\), Jason Behnke\(^2\), Nathaniel Cady\(^2\), Ing-Nang Wang\(^1\) and Kurt A. McKean\(^1\)

Institutions: \(^1\) State University of New York at Albany, Biological Sciences

\(^2\) State University of New York at Albany, College of Nanoscale Science & Engineering

*Biofilm assay was performed by Jason Behnke. All other data presented in this chapter were collected by Heather Lindberg and Kurt McKean*
Abstract

In nature, pathogen fitness is determined not only by interactions with its primary host, but also by interactions with competitors and hyperparasites. Adaptation in response to these diverse selective pressures may be constrained by the pleiotropic effects of traits, which improves performance in one setting while reducing performance in another. Such trade-offs may be important in determining the precise nature of the interaction between a pathogen and its primary host, including patterns of pathogen virulence. To study how hyperparasites can affect pathogen virulence we selected for populations of *Pseudomonas aeruginosa* which were resistant to a hyperparasite, a type IV pilin-binding lytic bacteriophage (phage). Populations quickly evolved phage resistance and the likely mechanism of resistance was the selective loss of type IV pili. In infections established in a *Drosophila melanogaster* host, phage-resistant lines showed reduced virulence and exhibited reduced within-host population growth. The loss of type IV pili also lead to the reduction of biofilm formation and loss of twitching motility, both of which are known *P. aeruginosa* virulence factors. In addition, resistant strains showed reduced fitness in nutrient poor, but not nutrient rich media. Overall our results indicate that pathogen adaptation in response to selection by a natural enemy, a lytic phage, affects virulence in its primary host. Furthermore, the observed trade-offs may be understood through the specific pleiotropic effects of the evolved mechanism of defense. Understanding how hyper-parasitism can alter pathogen virulence is important for gaining a better understanding of not only host pathogen interactions but also the evolution of pathogen virulence. Furthermore, understanding how hyperparasites alter pathogen virulence shows their potential use as biological control agents.
**Introduction**

In their struggle for existence organisms continuously face challenges by multiple natural enemies in the form of competitors, predators and parasites. The response to selection by multiple enemies is made complex if adaptation to one natural enemy is not independent of adaptation to another. Improved defense against one natural enemy could often have a correlated effect of improving defense against another. For example, improved running speed in response to a selection by a specific predator would likely improve survival when faced with other predators as well. Alternatively, improved defense against one natural enemy may come at a cost of reduced defense against another. For example in the waterflea *Daphnia magna*, reductions in negative phototactic behavior reduces the risk of parasitism *Pasteuria ramosa* but increases the risk of predation by visually hunting predators who patrol higher in the water column (Decaestecker, De Meester, and Ebert 2002). Such tradeoffs in defense could constrain adaptive evolution with selection favoring genotypes providing an advantageous compromise between the competing needs of the organism with respect to fitness.

Pathogens are an important class of natural enemy and the costs of pathogen defense are well studied in plants and animals (McKean and Lazzaro 2011; Schmid-Hempel 2005; Rolff and Siva-Jothy 2003; Martin, Hawley, and Ardia 2011; Schulenburg et al. 2009; Lazzaro and Little 2009). The presence of such costs can constrain the evolution of improved defense, contribute to the maintenance of disease susceptibility in populations and affect the appearance of pathogen virulence. Pathogens themselves face trade-offs affecting the evolution of their virulence. The classic trade-off model (Anderson and May 1982; Ewald 1983) proposes that virulence evolution is guided by a
trade-off with transmission (Alizon et al. 2009). Virulence evolution is complicated, however, when pathogens evolve in ecological settings involving natural enemies beyond a single host (and its immune defenses). In multiple infections, for example, virulence is predicted to increase or decrease depending upon the nature of the competitive interaction between strains. For pathogens with multi-host life cycles, adaptation to one host often comes at a cost of reduced performance in the other, as revealed by serial passage experiments (Ebert 1998). Lastly, pathogens themselves could face their own pathogen pressure in the form of hyperparasitism.

