Eco-immunology of laboratory-adapted *Drosophila melanogaster* populations

Aabeer Kumar Basu

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Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

Knowledge city, Sector 81, SAS Nagar, Manauli PO, Mohali 140306, Punjab, India.

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DECLARATION

The work presented in this thesis has been carried out by me under the guidance of Prof. N. G. Prasad at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali. This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly with due acknowledgment of collaborative research and discussions. This thesis is a bona fide record of original work performed by me and all sources listed within have been detailed in the bibliography.

Aabeer Kumar Basu

(Ph17032)

In my capacity as supervisor of the candidate's thesis work, I certify that above statements by the candidate are true to the best of my knowledge.

Prof. N.G. Prasad

Department of Biological Sciences

IISER Mohali

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Contents

Synopsis: Eco-immunology of laboratory-adapted *Drosophila melanogaster* populations

In this thesis, I detail the experiments that I carried out to explore various eco-immunological phenomenon using laboratory adapted populations of *Drosophila melanogaster* (order: Diptera) and various bacterial pathogens that are known to infect this species in the wild. The **1st chapter** of this thesis introduces some of the key tenets of the existing eco-immunology literature to help put the rest of the thesis in context. The **2nd chapter** introduces the study system: the various fly populations and pathogens used in the experiments reported throughout the various chapters detailed below.

Effect of bacterial infection on female fecundity

Costs associated with immune defenses are best measured as life history trade-offs, with reproduction-immunity trade-off being one of the frequently explored phenomena in insects (Lawniczak et al 2007, Schwenke et al 2016). Reproduction-immunity trade-off can manifest in either direction: an infected host is expected to exhibit reduced reproductive output, and an organism investing vigorously towards reproduction is expected to exhibit compromised immunocompetence. These observations are hypothesized to be driven by differential allocation of limiting resources to both processes (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Schmid-Hempel 2003). Reproduction in an infected host can be further compromised due to damage to reproductive tissue caused by either pathogen virulence factors or immunopathology (Hurd 2001, Frank et al 2008).

An alternative expectation is that due to impending pre-mature mortality, brought about by infection, an infected host will increase its reproductive output to compensate to loss of future

opportunity to reproduce (Minchella 1985, Parker et al 2011). Previous studies exploring the effect of bacterial infection on fecundity of *D. melanogaster* females have reported mixed outcomes, with certain studies reporting an increase in reproductive output (Hudson et al 2020) while others report a reduction (Brandt and Schneider 2007, Linder and Promislow 2009) or lack of any effect (Kutzer and Armitage 2016, Kutzer et al 2018). The reported outcomes seem to be determined by, at least partially, the identity of the infecting pathogen, but other factors, such as the route of infection and the host diet, may also have an important role.

The **3rd chapter** of this thesis explores the effect of bacterial infection on reproductive output of *D. melanogaster* females, when infected with three different bacteria. In these experiments, I tested if the effect of bacterial infection on female fecundity was determined by (a) pathogen identity, (b) infection outcome (whether the host survives or dies of infection), and (c) post-infection lifespan in case of the dying females. The results indicate that neither infection status, nor infection outcome, nor post-infection lifespan are good predictors of female fecundity. Furthermore, pathogen identity is a reliable predictor of bacterial infection-induced change in fecundity only at the level of population means, but not at the level of individual females. In conclusion, neither of the tested factors seem to be the sole determining factor for fecundity of *D. melanogaster* females infected with bacterial pathogens.

Resource limitation and resistance to bacterial infection

The environment of the host determines both the efficiency with which a host can mount an immune defense when infected and the costs associated with this defense (Sandland and Minchella 2003, Lazzaro and Little 2011). Host access to nutrition, in terms of both quantity and quality, is one key environmental variable that can influence infection outcomes (Adamo 2021, Cotter and Al Shareefi 2021). The simplest way poor nutrition can dictate host response to infection is by compromising the host capacity to mount a suitable immune defense due to

lack of resources. Recent studies have suggested that every component of the immune system may not be equally compromised by resource limitation (Adamo et al 2016, Adamo 2017); this implies that the observed effect of resource limitation on immune defense will be pathogen specific. Further complications can arise because limiting host access to nutrition also implies starving the pathogen of resources necessary for its proliferation within the host body (Pike et al 2020), and accordingly, various cases have been reported where reduced intake of nutrition improves post-infection host fitness (Hite et al 2019).

The **4th chapter** of this thesis explores the effects of resource limitation on resistance to bacterial infection in *D. melanogaster* females. In the first experiment, I tested the effect of resource limitation on survival of females when infected with five different bacteria. Resource limitation was implemented either by starving the females (leading to a global unavailability of resources) or by enforcing investment towards reproduction via mating (which is known to drive re-allocation of resources away from immune function and towards reproduction; Schwenke and Lazzaro 2017). The results indicate that (a) starvation increases post-infection mortality for four out of five bacteria used for infection, with the degree of increase being dependent upon pathogen-identity, and (b) sexual activity also increases post-infection mortality for all pathogens, with the degree of increase again being dependent upon pathogen-identity.

In a follow-up experiment, I tested the effect of either mode of resource limitation on within host bacterial proliferation. The results indicate that limiting host access to resources by either method leads to a significant increased rate of systemic bacterial proliferation. Importantly, significant increase in systemic bacterial proliferation following starvation is observed even in case of the pathogen for which starvation does not lead to an increase in post-infection mortality.

In conclusion, I have demonstrated here that resource limitation reduces host resistance (i.e., host ability to restrict systemic pathogen proliferation) to bacterial infections, thereby making the host more susceptible to infection. In one exceptional case, reduced resistance was not accompanied by an associated increase in host mortality. I hypothesize, that it is possible for starvation to increase host tolerance for this bacterium while simultaneously reducing resistance, thereby leading to zero change in mortality. Similar proposal has been made by previous studies working with murine and insect model systems (Stahlschmidt et al 2015, Clough et al 2016, Miller and Cotter 2018).

Trans-generational effects of bacterial infections

Given that the omnipresence of pathogens imposes a strong, ever-present selection on the hosts to evolve better and better defense mechanisms, it is surprising that empirical studies still record variation in host susceptibility to pathogens. Some of the frequently cited reasons for this observation include genetic variation within the host or the pathogen populations, antagonism amongst host defense mechanisms, and dependence of immune traits on host environment (Schmid-Hempel 2003, Lazzaro and Little 2009). Another potential source of variation in expression of immune defenses, and therefore host susceptibility, can be due to prior experience with pathogens, both within and across generations. Trans-generational effects are known to influence the expression of various organismal traits (Yin et al 2019), including immune function (Roth et al 2018, Tetreau et al 2019).

The **5th chapter** of this thesis explores the trans-generational effects of pathogenic bacterial infections in *D. melanogaster*. In a series of five experiments, I tested if infecting parental generation flies with bacteria affected post-infection survival and reproductive output of flies from the offspring generation(s). I further tested if any change in offspring post-infection

survival can be explained by change in resistance (i.e., host ability to restrict systemic pathogen proliferation) to infection.

The observations from these experiments were that (a) exposing parents to bacterial infections increases offspring defense (observed as an increase in post-infection survival) against both *homologous* (i.e., when the parents and the offspring are challenged with the same bacteria) and *heterologous* (i.e., when the parents and the offspring are challenged with different bacteria) infections; (b) increase in offspring defense against heterologous infections is dictated by offspring sex, but not by the Gram-character of the bacteria used for infecting either the parents or the offspring; (c) increase in offspring defense against infections, by virtue of parents also being infected, is transient: the effect decays after the first offspring generation and is also only observed in offspring that are produced by the parents temporally close to point of infection; (d) exposing parents to bacterial infections has no impact on offspring reproduction (female fecundity and male competitive fertilization success); and, (e) exposing parents to bacterial infections increases the offspring's ability to resist systemic proliferation of pathogens which underlies the observed increase in defense (post-infection survival).

In conclusion, trans-generational experience with pathogenic infection increases host defense (post-infection survival) against the same and other pathogens, with additional nuances. This increase in post-infection survival stems from increased resistance to infection.

Experimental evolution for increased post-infection survival

Host resistance and tolerance complementarily contribute towards post-infection fitness of the host (Raberg et al 2009, Raberg and Stjernman 2012). Host resistance includes various mechanisms that restrict systemic proliferation of the pathogen, while host tolerance constitutes of processes that minimize the damage caused by infection on the host body. Both wild and laboratory populations are known to harbor genetic variation for both traits (viz., Hansen and

Koella 2003, Raberg et al 2007, Lefevre et al 2011), and many large effect mutations have been identified in *D. melanogaster* that effect either or both traits (viz., Ayres and Schneider 2008, Prakash et al 2022). Various theoretical models have addressed the conditions under which resistance and/or tolerance is expected to evolve (Singh and Best 2021, Roy and Kirchner 2000), but empirical test for evolution of either strategy is rare (viz. Silva 2021). Using a set of replicate *D. melanogaster* populations experimentally evolved for increased post-infection survival following infection with the Gram-positive bacterium *Enterococcus faecalis*, and their ancestrally paired control populations (Singh et al 2021), the **6th chapter** of this thesis explores whether resistance or tolerance has evolved in the selected populations.

When infected with *E. faecalis*, the selected populations exhibited a 70 to 80 percent reduction in post-infection mortality, compared to the control populations, when tested after 65 and 75 generations of forward selection. Improvement in post-infection survival was similar in both sexes. Flies from the selected and the control populations carried similar bacterial loads at the time of their death. Bacterial load upon death (BLUD) has been suggested by recent studies as a suitable proxy for tolerance in *D. melanogaster* (Duneau et al 2017). The lack of any difference in BLUD between the selected and the control populations can therefore be interpreted as no change in tolerance in response to selection. Furthermore, the flies from the selected populations were better at slowing down systemic bacterial proliferation, compared to the flies from the control populations, especially during the acute phase of infection. This suggests that flies from selected populations are more resistant compared to flies from the control populations. Therefore, one may conclude that resistance, but not tolerance, evolves in response to directional selection for increased post-infection survival.

I further observed the presence of a cost of *immune deployment*, but no cost of *immune maintenance*, in the selected populations (*sensu* McKean et al 2008). In absence of any infection, the females from the selected and the control populations had comparable fecundity.

When infected with *E. faecalis*, the females from the selected populations exhibited a reduction in fecundity (compared to uninfected females from the selected population). The fecundity of females from the control populations remained unaffected by infection. Additionally, the effect of infection on female fecundity, when present, was observed only during the acute phase of infection. During the chronic phase of infection, female fecundity was affected by neither selection history nor infection status.

My results therefore demonstrate that increased host resistance evolves in *D. melanogaster* populations in response to selection for increased post-infection survival following infection with bacterial pathogens.

Summary

A few general conclusions can be derived from the results obtained from various experiments distributed across the different chapters of this thesis. First, differences in host resistance (i.e., the host ability to prevent systemic pathogen proliferation) can explain observed differences in host post-infection survival under many different contexts. Such contexts include resource limitations (chapter 4), parental effects (chapter 5), and adaptation to selection for increased post-infection survival (chapter 6).

Second, effect of bacterial infection on fly fecundity is difficult to predict, and depends upon a complex interaction between pathogen identity, infection outcome, and some unexplainable source of inter-individual variation (chapter 3). Phase of infection (acute vs. chronic) does determine the effect of bacterial infection on female fecundity (chapter 6).

Third, parental experience with pathogens can improve offspring defense against pathogens in a non-specific manner without imposing any reproductive costs (chapter 5).

And fourth, hosts evolve increased resistance when experimentally evolved for increased post-infection survival (chapter 6).

The **7th chapter** of this thesis elaborates on these points and discusses them in broader context of the existing eco-immunology literature.

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1. Introduction

The field of eco-immunology deals with elucidating the various ecological factors that determine host capacity to defend itself from infections, and therefore shape the evolution of immune function (Schulenberg et al 2009). Insects, including *Drosophila melanogaster*, have served as excellent model hosts for studying the ecological and evolutionary aspects of host immune function (Dionne and Schneider 2008, Rolff and Reynolds 2009). Over the last two and a half decades, various studies have explored the physiological, ecological, and evolutionary aspects of insect immune defenses (Armitage and Milutinovic 2022). Building upon pre-existing research, during my PhD, I made an attempt to explore some of the yet unanswered (relatively speaking) question in eco-immunology, which I found to be interesting. To this effect, I used lab adapted *D. melanogaster* populations and their bacterial pathogens as a model host-pathogen system. During my PhD, I focused primarily on four phenomena, namely

a. effect of bacterial infection on host reproductive capacity,
b. effect of resource limitation on host response to bacterial infection,
c. trans-generational effects of bacterial infections, and,
d. experimental evolution of host immune function under laboratory conditions.

In this thesis, I report and discuss the various experiments undertaken by me with the aim of exploring the four above-mentioned phenomenon. I discuss, very briefly, some of the key tenets of eco-immunology below, with some examples from studies in *D. melanogaster*, that help put my various chapters in context. A detailed introduction to individual research

questions is provided at the beginning of individual chapters; I have focused on only some of the common elements here.

The challenge: Which host is more immune?

In studies focusing on eco-immunological questions, comparing immunocompetence of different groups of hosts is a necessary step. But ranking hosts in order of their immunocompetence, however it may be defined, is often difficult (Adamo 2004). Studies often focus on measuring within-host pathogen loads as a proxy of immune function, or focus on measuring certain components of the host immune system. The problem with these approaches is that, one, within host pathogen loads do not always predict hot survival (viz. Corby-Harris an Promislow 2007), and two, various components of the immune system do not always change in concert with one another when the host is subjected to any phenotypic or genetic manipulation (viz. Short et al 2012, Ayres and Schneider 2008, Ayres and Schneider 2009). In such a scenario, measuring post-infection survival provides somewhat of a solution to the problem of ranking hosts in terms of post-infection fitness. Still, defense against different pathogens, and therefore post-infection survival, do not always correlate with one another (McKean and Lazzaro 2011, Chambers et al 2012). Hence, the best way to compare immunocompetence of two groups of hosts seems to be to measure their post-infection survival against multiple pathogens.

Throughout all experiments reported in this thesis, post-infection survival has been my primary measure for comparing immune function of hosts, be it across populations or across treatments. In most experiments (chapters 3-5), I have subjected flies to infection with multiple bacterial pathogens and measured their post-infection survival. In addition to survival, as and when required, I have also measured within-host pathogen levels, to quantify host resistance, and

post-infection reproductive output of hosts, as a measure of fitness of infected hosts beyond survival.

The *assumption*: Defense against pathogens comes with associated costs.

One of the central assumptions of eco-immunology is that defense against pathogens often impose costs on the host organism (Sheldon and Verhulst 1996, Schulenberg et al 2009). These costs can manifest in various forms, and thus have been classified by previous authors in numerous ways (Schmid-Hempel 2003, Schmid-Hempel 2005, Sadd and Siva-Jothy 2006, McKean et al 2008, McKean and Lazzaro 2011). While discussing costs of immune defense, most studies implicitly assume that the cost stems from host resistance to diseases, but this is not necessarily correct, and both cost of resistance and tolerance can manifest in a host, and it is often difficult to separately measure these costs (Simms and Triplett 1994).

Costs of disease resistance can either be ecological or physiological, the latter of which takes various forms. The primary ecological cost of resistance is that evolution of increased resistance in the host imposes selection on the pathogen to become more virulent (Miller et al 2006). This is because increased host resistance reduces pathogen prevalence, both within an individual host and at the population level, thereby imposing selection on pathogens to become more prevalent and therefore more virulent.

The most common form of physiological cost of resistance is trade-off between host immune function and other physiological process and life-history traits (Schmid-Hempel 2005, McKean and Lazzaro 2011), driven by allocation of limiting resources between different organismal functions. A key aspect of such trade-offs is that they can manifest either in absence or in presence of a pathogenic infection (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Rolf and Siva-Jothy 2003, Schmid-Hempel 2005). Costs of *immune maintenance* are constitutively paid costs that manifest in absence of an infection (McKean et al 2008). Such

costs are driven by resources invested towards developing a functional immune system and towards mounting the constitutively activate immune responses. On the other hand, costs of *immune deployment* are paid only in the event of an infection (McKean et al 2008). These costs are driven by resources invested towards mounting inducible immune defenses to counter an invading pathogen.

Trade-offs between defense against pathogens and other organismal functions can also stem from functional or structural constraints. For example, increasing the ability to absorb nutrition via the gut wall can make hosts more susceptible to food borne pathogens (Vijendravarma et al 2015). Another manifestation of cost of resistance is immunopathology, whereby the host resistance mechanisms cause harm to the host tissue in addition to killing the pathogens (Sadd and Siva-Jothy 2006, Graham et al 2021). Immunopathology can harm internal organs of the hosts, and have long term effects such as accelerated aging in hosts recovered from early life infection challenge (Khan et al 2017).

A simple and common method of quantifying costs of immune deployment is to compare the reproductive output of uninfected and infected hosts. Irrespective of the underlying mechanism that drives the cost, be it resource-based trade-offs or immunopathology, infection-induced reduction in host reproductive output is the expected outcome (Lawniczak et al 2007, Schwenke et al 2016). Additionally, pathogen-derived virulence factors can add to this reduction of host reproductive output in case of pathogenic infections (see detailed introduction in chapter 3). But an alternative scenario is also possible, as predicted by life-history theory. An infected host, faced with imminent mortality brought about by a pathogenic infection, can increase its reproductive output to compensate for loss of future bouts of reproduction (Minchella 1985). This terminal investment response (Clutton-Brock 1984) can help hosts maintain their fitness (Parker et al 2011).

In **chapter 3** of this thesis, I explore the effect of pathogenic bacterial infections on fecundity of female *D. melanogaster*. Numerous studies have previous addressed this very question using female flies but have yielded mixed results which are equivocal in support of either of the above stated hypothesis, with some also reporting no change in reproductive output following infection (see detailed introduction in chapter 3). Certain obvious factors can contribute to the differences in outcome of these studies, such as type of pathogen used, phase of infection, route of infection, etc. Using different bacterial pathogens, I tested if (a) virulence of the pathogen (i.e., what proportion of infected hosts die and how quickly) and (b) infection outcome (i.e., whether a host dies or survives the infection challenge) have any bearing on the fecundity of individual females. Briefly, my results suggest that infected females exhibit a high degree of inter-individual variability in fecundity, that cannot be explained by either of the tested factors.

The *observation*: Hosts vary in susceptibility to infections.

Given the ever-present threat of infection from various pathogens, the hosts are under constant selection for evolving defense mechanisms that counter infections. But despite this continued selection pressure, variation among hosts for susceptibility to infections is frequently observed, and therefore deserves some explanation (Schmid-Hempel 2003). The primary source of variation in immune defenses is expected to be host genetics. Numerous studies have demonstrated that *D. melanogaster* genotypes differ with respect to how susceptible they are to infections and how efficient they are in controlling within host pathogen proliferation (viz. Lazzaro et al 2004, Lazzaro et al 2006, Hotson et al 2015, Howick and Lazzaro 2017). Genetic variability for host resistance to pathogens is maintained in host populations is mainly maintained due to the various kinds of costs associated with disease resistance (Schmid-Hempel 2003). Variation can also be maintained if hosts by virtue of being better defended against one particular pathogen is more or less defended against a different pathogen (McKean and Lazzaro 2011). In addition to host genetics, variation in defense response can also be

determined by the physiological state of the host. Any factor that modifies the physiological state of the host can in essence determine the host's ability to defend itself against pathogens. Such factors in *D. melanogaster* include host age (Corbally and Regan 2022), host sex (Rolf 2002, Belmonte et al 2020), mating status (Lawniczak et al 2007, Schwenke et al 2016), etc. The environment the host inhabits can also be a major determining factor for host's defense against pathogens (Lazzaro and Little 2008). Both abiotic (nutrition, temperature, etc.) and the biotic (intraspecific and interspecific competitors, predators, earlier pathogen exposure in both ecological and evolutionary timescale, etc.) environment of the hosts can determine host immunocompetence. Environment and gene-by-environment interactions are known to determine response to infection in *D. melanogaster* (viz. McKean et al 2008, Kutzer et al 2018).

In **chapter 4** of this thesis, I explore the role of resource limitation on response to bacterial infections in female flies. Resource limitation was imposed in two ways: starvation and change in mating status. Starvation represents a modification of the abiotic environment of the host, that deprives the host of necessary resources it requires to mount a suitable immune response. Change in mating status represents a modification of the physiological state of the hosts: female flies after mating prioritize the allocation of available resources towards production of eggs, which deprives the immune system of necessary resources. Therefore, both these manipulations are expected to have a negative impact on host defense against pathogens. My results suggest that both modes of resource limitation make flies more susceptible to bacterial infections. The increase in susceptibility is driven by reduced host capacity to restrict systemic pathogen proliferation, and degree to which susceptibility increases is pathogen specific.

In **chapter 5** of this thesis, I explore if experience with pathogenic infection in previous generations can influence host defense against bacterial infections. My results suggest that parental, but not grand-parental, exposure to bacterial infections makes focal hosts better at surviving pathogenic challenges. This betterment neither universal nor highly specific, but

depends on various factors such as the identity of the pathogens used to infect both the parent and the offspring (focal hosts) and on host sex. The increase defense against pathogenic challenges, by virtue of parental exposure to infection, is driven by increased host resistance, and interestingly does not entail any reproductive costs on part of the hosts.

Resistance vs. tolerance

The fitness of an infected host is dependent on both the host's ability to restrict systemic pathogen proliferation (i.e., resistance) and the host's ability to mitigate infection-induced damages to its soma (i.e., tolerance), be it due to the action of the pathogens or immunopathology cause by its own immune response (Raberg et al 2009, Medzhitov et al 2012, Kutzer and Armitage 2016, Lissner and Schneider 2018). These two strategies have different eco-evolutionary dynamics (Raberg and Stjernman 2012), and can have important consequence both at the level of individual hosts and at an ecological scale. For example, increased host resistance selects for increased virulence in pathogens (Miller et al 2006), while increased host tolerance increases pathogen prevalence (Roy and Kirchner 2000) which can lead to increased pandemics (Seal et al 2021).

In **chapter 6** of this thesis, I explore which of these two strategies evolve when hosts are experimentally evolved to increase post-infection survival. Experimental evolution has proved to be an excellent tool for elucidating the features of *Drosophila*-bacteria interactions in evolutionary time. Previous studies have demonstrated presence of trade-offs (Ye et al 2009, Ma et al 2012), absence of trade-offs (Faria et al 2015, Gupta et al 2016, Singh et al 2022a, Singh et al 2022b), evolution of specific defenses (Martins et al 2013), evolution of generic defenses (Singh et al 2021), adaptation to co-evolution of pathogen (Ahlawat et al 2022), etc. I selected fly populations for increased survival after being infected with a Gram-positive bacterial pathogen, *Enterococcus faecalis*. My results suggest that selection for increased post-

infection survival leads to evolution of increased resistance, but not tolerance, in the host populations.