Hyperparasites are parasites of parasites that are on their own incapable of infecting the primary host. Hyperparasites may act directly or indirectly to limit the effect of the pathogen on the primary host population. Direct effects of hyperparasites reducing pathogen virulence occur when infected pathogens have reduced growth rates (Hochberg 1989; Taylor et al. 1998). For example, North American chestnut tree (Castanea dentata) populations were devastated by the fungal pathogen Cryphonectria parasitica after its introduction from Asia in the 1800’s (Anagnostakis 1987). Chestnut blight had much less of an impact on European chestnuts (Castanea sativa) primarily because of the direct effect of infection by a hyperparasite, Cryphonectria, (a dsRNA hypovirus) on C. parasitica (Anagnostakis 1987, 1982; Van Alfen et al. 1975). These direct effects of hyperparasites are the basis of biological control efforts, including the control of insect pests (Hajek 2004), the control of pathogenic bacteria in the context of bacteriophage therapy (Summers 2001) or the use of mycoviruses similar to C. parasitica in the control of fungal pathogens of plants (Nuss 2005).
Hyperparasites could indirectly affect pathogen virulence if a correlated response to the evolution of resistance to the hyperparasite is a change in the interaction of the pathogen with its host. While the correlated response could be positive or negative, the expectation is of a trade off given that resistance mechanisms often come at some cost (McKean and Lazzaro 2011; Schmid-Hempel 2005; Rolff and Siva-Jothy 2003; Martin, Hawley, and Ardia 2011; Schulenburg et al. 2009; Lazzaro and Little 2009; Bohannan et al. 2002). Costs of resistance are manifest in the absence of the hyperparasite and could therefore affect the pathogen host interaction even in the absence of direct control.

Lytic bacteriophages (phage) are viral parasites of bacteria and important regulators of bacterial population growth. In response to this selection pressure, bacterial populations may evolve resistance to the phage in the form of restriction enzymes, CRISPR-mediated immunity, or through the selective loss of cell surface receptors used by the phage for adsorption to the host cell. The costs of resistance to phage have been studied in a number of ecological contexts (Bohannan et al. 2002), including the within host environment, where phage act as hyperparasites of pathogenic bacteria. These studies found that phage-resistant bacteria were less virulent than phage-susceptible bacteria (Flyg, Kenne, and Boman 1980; Heierson et al. 1986; Laanto et al. 2012).

As with other trade-offs, antagonistic pleiotropy is the primary genetic mechanism promoting the correlated response to selection. It is often the case, however, that the pleiotropic nature of alleles contributing to observed trade-offs is left unexplored. Understanding how the costs of resistance arise is important for understanding how interactions with hyperparasites may affect pathogen virulence, in predicting evolutionary
outcomes and, from a more practical point of view, as essential information needed to better design biological control measures such as phage therapy.

Here we report results from experiments evaluating how the evolution of resistance to hyperparasitic lytic bacteriophage affects an opportunistic pathogen, *Pseudomonas aeruginosa*, during infections in a *Drosophila melanogaster* host. We first test whether reduction in within-host growth and virulence follows the evolution of resistance to the hyperparasite in replicate populations of *Pseudomonas aeruginosa*. Second, we test whether the reduced virulence is associated with changes in known virulence mechanisms.

**Materials and Methods**

**Phage, bacterial strains and flies**

The phage used in the experiments was isolated by H. Wilson from a sewer water sample collected at a waste water treatment plant in Menands NY and is referred to as HWPB-1. Details of the phage characterization can be found in Chapter 1. Briefly, HWPB-1 is a *Siphoviridae* class phage, with sequence homology to M6, MP1412 (Bae et al. 2012) and YuA phages (Ceyssens et al. 2008). HWPB-1 uses the type IV pilus for adsorption to the *P. aeruginosa* host cell.

The bacterial strains used for this study were MPAO1 and PA4525 (*a pilA* transposon mutant lacking the type IV pilus) acquired from the University of Washington mutant transposon library (Jacobs et al. 2003).
Flies used in the experiment were from a laboratory population established in 2008 with flies caught at Indian Ladder farms in Voorheesville NY. The population was maintained as a large outbred population kept at 25°C with a 12:12 light cycle.

**Experimental evolution of phage-resistant *Pseudomonas aeruginosa***.

Experimental evolution of phage resistance was carried out in 16 replicate populations by serial passage of phage-susceptible MPAO1 populations in the presence of HWPB-1. Initial cultures of $10^8$ cfu MPAO1 suspended in 100μl of LB broth and 100μl of a $10^9$ pfu ml$^{-1}$ were added to 2 ml of LB broth and allowed to grow for 24 hours in a shaking incubator at 30°C. Thereafter, 100μl of the MPAO1/phage culture was serially transferred to 2 ml of fresh LB broth every 24 hours. The presence of phage in the culture was checked after every other transfer. In addition to checking for the presence of phage, the bacterial population was also checked for phage resistance every other transfer. The cultures were checked for resistance by addition of 100 μl of $10^9$ pfu ml$^{-1}$ of the original HWPB-1 stock solution to a spread plate of the bacteria. Populations resistant to HWPB-1 were then streak-plated and a single colony chosen from each of the 16 replicate populations, again checked for phage resistance and stored in 10 % glycerol at -80°C until their use in further experiments.

**Characterization of phage-resistant bacteria**

**Virulence and within-host fitness.** To examine the hypothesis that a cost of phage resistance is a reduction in within host fitness and virulence, we established experimental infections in a *D. melanogaster* host. We compared the within-host growth rates (fitness) and the time until death following infection (virulence) of phage-resistant bacteria to
ancestral phage-susceptible MPAO1. Infections were established in adult females collected from vials maintained at a low larval density by placing 10 males and 10 females in a vial for 24 hours. Infections were carried out by piercing the thorax with a 0.1 mm diameter minutien pin (Fine Science Tools, Foster City, CA), which had been dipped into a culture containing one of 16 phage-resistant strains, or with ancestral phage-susceptible MPAO1. Bacterial cultures were grown to log-phase, diluted to a common optical density (average across experiments, OD$_{610} = 0.331 ± 0.029$) and then diluted one hundred fold prior to infection. On each of 4 separate days of infection, 50 females, housed 10 per vial, were injected with each strain. Virulence was assayed in four of these vials (on each day, for a total of 160 flies) by recording the time of death every 4 hours for 2 days. Flies in the fifth vial were assayed for bacterial counts 18 hours after infection. In these assays three sets of two females from each vial were placed in a sterile 1.5 ml centrifuge tube containing 500 μl of sterile LB, and homogenized. The homogenate was then diluted ten-fold and 50 μl plated on LB agar plates using an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda, MD). These plates were incubated overnight at 30°C and the number of colony forming units (cfu) counted using the Q-count detection system (Spiral Biotech, Bethesda, MD). A total of 24 plates were plated for each of the 17 bacterial strains (16 phage resistant plus ancestral MPAO1).

**Bacterial growth in media.** To determine if bacterial fitness in non-host environments was affected by the evolution of phage resistance, we estimated growth rates of evolved phage-resistant lines and ancestral phage-susceptible MPAO1 in both nutrient rich LB media as well as nutrient-limiting minimal media supplemented with 0.04 g/ml glucose. In these assays, log-phase cells (MPAO1 or the sixteen phage resistant mutants) were
diluted to a common optical density (OD$_{610} = 0.10 \pm 0.01$) and 1 ml of the culture spun down and resuspended in 1 ml of M9 minimal media. The resuspended culture was then diluted 1/100, and 100μl placed in a well of a 24 well Falcon plate containing 1.9 ml of either LB broth or minimal media. A cfu count was immediately taken using the Autoplate 4000 as described above. The plates were then placed in a 30°C shaking incubator and cfu again estimated after 6 hours of bacterial growth. Fitness was calculated as the malthusian parameter, $m_i = \ln[N_i(6)/N_i(0)]/6$, where $m_i$ is the absolute fitness of strain $i$, and $N_i$ is the cfu estimate of strain $i$ at time 0 or at 6 hours after inoculation. The fitness of each strain was estimated in 6 independent replicates in each environment.

**Twitching motility and biofilm assays.** Because HWPB-1 shows sequence similarity to other pilin-binding phage and is unable to establish an infection in unpiliated pilA mutants we sought to determine if the evolution of phage resistance affected twitching motility and biofilm formation. Loss of the type IV pili (the most common mechanisms of resistance to pilin-binding phage) leads to complete loss of twitching motility and a reduction in biofilm formation, both traits known to affect virulence during infections in *D. melanogaster* and in other hosts as well (Comolli et al. 1999; Potvin et al. 2003; O'Toole and Kolter 1998; Cady et al. 2012).

Twitching motility was assayed in three independent replicates in the 16 phage-resistant strains, the ancestral phage-susceptible MPA01 as well as pilA transposon mutant PA4525 by stab inoculation of log-phase cells diluted to a common optical density (OD$_{610} = 0.074 \pm 0.003$) into 1% agar LB plates. These plates were then incubated overnight and the next day inspected for the faint halo at the interstitial surface
between the agar and the plate that is indicative of active twitching motility (Semmler, Whitchurch, and Mattick 1999).