2. General methods

Host populations

LH baseline population

The LH baseline population is a large, outbred laboratory adapted population of *Drosophila melanogaster* (Chippindale and Rice 2001, Prasad et al 2007, Nandy et al 2012). The LH population is maintained on a 14-day discrete generation cycle, at 25 ^{O}C temperature and 12:12 hour light-dark cycle, on cornmeal-molasses-yeast medium, at a census size of about 1900 adults. The flies are maintained in vials (95 mm height × 25 mm diameter); each generation starts with setting up of 60 vials with 150 eggs each on 8-10 ml of food medium. 12 days post-egg laying, by which time most adults have eclosed, adults from different vials are mixed together and redistributed into 60 vials with 16 females and 16 males in each vial. The vials are supplied with limiting live dietary yeast supplement, and on 14th day post-egg laying, the adults are transferred to fresh vials and allowed to oviposit for 18 hours to start the next generation. Vials with eggs greater than 150 undergo egg-culling to maintain the specified egg density in vials and avoid crowding.

Blue Ridge Baseline (BRB) populations

The BRB populations consist of five replicate, large, out-bread *Drosophila melanogaster* population. The Blue Ridge Baseline (BRB) populations were originally established by hybridizing 19 wild-caught iso-female lines (Singh et al 2015) and has been maintained since then as outbred populations on a 14-day discrete generation cycle with census size of about 2800 adults each generation. Every generation, eggs are collected from population cages

(plexiglass cages: 25 cm length × 20 cm width × 15 cm height) and dispensed into vials (25 mm diameter × 90 mm height) with 6-8 ml banana-jaggery-yeast food medium, at a density of 60-80 eggs per vial. 40 such vials are set up; the day of egg collection is denoted as day 1. The vials are incubated at 25 ^{O}C, 50-60% RH, 12:12 hour LD cycle; under these conditions the egg-to-adult development time for these flies is about 9-10 days. On day 12 post egg collection, all adults are transferred to a new population cage, and provided with fresh food plates supplemented with *ad libitum* live yeast paste. On day 14, the cage is provided with fresh food plate for oviposition, and 18 hours later eggs are collected from this plate to begin the next generation.

Table 2.1. Composition of food medium used to maintain different fly populations, per 1 litre of food medium.

Ingredients	**Banana-jaggery-yeast medium**	**Cornmeal-molasses-yeast medium**
Banana	205 g	-
Barley	25 g	-
Cornmeal	-	100 g
Jaggery	35 g	-
Molasses	-	100 ml
Yeast	36 g	41.2 g
Agar	12.4 g	14.8 g
Water	1000 + 180 ml	1000 + 100 ml
Ethanol (used to inactivate the yeast)	22 ml	-
p-Hydroxy methyl benzoate	2.4 g	2.25 g
Ethanol (used to dissolve benzoate)	23 ml	22.5 ml
Propionic acid	-	8 ml

Experimentally evolved populations: EPN selection regime

Replicate *Drosophila melanogaster* populations were evolved parallelly, subjecting some to selection for increased post-infection survival, while maintaining others as either procedural or uninfected controls. The experimental evolution set-up consisted of 12 populations, distributed into 3 selection regimes (Singh et al 2021):

(a) **E_{1-4} populations**: selected for increased post-infection survival. Every generation 200 females and 200 males per replicate population are infected (see below for details of infection procedure) with *Enterococcus faecalis* and survivors are allowed to reproduce after 96 hours post-infection.

(b) **P_{1-4} populations**: controls for infection procedure. Every generation 100 females and 100 males per replicate population are sham-infected and survivors are allowed to reproduce after 96 hours post-sham-infection.

(c) **N_{1-4} populations**: uninfected controls. Every generation 100 females and 100 males are simply subjected to CO_2 anaesthesia; there is zero mortality in these populations before the reproduction window.

All the populations were derived from four replicate baseline populations, Blue Ridge Baselines (BRB_{1-4}). The E_1, P_1, and N_1 populations were derived from BRB_1, the E_2, P_2, and N_2 populations were derived from BRB_2, and so on. Selected populations with the same numeric subscript therefore share a recent common ancestor and are part of the same ancestral 'block'. Blocks are handled on separate days for both regular maintenance and for experiments.

All the populations are maintained on a 16-day discrete generation cycle (day 1 being the day of egg collection). Infections happen on day 12 every generation. Flies are maintained on standard banana-jaggery-yeast medium, and are housed in an incubator at 25 ^{O}C with a 12:12 hours LD cycle and 50% relative humidity. For experiments, flies were generated and handled

in a fashion that closely resembles the regular maintenance regime for these populations. On day 1, eggs are collected from each of the 12 populations, at a density of 60-80 eggs per vial (25 mm diameter × 90 mm height), in 6-8 ml of standard food medium; 10 vials are set-up per population. The vials are incubated under the constant environmental conditions listed above, and the eggs develop into adults by day 10. The adults remain in these vials till day 12, when the adult flies are subjected to the corresponding selection regime according to their population identity. By this point of time, all adults are sexually mature and have mated at least once. After being subjected to selection (infected, sham-infected, or uninfected), adult flies are housed in plexiglass cages (14 cm length × 16 cm width × 13 cm height); each population is housed in a separate cage. Each cage is supplied with fresh food medium, poured into a 60 mm Petri plate. The flies remain in these cages for the next 96 hours, with fresh food medium provided to them every 48 hours. On day 16 (96 hours after selection is imposed), each cage is provided with a fresh food plate for the surviving flies to lay eggs on. On day 17 (18 hours after start of egg laying), eggs are collected from these plates to start the next generation. Day 17 of the previous generation becomes the day 1 of the following generation.

During regular maintenance, there is negligible mortality in the P_{1-4} populations (<2%) and no mortality in the N_{1-4} populations, from the point of handling of adults on day 12 till the start of oviposition window in day 16. During the selection process, the mortality of flies from E_{1-4} populations are maintained at about fifty percent. To this effect, the flies were infected at an infection dose of OD_{600} = 0.8 (see below for more details) between generations 1 and 20 of forward selection. Thereafter, the flies were infected with OD_{600} = 1.0 from generation 21 to 40, with OD_{600} = 1.2 from generation 41 to 60, and with OD_{600} = 1.5 from generation 61 onwards.

Pathogens

All experiments reported in this thesis used entomopathogenic bacteria that are frequently used for eco-immunological experiments in *Drosophila melanogaster* (Troha and Buchon 2019). A list of all the bacterial pathogens used is provided in table 2.2.

Storage and handling of bacterial pathogens

The bacterial isolates are preserved as glycerol stocks at -80 ^{O}C. To obtain live bacterial cells for infections, 10 ml lysogeny broth (Luria Bertani Broth, Miler, HiMedia) is inoculated with the glycerol stocks of the necessary bacterium and incubated overnight with aeration at the right temperature. 100 microliters from this primary culture are inoculated into 10 ml fresh lysogeny broth and incubated for the required amount of time to obtain confluent cultures. The bacterial cells are pelleted down using centrifugation and resuspended in sterile $MgSO_4$ (10 mM) buffer at optical density (OD_{600}) of 1.0 or at any other necessary dosage. In chapters 3 – 5, all experimental infections were carried out using bacterial suspension at OD_{600} = 1, for all pathogens used. For experiments in chapter 6, experimental infections were carried out using bacterial suspension at OD_{600} = 1.5.

Infection protocol

Flies are infected, under light CO_2 anaesthesia, by pricking them on the dorsolateral side of their thorax with a 0.1 mm Minutien pin (Fine Scientific Tools, USA) dipped in the bacterial suspension. Sham-infections (injury controls) are carried out in the same fashion, except by dipping the pins in sterile $MgSO_4$ (10 mM) buffer. Uninfected controls are subjected only to temporary light CO_2 anaesthesia.

Table 2.2. List of bacterial pathogens used in various experiments reported in this thesis.

Bacteria	**Growth conditions**	**Source**	**Literature reference**
Bacillus thuringiensis	30 °C, lysogeny broth	DSMZ, Germany (catalogue no.: DSM2046)	-
Enterococcus faecalis	37 °C, lysogeny broth	Lazzaro Lab, Cornell Unversity	Lazzaro et al 2006
Erwinia carotovora carotovora (strain Ecc15)	30 °C, lysogeny broth	Sucena Lab, IGC Portugal	Martins et al 2013, Troha and Buchon 2019
Providencia rettgeri	37 °C, lysogeny broth	Lazzaro Lab, Cornell University	Short and Lazzaro 2010
Pseudomonas aeruginosa	37 °C, lysogeny broth	MTCC, India	-
Pseudomonas entomophila (strain L48)	27 °C, lysogeny broth	Cornelis Lab, Vrije Universiteit Brussel, Belgium	Vodovar et al 2005
Serratia marcescens	37 °C, lysogeny broth	Sucena Lab, IGC Portugal	Martins et al 2013
Staphylococcus succinus (strain PK-1)	37 °C, lysogeny broth	-	Singh et al 2016

Statistical analysis

All statistical analyses of the data were carried out using R statistical software (R Core Team 2021). Data reported in chapter 3 was analyzed using R v3.6.3, and data reported in chapters 4 to 6 were analyzed using R v4.1.0. The method for analyses of data from each individual experiment is detailed within the respective chapters. Table 2.3 lists the various R packages, and the relevant functions, that were used for analyzing data across all chapters. All graphs, other than the survival curves, were created using the R package ggplot2.

Table 2.3. List of R packages and functions used for data analysis.

Function	**Package**	**Usage**
coxme()	coxme	Mixed-effects Cox-proportional hazards models
survfit()	survival	Deriving survival function
ggsurvplot()	survminer	Plotting of survival curves using Kaplan-Meier method
pairwise_survdiff()	survminer	Pair-wise Log-rank test for comparing survival across treatments
anova()	base R	Analysis of variance (ANOVA, type III)
lmer()	lmerTest	Mixed-effect general linear models
ranova()	lmerTest	Significance tests for random effects in an ANOVA
lsmean(adjust = "tukey")	emmeans	Tukey's HSD
leveneTest(centre = "median")	car	Levene's test
Anova()	car	Analysis of deviance, implemented on a *coxme()* model

3. Effect of bacterial infection on female fecundity

Introduction

Omnipresence of pathogens and parasites impose a strong selection pressure on hosts to evolve mechanisms of defense. Such defense mechanisms go far beyond the canonical anatomical and physiological defenses, and include alternative strategies that either help alleviate risk of infection or help mitigate the consequences. Fecundity compensation, that is the post infection increase in reproductive effort of the host, is one such alternative strategy that helps hosts maintain their evolutionary fitness (Parker et al 2011). Since increased reproductive effort maximizes immediate reproductive output at the cost of future chance of reproduction (Stearns 1972), organisms under benign conditions are expected to pace out their reproductive schedule so as to maximize their life-time reproductive success (Fisher 1930, Williams 1957). Under circumstances which lead to pre-mature death, such as a lethal infection, future opportunities of reproduction are compromised and organisms should, in theory, maximize their immediate reproductive effort (Williams 1966, Clutton-Brock 1984, Minchella 1985). Minchella and Loverde (1981) first demonstrated this phenomenon in snails infected with castrating trematode parasites, where hosts increased their immediate reproductive output in response to parasitic infection.

An infection is also detrimental to the physiology of the host organism. One, mounting an immune response requires investing energy and resources that could otherwise have been utilized elsewhere, such as towards reproduction (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Schmid-Hempel 2005). Two, infection leads to somatic damage caused by

the virulence factors produced by parasites and pathogens (Hurd 2001, Frank and Schmid-Hempel 2008). And three, the immune response mounted by the host often causes collateral somatic damage to the host, leading to immunopathology (Sadd and Siva-Jothy 2006). Altogether this suggests that post-infection fitness of hosts depends on its ability to restrict the systemic propagation of the parasite/pathogen, plus the host's capacity to continue to maintain physiological functionality during and after recovery from the infection (resistance and tolerance, *sensu* Raberg et al 2009). Reallocating resources towards mounting an immune defense can lead to reduced reproductive effort during acute infection (Howick and Lazzaro 2014), and lingering somatic damage can keep reproductive effort to a minimum even after recovery. Fecundity compensation, as described above, therefore might not be the observed strategy in case of all hosts on every occasion, and will depend on the features of the specific host-pathogen system being studied.

The choice of strategy is likely to depend on the balance between the actual risk of mortality and the level of somatic damage incurred by the host. A greater risk of mortality should induce a stronger fecundity compensation response, thereby increasing reproductive effort, while reproductive effort should decline proportionately with increasing somatic damage. This balance can vary at the level of individual hosts, causing the mean population behavior to not be a true reflection of the individual variation in strategies. In fact, increased and decreased reproductive effort can be viewed as two ends of a continuum – instead of a dichotomous choice – with each individual host opting for an optimal level of reproductive effort based on their proximate circumstances.

Furthermore, post-infection reduction in host reproductive effort may also be driven by leeching of resources by the pathogen/parasite, damage to reproductive tissue, or manipulation of the host physiology by virulence factors produced by the pathogen/parasite (Hurd 2001). Thus, post-infection reduction in host reproductive effort can also be a consequence of the

infection process (presence of pathogen), independent of the host response to infection. To differentiate between post-infection phenotypes that are driven by pathogen manipulation of the host and those caused by host immune response, previous studies have often used attenuated pathogens or pathogen-like proxies (bacteria-derived lipopolysaccharides, plastic beads, etc.; viz. Moret and Schmid-Hempel 2000), arguing that such proxies stimulate the host immune system without causing any infection-related pathologies. While experiments with live pathogens may fail to tease apart host's response to pathogens from pathogen's manipulation of the host, experiments with pathogen-like proxies may not induce any fitness effects, both physiological and reproductive. Furthermore, given that mounting an immune response is costly to the host, hosts are under pressure to evolve mechanisms that differentiate real infections from false alerts. Thus, results obtained from experiments using attenuated pathogens and proxies are difficult to interpret. The results of experiments are thus likely to depend upon the exact biology of the interacting host and pathogen (Forbes 1993), on the physiological capability of the host to modify its own reproductive effort, and on whether such modifications of reproductive effort will materialize into benefits in terms of immune function (Javois 2013), among various other factors.

Previous studies exploring the effect of parasites and pathogens on reproductive behavior of *Drosophila melanogaster* have reported diverse outcomes, depending partially upon the type of infectious agent used in the experiments. Flies having successfully survived a parasitoid attack as larvae have reduced fecundity as adults (Carton and David 1983, Fellowes 1999). Flies infected with Drosophila C Virus exhibit genotype and infection route dependent increase or decrease in reproductive output (Gupta at al 2017). Infection with bacterial pathogens have been demonstrated to increase (Hudson et al 2019), reduce (Brandt and Schneider 2007, Linder and Promislow 2009), or maintain fecundity at an unaltered level (Kutzer and Armitage 2016, Kutzer et al 2017). The reasons for this diversity of outcomes can be multiple, including host

susceptibility to pathogens used (Stephenson 2019), infection route (Martins et al 2013, Beherens et al 2014), genotypic differences in host strains and possible interactions with environmental factors (McKean et al 2008, Vale and Little 2012). Another variable that can affect experimental outcomes is whether reproductive effort is measured during the acute or the chronic phase of infection (*sensu* Howick and Lazzaro 2014). Infection survivors continue to have a low level of systemic pathogen presence which have life history consequences (Chambers et al 2019), although one study reported infection survivors have similar fecundity as to the controls following recovery from a bacterial infection (Gupta et al 2015).

In this chapter, I challenged *Drosophila melanogaster* females with three pathogenic bacteria, (a) *Bacillus thuringiensis*, (b) *Pseudomonas aeruginosa*, and (c) *Seratia marscesens*, and quantified their change in post-infection reproductive output, during the acute phase of infection, compared to uninfected controls. The aim of the study was to identify the effect of (a) pathogen identity, (b) infection outcome, and (c) time of death, on post-infection reproductive effort. Pathogen identity represents differences in pathogen virulence factors, host defence mechanisms, associated costs and immunopathology. Therefore, I expected that pathogen identity will be a strong determining factor for post-infection reproductive effort. Infection outcome, that is survival versus death, is the ultimate determinant of fitness at the level of individual hosts. Hosts that succumb to infection lose out on future opportunities to reproduce, and therefore are expected to modulate their current reproductive effort differently than hosts that recover from infection. And finally, individual hosts that die within a short period following infection are expected to exhibit a greater increase in reproductive effort compared to hosts that die relatively later.

Materials and methods

Study system

Flies from BRB2 population were used for the experiments reported in this chapter. Please refer to chapter 2 for details of origin and maintenance of this population.

Pathogens

Three bacterial pathogens were used across all experiments reported in this chapter:

a. *Bacillus thuringiensis*,
b. *Pseudomonas aeruginosa*, and
c. *Serratia marcescens*.

Generation of experimental flies

Eggs were collected from BRB2 population cages and distributed into food vials with 8 ml of standard food medium at a density of 70 eggs per vial. These vials were incubated as per general maintenance. Twelve days post egg-laying flies were flipped into fresh food vials and hosted for two more days before experimentation. This ensured all focal females were 4-5 day old, sexually mature and inseminated, at the time of infections. Flies were again flipped into fresh food vials 6 hours before being subjected to experimental treatments (as described below).

Experimental design

Experiment 1. Focal females were randomly distributed into five treatments: (a) infected with *Bacillus thuringiensis* (*Bt*), (b) infected with *Pseudomonas aeruginosa* (*Pa*), (c) infected with *Serratia marcescens* (*Sm*), (d) sham-infected controls, and (e) uninfected controls. The entire experiment was independently replicated thrice. Flies were placed in fresh food vials after being subjected to respective treatments. For each treatment 10 vials were set up, each with 8 females for oviposition; each vial was used as a unit of replication. The vials were monitored

every 2 hours to record any mortality, for 24 hours post-infection, divided into two consecutive 12-hour windows. Flies alive at the end of first 12-hour window were flipped into fresh food vials (one-to-one mapping of vial identity), and flies alive at the end of 24 hours were discarded (censored). The number of eggs in each vial was counted at the end of respective 12-hour windows. The vials were then incubated under standard maintenance conditions for the eggs to develop into adults, and 12 days after the oviposition period, all adult progeny were counted under light CO_2 anesthesia and transferred to fresh food vials.

Standardized reproductive output of females in each vial was calculated as,

$$Standardized\ egg\ count = \frac{Total\ number\ of\ eggs\ layed\ over\ 24\ hours}{Summation\ of\ lifespan\ of\ all\ females\ in\ the\ vial}$$, and

$$Standardized\ progeny\ count = \frac{Total\ number\ of\ adult\ progeny\ produced}{Summation\ of\ lifespan\ of\ all\ females\ in\ the\ vial}$$.

Standardization was carried out to account for the differences in post-infection survival time of females in various treatments (see RESULTS for more details). *Progeny pre-adult viability* for each vial was calculated as,

$$Pre\ adult\ viability = \frac{Total\ number\ of\ adult\ progeny\ produced}{Total\ number\ of\ eggs\ laid}$$.

Following progeny counts, 4-5-day old adult progeny were pooled together according to treatments and distributed to fresh food vials with 5 females and 5 males in each vial; 10 such vials were set up per *maternal* treatment per replicate. These flies were allowed to oviposit for six consecutive days (by flipping them into fresh food vials every day) to obtain an estimate of *offspring early-life fecundity*. These vials were incubated at standard maintenance conditions, and the number of progeny in these vials were counted 12 days post-oviposition.

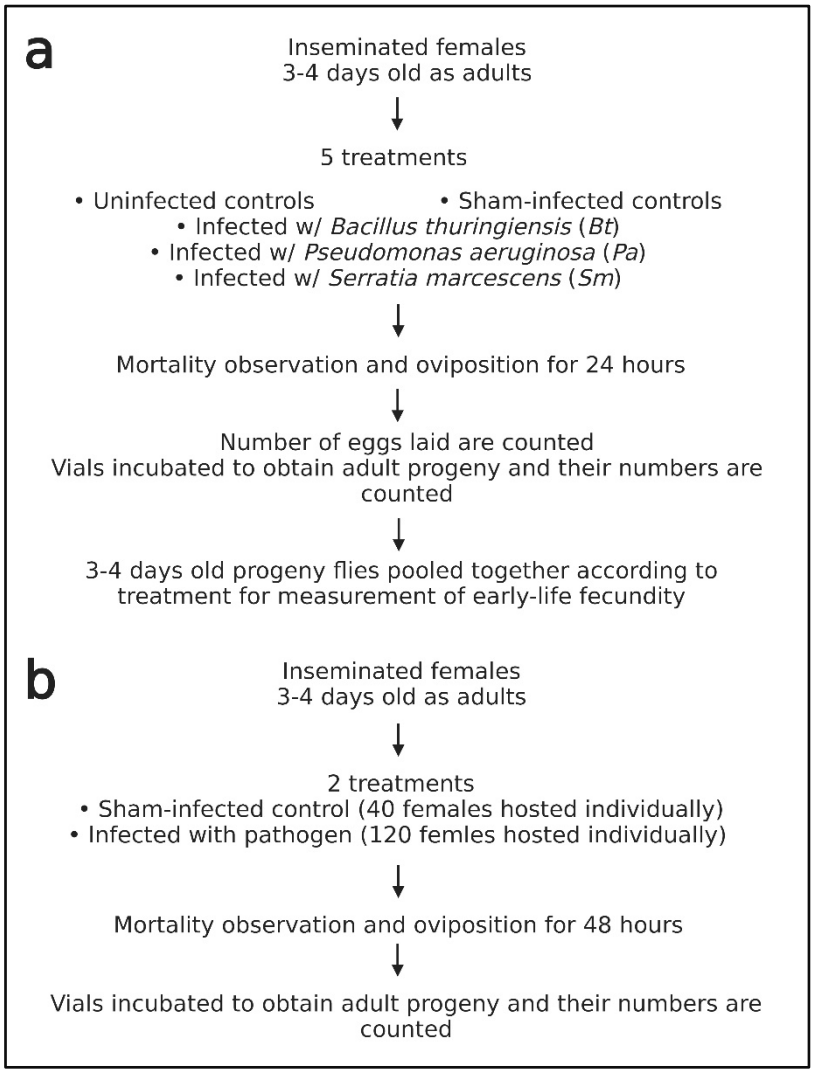
Experiment 2. Focal females were randomly distributed into two treatments: (a) infected with bacteria, and (b) sham-infected controls. For infected treatment, 120 females were individually

hosted in vials for oviposition, while for sham-infected controls 40 females were hosted individually. The experiment was replicated thrice with each pathogen. (Due to a handling accident, one replicate with Sm had sample size of 60 and 30 females for infected and sham-infected treatments, respectively.) The vials were monitored every 2 hours for any mortality, for 48 hours post-infection, after which the alive flies were discarded. The vials were then incubated under standard maintenance conditions for the eggs to develop into adults, and 12 days later the number of adult progeny was counted for each individual female.