The ability of the strains to form a biofilm was assayed by comparing the amount of cells adhered to the well walls of a 96 well plate over a 24 hour period using a technique modified from (Cady et al. 2012). In the assay, overnight cultures were rinsed with phosphate buffered saline (PBS) and re-suspended in M9 minimal media to a target of OD$_{600}$= 0.050. Each well of a 96-well plate (Corning clear bottomed polystyrene plates) was filled to a final volume of 100 µl and the OD$_{600}$ measured using a Tecan plate reader (Infinite M200) prior to incubating. After a 24 hour incubation period another OD$_{600}$ measurement was taken to determine planktonic cell growth. The media was then gently removed and the wells were washed twice with 150 µl of PBS using a multichannel pipette leaving the biofilm attached to the well of the plate. The remaining biofilm was then stained using 100 µl of a 0.2 weight % crystal violet solution for 15 minutes at room temperature. The crystal violet was then removed from the wells, rinsed 4 times, and then re-suspended using 95% ethanol. The plate was then read at an OD$_{600}$ to determine biofilm formation.

**Data Analyses**

All statistical analyses were performed using JMP 10 software. The significance of bacterial strain on virulence was analyzed using a Cox proportional hazards model while the significance of strain on within-host growth, *in vitro* fitness and biofilm formation were analyzed using a standard least squares model. Correlations were done using a bivariant analysis. Given the multiple comparisons done in each assay, all
pairwise comparisons with the ancestral strain were corrected for multiple comparisons using a Bonferroni correction (Rice 1989).

**Results**

**Phage resistant mutants lose twitching motility.** As expected if the mechanism of resistance to HWPB-1 was the selective loss of the type IV pili, all of the phage resistant lines completely lost their twitching motility (Figure 1).

**Phage resistant *P. aeruginosa* are less virulent than the ancestral phage susceptible *P. aeruginosa.*** As a class, phage-resistant *P. aeruginosa* were significantly less virulent in *D. melanogaster* than their phage-susceptible ancestor (Proportional Hazards model $\chi^2 = 9.84$, df=1, $p=0.0017$; Figure 2 A). There was significant variation in virulence among phage resistant lines (Proportional Hazards model $\chi^2 = 88.6718$, df=15, $p<0.0001$). The virulence of phage-resistant lines was always less than ancestral MPAO1; although only 8 of the 16 pairwise comparisons were statistically significant.

In addition to being less virulent, as a class the phage-resistant lines showed slower within-host population growth than the ancestral phage-susceptible line (ANOVA: $F_{1,425}=4.92$, $p=0.027$). Again, there was significant variation among the mutant strains (ANOVA: $F_{15,105}=1.7326$, $p=0.0437$). All but one of the phage resistant strains showed a reduction in within-host growth although in pairwise comparisons this was statistically significant in only four comparisons (Figure 2B). Lastly, we found a significant negative correlation between virulence and the within host growth rate ($r=-0.545$ $p=0.024$; Figure 2C) as expected if population the size of the bacterial population is a primary determinant of fly mortality.
**Phage resistant P. aeruginosa have reduced biofilm production.** As a class, phage-resistant bacterial strains had a reduction in their ability to produce biofilm when compared to the ancestral MPA01 (ANOVA $F_{1,78} = 23.435 \ p<0.0001$). There was also significant variation among the mutant strains (ANOVA $F_{15,105} = 4.6541 \ p<0.0001$) All of the phage resistant strains showed a decrease in the ability to form biofilm, although in pairwise comparisons, this was only significant in eleven comparisons (Figure 3)

**Phage resistant P. aeruginosa have reduced fitness in minimal media.** The *in vitro* fitness of each of the bacterial lines was assessed in both nutrient rich LB broth as well as minimal media. The phage-resistant lines had similar growth rates to the ancestral phage susceptible strain in LB broth (ANOVA $F_{1,110} = 1.026 \ p=0.313$). In addition, there was no significant variation seen among the resistant strains (ANOVA $F_{15,105} = 1.0318 \ p=0.4293$). While all but two strains showed lower absolute fitness than the ancestral strain, it was only a significant decrease in one strain. In minimal media, the phage resistant strains as a class show a significantly decreased fitness compared to the ancestral strain (ANOVA $F_{1,84} = 4.748 \ p=0.032$). There was no significant variation seen within the phage resistant strains (ANOVA $F_{15,79} = 1.1021 \ p=0.3685$). While all but one strain has lower absolute fitness compared to the ancestral strain, only six of the pairwise comparisons are significant.