Standardized reproductive output for each individual female was calculated as,

$$Standardized\ progeny\ count = \frac{Number\ of\ offspring\ produced}{Lifespan\ of\ the\ female\ in\ the\ vial}.$$

Figure 3.0. Schematic representing the experimental design for (a) experiment 1, and (b) experiment 2.



Statistical analysis

Experiment 1. Survival data was analyzed using mixed-effects Cox proportional hazards model, with 'Treatment' as a fixed factor and 'Replicate' as a random factor. Reproductive output, progeny viability, and progeny early-life fecundity was modeled using linear models (as described below) and subjected to significance testing using type III analysis of variance (ANOVA). Post-hoc pairwise comparisons, wherever necessary, was carried using Tukey's HSD method.

Standardized egg count ~ Treatment + (1|Replicate)

Standardized progeny count ~ Treatment + (1|Replicate)

Progeny pre-adult viability ~ Maternal treatment + (1|Replicate)

Progeny early-life fecundity ~ Day + Maternal treatment + (1|Replicate)

Experiment 2. Reproductive output data was modeled using a linear model (as described below) and subjected to significance testing using type III ANOVA. Post-hoc pairwise comparisons, wherever necessary, was carried using Tukey's HSD method.

Standardized progeny count ~ Category + (1|Replicate)

'Category' denoted the combination of infection status and infection outcome, and consists of three levels: sham-infected females, infected-alive females, and infected-dead females. Effect of time on death on reproductive output of infected-dead females was similarly analyzed with type III ANOVA using the following linear model:

Standardized progeny count ~ Time of death + (1|Replicate)

Comparison of variances across 'category' was carried out using Levene's test after pooling data from all three replicates for each pathogen.

Results

Effect of pathogen identity on female post-infection fecundity

In the first experiment, I infected 4-5-day old, inseminated females with three bacteria: *Bacillus thuringiensis* (hereafter *Bt*), *Pseudomonas aeruginosa* (hereafter *Pa*), and *Serratia marcescens* (hereafter *Sm*); I maintained sham-infected and uninfected controls along with the infected treatments. After infections, the females of each treatment were hosted in vials in groups of 8, with 10 vials per treatment. The entire experiment was independently replicated thrice. I monitored the mortality in these vials, every 2 hours, for 24 hours post-infection, covering the acute phase of infection of all three pathogens. As a measure of reproductive output, I counted the total number of eggs in each vial, laid by 8 females in 24 hours, and also the total number of adult progeny that developed from the eggs. This provided us with an additional measure of fitness: pre-adult viability (proportion of eggs that successfully developed into adults) of the progeny produced by the infected females.

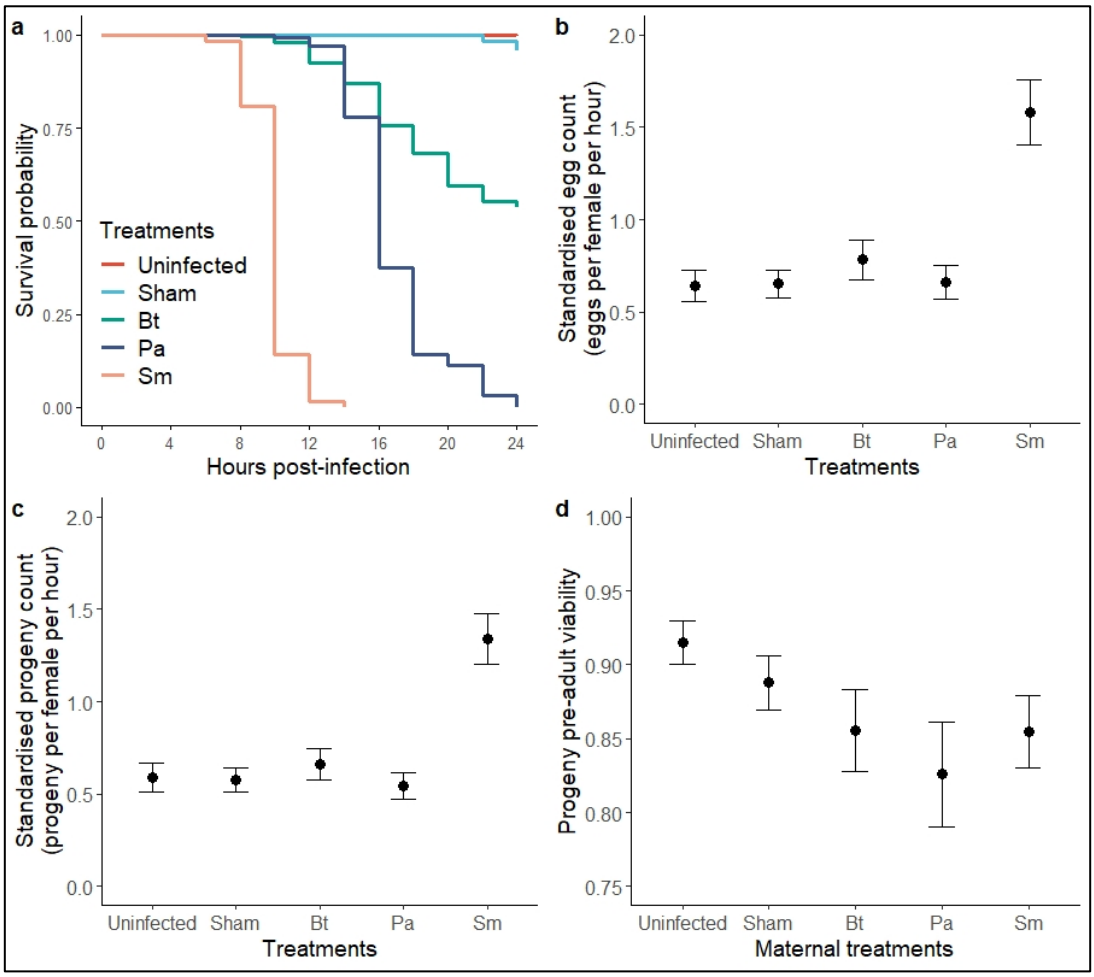
All three pathogens used imposed significant mortality upon the infected females compared to the uninfected controls (figure 3.1.A). All females infected with *Sm* (hazard ratio, 95% confidence interval: 31895.33, 4333.172-234773.08) and *Pa* (HR, 95% CI: 936.66, 130.79-6707.86) died because of infection within 12 and 24 hours of infection, respectively, while about half of all females infected with *Bt* (HR, 95% CI: 156.17, 21.80-1118.65) died of infection within the observation period. Females that were sham-infected (HR, 95% CI: 7.98, 0.99-63.78) did not show significant difference in mortality compared to uninfected controls.

Since the lifespan of females in each treatment was different from one another, instead of directly comparing the absolute number of eggs laid (or progeny produced), I divided the total number of eggs (or progeny) in each vial with the summation of the hours survived (survival time right-censored at 24 hours post-infection for surviving females) by the females in that vial.

I call this the "standardised reproductive output" and use this value as the subject of analysis. This value is essentially the measure of the number of eggs (or progeny) per female per hour, when the females are either infected with pathogens, or sham-infected, or left uninfected.

Infection treatment had a significant effect on standardised reproductive output, in terms of both eggs laid ($F_{4,147}$: 58.778, $p < 2.2$ e-16; figure 3.1.B) and progeny produced ($F_{4,147}$: 61.338, $p < 2.2$ e-16; figure 3.1.C). Post-hoc pairwise comparison using Tukey's HSD indicated that *Sm*-infected females (least-square mean, 95% CI: 1.580, 1.408-1.752) laid a significantly greater number of eggs per female per hour compared to uninfected (LS mean, 95% CI: 0.641, 0.469-0.813), sham-infected (LS mean, 95% CI: 0.653, 0.481-0.825), *Bt*-infected (LS mean, 95% CI: 0.783, 0.611-0.955), and *Pa*-infected (LS mean, 95% CI: 0.662, 0.490-0.834) females; the other four experimental treatments did not differ from one another significantly in terms of number of eggs laid (table 3.1.A). Similarly, *Sm*-infected females (LS mean, 95% CI: 1.337, 1.207-1.467) produced a significantly greater number of progeny per female per hour compared to uninfected (LS mean, 95% CI: 0.589, 0.458-0.719), sham-infected (LS mean, 95% CI: 0.578, 0.448-0.708), *Bt*-infected (LS mean, 95% CI: 0.663, 0.533-0.794), and *Pa*-infected (LS mean, 95% CI: 0.545, 0.414-0.675) females; the other four experimental treatments did not differ from one another significantly in terms of number of progeny produced (table 3.1.B).

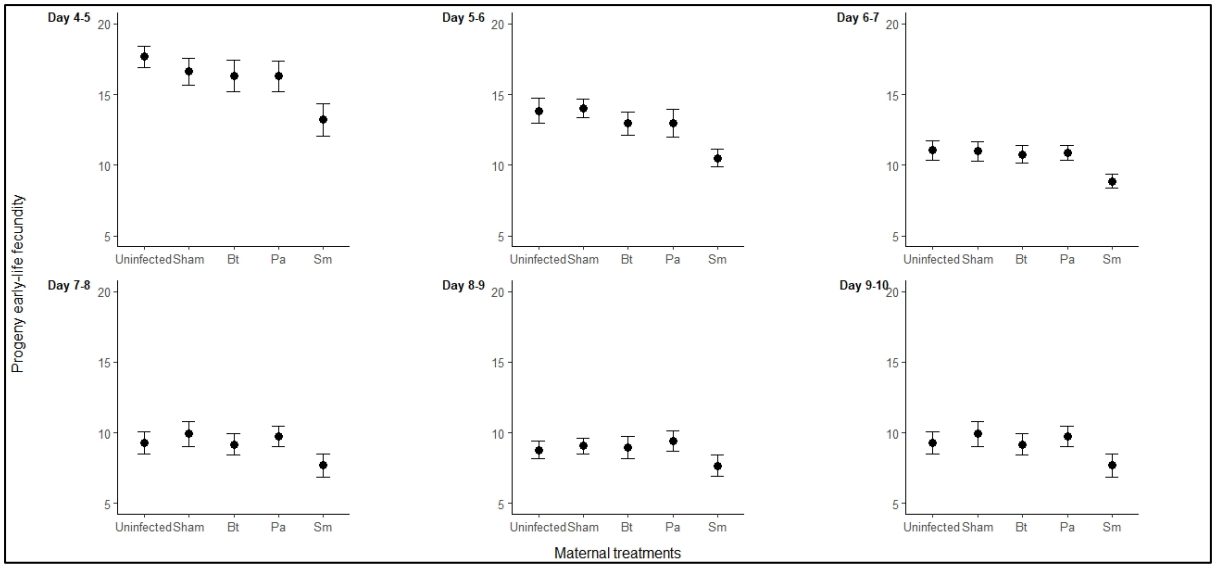
Figure 3.1. Effect of infection with different bacterial pathogens on (A) survival, (B) number of eggs produced (mean ± 95% CI), (C) number of progeny produced (mean ± 95% CI), and (D) pre-adult viability of progeny (mean ± 95% CI), of *Drosophila melanogaster* females. All plots are created after pooling data from all three experimental replicates.



Infection treatment had a significant effect on progeny pre-adult viability ($F_{4,150}$: 7.985, p = 7.304 e-06; figure 3.1.D). Post-hoc pairwise comparison using Tukey's HSD indicated that progeny of *Bt*-infected (LS mean, 95% CI: 0.856, 0.830-0.881), *Pa*-infected (LS mean, 95% CI: 0.826, 0.800-0.851), and *Sm*-infected (LS mean, 95% CI: 0.855, 0.829-0.880) females had significantly less pre-adult viability compared to progeny of uninfected females (LS mean, 95% CI: 0.915, 0.889-0.940). There was no difference in viability between progeny of sham-infected (LS mean, 95% CI: 0.888, 0.863-0.914) and uninfected females. Progeny of *Pa*-infected females also had less viable compared to progeny of sham-infected females (table 3.1.C).

To have an estimate of the effect of infecting females with different pathogenic bacteria on the fitness of their progeny, I measured the early-life fecundity of the progeny, beginning at day 4-5 of adulthood till day 10-11 of adulthood. I pooled all progenies produced by all 80 females in each treatment (8 females × 10 vials) and randomly samples 60 males and 60 females, housing them in groups of 6 males and 6 females, setting up 10 vials per *maternal* treatment. I counted the number of progenies produced by these flies over the next six days, counting the progenies per day separately, and using that as the subject of analysis. The day of count (age of the flies) had a significant effect on early-life fecundity of progeny flies ($F_{1,897}$: 488.713, p < 2.2 e-16; figure 3.2). Progeny early life fecundity was also significantly affected by *maternal* treatment ($F_{4,897}$: 31.427, p < 2.2 e-16; figure 3.2). Post-hoc pairwise comparison using Tukey's HSD indicated that progeny of *Sm*-infected females (LS mean, 95% CI: 9.56, 8.52-10.06) had significantly lesser fecundity compared to progeny of uninfected (LS mean, 95% CI: 12.08, 11.04-13.1), sham-infected (LS mean, 95% CI: 12.06, 11.02-13.1), *Bt*-infected (LS mean, 95% CI: 11.59, 10.55-12.6), and *Pa*-infected (LS mean, 95% CI: 11.79, 10.75-12.8) females; the other four *maternal* treatments did not differ from one another significantly in terms of progeny fecundity (table 3.1.D).

Figure 3.2. Effect of *maternal* infection treatment on progeny early-life fecundity (mean ± 95% CI); fecundity measured on each day shown separately. All plots are created after pooling data from all three experimental replicates.



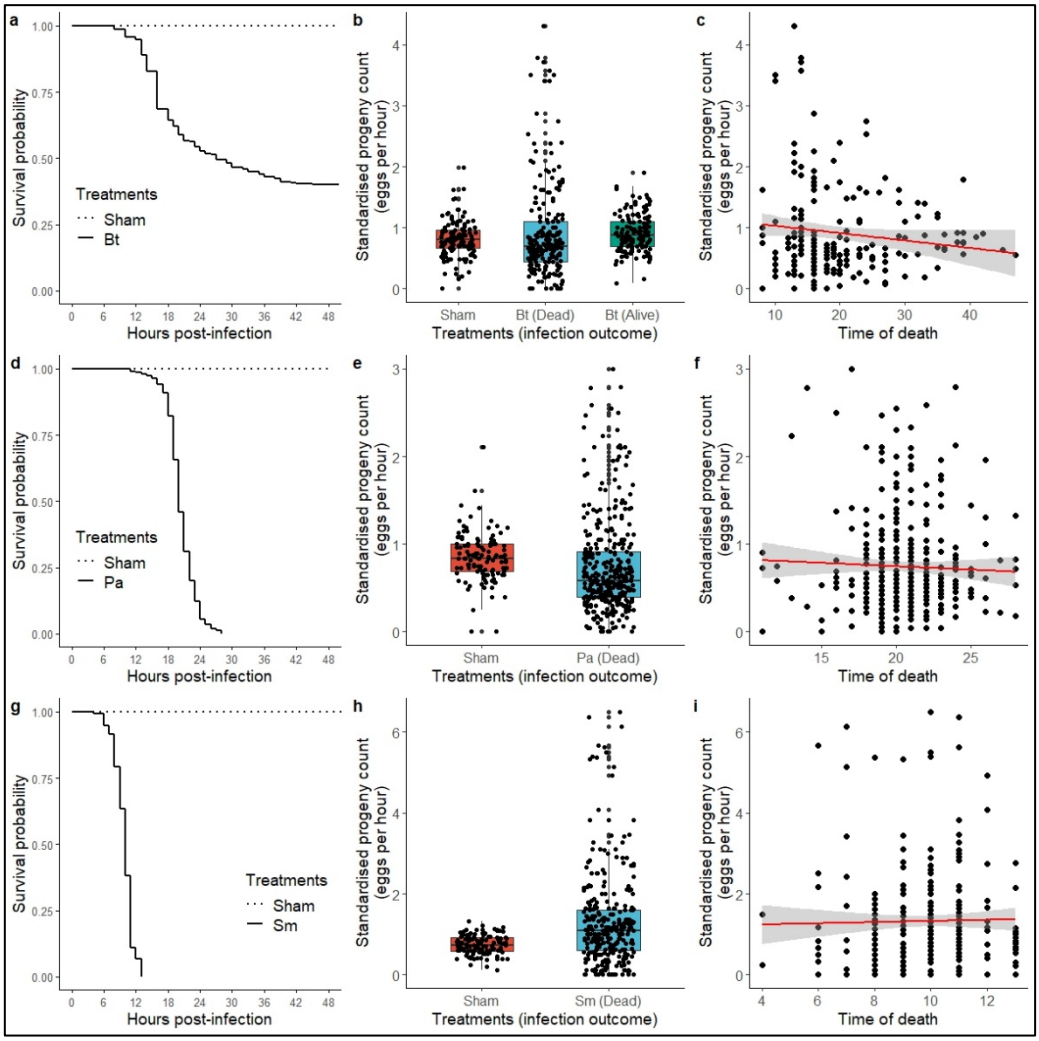
Effect of infection outcome and time of death on female post-infection fecundity

In the first experiment, all females infected with *Sm* and *Pa* died of infection, while only half of *Bt*-infected females died (figure 3.1.A). In the second experiment, I tested if the outcome of infection (survival vs. death), and the time of death for individual females, had any effect on reproductive fitness of the females. I housed infected and sham-infected females individually in food vials after infection, and monitored their mortality every 2 hours for 48 hours post-infection. The experiment was independently replicated thrice for each bacterium used: *Bt*, *Pa*, and *Sm*. I counted the number of progeny produced by individual females in the span of 48 hours (or till the time the female died) as a measure of reproductive output. To account for differences in lifespan (number of hours survived by an infected female; survival time right-censored at 48 hours post-infection for females that didn't die within that time), I divided the number of progeny produced by a female by the hours survived, and used this "standardised reproductive output" as subject of analysis. Since in the first experiment, between treatment differences in standardised reproductive output did not change based on whether I focused on the number of eggs or the number of progeny, in this experiment I only counted the number of progeny produced.

Similar to the first experiment, only about half of *Bt*-infected females, while all of *Sm*- and *Pa*-infected females, died due to infection (figure 3.3.A, 3.3.D, 3.3.G). For *Bt*-infected females, infection outcome did not have a significant effect on mean standardised reproductive output of the females ($F_{2,475}$: 1.4701, p = 0.2309); the infected-dead females (LS mean, 95% CI: 0.926, 0.683-1.17), the infected-alive females (LS mean, 95% CI: 0.895, 0.654-1.14), and the sham-infected females (LS mean, 95% CI: 0.817, 0.577-1.06) had comparable mean standardised reproductive output (figure 3.3.B). Infection outcome significantly affected the variance in standardised reproductive output (Levene's test, $F_{2,475}$: 20.808, p = 2.174 e-09), with infected-dead females exhibiting greater variance compared to both infected-alive and sham-infected

females (figure 3.3.B). Within infected-dead female, time-of-death had a significant effect on standardised reproductive output ($F_{1,210}$: 6.3233, p = 0.01267), with reproductive output having a mild negative correlation with time-to-death (coefficient, 95% CI: -0.01733, -0.03109 – -0.00355; η^2, 90% CI: 0.03, 0.00-0.08; figure 3.3.C).

Figure 3.3. Effect of infection outcome on post-infection reproductive output of *Drosophila melanogaster* females, infected with *Bacillus thuringiensis* (A: survival, B: effect of infection outcome on progeny count, C: effect of time of death on progeny count for females that dies of infection), *Pseudomonas aeruginosa* (D: survival, E: effect of infection outcome on progeny count, F: effect of time of death on progeny count for females that dies of infection), and *Serratia marcescens* (G: survival, H: effect of infection outcome on progeny count, I: effect of time of death on progeny count for females that dies of infection). All plots are created after pooling data from all three experimental replicates.



For *Pa*-infected females (all of which died following infection; figure 3.3.D), infection outcome had a significant effect on standardised reproductive output ($F_{1,474}$: 4.3739, p = 0.03703), with infected-dead females (LS mean, 95% CI: 0.743, 0.618-0.867) producing less number of progeny compared to sham-infected females (LS mean, 95% CI: 0.851, 0.721-0.981; figure 3.3.E). Infection outcome significantly affected the variance in standardised reproductive output (Levene's test, $F_{2,474}$: 19.795, p = 1.075 e-05), with infected-dead females exhibiting greater variance compared to sham-infected females (figure 3.3.E). Time-of-death did not have a significant effect on standardised reproductive output ($F_{1,357}$: 0.511, p = 0.4503; figure 3.3.F).

For *Sm*-infected females (all of which died following infection; figure 3.3.G), infection outcome had a significant effect on standardised reproductive output ($F_{1,408}$: 25.5, p = 6.684 e-07), with infected-dead females (LS mean, 95% CI: 1.28, 0.747-1.81) producing greater number of progeny compared to sham-infected females (LS mean, 95% CI: 0.72, 0.207-1.23; figure 3.3.H). Infection outcome significantly affected the variance in standardised reproductive output (Levene's test, $F_{2,408}$: 40.875, p = 4.444 e-10), with infected-dead females exhibiting greater variance compared to sham-infected females (figure 3.3.H). Time-of-death did not have a significant effect on standardised reproductive output ($F_{1,290}$: 0.1505, p = 0.6983; figure 3.3.I).

Discussion

Fecundity compensation (or, terminal investment) theory in its simplest form hypothesises that an infected host facing impending death would increase its immediate reproductive effort to compensate for the loss of future opportunities to reproduce (Clutton-Brock et al 1984, Minchella 1985, and Parker et al 2011). This hypothesis can be sub-structured into testable predictions, such as

a. hosts infected with a lethal pathogen would exhibit increased reproductive effort compared to hosts infected with a pathogen that does not kill all of the infected individuals;
b. in case of pathogens for which all hosts do not succumb to infection, hosts that die due to infection would increase their reproductive effort compared to hosts that survive the infection; and,
c. among hosts that succumb to infection, there will be a negative correlation between reproductive effort and time of death.