**Discussion**

Here we have shown that the evolution of resistance to hyperparasitic bacteriophage is not independent of the bacterial pathogen’s interaction with its host, but instead shows evidence of a trade off. Reduced within-host fitness and reduced virulence
were correlated responses to the evolution of resistance to pilin-binding bacteriophage HWPB-1 in the opportunistic pathogen *Pseudomonas aeruginosa* (Figure 2). Among the phage-resistant lines there was significant variation in the extent of the experienced cost of resistance, as revealed by both the reduced within-host fitness and observed decline in virulence (Figure 2A and B). It appears that much of the decline in virulence is due to the reduced within-host population growth, as revealed by a significant negative correlation between these two traits.

The observation of among-line variation in the cost of resistance begs the question of whether cost-free resistance to hyperparasitic phage could evolve. All but one of the lines had lower average within host growth than phage-susceptible MPAO1 (Figure 2B). Line ‘N’ had slightly higher within host growth than MPAO1 (N = 12.57, vs. MPAO1 = 12.46) however this difference was not statistically significant. Furthermore, line N did have lower virulence in the fly (N vs. MPAO1 $\chi^2=4.4605$ df=1 p=0.0347) suggesting that the slightly higher observed average within host growth may have been artifactual. A further 10 lines did not show a significant deviation in virulence from MPAO1 in pairwise comparisons even though their average within host growth was observed to be lower. It is possible that the continued evolution in lines showing the greatest cost of resistance would show an eventual allelic replacement by less costly resistance mutations. Furthermore, previous studies looking at costs of resistance to bacteriophage have found the evolution of compensatory mutations can evolve to mitigate such costs (Lenski 1988).

The evolution of resistance to phage using the type IV pili for adsorption to the host cell can occur through selective loss of the pili or through hyperpiliation (Brockhurst, Buckling, and Rainey 2005; Deziel, Comeau, and Villemur 2001; Haussler
et al. 2003). Loss of type IV pili results in a complete lack of twitching motility (Semmler, Whitchurch, and Mattick 1999), which is also reduced, but not eliminated, in hyperpiliated P. aeruginosa (Deziel, Comeau, and Villemur 2001). The complete lack of twitching motility observed in all 16 of the evolved phage-resistant lines (Figure 1) suggests that the mechanism of resistance that evolved was the selective loss of the type IV pilus. Furthermore, a round colony morphology was also observed in all of the 16 phage-resistant lines, again consistent with the loss of the pilus, and not hyperpiliation, which results in rough irregular colony formation (Brockhurst, Buckling, and Rainey 2005; Deziel, Comeau, and Villemur 2001; Haussler et al. 2003).

Previous studies have found that loss of the type IV pili results in decreased virulence in flies (Potvin et al. 2003; D'Argenio et al. 2001b) as well as other hosts. Antagonistic pleiotropy is the primary genetic mechanism mediating trade offs (Roff 2002) and loss of the type IV pili has a number of known pleiotropic effects on traits likely to affect the interaction of the pathogen with its host. The type IV pili play an important role in the initial adherence and colonization of the host (Hahn 1997). Motility itself may be important during infections by allowing bacterial cells to move into protected niches within the host or escape immune responses (Drake and Montie 1988; Josenhans and Suerbaum 2002; Krukonis and DiRita 2003; Chiang and Burrows 2003) although motility may also come at a cost, increasing the likelihood of contact with phagocytes (Tomita and Kanegasaki 1982). Consistent with the role of twitching motility itself being important, experimental evolution of increased twitching motility had a correlated response of increasing virulence in infections in waxmoth larvae, Galleria mellonella (Taylor and Buckling 2011), while twitching motility defective mutants had
an impaired ability to translocate across the corneal epithelium in an eye infection model in rabbit corneal epithelial tissue (Alarcon, Evans, and Fleiszig 2009).

Lastly, selective loss of the type IV pili is known to affect the expression of other virulence mechanisms and in particular, biofilm formation (O'Toole and Kolter 1998; Klausen et al. 2003). Biofilms are matrix-enclosed aggregates of microbial cells (Hall-Stoodley, Costerton, and Stoodley 2004) and often play an important role during infections because of their relative imperviousness to antibiotics and host immune defenses (Costerton, Stewart, and Greenberg 1999; Davies 2003). A number of previous studies found that twitching motility defective mutants show a reduced ability to form a biofilm (O'Toole and Kolter 1998; Deziel, Comeau, and Villemur 2001; Klausen et al. 2003). Consistent with these results, we found that evolved phage-resistant lines had reduced ability to form biofilm (Figure 3). We have recently found that organosulfur compounds acting to reduce biofilm formation reduce the within-host growth and virulence of *P. aeruginosa* when used to treat systemic infections (Cady et al. 2012), which suggests that the decreased biofilm formation in our evolved lines may play an important role in the observed decrease in virulence. Interestingly, in an oral infection model in *D. melanogaster*, mutants completely lacking the ability to form a biofilm had increased virulence while a hyperbiofilm strain had decreased virulence compared to wild-type PAO1 (Mulcahy et al. 2011), suggesting either that biofilm formation has different effects on the infection process depending on the site of infection or that there may be an optimal level of biofilm formation. However, while evolved phage-resistant lines did show reduced biofilm formation and there was significant among-line variation in this decline, the correlations of trait values across lines with within host fitness and

97
virulence were not significant (within host fitness: $r = 0.2983$, $p = 0.2444$; virulence: $r = 0.3046$, $p = 0.2343$).

Loss of cell surface receptors in response to selection by bacteriophage can also affect the ability of cells to acquire nutrients (Bohannan et al. 2002; Jessup and Bohannan 2008). The type IV pilus has no known function in the direct uptake of nutrients; however the loss of the pilus could impair nutrient uptake if there are substantial changes in the structure and formation of the bacterial cell wall or if the lack of motility limits nutrient acquisition. If this is the case, then in environments where nutrients are in very limited supply we may expect trade-offs to be more apparent. Consistent with this hypothesis, we found that evolved phage-resistant lines had decreased fitness in nutrient poor, but not nutrient rich, media when compared to ancestral phage-susceptible line. To the extent that the within host environment is nutrient poor with respect to the needs of the growing population of *P. aeruginosa*, this could also go to explain the reduced virulence. However, like for biofilm formation, there was not a significant correlation between fitness in the either nutrient rich or nutrient poor media and virulence of within-host growth (nutrient rich: $r = 0.111$, $p = 0.62558$, nutrient poor: $r = 0.6204$, $p = 0.8130$).

The other genetic mechanism promoting the observation of trade-offs is linkage disequilibrium (Roff 2002). In the context of the experiments described here, if resistance mutations that eventually fixed in each population occurred on a genetic background containing a deleterious allele or alleles, then the hitch-hiking of these alleles would result in the correlated response to selection in other traits (Kawecki 1994; Buckling et al. 2007; Rose, Nusbaum, and Chippendale 1996). While we cannot completely eliminate this hypothesis, it is unlikely to explain our results for 3 reasons. First, in the nutrient rich
environment there was no apparent cost of resistance which would be expected if hitchhiking alleles had general effects on depressing fitness. Second, there was no correlation across the different measures of performance which would be expected if there was the fixation of deleterious alleles with more general effects on fitness. Lastly, our results are broadly consistent with transposon mutagenesis studies of type IV pili in *P. aeruginosa*, suggesting that it is the pleiotropic effect of losing type IV pili that leads to the observed trade-offs in our lines and not an effect of the hitchhiking of deleterious mutations in the genetic background.

Organisms in nature face a multitude of threats from a variety of natural enemies and this study adds to growing literature describing trade-offs in defense against different natural enemies (Decaestecker, De Meester, and Ebert 2002; Rigby and Jokela 2000; Yin et al. 2011; Poitrineau, Brown, and Hochberg 2003). Parasites and pathogens face multiple natural enemies in the form of competitors, predators, hyperparasites, and multiple hosts. Trade-offs in defense against different natural enemies have been reported for pathogens (Alizon et al. 2009; Taylor et al. 1998). Very often these trade-offs cause deviations in the levels of virulence predicted from the trade-off model (Alizon et al. 2009). As an ecological interaction, hyper-parasitism has been neglected relative to the frequency of its occurrence in nature (Taylor et al. 1998; Morris and Freeman 2010, Levin and Bull 2004; Levin and Bull 1996; Beddington and Hammond 1977). The direct and indirect consequences of the interaction with these natural enemies is therefore important for gaining a better understanding of the pathogen host interaction, the evolution of pathogen virulence as well as practical uses in the use of hyperparasites in biological control efforts (Hochberg 1989; Hajek 2004; Levin and Bull 2004).
Figure 1. Twitching motility assay. On 1% agar plate, each of the 16 mutants, MPAO1 and a type IV pili knockout mutant were plated (pilA). MPAO1 is the only culture exhibiting twitching motility (labeled as PAO1 in picture).
A) LT 50 (hrs) ± s.e.