Tests of theoretical predictions of change in investment towards reproduction, in response to various intrinsic and extrinsic variables, hinge on accurate estimation of reproductive effort (proportion of total available resources that is invested towards reproduction), which is often difficult to measure (Hirshfield and Tinkle 1975). Studies exploring infection induced changes in reproductive investment subvert this problem using uninfected controls. The uninfected controls represent an optimal reproductive output given a certain level of accessible resources and residual reproductive value. Resultantly in such studies a change in reproductive output in infected hosts compared to controls can be interpreted in light of the fecundity compensation/terminal investment theory (Clutton-Brock et al 1984, Minchella 1985, and Parker et al 2011). In this study I use '*standardised* reproductive output' (number of eggs, or progeny, normalised by the post-infection time-to-death of the females) as a proxy of

reproductive effort. The lifespan of infected females in our experiments vary greatly depending upon the pathogen used for infection (figure 3.1.A), and thus a direct comparison of absolute egg or progeny count is not suitable.

Briefly, in this study I investigated how infection with three entomopathogenic bacteria, which differ from one another with respect to the level of mortality imposed on the host, affect the reproductive output of female *Drosophila melanogaster*. Additionally, I explored the effect of *maternal* infection on pre-adult viability and early-life fecundity of the progeny. I further investigated if infection outcome (death vs. survival), and the time of death, differentially affected the reproductive output of individual infected females. Our key findings are as follows:

The effect of infection on mean reproductive output is pathogen dependent (figures 3.1.B-C). Females infected with *Serratia marcescens* (hereafter *Sm*) produce a greater number of eggs (and progeny) compared to uninfected control females, after accounting for differences in post-infection lifespan. Females infected with *Bacillus thuringiensis* (hereafter *Bt*) or *Pseudomonas aeruginosa* (hereafter *Pa*) have reproductive output similar to controls.

The effect of *maternal* infection on progeny life-history is different for each trait measured. Progeny pre-adult viability was reduced by infection with all three pathogens, with the greatest reduction seen in progeny of *Pa*-infected females (figure 3.1.D). On the other hand, progeny early-life fecundity was compromised only in case of progeny of *Sm*-infected females; progeny of *Bt*- and *Pa*-infected females had fecundity comparable to progeny of uninfected females (figure 3.2).

Females that succumb to infection exhibit greater variability in reproductive output, compared to control females and females that survive the infection, irrespective of the pathogen used for infection (figures 3.3.B, 3.3.E, and 3.3.H). This variability in reproductive output is not explained by time of death in *Pa*- and *Sm*-infected female (figures 3.3.F and 3.3.I); for *Bt*-

infected females there is a negative correlation between time of death and *standardised* reproductive output, but with a very low effect size (figure 3.3.C). Females that survive the infection have reproductive output comparable to controls in terms of both mean and variance (comparison possible for *Bt*-infected females only).

Forbes (1993) classified host-pathogen systems based on whether acute infection had any negative effect on current reproduction (possibly due to somatic damage to the reproductive tissue or leeching of resources) and future reproductive potential (brought about by host death or permanent somatic damage) of the host. Increased reproductive output is the predicted outcome only if future reproductive potential is compromised, but without any negative effect on current reproduction (Forbes 1993). An observed reduction in reproductive output during acute infection can thus be because of (a) pathogen leeching resources from the host or manipulating host physiology to reduce fecundity (Hurd 2001), (b) damage to reproductive tissue by the pathogen (Brandt and Schneider 2007) or by the host immune defence itself (Sadd and Siva-Jothy 2007), or (c) rerouting of resources meant for reproduction towards immune defence by the host (McKean et al 2008); although such reallocation of resources in either direction may not always translate into greater fitness benefits (Javois 2013).

Amongst the three pathogens used in this study, infection with two (*Pa* and *Sm*), is absolutely lethal, while about half of females infected with *Bt* survive acute infection (figure 3.1.A). Therefore, *Sm*- and *Pa*-infected females have zero future reproductive potential, while *Bt*-infected females can continue to reproduce post-recovery, assuming that there is no lingering somatic damage. *Drosophila melanogaster* flies never clear out infecting pathogens from their system completely (Duneau et al 2017), and a chronic, low level of pathogens continue to persist with in the fly body, which requires some investment into immune function on part of the host to keep in check (Chambers et al 2019). It is therefore a possibility that *Bt*-infected females may never regain uninfected levels of reproduction even post-recovery, but given that

Bt-infected females that survive the infection continue to reproduce at levels of control females even during acute infection period (figure 3.3.B), this is an unlikely possibility. *Bt*-infected females should therefore invest towards immune defence and not towards increasing immediate reproductive output, to maximise chances of survival and opportunity of future reproduction, as I see in the results from our experiment (figure 3.1.B and 3.1.C).

Based on the arguments outlined above it is expected that females would increase their reproductive effort after being infected with *Sm* and *Pa*, but I observe an increase in reproductive output only in case of *Sm*-infected females (figure 3.1.B and 3.1.C). The absence of any change in reproductive output of *Pa*-infected females may be driven by many possible reasons, including damage to reproductive tissue, and exploitation or manipulation of host by the pathogen; I rule out resource reallocation driven costs since I have argued above that when infection guarantees lethality, investment away from reproduction is counter-productive. Since I directly did not measure damage to reproductive tissue, I cannot choose with sanguinity between the different possibilities listed above based on the data at hand.

Progeny of infected females, independent of the infecting pathogen suffered from reduced pre-adult viability; progeny of *Pa*-infected females exhibited the greatest reduction (figure 3.1.D). Perrin et al (1996) have proposed that when *maternal* infection compromises progeny viability, increasing progeny production is not a suitable strategy for an infected host; this may be another explanation for why *Pa*-infected females do not increase reproductive output despite of guaranteed lethality due to infection. Reduced viability of progeny of Pa-infected females have been reported in other previous studies (viz. Ye et al 2009; but see Hudson et al 2019). Reduced progeny viability can lead to a progeny quantity vs. progeny quality trade-off (Stearns 1989), making investment into progeny quality, instead of increasing progeny number, a potential strategy for *Pa*-infected females.

Infection with *Pa* has been previously demonstrated to both increase (Hudson et al 2019) and suppress (Linder and Promislow 2009) reproduction in females. A host's response to the same pathogen can change because of the route of infection (Matins et al 2013, Gupta et al 2017), which can be a possible reason behind different observations in different studies: Hudson et al (2019) infected flies via oral route, while in this study and in that of Linder and Promislow (2009) flies were infected via septic injury to the thorax. A systemic infection is more likely to reach the reproductive tissues, via the haemolymph, than an oral infection, which first has to colonise the gut and breach the gut lining to enter into circulation.

Infected females that died of infection, irrespective of the pathogen used for infection, exhibited greater inter-individual variability in reproductive output compared to control (sham-infected) females and females that survived the infection (figures 3.3.B, 3.3E, and 3.3.H). The observed difference in the mean reproductive outputs of the infected and control females remained consistent across both experiments, suggesting that pathogen identity is a reliable predictor of post-infection reproductive output at the population level. Females that survived the infection with *Bt* had reproductive output similar to that of controls (figure 3.3.B), in terms of both population mean and inter-individual variability, suggesting that infected females may be able to judge their own prognosis and invest into reproduction accordingly. Since no females infected with either *Pa* or *Sm* survived the infection, I cannot conclude if this observation is generalizable for all other pathogens for which mortality is less than hundred percent. What seems puzzling therefore is why don't females that succumb to infection, irrespective of pathogen identity, increase their reproductive output?

Based on the earlier discussion, individual females that succumb to infection are expected to increase their reproductive output. This increase should happen irrespective of pathogen identity, driven only by the risk of mortality, except in a case where infection compromises current reproductive capacity. Contrary to this expectation, I see that the reproductive output

of infected-dead females for each pathogen ranges from zero to extremely high values; with some females reproducing less compared to the controls and other reproducing far more in excess (figures 3.3.B, 3.3E, and 3.3.H). Therefore, in canonical sense, I see some females exhibiting 'cost of immunity' while other females exhibiting 'fecundity compensation' when infected with the very same pathogen. This variation in reproductive output seems to be independent of both pathogen identity and time of death.

The observed inter-individual variability in reproductive output of females that die of infection may purely be stochastic, without any consequence in terms of evolutionary outcomes (Steiner and Tuljapurkar 2012). Alternatively, the heterogeneity may reflect variation in individual female quality (Wilson and Nussey 2010) and physiological state (McNamara and Houston 1996). The physiological state of an individual is a potent predictor of its residual reproductive value, and all else being equal, can therefore influence infection-induced changes in reproductive effort (Duffield et al 2012). A third possibility is that the heterogeneity is a consequence of host variation, genetic or otherwise, in response to infection, in terms of both resistance and tolerance (Raberg et al 2009, Vale and Little 2012, Parker et al 2014, Kutzer et al 2018). Further empirical exploration is necessary to disentangle these potential causes of inter-individual variation.

To summarize, I find that lethal infections do not always induce an increased investment towards immediate reproduction in female *Drosophila melanogaster*; females infected with only one out of two pathogens that imposed hundred percent mortality increased their reproductive effort. Furthermore, females dying of infection do not have greater reproductive effort compared to females that survive the infection, and reproductive effort had a negative correlation with time of death in case only one out of three pathogens used in this study. These

findings suggest that the mechanistic interaction between a host and a pathogen has a greater influence on host reproductive effort, compared to infection status, infection outcome, and mortality risk on the host by the pathogen. Additionally, my results suggest that pathogen identity is a reliable predictor of bacterial infection induced change in reproductive effort of the females at the level of population means, but pathogen identity does not predict reproductive output of individual females. Females infected with all three pathogens used in this study have overlapping range of reproductive output. Furthermore, maternal infection can affect progeny life-history traits, but the effect is specific to individual traits. In conclusion, dichotomy of 'cost of immunity' versus 'fecundity compensation' is too narrow in scope to account for all nuances involved in post-infection change in reproductive effort.

Tables

Table 3.1. Post-hoc pairwise comparisons using Tukey's HSD for significant effects reported for fixed factors in various type-III ANOVA reported in the 'Results' section.

Pairwise comparison	Estimate	SE	DF	t-ratio	p-value
(A) Effect of infection treatment on standardized egg count					
Uninfected - Bt	-0.14222	0.0757	151	-1.880	0.3327
Uninfected - Pa	-0.02071	0.0757	151	-0.274	0.9988
Uninfected - Sham	-0.01165	0.0757	151	-0.154	0.9999
Uninfected - Sm	**-0.93912**	**0.0757**	**151**	**-12.412**	**<.0001**
Bt - Pa	0.12151	0.0757	151	1.606	0.4960
Bt - Sham	0.13057	0.0757	151	1.726	0.4214
Bt - Sm	**-0.79690**	**0.0757**	**151**	**-10.532**	**<.0001**
Pa - Sham	0.00906	0.0757	151	0.120	1.0000
Pa - Sm	**-0.91841**	**0.0757**	**151**	**-12.138**	**<.0001**
Sham - Sm	**-0.92747**	**0.0757**	**151**	**-12.258**	**<.0001**
(B) Effect of infection treatment on standardized progeny count					
Uninfected - Bt	-0.0749	0.0614	151	-1.220	0.7399
Uninfected - Pa	0.0439	0.0614	151	0.715	0.9528
Uninfected - Sham	0.0108	0.0614	151	0.175	0.9998
Uninfected - Sm	**-0.7483**	**0.0614**	**151**	**-12.193**	**<.0001**
Bt - Pa	0.1187	0.0614	151	1.935	0.3035
Bt - Sham	0.0856	0.0614	151	1.395	0.6318
Bt - Sm	**-0.6734**	**0.0614**	**151**	**-10.973**	**<.0001**
Pa - Sham	-0.0331	0.0614	151	-0.540	0.9830
Pa - Sm	**-0.7922**	**0.0614**	**151**	**-12.908**	**<.0001**
Sham - Sm	**-0.7591**	**0.0614**	**151**	**-12.368**	**<.0001**
(C) Effect of *maternal* infection treatment on progeny pre-adult viability					
Uninfected - Bt	**0.059137**	**0.0174**	**151**	**3.403**	**0.0075**
Uninfected - Pa	**0.088922**	**0.0174**	**151**	**5.117**	**<.0001**
Uninfected - Sham	0.026788	0.0174	151	1.542	0.5372
Uninfected - Sm	**0.059889**	**0.0174**	**151**	**3.446**	**0.0065**
Bt - Pa	0.029786	0.0174	151	1.714	0.4285
Bt - Sham	-0.032349	0.0174	151	-1.862	0.3426

Bt - Sm	0.000752	0.0174	151	0.043	1.0000
Pa - Sham	**-0.062134**	**0.0174**	**151**	**-3.576**	**0.0042**
Pa - Sm	-0.029033	0.0174	151	-1.671	0.4551
Sham - Sm	0.033101	0.0174	151	1.905	0.3192
(D) Effect of *maternal* infection treatment on progeny early-life fecundity					
Uninfected - Bt	0.4849	0.267	902	1.817	0.3643
Uninfected - Pa	0.2900	0.267	902	1.086	0.8136
Uninfected - Sm	**2.5128**	**0.267**	**902**	**9.415**	**<.0001**
Uninfected - Sham	0.0146	0.267	902	0.055	1.0000
Bt - Pa	-0.1950	0.267	902	-0.730	0.9494
Bt - Sm	**2.0278**	**0.267**	**902**	**7.598**	**<.0001**
Bt - Sham	-0.4703	0.267	902	-1.762	0.3965
Pa - Sm	**2.2228**	**0.267**	**902**	**8.328**	**<.0001**
Pa - Sham	-0.2754	0.267	902	-1.032	0.8407
Sm - Sham	**-2.4982**	**0.267**	**902**	**-9.360**	**<.0001**

4. Resource limitation and resistance to bacterial infections

Introduction

Maintaining a functional immune system and successfully deploying it in event of a pathogen challenge comes at considerable cost to the host organism in terms of energy and resources (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Schmid-Hempel 2003). Immune function has thus been canonically considered to be contingent upon hosts' access to resources, and physiological trade-offs resulting from allocation of limiting resources among different organismal functions (Schmid-Hempel 2005, McKean and Lazzaro 2011). Based on these assumptions suppression of immune system is predicted to occur under stressful conditions, such as reduced access to nutrition (Sandland and Minchella 2003). But recent studies point towards a picture more complex than a simple case of immune suppression in low resource environments (reviewed in Adamo 2017, Cotter and Al Shareefi 2022).

A resource limited host may not exhibit an increased susceptibility to infections on every occasion because of multiple possible reasons. Even when trade-offs do exist, resource allocation priorities can change depending upon the availability of resources (Ng'oma et al 2017). When under duress, it may be advantageous for an organism to invest into somatic defence (including immune function), thereby prolonging life-span, rather than investing towards other faculties such as reproduction. Additionally, instead of a global downregulation of the immune system, resource deprivation can induce a restructuring of the immune network to a new stable state that helps maximise immune defence under resource limited conditions (Adamo et al 2016, Adamo 2021). Such restructuring, in principle, can lead to pathogen

specific infection outcomes. Furthermore, because the pathogen is also dependent on the host for resources for its own proliferation, reducing host access to resources can also affect within host pathogen growth, thereby influencing infection outcome (Pike et al 2019). Reduced uptake of nutrition can often – but not always – be beneficial for infected hosts (Hite et al 2020), and infected hosts do also modify their diet to suit immediate energetic requirements (Abbott 2014).

The effect of resource limitation on insect immune function can be dependent on both the severity of limitation (ranging from dilution of nutrition to complete starvation) and on the specific nutritional component missing from the diet (Adamo et al 2016, Ponton et al 2013). Physiological consequences of starvation in *Drosophila melanogaster* have been studied in great detail from the viewpoint of stress resistance and life-history processes (reviewed in Prasad and Joshi 2003, Rion and Kawecki 2007), but the effect of starvation on immune function is relatively less well studied. *Relish* deficient flies survive better following infection with *Escherichia coli* and *Erwinia carotovora* (but not when infected with *Enterococcus faecalis*) when subjected to a short period of starvation before infection (Brown et al 2009). Negative effect of starvation on immune function has been reported in other insects (Brown et al 2000, Siva-Jothy and Thompson 2002), but these negative consequences of starvation are specific to only certain components of the immune system and are reversible if the insects are allowed to feed again (Siva-Jothy and Thompson 2002).

Effects of poor nutrition in *D. melanogaster* have primarily been investigated via experiments where the diet of the flies is restricted to low levels of nutrition or a particular ingredient (protein, carbohydrate, etc.) is limiting in the diet. Flies subjected to dietary restriction exhibit increased survival when infected with *Lactococcus lactis* and *Pseudomonas aeruginosa* (Burger et al 2007), and *Salmonella typhimurium* (Ayres and Schneider 2009), but decreased survival when infected with *Listeria monocytogenes* (Ayres and Schneider 2009). Defence against *E. faecalis* is unaffected by dietary restriction (Ayres and Schneider 2009). Flies on low

protein diets have increased susceptibility to infection by *Pseudomonas entomophila* (Kutzer et al 2018) but exhibited reduced mortality when infected with *P. aeruginosa* and *Staphylococcus aureus* (Lee et al 2017). Additionally, flies on a diet with low protein-to-carbohydrate ratio survive better when infected with *Micrococcus luteus* compared to flies on a normal diet (Ponton et al 2020). In case of both dietary restriction and low protein diets the effect of experimental manipulation is pathogen specific.

The effect of resource limitation on post-infection fitness of the host may be mediated via its effect on within-host pathogen levels. Since pathogens are dependent on the host for acquiring resources necessary for proliferation, a resource limited host also logically implies a resource limited pathogen (Pike et al 2019, Cressler et al 2014). Accordingly, within-host sporulation of the microsporidian parasite *Vavraia culisis* increases with increasing access to nutrition of its host *Aedes aegypti* (Bedhomme et al 2004). In *D. melanogaster*, a high carbohydrate diet similarly increases within-host levels of *Providencia rettgeri* (Unckless et al 2015). On the other hand, low protein diets lead to greater pathogen burden for *E. coli* and *Lactococcus lactis* (Kutzer and Armitage 201), but lower pathogen load in case of *P. aeruginosa* and *S. aureus* (Lee et al 2017) in flies. *Relish* deficient flies, when subjected to starvation before infection, carry low levels of pathogen burden when infected with *E. coli* and *E. carotovora* (Brown et al 2009). The effect of limited nutrition on within-host pathogen levels is also apparently mixed, and pathogen specific. Therefore, while reduced access to resources/nutrition can compromise a host's ability to mount an immune response, it can also compromise the pathogens capacity to proliferate, making the outcome of infection (in terms of host survival) dependent on whether the host immune system or the pathogen's capacity to proliferate is more affected by lack of resources (Pike et al 2019, Cressler et al 2014).

Sexual activity (mating) leads to increased susceptibility to bacterial infections in *D. melanogaster* females, and mated females also carry a greater systemic pathogen burden

(Fedorka et al 2007, Short and Lazzaro 2010, Imroze and Prasad 2011, Short et al 2012). This post-mating immune-suppression is part of reproduction-immunity trade-off observed in many insects and other invertebrates (Lawniczak et al 2007, Schwenke et al 201)] although not in all insects (Oku et al 2019). Post-mating immune suppression is driven by reallocation of resources away from somatic defence and towards reproduction, primarily production of eggs. Consequently, females lacking a functioning germline do not exhibit post-mating immune suppression (Short et al 2012). Different components of the male seminal fluid (sperms and accessory gland proteins, especially sex peptide) play an important role in suppressing female immunity (Short et al 2012). Sex peptide transferred by males during mating increases synthesis of Juvenile Hormone in females, which reduces a female's ability to mount an immune response against bacterial infection, leading to greater post-infection mortality (Schwenke and Lazzaro 2017). In *D. melanogaster*, Juvenile Hormone dictates investment towards reproduction (Flatt et al 2005) and also suppresses expression of anti-microbial peptide genes (Flatt et al 2008). Mating can also slow down translation, leading to a delay in mounting of an immune response against bacterial pathogens (Gupta et al 2021). All together these suggest that mating induced immune suppression in flies can be used as a stand-in model for a resource deprived immune system.

In the experiments reported here, I explored how starvation and sexual activity (mating) – individually or in concert – affect post-infection survival of female *D. melanogaster* flies when challenged with five different bacterial pathogens (three Gram-negative bacteria: *Providencia rettgeri*, *Pseudomonas entomophila*, *Erwinia c. carotovora*; and two Gram-positive bacteria: *Enterococcus faecalis* and *Staphylococcus succinus*). I also quantified the within-host levels of bacteria following infection to test for the effect of resource limitation on systemic pathogen levels. Based on previously published results, both theoretical and empirical (as described above), I predicted that while both starvation and mating will increase post-infection mortality,

mating will increase bacterial levels within the host, but starvation will reduce the same. The results suggest that starvation and mating can both compromise post-infection survival of the host and encourage within-host pathogen proliferation, albeit in a pathogen dependent manner.

Materials and methods

Study system

Flies from the LH baseline population were used in the experiments described in this chapter. Please refer to chapter 2 for a detailed description of maintenance of the LH baseline population.

Derivation of experimental flies

2-3 day old adults were transferred to plexiglass cages (14 cm length × 16 cm width × 13 cm height) at a density of 1000-1200 flies, and the cages are provided with standard food medium in 60 mm Petri plates. For collection of eggs for setting up experiments, cages are provided with a fresh food plate, supplemented with *ad libitum* live yeast supplement, for 48 hours. This is done to encourage egg production and laying in the females. Following this, a fresh food plate is provided to the cages and 12-14 hours later eggs are collected off these plates (using moist paint brushes on 1.5% agar gel) and seeded into food vials (with 8-10 ml of food medium) at an exact density of 150 eggs per vial. The number of vials set up in this manner depends upon the requirement for a particular experiment. These vials are then incubated under standard conditions (detailed above) for egg to mature into larvae and then into adults. On 10^{th} day after egg laying, during the eclosion peak, adults are collected as virgins within 5-6 hours of eclosion, and housed in single-sex vials (each with 1-2 ml of food medium) at constant density of 8 females per vial or 10 males per vial. Flies are housed in these vials till further manipulation/experimentation.

Pathogens

Five pathogens were used in total across the two experiments reported in this chapter:

a. *Enterococcus faecalis* (Lazzaro et al 2006),

b. *Erwinia carotovora carotovora*, strain Ecc15 (Martins et al 2013),

c. *Providencia rettgeri* (Short and Lazzaro 2010),

d. *Pseudomonas entomophila*, strain L48 (Vodovar et al 2005, Mullet et al 2012), and,

e. *Staphylococcus succinus*, strain PK-1 (Singh et al 2016).