MPAO1 vs. phage resistant p=0.0017

B) ln(bacterial load) ± s.e.

MPAO1 vs. phage resistant p=0.027
Figure 2: Outcome of infection with either phage resistant bacteria or MPAO1. A) LT$_{50}$ after infection (hours). Mortality was assessed every four hours for 48 hours. Flies alive after 48 hours were censored for analysis. Data was analyzed using a Cox’s proportional hazards model. Flies injected with phage-resistant bacteria died significantly slower when compared to flies injected with MPAO1 ($\chi^2 = 9.84$, df=1, $p=0.0017$). B) Bacterial load recovered from flies 18 hours after infection. Phage-resistant bacteria grew slower within flies when compared to MPAO1 (ANOVA $F_{1,425}=4.92$, $p=0.027$). In figures A and B, MPAO1 is in black, light grey bars were $p<0.05$ in pairwise comparisons with MPAO1, and dark grey bars are $p<0.05$ in pairwise comparisons with MPAO1 after corrections for multiple comparisons. C) Correlation of LT$_{50}$ and bacterial load. The LT$_{50}$ of the phage resistant mutants is negatively correlated with the within host growth rate ($r=-0.545$, $p=0.024$). The ancestral MPAO1 is circled.
Figure 3: Biofilm production of MPAO1 and phage resistant *Pseudomonas aeruginosa*. Phage resistant mutants have decreased biofilm production compared to the ancestral MPAO1 strain (ANOVA $F_{1,78} = 23.435$ $p<0.0001$). The ancestral phage-susceptible MPAO1 is in black. Dark grey bars, $p < 0.05$ after correcting for multiple comparisons.
Figure 4: *In vitro fitness of phage resistant mutants and MPAO1*. Malthusian fitness of each bacterial strain was assessed using the initial bacterial concentration and the concentration after 6 hours of growth. A) Malthusian Fitness in nutrient rich LB media. Test of phage resistant *P. aeruginosa* vs MPA01 (ANOVA $F_{1,110} = 1.026$ $p=0.313$) B) Malthusian Fitness in minimal media. Test of phage resistant *P. aeruginosa* vs MPA01 (ANOVA $F_{1,84} = 4.748$ $p=0.032$) In both figures MPA01 is black, light grey phage resistant lines $p<0.05$ in pairwise comparisons and dark grey bars $p<0.05$ after corrections for multiple comparisons.
Conclusion

The renewed interest in phage therapy over the last thirty years has greatly increased both the knowledge of basic phage biology as well as bacteria/phage interactions. Much of the phage therapy research that has been done has followed a single model: researchers obtain one or two newly isolated phages and test these phages for their ability to treat various bacterial infections using an animal host, generally the mouse model system (Atterbury et al. 2007b; McVay, Velasquez, and Fralick 2007; Watanabe et al. 2007; Wang et al. 2006; Chibani-Chennoufi et al. 2004; Biswas et al. 2002; Barrow, Lovell, and Berchieri 1998; Soothill 1992; Smith and Huggins 1982). My research aimed to take a slightly different, but more systematic, approach by utilizing six different phages in an attempt to find a way to easily and quickly discern phage therapeutic efficacy \textit{in vitro}. In addition, my research utilized an invertebrate model system, \textit{Drosophila melanogaster}, rather than the typical mouse model. By using \textit{D. melanogaster}, not only could I use a larger sample sizes for each assay, but I could also observe the bacteria/phage interaction within a living host that had an innate immune response. The results found within this system can then inform decisions about which phages may be of high therapeutic value and warrant more testing in the more complex mouse model system which contains both the innate and acquired immune systems.

Overall, this research shows the validity of using \textit{D. melanogaster} as a model system to study different aspects of the phage/bacteria interaction and phage therapy. Within our model system, not only was the \textit{P. aeruginosa} infection highly lethal, but it was also an acute infection, killing untreated flies within 24-30 hours. All six of our isolated phages were able to significantly extend the life span of the infected flies,
although there was significant variation among the therapeutic efficacies. Overall, the acute nature of the *P. aeruginosa* infection made delaying phage treatment to six hours post infection a late treatment model of phage therapy. By six hours post infection flies had already been infected for approximately one fourth of the typical survival time with a *P. aeruginosa* infection, given death without treatment occurs within 24-30 hours.

Studying how the bacteria and phage interacted within a host system after the infection is fully established allows the results to be viewed in a more realistic clinical light than if the flies had been treated with phage prior to the bacteria infection becoming established within the host.