Systemic bacterial load estimation. To measure the systemic bacterial load**,** infected females are first surface sterilised using 70% ethanol for 1 minute and 30 seconds, twice. Females are then washed in sterile distilled water for 30 seconds and dried using autoclaved tissue paper. Females are then transferred individually to 1.5 ml vials (micro-centrifuge tubes) containing 50 or 75 microliters (depending upon pathogen used for infection) of sterile $MgSO_4$ (10 mM) buffer. Females are homogenised in these vials using a motorised pestle for 50-60 seconds. This homogenate is serially diluted (1:10 dilutions) 8 times in sterile $MgSO_4$ (10 mM) buffer. 10 microliters from each dilution, and the original homogenate, are spotted onto a lysogeny agar plate (2% agar, Luria Bertani Broth, Miler, HiMedia). The plates are incubated at required temperature for 8-12 hours (depending upon pathogen used for infection), and the number of colony forming units (CFUs) in each dilution is counted. The number of CFUs in the *countable* dilution ($30 \leq CFUs \geq 300$) is multiplied by appropriate dilution factor to obtain the bacterial load for each individual female.

Experiment design

Experiment 1. Effect of starvation and sexual activity on post-infection survival of females.

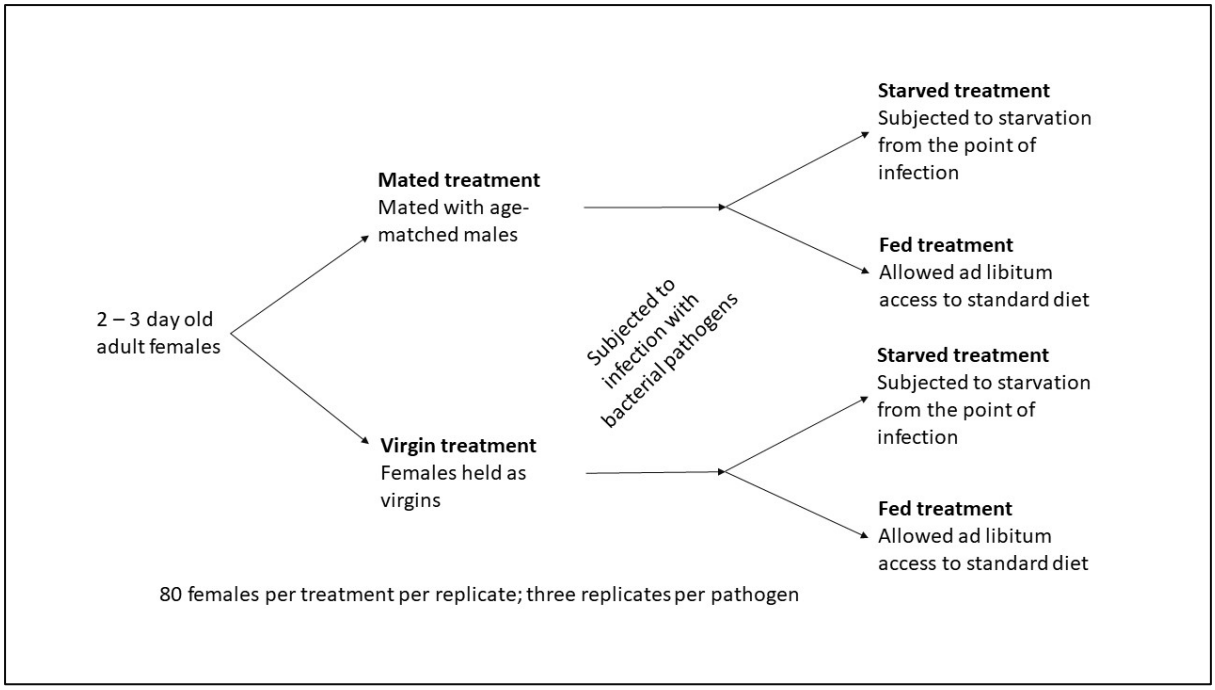
Virgin females and males were obtained following the protocol described above. On day 12 after egg laying, half of the females were randomly assigned to ‘virgin’ treatment and the rest to ‘mated’ treatment. Females in the ‘mated’ treatment were combined with males in fresh food vials (1-2 ml standard food medium) in groups of 8 females and 10 males per vial, and allowed

to mate for 4 hours (it was visually confirmed that each female had mated at least once). Following this, the females were lightly anaesthetised and infected with bacterial pathogens (or sham-infected) following the infection protocol described above; males were discarded. Females from the 'virgin' treatment were similarly infected (or sham-infected). Following infections, half of the females from both these treatments were housed in vials with 1-2 ml of standard food medium ('fed' treatment), and the remaining were housed in vials with 1-2 ml 2% non-nutritive agar gel ('starved' treatment). This produced four experimental treatments:

a. Virgin, Fed (VF): 10 vials of infected flies and 5 vials of sham-infected females, each vial with 8 females;
b. Virgin, Starved (VS): 10 vials of infected flies and 5 vials of sham-infected females, each vial with 8 females;
c. Mated, Fed (MF): 10 vials of infected flies and 5 vials of sham-infected females, each vial with 8 females; and,
d. Mated, Starved (MS): 10 vials of infected flies and 5 vials of sham-infected females, each vial with 8 females.

Note that in this experiment females were subjected to starvation from the time of infection. The vials were monitored for mortality every 4-6 hours, for 96 hours post-infection (HPI); alive flies were shifted to fresh food/agar vials at 48 HPI. This experiment was carried out for five bacterial pathogens – *E. faecalis*, *E. c. carotovora*, *P. rettgeri*, *P. entomophila*, and *S. succinus* – and replicated thrice for each pathogen. In each replicate, 320 females were subjected to infection (80 females x 4 treatments) and 160 females were subjected to sham-infection (80 females x 4 treatments).

Figure 4.0. Schematic depicting the experimental design for experiment 1. Experiment 2 followed a similar design, with the only difference being post-infection survival was measured in experiment 1, while within-host bacterial loads were quantified in experiment 2.



Experiment 2. Effect of starvation and sexual activity on systemic bacterial load in infected females.

Following a protocol identical to that of Experiment 1, females were distributed into four treatments (VF, VS, MF, and MS, as described above) and infected; 100 infected females in each treatment and 30 sham-infected females in each treatment. Following infections, females were housed in plexiglass cages (14 cm length × 16 cm width × 13 cm height), with all females from a particular treatment in a single cage; infected and sham-infected females were housed in separate cages. (Cages of 'fed' treatments were supplied with standard food medium in 60 mm Petri plates and cages of 'starved' treatments were supplied with 2% non-nutritive agar gel in 60 mm Petri plates.) At 4 and 10 HPI, 12 females were randomly aspirated out of cages for each treatment (for infected flies only) and the systemic bacterial load was measured for individual females following the CFU enumeration protocol described above. This experiment was carried out for three bacterial pathogens – *E. faecalis*, *P. rettgeri*, and *P. entomophila* – and replicated thrice for each pathogen. In each replicate, systemic bacterial load was measured for 72 individual females (12 females x 2 time-points x 4 treatments).

Statistical analysis

Post-infection survival data from experiment 1 was analysed using a mixed-effects Cox proportional hazards model, including 'treatments' as a fixed factor and 'replicate' as a random factor. Since sham-infected females exhibited negligible mortality (supplementary figure S3.1), survival data from only the infected females were included for analysis. Systemic bacterial load data from experiment 2 was analysed using type III analysis of variance (ANOVA) on log (base 2) transformed data, including 'treatment', 'HPI', and 'treatment × HPI' interaction as fixed factors, and 'replicate' as random factor. Post-hoc analysis for pairwise comparison was carried out using Tukey's HSD method.

Results

Effect of starvation and sexual activity on post-infection survival of females

In this experiment, I measured the effect of starvation and sexual activity – individually and in concert – on survival of female *Drosophila melanogaster* when infected with different entomopathogenic bacteria. Briefly, females were distributed into four treatments: virgin-fed (VF), virgin-starved (VS), mated-fed (MF), and mated-starved (MS); VS, MF, and MS represent the three experimental treatments where host's access to resources has been limited/manipulated. The five pathogens used for infection were *Enterococcus faecalis*, *Erwinia c. carotovora*, *Providencia rettgeri*, *Pseudomonas entomophila*, and *Staphylococcus succinus*.

When infected with *P. rettgeri*, all three resource limited treatments – VS (hazard ratio, 95% confidence interval: 4.799, 3.173-7.258), MF (HR, 95% CI: 1.81, 1.140-2.875), and MS (HR, 95% CI: 9.329, 6.242-13.941) females – exhibited decrease in survival compared to VF females (figure 4.1.A). Females from these three treatments also differed from one another in terms of post-infection survival, with MS females exhibiting greatest mortality, followed by VS and MF (figure 4.2, table 4.1.A).

Upon infection with *P. entomophila*, all three resource limited treatments – VS (HR, 95% CI: 1.431, 1.172-1.747), MF (HR, 95% CI: 1.822, 1.491-2.228), and MS (HR, 95% CI: 1.449, 1.184-1.774) females – exhibited decrease in survival compared to VF females (figure 4.1.B), but these treatments did not differ from one another in terms of post-infection survival (figure 4.2, table 4.1.B).

Following infection with *E. c. carotovora*, all three resource limited treatments – VS (HR, 95% CI: 1.667, 1.324-2.099), MF (HR, 95% CI: 1.873, 1.490-2.354), and MS (HR, 95% CI: 2.548, 2.044-3.176) females – exhibited decrease in survival compared to VF females (figure 4.1.C).

Additionally, MS females exhibited a significant greater mortality compared to VS and MF females, who did not differ from one another in terms of post-infection mortality (figure 4.2, table 4.1.C).

Following infection with *E. faecalis*, only MF (HR, 95% CI: 1.559, 1.253-1.940) females exhibited a reduction in post-infection survival compared to VF females; both VS (HR, 95% CI: 1.219, 0.978-1.518) and MS (HR, 95% CI: 1.223, 0.975-1.535) females exhibited survival similar to that of VF females (figure 4.1.D). Furthermore, there was no significant difference in mortality between MF, VS, and MS females (figure 4.2, table 4.1.D).

Upon being infected with *S. succinus*, all three resource limited treatments – VS (HR, 95% CI: 1.478, 1.633-1.879), MF (HR, 95% CI: 1.572, 1.246-1.982), and MS (HR, 95% CI: 1.474, 1.160-1.874) females – exhibited decrease in survival compared to VF females (figure 4.1.E), but these three treatments did not differ from one another in terms of post-infection survival (figure 4.2, table 4.1.E).

Figure 4.1. Survival of females from different experimental treatments after being subjected to infection with bacterial pathogens. Survival curves plotted using Kaplan-Meier method after pooling data from all three experimental replicates for each pathogen. **(A)** *Providencia rettgeri*. **(B)** *Pseudomonas entomophila*. **(C)** *Erwinia c. carotovora*. **(D)** *Enterococcus faecalis*. **(E)** *Staphylococcus succinus*. For a comparison of survival of infected and sham-infected females, please refer to figure S4.1.

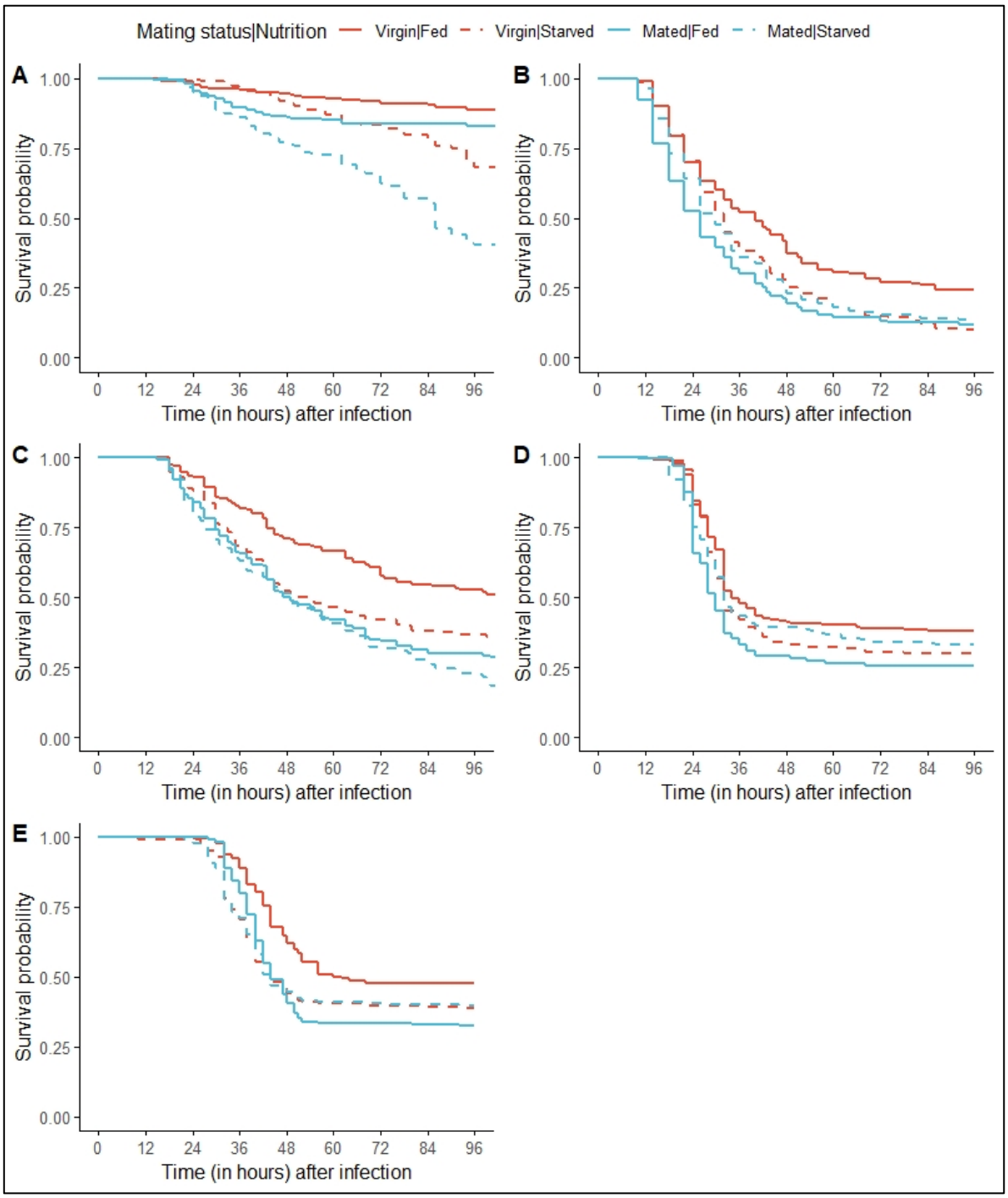
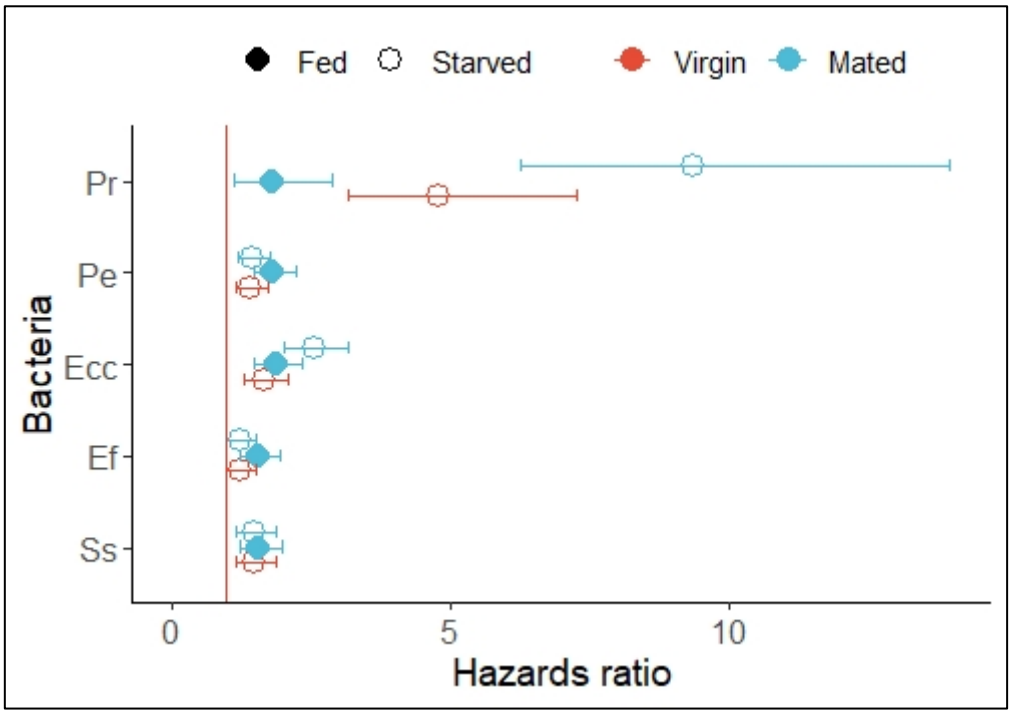


Figure 4.2. Hazard ratio (± 95% confidence intervals) of infected females from different experimental treatments, with respect to the '*virgin, fed*' treatment (represented by the red vertical line), across different bacterial pathogens. Hazard ratios calculated using a mixed-effect Cox proportional hazards model.



Effect of starvation and sexual activity on systemic bacterial load in infected females.

In this experiment, using an experimental set-up identical to the first experiment, I measured systemic bacterial load within infected female *Drosophila melanogaster*. Measurement of within-host bacterial load was carried out at two time points – at four and ten hours-post-infection (HPI) – following infection of females separately with three pathogenic bacteria: *Enterococcus faecalis*, *Providencia rettgeri*, and *Pseudomonas entomophila*.

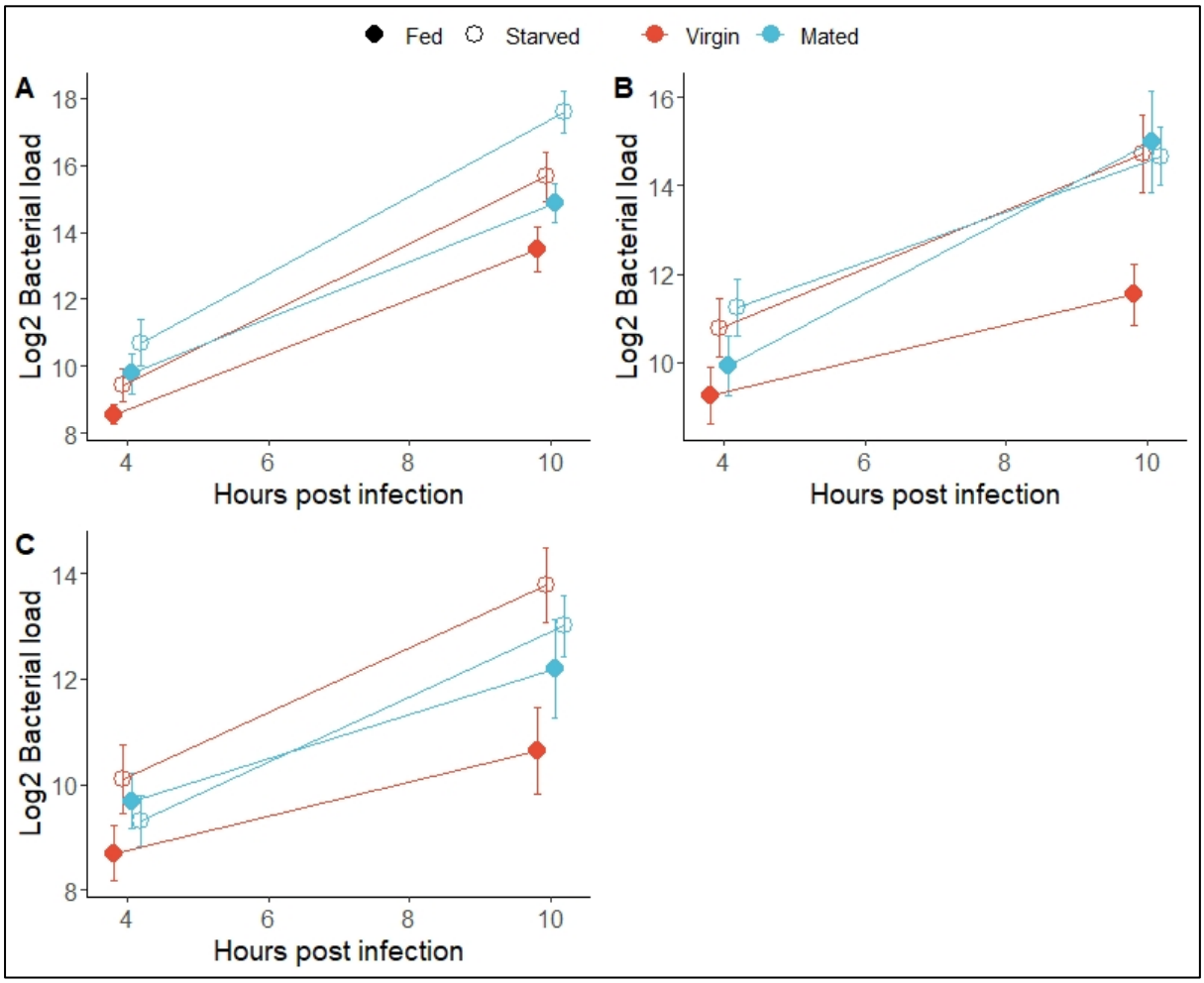
For females infected with *P. rettgeri*, systemic bacterial load was affected by treatment ($F_{3,285}$: 38.93, $p < 2.2e-16$), time post-infection (HPI; $F_{1,285}$: 789.48, $p < 2.2e-16$), and treatment × HPI interaction ($F_{3,285}$: 5.33, $p = 0.0014$) (figure 4.3.A). In terms of total bacterial load, VS (LS mean, 95% CI: 12.5, 12.1-13.0), MF (LS mean, 95% CI: 12.3, 11.9-12.8), and MS (LS mean, 95% CI: 14.1, 13.7-14.6) females carried significantly greater bacterial load compared to VF (LS mean, 95% CI: 11.0, 10.6-11.5) females (post-hoc analysis using Tukey's HSD; table 4.2.A). MS females also carried a significantly greater load compared to MF and VS females, which did not differ from one another (post-hoc analysis using Tukey's HSD; table 4.2.A). At 4 HPI, only the differences between VF and MS females, and VS and MS females, were significant (post-hoc analysis using Tukey's HSD; table 4.3.A); at 10 HPI, all pair-wise differences were statistically significant, except the difference between VS and MF females (post-hoc analysis using Tukey's HSD; table 4.3.A). Within each treatment, bacterial load always increased significantly with HPI (post-hoc analysis using Tukey's HSD; table 4.3.A).

For females infected with *P. entomophila*, systemic bacterial load was affected by treatment ($F_{3,285}$: 21.33, $p = 1.71e-12$), time post-infection (HPI; $F_{1,285}$: 206.31, $p < 2.2e-16$), and treatment × HPI interaction ($F_{3,285}$: 5.08, $p = 0.0019$) (figure 4.3.B). In terms of total bacterial load, VS (LS mean, 95% CI: 12.7, 11.75-13.7), MF (LS mean, 95% CI: 12.5, 11.48-13.5), and MS (LS mean, 95% CI: 13.0, 11.96-13.9) females carried significantly greater bacterial load

compared to VF (LS mean, 95% CI: 10.4, 9.39-11.4) females (post-hoc analysis using Tukey's HSD; table 4.2.B); the pair-wise differences between VS, MF, and MS females were not statistically significant. At 4 HPI, only the difference between VF and MS females was significant (post-hoc analysis using Tukey's HSD; table 4.3.B); at 10 HPI, VS, MF, and MS females had similar levels of systemic bacteria, and all three had greater bacterial load compared to VF females (post-hoc analysis using Tukey's HSD; table 4.3.B). Within each treatment, bacterial load always increased significantly with HPI (post-hoc analysis using Tukey's HSD; table 4.3.B).

For females infected with *E. faecalis*, systemic bacterial load was affected by treatment ($F_{3,285}$: 16.87, p = 4.08e-10), time post-infection (HPI; $F_{1,285}$: 165.02, p < 2.2e-16), and treatment × HPI interaction ($F_{3,285}$: 3.62, p = 0.01369) (figure 4.3.C). In terms of total bacterial load, VS (LS mean, 95% CI: 11.95, 11.42-12.5), MF (LS mean, 95% CI: 10.95, 10.42-11.5), and MS (LS mean, 95% CI: 11.16, 10.64-11.7) females carried significantly greater bacterial load compared to VF (LS mean, 95% CI: 9.67, 9.14-10.2) females (post-hoc analysis using Tukey's HSD; table 4.2.C). VS females also had significantly greater bacterial load compared to MF females, but the difference between VS and MS females, and MF and MS females was not statistically significant (post-hoc analysis using Tukey's HSD; table 4.2.C). At 4 HPI, females from all four treatments carried similar bacterial load (post-hoc analysis using Tukey's HSD; table 4.3.C); at 10 HPI, all pair-wise differences were statistically significant, except the differences between VS and MS females, and MF and MS females (post-hoc analysis using Tukey's HSD; table 4.3.C). Within each treatment, bacterial load always increased significantly with HPI (post-hoc analysis using Tukey's HSD; table 4.3.C).

Figure 4.3. Systemic bacterial load (mean ± 95% confidence intervals) in infected females from different experimental treatments. Graphs plotted after pooling data from all three experimental replicates for each pathogen. **(A)** *Providencia rettgeri*. **(B)** *Pseudomonas entomophila*. **(C)** *Enterococcus faecalis*.



Discussion

In this chapter, I tested for the effect of starvation and sexual activity (mating), individually or in concert, on post-infection survival and within-host bacterial load of *Drosophila melanogaster* females when infected with bacterial pathogens. The *D. melanogaster* females were divided into four experimental treatments: (a) virgin, fed (VF): *ad libitum* access to resources and no mating; (b) virgin, starved (VS): no access to resources and no mating; (c) mated, fed (MF): *ad libitum* access to resources with mating; and, (d) mated, starved (MS): no access to resources, but with mating. The females were thereafter infected with five bacterial pathogens – *Providencia rettgeri*, *Pseudomonas entomophila*, *Erwinia c. carotovora*, *Enterococcus faecalis*, and *Staphylococcus succinus* – and their post-infection survival was recorded in the first experiment. In the second experiment I measured the within-host bacterial load in infected females, but only for three of the bacterial pathogens: *P. rettgeri*, *P. entomophila*, and *E. faecalis*.

Among the flies with *ad libitum* access to resources (comparison between VF and MF females), sexual activity increased post-infection mortality for all the pathogens used for infection, but the difference in mortality between the virgin and mated females was pathogen specific (figure 4.2). Pathogen-specific effect of sexual activity on female immune function has been recorded in previous studies (Short et al 2012). In case of the three pathogens for which systemic pathogen load was measured, the mated females also carried greater systemic pathogen load compared to virgin females (figure 4.3). There is a congruency in results from the two experiments, with increased bacterial levels in the system explaining the increased mortality of mated females. Mated females carrying greater pathogen load has also been reported in previous studies using *D. melanogaster* (Fedorka et al 2007, Short and Lazzaro 2010, Short et al 2012, Schwenke and Lazzaro 2017). Therefore, I conclude that sexual activity leads to

reduced host resistance, i.e., the ability of the host to control systemic pathogen proliferation (*sensu* Raberg et al 2009), leading to reduced post-infection survival.

Decrease in host resistance (or alternatively, excess proliferation of bacteria in host system) following sexual activity in *D. melanogaster* females may be due to multiple possible reasons. One, mating leads to increased egg production, and depletion of the female fat body to provide resources for the same. Fat body is the primary site for production of anti-microbial proteins required for defence against systemic bacterial infections. Anti-microbial proteins are the primary defence against a few of the pathogens tested here, such as, *P. rettgeri* (Hanson et al 2019), *P. entomophila* and *E. c. carotovora* (Vodovar et al 2005). Depletion of fat body can therefore lead to decreased resistance to bacterial infections. Two, mating has been shown to slow down translation of genes in fat body cells, leading to a reduced expression of antimicrobial proteins and other genes relevant for defence against bacterial pathogens, especially *P. rettgeri* (Gupta et al 2021). Three, mating can lead to reduced immune-surveillance, because of the dual role of proteins involved in lipid transport: when bound to lipid molecules, proteins such as apolipophorins cannot perform their designated role in pathogen recognition (Adamo et al 2008, Palm et al 2012). Four, transfer of lipid reserves from the fat body to ovaries also implies increase in circulating resources, readily accessible to the bacterial pathogen, which can help the pathogen proliferate faster in the body of a mated female (Herren et al 2014).

Among virgin females (comparison between VF and VS females), starvation increased mortality in case of four out of five pathogens used for infection (figure 4.2), *E. faecalis* being the only exception. Similar to the effect of sexual activity, the degree of change in post-infection mortality of females brought about by starvation was pathogen specific. Also, for all three pathogens for which systemic bacterial loads were measured, VS females carried greater bacterial load compared to VF females (figure 4.3), indicating a reduction of host resistance

(*sensu* Raberg et al 2009). Resource limitation can lead to restructuring of the immune system (Adamo et al 2016, Adamo 2021), which may lead to the effects of resource limitation on insect immune function being pathogen specific. This can explain the pathogen specific results in the experiments presented here. Additionally, the constitutive components of the insect immune system may be less affected by limitation of nutrition compared to the inducible components of the immune system (Adamo 2017). Phagocytosis, a primarily constitutive defence mechanism, is important for defence against *E. faecalis* (Nehme et al 2011) in *D. melanogaster*, while defence against *P. rettgeri* is primarily mediated by diptericin (Hanson et al 2019), an inducible antimicrobial peptide. This might explain why survival following infection with *E. faecalis* is unaffected by starvation, while starved females die more when infected with *P. rettgeri*. This line of reasoning can be extended to include the other two Gram-negative bacteria used in these experiments, *P. entomophila* and *E. c. carotovora*, since defence against these two bacteria is also mediated by inducible anti-microbial peptides in *D. melanogaster* (Vodovar et al 2005).

Interestingly, VS females carried significantly greater pathogen load compared to VF females when infected in *E. faecalis* (figure 4.3.C), although there was no significant difference in mortality between these two treatments (figures 4.1.D and 4.2). Differences in post-infection survival can be caused by change in host resistance or host tolerance (i.e., the ability of the host to deal with infection induced damage to the soma; *sensu* Raberg et al 2009). Single gene mutations in *D. melanogaster* can affect resistance and tolerance to bacterial infections independent of one another (Ayres and Schneider 2008), and manipulation of diet can have similar discordant effects on host resistance and tolerance in crickets (Stahlschmidt et al 2015), burying beetles (Clough et al 2016), and mice (Kuraishi et al 2013). Therefore, I propose that starvation probably leads to increased tolerance to bacterial infections in flies, especially in case of *E. faecalis* infections.

Production of reactive oxygen species is part of the *D. melanogaster* defence repertoire against various bacterial pathogens (Chakrabarti et al 2020), especially *E. faecalis*, which can lead to immunopathology (damage to the host tissue caused by the host immune system; *sensu* Sadd and Siva-Jothy 2006, Pursall and Rolf 2011). Starvation is known to induce increased production of antioxidants in Carob moth larvae (Farahani et al 2020). Therefore, it is possible that in *D. melanogaster* too, starvation might protect flies from harmful effects of reactive oxygen species, leading to increased tolerance and decreased immunopathology, culminating into improved post-infection survival even with high systemic pathogen loads. The possibility of disease tolerance being contingent upon availability of resources has also been suggested by various previous studies (reviewed in Budischak et al 2018).

There is a disagreement between results from post-infection survival assay and systemic pathogen load measurements in a few other instances. For example, VS females die more compared to MF females when infected with *P. rettgeri* even though they carry similar bacterial loads, and VS females die equally as that of MF females when infected with *E. faecalis* even though in this case VS females carry significantly greater pathogen load. Extending my previous argument, these observations can be explained if starvation had differential effects on resistance and tolerance to different pathogens used in these experiments. One must note that I did not measure bacterial load of dying flies, and therefore have no direct measure of disease tolerance, such as bacterial load upon death (BLUD, *sensu* Duneau et al 2017). My inference of differences in tolerance between treatments is based on discordance between observed systemic bacterial load and observed mortality rate (similar to Ayres and Schneider 2008).

An alternative to increase in host tolerance can be reduction in pathogen virulence. Lack of host nutrition has been shown to increase virulence in viral pathogens of vertebrates (Beck et al 2004). Virulence of a pathogen is a function of its ability to grow within the host (proliferation) and capacity to damage the host (pathogenicity; Wollein-Waldetoft et al 2020).

My results (figure 4.3) clearly show that pathogen proliferation increases when hosts are starved. There is no evidence in literature yet that pathogenicity of an entomopathogenic bacteria can be influenced by environmental factors experienced by the host. Therefore, *for the present discussion*, I discount the possibility that host-starvation induced increase in bacterial virulence explains my observations.

A meta-analysis by Pike and colleagues (2019) revealed that increasing hosts' access to nutrition increases within host pathogen fitness in invertebrates, including insects, suggesting that increased host nutrition fuels greater within-host pathogen proliferation. Previous results from studies on *D. melanogaster* show that the effect of manipulating host nutrition can both increase and decrease within-host pathogen levels depending upon pathogen identity (Brown et al 2009, Lee et al 2017, Unckless et al 2017, Kutzer and Armitage 2016). Results from my experiments show that for all three pathogens for which bacterial load measurements were made, starved females carried a greater pathogen load compared to females fed ad libitum (comparison of VS and MS females with VF females). Pathogen's access to resources for proliferation is limited by host's access to nutrition, but here we observe that starved hosts carry a greater bacterial load. This further suggests that the observed results are driven by starvation-induced reduction in host resistance, and additionally, for at least these three pathogens, limiting host resources does not have a negative effect on the pathogen's capacity to proliferate. My results align well with the predictions of Cressler and colleagues (Cressler et al 2014), who predicted that pathogens have a competitive advantage at low and intermediate resource levels, while the host immune system benefits when resources are abundant.

Lastly, my results suggest that although starvation and mating individually do lead to increased post-infection mortality and higher systemic bacterial loads, subjecting females to a dual treatment of starvation and mating does not significantly alter their immune phenotypes compared to single treatments: in case of all but two pathogens (*P. rettgeri* and *E. c.*

carotovora), MS females have similar survival and bacterial load as that of VS and MF females (figure 4.2 and 4.3; tables 4.1 and 4.2). I hypothesize that this is driven by the fact that *D. melanogaster* females stop egg production when subjected to starvation (Bownes et al 1988, Terashima et al 2004), making MS females physiologically similar to VS females. Previous studies have shown that *D. melanogaster* females lacking a germline do not exhibit post-mating immune suppression (Short et al 2012; but see Fedorka et al 2007), and such females also respond differently in terms of gene expression patterns (compared to females with a functioning germline) when subjected to either mating or immune challenge (Short and Lazzaro 2013, Rodrigues et al 2021). Starvation induced suspension of reproduction can therefore alleviate the negative effect of mating on female immune function, but in a pathogen specific manner.

To summarize, in the present study I focused on how different modes of resource limitation – starvation (global unavailability of resources) and sexual activity (reallocation of resources away from somatic defence and into reproduction) – affect infection outcome (survival) and within-host pathogen levels in *Drosophila melanogaster* females. Results show that mated (sexually active) females have reduced resistance to bacterial infection, which manifests as increased post-infection mortality. Starvation can also lead to reduced resistance, but conditional upon the mating status of the female fly. Additionally, starvation can increase tolerance to bacterial infection, in a pathogen dependent manner. Therefore, my results suggest that the lack of resources to the immune system, whether it is because of unavailability of nutrition or because of reallocation of resources away from the immune system, can compromise host's ability to resist systemic pathogen proliferation, but the ultimate infection outcome also depends upon the change in host's tolerance to infection brought about by resource limitation.

Tables

Table 4.1. Pair-wise comparison between post-infection survival of females from all four treatments infected with different bacterial pathogens using Log-rank analysis, with p-values adjusted for multiple comparisons using the Benjamini-Hochberg method.

	Virgin, Fed	Virgin, Starved	Mated, Fed	Mated, Starved
a. Providencia rettgeri				
Virgin, Fed	-			
Virgin, Starved	**2.5e-16**	-		
Mated, Fed	**0.013**	**4.9e-09**	-	
Mated, Starved	**< 2e-16**	**5.3e-09**	**< 2e-16**	-
b. Pseudomonas entomophila				
Virgin, Fed	-			
Virgin, Starved	**0.00072**	-		
Mated, Fed	**1.5e-06**	0.06873	-	
Mated, Starved	**0.00072**	0.84846	0.09785	-
c. Erwinia carotovora carotovora				
Virgin, Fed	-			
Virgin, Starved	**2.4e-05**	-		
Mated, Fed	**2.9e-07**	0.322	-	
Mated, Starved	**< 2e-16**	**2.0e-05**	**0.003**	-
d. Enterococcus faecalis				
Virgin, Fed	-			
Virgin, Starved	0.09729	-		
Mated, Fed	**0.00048**	0.05039	-	
Mated, Starved	0.10366	0.99898	0.09390	-
e. Staphylococcus succinus				
Virgin, Fed	-			
Virgin, Starved	**0.003**	-		
Mated, Fed	**0.00016**	0.76008	-	
Mated, Starved	**0.00340**	0.98151	0.76008	-

Table 4.2. Post-hoc pair-wise comparisons of the effect of 'treatment' on systemic bacterial load in infected females using Tukey's HSD.

Pairwise comparison	Estimate	SE	DF	t-ratio	p-value
a. Providencia rettgeri					
Virgin Fed - Mated Fed	**-1.304**	**0.295**	**292**	**-4.421**	**0.0001**
Virgin Fed - Mated Starved	**-3.133**	**0.295**	**292**	**-10.622**	**<.0001**
Virgin Fed - Virgin Starved	**-1.518**	**0.295**	**292**	**-5.146**	**<.0001**
Mated Fed - Mated Starved	**-1.829**	**0.295**	**292**	**-6.202**	**<.0001**
Mated Fed - Virgin Starved	-0.214	0.295	292	-0.725	0.8870
Mated Starved - Virgin Starved	**1.615**	**0.295**	**292**	**5.476**	**<.0001**
b. Pseudomonas entomophila					
Virgin Fed - Mated Fed	**-2.087**	**0.368**	**292**	**-5.676**	**<.0001**
Virgin Fed - Mated Starved	**-2.568**	**0.368**	**292**	**-6.984**	**<.0001**
Virgin Fed - Virgin Starved	**-2.362**	**0.368**	**292**	**-6.423**	**<.0001**
Mated Fed - Mated Starved	-0.481	0.368	292	-1.308	0.5587
Mated Fed - Virgin Starved	-0.275	0.368	292	-0.747	0.8779
Mated Starved - Virgin Starved	0.206	0.368	292	0.561	0.9435
c. Enterococcus faecalis					
Virgin Fed - Mated Fed	**-1.277**	**0.329**	**292**	**-3.877**	**0.0007**
Virgin Fed - Mated Starved	**-1.493**	**0.329**	**292**	**-4.534**	**<.0001**
Virgin Fed - Virgin Starved	**-2.278**	**0.329**	**292**	**-6.916**	**<.0001**
Mated Fed - Mated Starved	-0.216	0.329	292	-0.657	0.9131
Mated Fed - Virgin Starved	**-1.001**	**0.329**	**292**	**-3.038**	**0.0138**
Mated Starved - Virgin Starved	-0.784	0.329	292	-2.382	0.0829

Table 4.3. Post-hoc pair-wise comparisons of the effect of 'treatment × HPI' interaction on systemic bacterial load in infected females using Tukey's HSD.

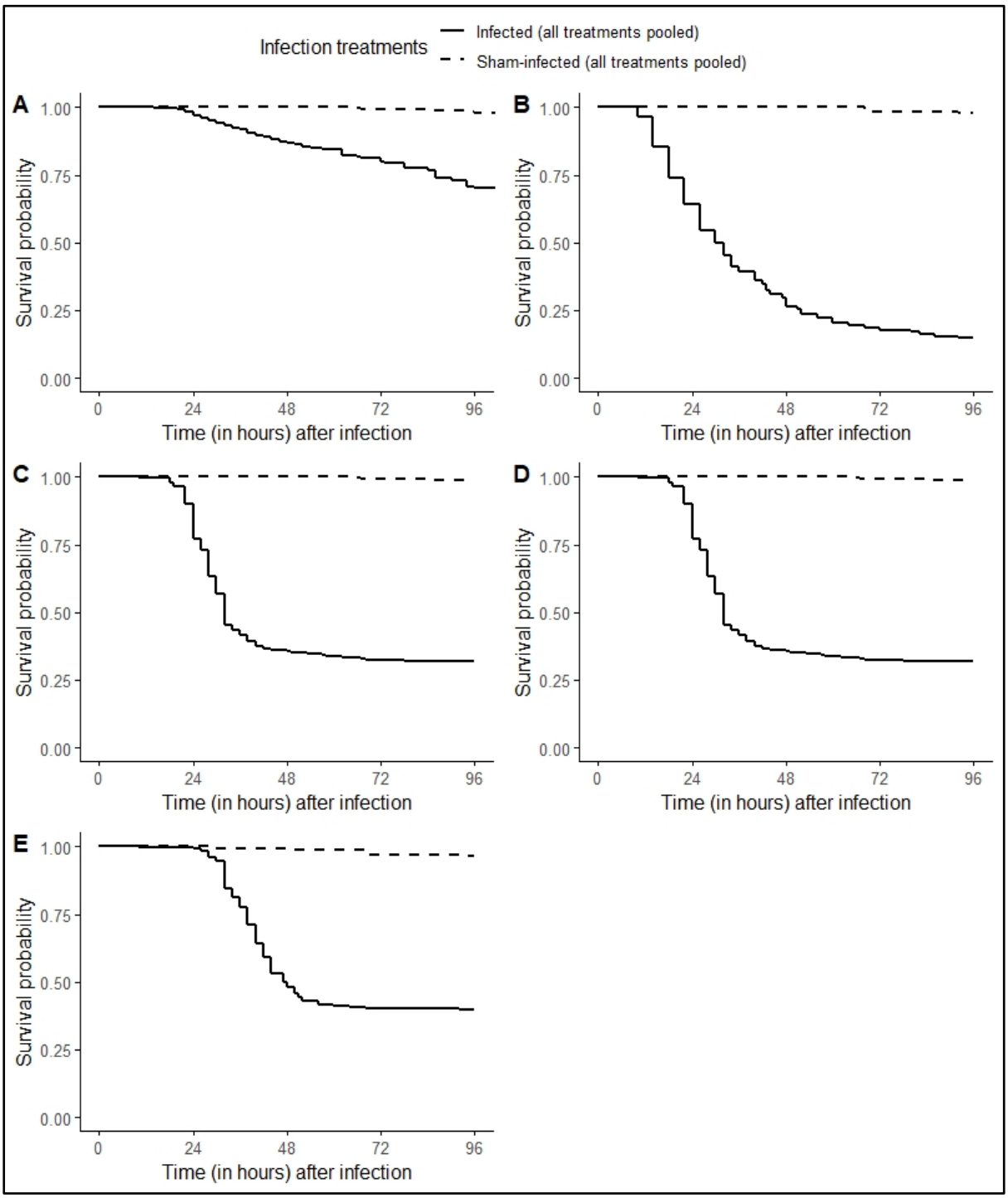
Pairwise comparison	Estimate	SE	DF	t-ratio	p-value
a. Providencia rettgeri					
Virgin Fed 4 - Mated Fed 4	-1.236	0.417	292	-2.963	0.0644
Virgin Fed 4 - Mated Starved 4	**-2.144**	**0.417**	**292**	**-5.140**	**<.0001**
Virgin Fed 4 - Virgin Starved 4	-0.864	0.417	292	-2.072	0.4355
Virgin Fed 4 - Virgin Fed 10	**-4.933**	**0.417**	**292**	**-11.825**	**<.0001**
Virgin Fed 4 - Mated Fed 10	**-6.305**	**0.417**	**292**	**-15.114**	**<.0001**
Virgin Fed 4 - Mated Starved 10	**-9.055**	**0.417**	**292**	**-21.707**	**<.0001**
Virgin Fed 4 - Virgin Starved 10	**-7.104**	**0.417**	**292**	**-17.030**	**<.0001**
Mated Fed 4 - Mated Starved 4	-0.908	0.417	292	-2.177	0.3688
Mated Fed 4 - Virgin Starved 4	0.372	0.417	292	0.891	0.9867
Mated Fed 4 - Virgin Fed 10	**-3.697**	**0.417**	**292**	**-8.862**	**<.0001**
Mated Fed 4 - Mated Fed 10	**-5.069**	**0.417**	**292**	**-12.151**	**<.0001**
Mated Fed 4 - Mated Starved 10	**-7.819**	**0.417**	**292**	**-18.744**	**<.0001**
Mated Fed 4 - Virgin Starved 10	**-5.868**	**0.417**	**292**	**-14.067**	**<.0001**
Mated Starved 4 - Virgin Starved 4	**1.280**	**0.417**	**292**	**3.068**	**0.0479**
Mated Starved 4 - Virgin Fed 10	**-2.789**	**0.417**	**292**	**-6.685**	**<.0001**
Mated Starved 4 - Mated Fed 10	**-4.161**	**0.417**	**292**	**-9.974**	**<.0001**
Mated Starved 4 - Mated Starved 10	**-6.911**	**0.417**	**292**	**-16.567**	**<.0001**
Mated Starved 4 - Virgin Starved 10	**-4.960**	**0.417**	**292**	**-11.890**	**<.0001**
Virgin Starved 4 - Virgin Fed 10	**-4.068**	**0.417**	**292**	**-9.753**	**<.0001**
Virgin Starved 4 - Mated Fed 10	**-5.440**	**0.417**	**292**	**-13.042**	**<.0001**
Virgin Starved 4 - Mated Starved 10	**-8.191**	**0.417**	**292**	**-19.635**	**<.0001**
Virgin Starved 4 - Virgin Starved 10	**-6.240**	**0.417**	**292**	**-14.958**	**<.0001**
Virgin Fed 10 - Mated Fed 10	**-1.372**	**0.417**	**292**	**-3.289**	**0.0247**
Virgin Fed 10 - Mated Starved 10	**-4.122**	**0.417**	**292**	**-9.882**	**<.0001**
Virgin Fed 10 - Virgin Starved 10	**-2.171**	**0.417**	**292**	**-5.205**	**<.0001**
Mated Fed 10 - Mated Starved 10	**-2.750**	**0.417**	**292**	**-6.593**	**<.0001**
Mated Fed 10 - Virgin Starved 10	-0.799	0.417	292	-1.916	0.5408