In attempting to predict how well phages will be able to treat a *P. aeruginosa* infected *D. melanogaster* by assessing specific life history traits characterized *in vitro*, we were moderately successful. While we were not able to identify a specific life history trait that was predictive of phage therapeutic efficacy, the combination of those life history traits, as seen by the *in vitro* fitness assay, was predictive of the phages’ therapeutic efficacy. This result is similar to that by Bull *et al.* (2010) where they found *in vitro* fitness of phages in serum was predictive of therapeutic efficacy in mice. Finding similar results in two different media suggests that relationships between specific phage traits and *in vivo* efficacy may be dependent on the model system used. Another possible explanation for the difference is that there has not been enough data collected regarding correlations between phage traits and *in vivo* efficacy to determine which medium to use *in vitro* to obtain the best correlation.

When looking at the *in vivo* dynamics of two of the six phages (HWPB-2 and HWNPB-3), it appears the rapid proliferation of the phages early in treatment may be key
to the effectiveness of the phage treatment. Despite eventually attaining similar phage levels within the flies and having similar bacterial loads throughout infection, the flies treated with HWPB-2 had much higher phage titers early in infection. These higher titers early in infection may have led to a decrease in the overall bacterial load seen by the flies throughout the course of infection. This decrease may have led to a decrease in bacterial debris and toxins released by the bacteria, which may have aided the flies in being able to survive the infection longer.

Our attempt to increase the therapeutic efficacy of the phages through modification of the life history traits was successful in that we were able to significantly modify both the phage life history traits, and the phages’ overall *in vitro* fitness. Given the correlations seen between *in vitro* fitness and therapeutic efficacy, we expected the phage lines that were serially transferred in liquid culture and showed higher *in vitro* fitness to have higher therapeutic efficacy in *D. melanogaster*. While we obtained the expected result with the HWNPB-3 evolved lines, the decrease in therapeutic efficacy we obtained from the HWPB-2 lines was unexpected. This result is most likely due to a therapeutic threshold for HWPB-2 within *D. melanogaster*. This threshold is most evident in the MOI study of the two ancestral phages, where increasing the MOI of the phage above 0.1 did not increase the therapeutic efficacy of the treatment any further. This type of therapeutic threshold has been seen previously in mice by Wang *et al.* (2006), and is likely caused by either a buildup of toxins, or the bacteria becoming refractory to the phage, either through the formation of biofilm, or by the evolution of phage resistant bacteria.
While we did see the evolution of phage resistance in several of the assays, the resistant population was always a subpopulation of the total population seen within the flies. We only observed the evolution of resistant bacteria for HWPB-2, and not for HWNPB-3 within *D. melanogaster*. This difference of evolution within the fly may be due to the variable cost of resistance between the two phage types. In fact, we see variable costs of resistance within phage resistant bacterial strains which are all resistant to the same pilin binding phage (HWPB-1). Resistance to the phage decreased the bacteria’s ability to form biofilm, presumably due to the loss of the type IV pilus, which is utilized by the bacteria for biofilm formation as well as the receptor utilize by the phage for entry into the cell (Mattick 2002). This loss of the receptor also caused a decreased growth rate within the fly, which resulted in a reduced virulence within the fly as well. A similar reduction in growth rate was found when the bacteria were tested in M9 minimal media, but not in nutrient rich LB broth, which may be due to a modification of the cell wall in response to the loss of the type IV pilus.

Overall, this research increases the knowledge of basic bacteria/phage interactions within a host organism. We found that not only can phage *in vitro* fitness be predictive of therapeutic efficacy in *D. melanogaster*, but also that through very simple serial transfer techniques; we can alter phage life history traits and ultimately, phage therapeutic efficacy. Being able to predict therapeutic efficacy can decrease the time it takes to preliminarily identify a potential therapeutic phage. The serial transfer techniques potentially increase the therapeutic efficacy of phages which have already been identified as viable therapeutic options, either through *in vivo* testing or through genomic analysis, thereby increasing the potential uses for those phages. In addition, we found that the cost
of being phage resistant can be highly variable, even within bacterial resistance to a single phage. Not only is the cost of resistance, as measured by within host growth and virulence, variable within the *D. melanogaster* environment, but that cost can be linked to a reduction in biofilm formation and *in vitro* growth within a nutrient poor environment as well. Understanding both the cost of phage resistance as well as the variability in that cost of resistance is important in potentially using phage for the biological control of bacteria.
 References