Mated Starved 10 - Virgin Starved 10	**1.951**	**0.417**	**292**	**4.677**	**0.0001**
b. Pseudomonas entomophila					
Virgin Fed 4 - Mated Fed 4	-0.6918	0.52	292	-1.330	0.8867
Virgin Fed 4 - Mated Starved 4	**-1.9937**	**0.52**	**292**	**-3.834**	**0.0038**
Virgin Fed 4 - Virgin Starved 4	-1.5357	0.52	292	-2.953	0.0661
Virgin Fed 4 - Virgin Fed 10	**-2.2905**	**0.52**	**292**	**-4.404**	**0.0004**
Virgin Fed 4 - Mated Fed 10	**-5.7733**	**0.52**	**292**	**-11.101**	**<.0001**
Virgin Fed 4 - Mated Starved 10	**-5.4333**	**0.52**	**292**	**-10.448**	**<.0001**
Virgin Fed 4 - Virgin Starved 10	**-5.4789**	**0.52**	**292**	**-10.535**	**<.0001**
Mated Fed 4 - Mated Starved 4	-1.3019	0.52	292	-2.503	0.1981
Mated Fed 4 - Virgin Starved 4	-0.8439	0.52	292	-1.623	0.7363
Mated Fed 4 - Virgin Fed 10	**-1.5987**	**0.52**	**292**	**-3.074**	**0.0471**
Mated Fed 4 - Mated Fed 10	**-5.0815**	**0.52**	**292**	**-9.771**	**<.0001**
Mated Fed 4 - Mated Starved 10	**-4.7415**	**0.52**	**292**	**-9.117**	**<.0001**
Mated Fed 4 - Virgin Starved 10	**-4.7871**	**0.52**	**292**	**-9.205**	**<.0001**
Mated Starved 4 - Virgin Starved 4	0.4580	0.52	292	0.881	0.9876
Mated Starved 4 - Virgin Fed 10	-0.2968	0.52	292	-0.571	0.9992
Mated Starved 4 - Mated Fed 10	**-3.7797**	**0.52**	**292**	**-7.268**	**<.0001**
Mated Starved 4 - Mated Starved 10	**-3.4396**	**0.52**	**292**	**-6.614**	**<.0001**
Mated Starved 4 - Virgin Starved 10	**-3.4852**	**0.52**	**292**	**-6.702**	**<.0001**
Virgin Starved 4 - Virgin Fed 10	-0.7548	0.52	292	-1.451	0.8322
Virgin Starved 4 - Mated Fed 10	**-4.2377**	**0.52**	**292**	**-8.148**	**<.0001**
Virgin Starved 4 - Mated Starved 10	**-3.8976**	**0.52**	**292**	**-7.495**	**<.0001**
Virgin Starved 4 - Virgin Starved 10	**-3.9432**	**0.52**	**292**	**-7.582**	**<.0001**
Virgin Fed 10 - Mated Fed 10	**-3.4828**	**0.52**	**292**	**-6.697**	**<.0001**
Virgin Fed 10 - Mated Starved 10	**-3.1428**	**0.52**	**292**	**-6.043**	**<.0001**
Virgin Fed 10 - Virgin Starved 10	**-3.1884**	**0.52**	**292**	**-6.131**	**<.0001**
Mated Fed 10 - Mated Starved 10	0.3400	0.52	292	0.654	0.9980
Mated Fed 10 - Virgin Starved 10	0.2944	0.52	292	0.566	0.9992
Mated Starved 10 - Virgin Starved 10	-0.0456	0.52	292	-0.088	1.0000
c. Enterococcus faecalis					

Virgin Fed 4 - Mated Fed 4	-0.991	0.466	292	-2.128	0.3994
Virgin Fed 4 - Mated Starved 4	-0.613	0.466	292	-1.315	0.8926
Virgin Fed 4 - Virgin Starved 4	-1.413	0.466	292	-3.033	0.0530
Virgin Fed 4 - Virgin Fed 10	**-1.939**	**0.466**	**292**	**-4.163**	**0.0011**
Virgin Fed 4 - Mated Fed 10	**-3.502**	**0.466**	**292**	**-7.518**	**<.0001**
Virgin Fed 4 - Mated Starved 10	**-4.313**	**0.466**	**292**	**-9.260**	**<.0001**
Virgin Fed 4 - Virgin Starved 10	**-5.082**	**0.466**	**292**	**-10.910**	**<.0001**
Mated Fed 4 - Mated Starved 4	0.379	0.466	292	0.813	0.9923
Mated Fed 4 - Virgin Starved 4	-0.421	0.466	292	-0.905	0.9855
Mated Fed 4 - Virgin Fed 10	-0.948	0.466	292	-2.035	0.4603
Mated Fed 4 - Mated Fed 10	**-2.511**	**0.466**	**292**	**-5.390**	**<.0001**
Mated Fed 4 - Mated Starved 10	**-3.322**	**0.466**	**292**	**-7.132**	**<.0001**
Mated Fed 4 - Virgin Starved 10	**-4.091**	**0.466**	**292**	**-8.782**	**<.0001**
Mated Starved 4 - Virgin Starved 4	-0.800	0.466	292	-1.718	0.6757
Mated Starved 4 - Virgin Fed 10	-1.326	0.466	292	-2.848	0.0876
Mated Starved 4 - Mated Fed 10	**-2.889**	**0.466**	**292**	**-6.203**	**<.0001**
Mated Starved 4 - Mated Starved 10	**-3.701**	**0.466**	**292**	**-7.945**	**<.0001**
Mated Starved 4 - Virgin Starved 10	**-4.469**	**0.466**	**292**	**-9.595**	**<.0001**
Virgin Starved 4 - Virgin Fed 10	-0.526	0.466	292	-1.130	0.9498
Virgin Starved 4 - Mated Fed 10	**-2.089**	**0.466**	**292**	**-4.485**	**0.0003**
Virgin Starved 4 - Mated Starved 10	**-2.901**	**0.466**	**292**	**-6.227**	**<.0001**
Virgin Starved 4 - Virgin Starved 10	**-3.669**	**0.466**	**292**	**-7.877**	**<.0001**
Virgin Fed 10 - Mated Fed 10	**-1.563**	**0.466**	**292**	**-3.355**	**0.0200**
Virgin Fed 10 - Mated Starved 10	**-2.374**	**0.466**	**292**	**-5.097**	**<.0001**
Virgin Fed 10 - Virgin Starved 10	**-3.143**	**0.466**	**292**	**-6.747**	**<.0001**
Mated Fed 10 - Mated Starved 10	-0.811	0.466	292	-1.742	0.6600
Mated Fed 10 - Virgin Starved 10	**-1.580**	**0.466**	**292**	**-3.392**	**0.0178**
Mated Starved 10 - Virgin Starved 10	-0.769	0.466	292	-1.650	0.7191

Supplementary figures

Figure S4.1. Comparison of survival of sham-infected females and females infected with **(A)** *Providencia rettgeri*, **(B)** *Pseudomonas entomophila*, **(C)** *Erwinia c. carotovora*, **(D)** *Enterococcus faecalis*, and **(E)** *Staphylococcus succinus*. Survival curves represent females of all treatments and replicates pooled together.



5. Parental effects of pathogenic bacterial infections

Introduction

It is common among hosts to exhibit variability in susceptibility to infections, at the level of species, populations, cohorts, and individuals. Although a major proportion of this variability is sourced from genetic differences, maintained via different evolutionary forces (Schmid-Hempel 2003), environmental factors (and gene-by-environment interactions) immensely contribute to this variability in susceptibility to diseases (Sandland and Minchella 2003, Lazzaro and Little 2008). In *Drosophila melanogaster*, the role of abiotic environment in modifying susceptibility to diseases is well documented, while role of biotic factors (such as predation, competition, etc.) is less explored. Previous experience with pathogens is one such biotic factor that can be a major determinant of host susceptibility of extant pathogen challenges. This is especially true for pathogen experience over an evolutionary time period, as demonstrated by multiple laboratory experimental evolution studies that suggest that hosts' adaptation to a particular pathogen not only modifies its susceptibility to that particular pathogen, but also to pathogens never encountered before (Fellowes et al 1999, Martins et al 2013, Biswas et al 2018, Singh et al 2021).

Experience with pathogens over relatively shorter periods of time, for example, within the same generation, can also influence host disease susceptibility. For example, *D. melanogaster* flies previously exposed to non-pathogenic bacterial infections in early life respond differently later when challenged with pathogenic infections depending upon the pathogen used for infection (Christofi and Apidianakis 2013, Kutzer et al 2019, Hidalgo and Armitage 2022). Host

susceptibility to infections can also change by virtue of their parents being exposed to pathogens, which has been repeatedly demonstrated in various insect species and other invertebrates (Milutinovic et al 2016, Contreras-Garduno et al 2016, Cooper and Eleftherianos 2017, Tetreau et al 2019, Prakash and Khan 2022), although not in *D. melanogaster* (Tetreau et al 2019). This phenomenon is reminiscent of parental effects driven improvement of offspring fitness, whereby offspring fair better in a stressful environment when their parents have also been exposed to the same environment (Yin et al 2019).

Parental effects of infection have been studied in insects and other invertebrates under the header of trans-generational immune priming. A characteristic feature of these studies is that the hosts from the parental generation are infected with a dead or attenuated pathogen, or some pathogen mimic (viz. bacteria derived LPS, micro-plastic beads, etc.) (Tetreau et al 2019). While this study design has its own merits, in that it can parse out the consequences of the host responding to infection from that of the manipulation of host physiology by pathogens, it overlooks one of the key consequences of infection: pathogenesis. In this study, I therefore explore the possibility of parental effects of pathogenic bacterial infections, using *D melanogaster* as a model host species. The only other study using *D. melanogaster* that subjected parents to pathogenic infections found that it did not lead to any change in offspring immune function (Linder and Promislow 2009).

In this chapter, I tested for the effects of infecting parents with pathogenic bacteria on post-infection survival of offspring flies. Two bacterial pathogens were used to test for possibility of parental effects in the experiments reported here: *Enterococcus faecalis* and *Erwinia carotovora carotovora*. Both these pathogens are known to infect flies and other insects in the wild (Lazzaro et al 2006, Troha and Buchon 2019), and the specific bacterial strains used here were also isolated from wild-caught Drosophila melanogaster flies (Lazzaro et al 2006, Troha

and Buchon 2019). This makes these pathogens suitable for use in experiments investigating parental effects (Tetreau et al 209).

I subjected flies to bacterial infection and found that their offspring exhibited increased survival when infected with the same bacterium. In certain cases, improved survival of the offspring was also observed for other pathogens, but in a pathogen-identity and host-sex dependent manner. Parental effects did not lead to increased susceptibility to any novel infections. A general property of parental effects is that offspring often pay a cost if they encounter a benign environment after their parents have been subjected to a stress. I find that subjecting parents to bacterial infection has no effect of offspring reproductive capacity, indicating an absence of costs. Furthermore, increased survival of offspring is driven by increased resistance to systemic infection.

Materials and methods

Study system

Flies from BRB2 population were used for the experiments reported in this chapter. Please refer to chapter 2 for details of origin and maintenance of this population.

Pathogens

Four bacterial pathogens were used across all experiments reported in this chapter:

d. *Enterococcus faecalis*,
e. *Erwinia carotovora cartotvora* (strain Ecc15),
f. *Bacillus thuringiensis*, and
g. *Pseudomonas entomophila* (strain L48).

Experiment design

Experiment 1: Effect of parental exposure pathogenic infection on offspring immune defense (post-infection survival) against the same pathogen.

Parental (P) generation. Eggs for the parental generation were collected from the BRB2 population cage, at a density of 60-80 eggs per vial with 6-8 ml of standard food medium (the same food medium used to maintain the BRB populations). These were maintained under the regular rearing environment for the BRB2 flies, and the eggs developed into adults by the 10^{th} day post-egg collection. 2–3-day old adult flies were randomly assigned to two treatments: 'infected parents' and 'control parents'. The flies in the 'infected parents' treatment (N = 200 females and 200 males) were subjected to infection with live bacterial pathogens following the protocol described above, while the flies in the 'control parents' treatment were subjected to

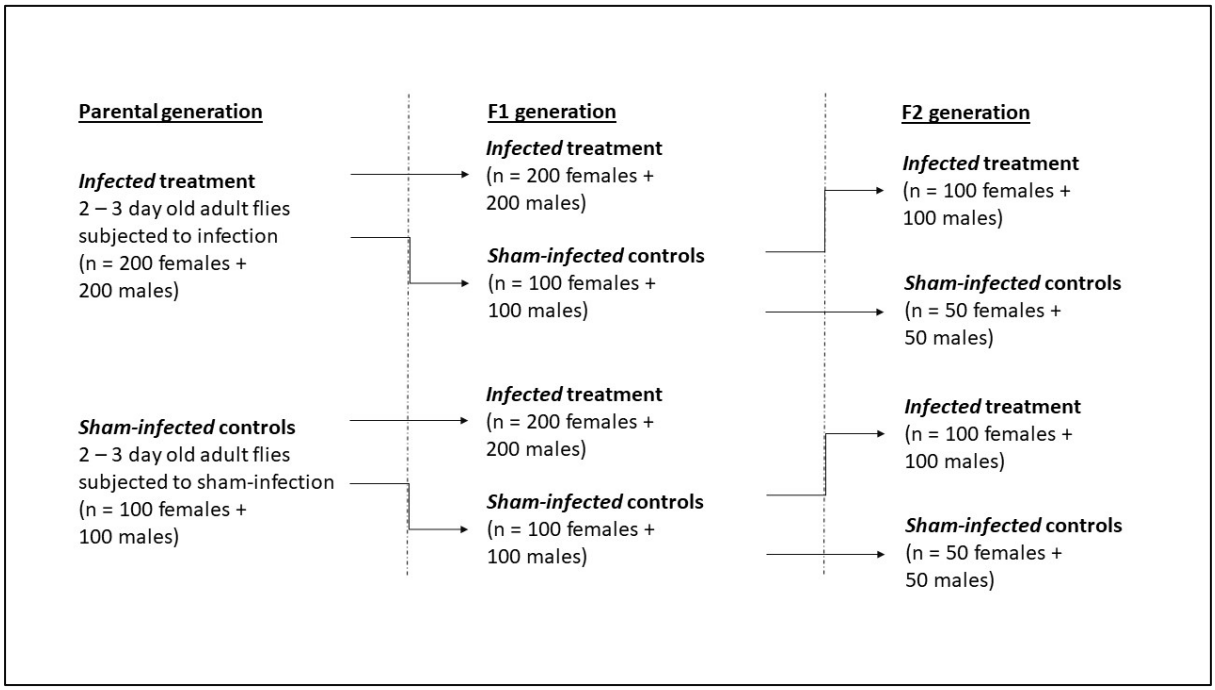
sham-infections. After infections the flies of each treatment were housed in separate plexiglass cages (dimensions) with ad libitum access to food. The experiment was set-up with two different pathogens: *Erwinia c. carotovora* and *Enterococcus faecalis*.

Offspring (F1) generation. Eggs for the F1 generation were collected from the flies of the parental generation, after the acute phase of infection was over. For the two replicates with *E. c. carotovora*, eggs were collected 96 hours post-infection (HPI), while for experiments with *E. faecalis*, eggs were collected for two replicates after 48 HPI and for two replicates after 96 HPI (the experimental set-up thus ran for total six replicates). Eggs were collected at a density of 60-80 eggs per vial with 6-8 ml of standard food medium and maintained under the regular rearing environment of the BRB2 flies; the eggs developed into adults by 10^{th} day post-egg collection. 2–3-day old adult F1 flies, from both parental treatments, were either subjected to infections (with the same pathogen used to infect the generation P flies for that particular replicate; N = 200 females and 200 males), or were subjected to sham-infections (N = 100 female and 100 males). Flies from each 'offspring treatment', for each 'parental treatment', were housed in separate plexiglass cages, and the mortality of the flies were monitored every 4-6 hours for 120 hours HPI.

Grand-offspring (F2) generation. Eggs for the F2 generation were collected from the sham-infected F1 fly cages (i.e., the parents of the F2 flies were not exposed to pathogens in the F1 generation). Eggs were collected at a density of 60-80 eggs per vial with 6-8 ml of standard food medium and maintained under the usual rearing environment of the BRB2 flies; the eggs developed into adults by 10^{th} day post-egg collection. 2–3-day old adult F2 flies, from both grand-parental (generation P) treatments, were either subjected to infections (with the same pathogen used to infect the generation P flies for that particular replicate; N = 100 females and 100 males), or were subjected to sham-infections (N = 50 female and 50 males). Flies from each 'grand-offspring (F2) treatment', for each 'grand parental (P) treatment', were housed in

separate plexiglass cages, and the mortality of the flies were monitored every 4-6 hours for 120 hours HPI.

Figure 5.0. Schematic depicting the experimental design for experiment 1. Experiments 2 and 3 followed a similar design, with necessary modifications to suit the assayed traits.



Experiment 2(a): Effect of infecting parents with one particular pathogen on offspring immune defense (post-infection survival) against other pathogens.

Parental (P) generation. The set-up for parental generation was identical to that of experiment 1 (outlined above). Parental generation common with experiment 2(b).

Offspring (F1) generation. Eggs for the F1 generation were collected from the flies of the parental generation, after the acute phase of infection was over. For the two replicates with *E. c. carotovora*, eggs were collected 96 hours post-infection (HPI), while for the two replicates with *E. faecalis*, eggs were collected after 48 HPI (the experimental set-up thus ran for total four replicates). Eggs were collected at a density of 60-80 eggs per vial with 6-8 ml of standard food medium and maintained under the regular rearing environment of the BRB2 flies; the eggs developed into adults by 10^{th} day post-egg collection. 2–3-day old adult F1 flies, from both parental treatments, were either subjected to infections with three novel pathogens (pathogens not used to infect the generation P flies; N = 60 females and 60 males for each pathogen) or were subjected to sham-infections (N = 60 females and 60 males). In replicates where generation P flies were infected with *E. c. carotovora*, the offspring generation flies from both parental treatments were infected with *Bacillus thuringiensis*, *E. faecalis*, or *Pseudomonas entomophila*. In replicates where generation P flies were infected with *E. faecalis*, the offspring generation flies from both parental treatments were infected with *B. thuringiensis*, *E. c. carotovora*, or *P. entomophila*. Flies from each 'offspring treatment', for each 'parental treatment', were housed in separate plexiglass cages, and the mortality of the flies were monitored every 4-6 hours for 120 hours HPI.

Experiment 2(b): Effect of parental exposure to infection with live pathogens on offspring reproductive capacity.

Parental (P) generation. The set-up for parental generation was identical to that of experiment 1 (outlined above). Parental generation common with experiment 2(a).

Offspring (F1) generation. Eggs for the F1 generation were collected from the flies of the parental generation, after the acute phase of infection was over. For the two replicates with *E. c. carotovora*, eggs were collected 96 hours post-infection (HPI), while for the two replicates with *E. faecalis*, eggs were collected after 48 HPI (the experimental set-up thus ran for total four replicates). Eggs were collected at a density of 60-80 eggs per vial with 6-8 ml of standard food medium and maintained under the regular rearing environment of the BRB2 flies; the eggs developed into adults by 10^{th} day post-egg collection. 2–3-day old adult F1 flies, from both parental treatments, were tested for their reproductive capacity: early-life fecundity (progeny output) for females and competitive fertilization success for males.

Assay for female early-life fecundity. 2–3-day old flies were sorted into vials at a density of 5 females and 5 males per vial, with 8 ml of standard food medium in each vial; 10 such vials were set up for offspring of each parental treatment. Flies were housed in these vials for 24 hours, during which the females laid eggs, and then transferred to a fresh food vials, with one-to-one mapping of vial identity. This procedure was repeated for 8 consecutive days. The vials with eggs were maintained under the regular rearing environment of the BRB2 flies; the eggs developed into adults by 10^{th} day post-egg laying. 12 days (post-egg laying) later the number of adult progenies in each vial was scored as a measure of reproductive output of females in that vial. The total number of progenies in the vials was divided by the number of alive females (usually 5, unless any female was lost during the transfer process) in the vial to obtain the measure of per capita female fecundity for each day.

Assay for male competitive fertilization success (CFS). Male offspring from each parental treatment (N = 50 males per parental treatment) were individually housed in standard food

vials, along with a BLst male and a pre-inseminated BLst female. (BLst is an outbred population, maintained under identical maintenance regime as BRB2. The BLst population is fully homozygous for the recessive eye-colour marker, *scarlet*.) The BLst flies were age matched with the focal males (offspring generation males). The vials were left undisturbed for 24 hours, so that the two males can compete for opportunity to inseminate the female and fertilize the eggs produce by it. 24 hours later both males were discarded, and the female was housed individually in a fresh food vial (with 8 ml of standard food medium) and allowed to lay eggs for 24 hours, following which the female was also discarded. The vials with eggs were maintained under the usual rearing environment of the BRB2 flies; the eggs developed into adults by 10^{th} day post-egg laying. 12 days (post-egg laying) later the adult progenies were scored on the basis of eye colour to determine paternity. The proportion of progenies sired by the focal male was considered as a measure of competitive fertilization success.

Statistical analyses

Effect of parental treatment on post-infection survival of flies from generations F1 and F2 was tested using mixed-effect Cox proportional hazards analysis. The mixed-effect model included parental infection treatment as a fixed factor and replicate identity as a random factor. Since negligible mortality occurred in sham-infected flies from generation F1 and F2 (figures S5.1 and S5.2), data from only the infected flies were subjected to survival analysis. Data for female and male offspring were analyzed separately. Female early-life fecundity data was analyzed using type-III analysis of variance (ANOVA). Parental infection treatment, offspring age, and their interaction were included as fixed factors, and replicate identity as a random factor. Male competitive fertilization success was analyzed using type-III ANOVA. Parental infection treatment was included as a fixed factor and replicate identity as a random factor.

Results

Effect of parental exposure to pathogenic infection on offspring immune defense (post-infection survival) against the same pathogen.

I infected generation P flies with *Erwinia c. carotovora* (along with sham-infected controls) and tested if parental infection treatment determined post-infection survival of flies from F1 and F2 generations, when infected with *E. c. carotovora*. In the F1 generation, offspring of infected parents had greater post-infection survival compared to offspring of sham-infected parents, in case of both female (hazard ratio, 95% confidence interval: 0.630, 0.529-0.752) and male offspring (HR, 95% CI: 0.534, 0.447-0.637). In the F2 generation, female grand-offspring of infected generation P flies did not differ in terms of survival from female grand-offspring of sham-infected generation P flies (HR, 95% CI: 1.099, 0.876-1.380). Male grand-offspring of infected generation P flies exhibited an increased risk of mortality compared to male grand-offspring of sham-infected generation P flies (HR, 95% CI: 1.284, 1.040-1.587).

I infected generation P flies with *Enterococcus faecalis* (along with sham-infected controls) and tested if parental infection treatment determined post-infection survival of flies from F1 and F2 generations, when infected with *E. faecalis*. We did this following two separate protocols independently: once, by collecting eggs for offspring generation after 48 hours post-infection (HPI), and in the other protocol, eggs were collected after 96 HPI. This was done to test if the time difference between infection and collection of eggs in generation P had any effect on post-infection survival of the offspring.

Following the first protocol (egg collection after 48 HPI), in the F1 generation, offspring of infected parents had greater post-infection survival compared to offspring of sham-infected parents, in case of both female (HR, 95% CI: 0.563, 0.742-0.673) and male offspring (HR, 95% CI: 0.535, 0.443-0.647). In the F2 generation, female grand-offspring of infected generation P

flies survived better compared to female grand offspring of sham-infected generation P flies (HR, 95% CI: 0.634, 0.492-0.816), but there was no survival difference in case of male grand-offspring (HR, 95% CI: 1.026, 0.790-1.332).

Following the second protocol (egg collection after 96 HPI), post-infection survival of flies in the F1 generation was not affected by parental treatment, in case of either females (HR, 95% CI: 1.005, 0.844-1.198) or males (HR, 95% CI: 1.053, 0.866-1.281). Similarly, in the F2 generation too, post-infection survival of flies was not affected by grand-parental (generation P) treatment, in case of either females (HR, 95% CI: 1.042, 0.800-1.358) or males (HR, 95% CI: 1.067, 0.796-1.429).

Figure 5.1. Post-infection survival of offspring (F1) generation flies after being subjected to infection with the same bacterial pathogen as the corresponding parental (P) generation flies. Survival curves plotted using Kaplan-Meier method after pooling data from both replicates for each pathogen. **(A)** *Erwinia c. carotovora*. **(B)** *Enterococcus faecalis*, egg collection from gen. P flies after 48 hours post-infection. **(C)** *Enterococcus faecalis*, egg collection from gen. P flies after 96 hours post-infection.

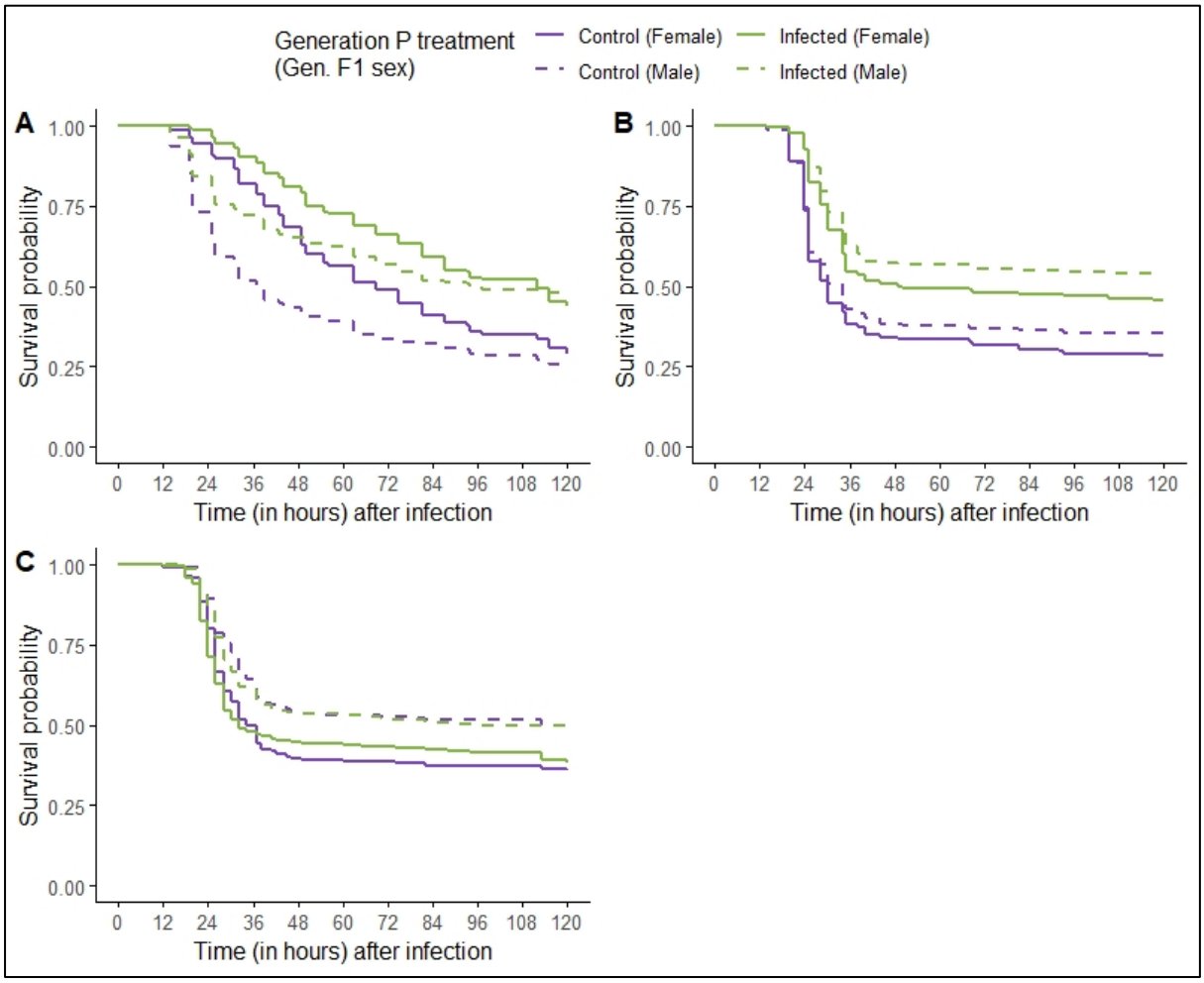
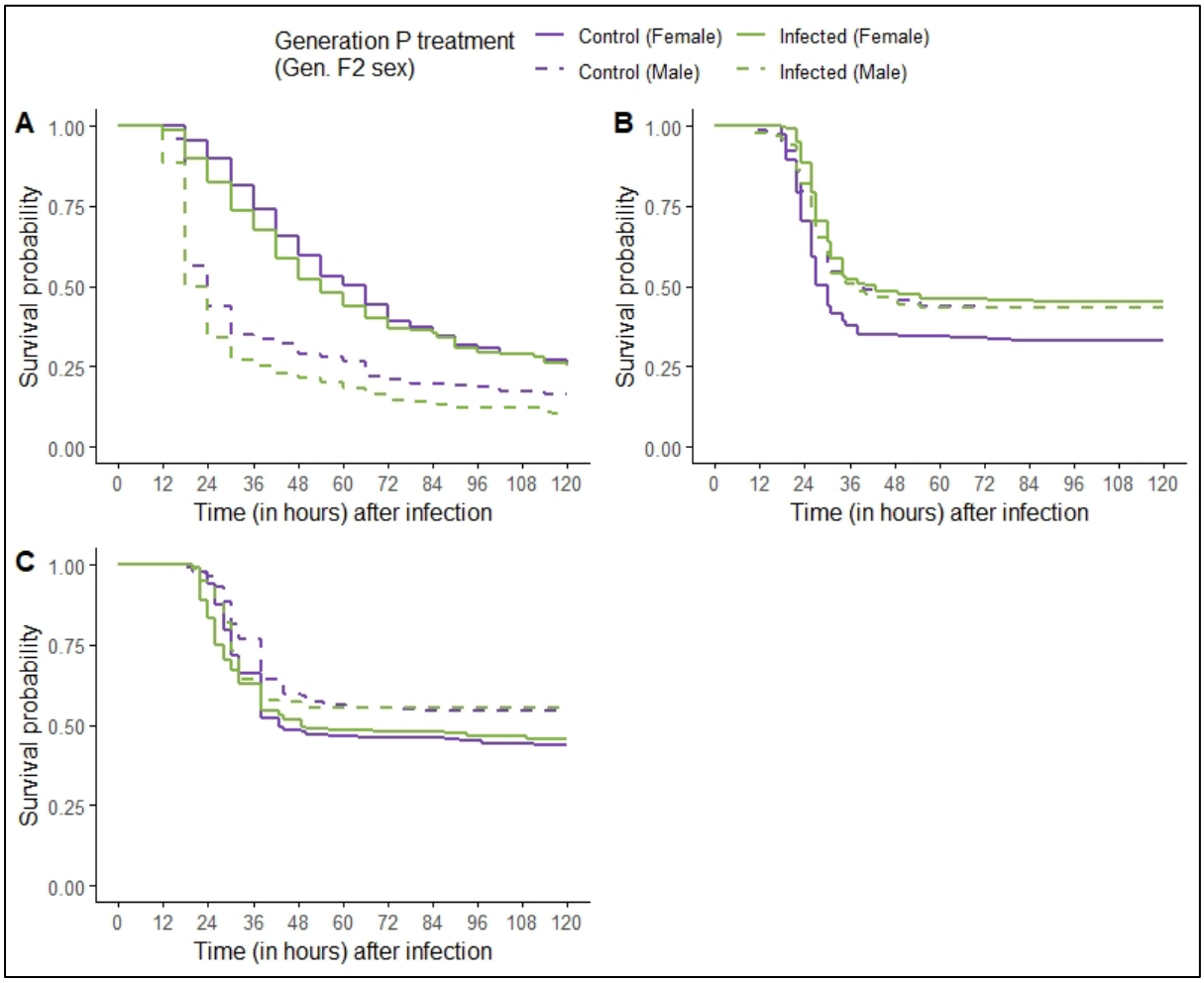


Figure 5.2. Post-infection survival of grand-offspring (F1) generation flies after being subjected to infection with the same bacterial pathogen as the corresponding parental (P) generation flies. Survival curves plotted using Kaplan-Meier method after pooling data from both replicates for each pathogen. **(A)** *Erwinia c. carotovora*. **(B)** *Enterococcus faecalis*, egg collection from gen. P flies after 48 hours post-infection. **(C)** *Enterococcus faecalis*, egg collection from gen. P flies after 96 hours post-infection.



Effect of infecting parents with one pathogen on offspring immune defense (post-infection survival) against other pathogens.

I infected generation P flies with *E. c. carotovora* (along with sham-infected controls) and tested if parental infection treatment determined post-infection survival of flies from F1 generation, when infected with three *novel* pathogens: *Bacillus thuringiensis*, *E. faecalis*, and *Pseudomonas entomophila*. Female F1 offspring of *E. c. carotovora* infected flies were not significantly different in terms of post-infection survival compared to female offspring of sham-infected flies when infected with either *B. thuringiensis* (HR, 95% CI: 0.831, 0.626-1.103) or *E. faecalis* (HR, 95% CI: 0.887, 0.657-1.198), or *P. entomophila* (HR, 95% CI: 1.132, 0.861-1.489). Male F1 offspring of *E. c. carotovora* infected flies survived significantly better compared to male offspring of sham-infected flies when infected with either *E. faecalis* (HR, 95% CI: 0.729, 0.535-0.993) or *P. entomophila* (HR, 95% CI: 0.618, 0.474-0.805), but not when infected with *B. thuringiensis* (HR, 95% CI: 0.864, 0.649-1.149).

I infected generation P flies with *E. faecalis* (along with sham-infected controls) and tested if parental infection treatment determined post-infection survival of flies from F1 generation, when infected with three *novel* pathogens: *B. thuringiensis*, *E. c. carotovora*, and *P. entomophila*. F1 offspring of *E. faecalis* infected flies were not significantly different in terms of post-infection survival compared to offspring of sham-infected flies when infected with either *B. thuringiensis*, in case of both females (HR, 95% CI: 1.263, 0.962-1.658) and males (HR, 95% CI: 0.774, 0.587-1.021), or *P. entomophila*, in case of both females (HR, 95% CI: 1.105, 0.839-1.454) and males (HR, 95% CI: 1.258, 0.954-1.659). When infected with *E. c. carotovora*, offspring of *E. faecalis* infected parents survived better compared to offspring of sham-infected parents, in case of both females (HR, 95% CI: 0.658, 0.485-0.892) and males (HR, 95% CI: 0.451, 0.340-0.597).

Figure 5.3. Post-infection survival of offspring (F1) generation flies after being subjected to infection with the *novel* bacterial pathogens, different from their parental (P) generation flies. Survival curves plotted using Kaplan-Meier method after pooling data from both replicates for each pathogen. **(A)** Bacillus thuringiensis. **(B)** *Enterococcus faecalis*. **(C)** *Pseudomonas entomophila*.

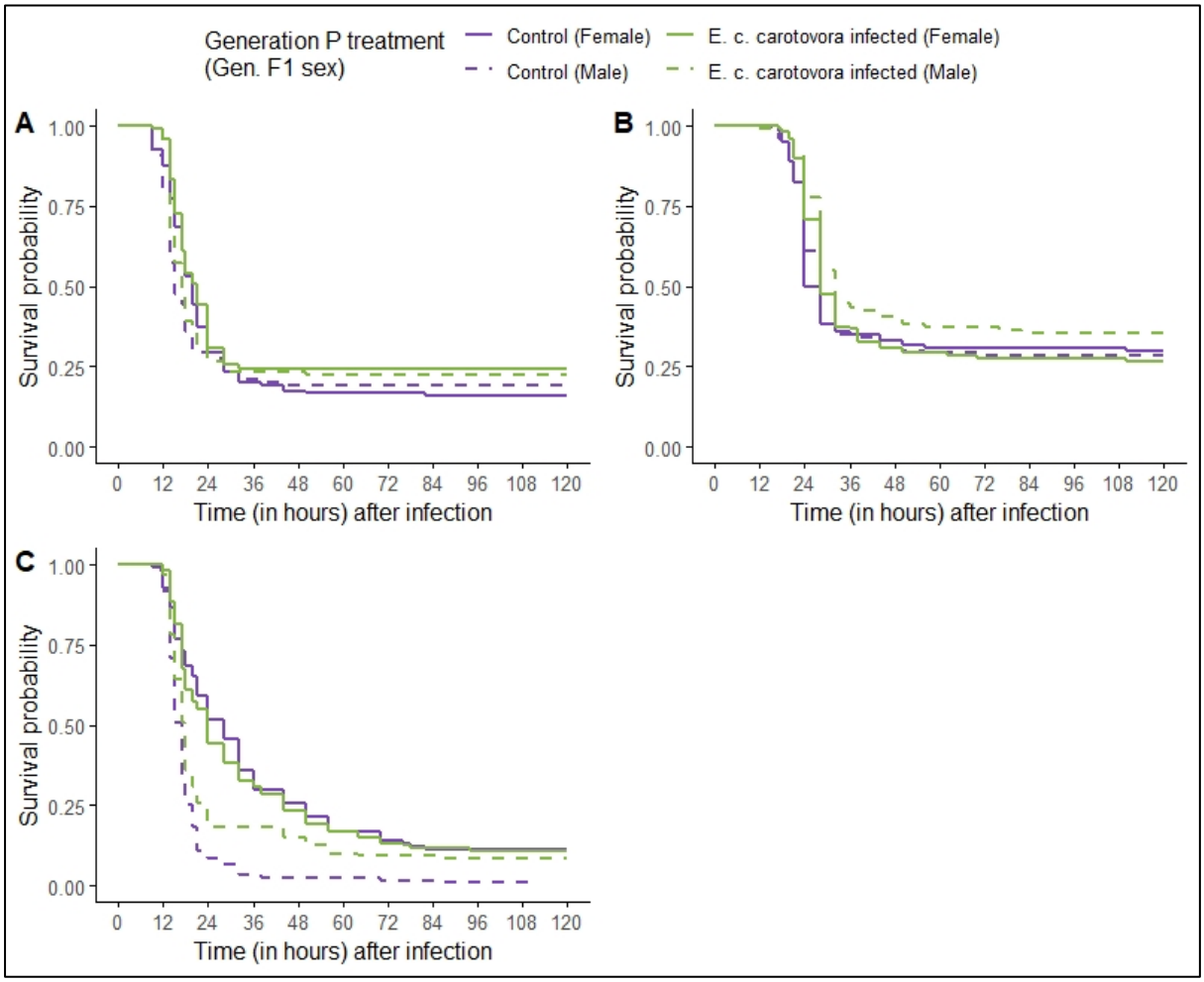
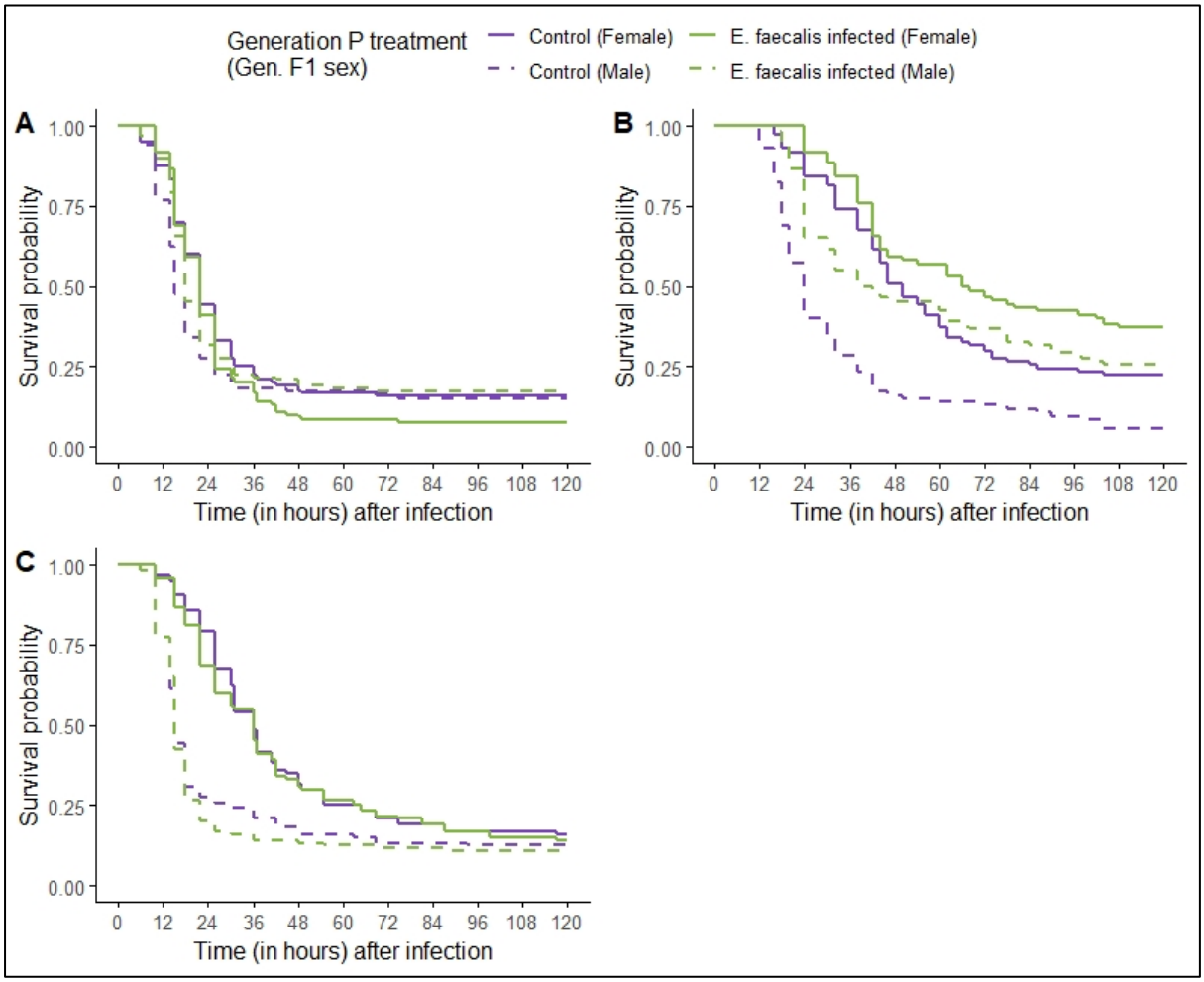


Figure 5.4. Post-infection survival of offspring (F1) generation flies after being subjected to infection with the *novel* bacterial pathogens, different from their parental (P) generation flies. Survival curves plotted using Kaplan-Meier method after pooling data from both replicates for each pathogen. **(A)** Bacillus thuringiensis. **(B)** *Erwinia c. carotovora*. **(C)** *Pseudomonas entomophila*.



Effect of parental exposure to pathogenic infection on offspring reproductive capacity.

I infected generation P flies with *E. c. carotovora* (along with sham-infected controls) and tested if parental infection treatment determined reproductive fitness of F1 offspring: female fecundity and male competitive fertilization success (CFS). Age of F1 female (the day of fecundity measurement) had a significant effect on per capita female fecundity ($F_{1,320}$ = 392.2122, p-value < 2.2e-16), but parental infection treatment did not have a significant effect ($F_{1,320}$ = 0.6158, p-value = 0.433), and neither did the interaction between age × parental treatment ($F_{1,320}$ = 0.0004, p-value = 0.984). Parental infection treatment had a significant effect on F1 male CFS ($F_{1,195}$ = 4.242, p-value = 0.041), with male offspring of infected parents being better at competing for fertilizations compared to male offspring of sham-infected parents.

I infected generation P flies with *E. faecalis* (along with sham-infected controls) and tested if parental infection treatment determined reproductive fitness of F1 offspring: female fecundity and male CFS. Age of F1 female (the day of fecundity measurement) had a significant effect on per capita female fecundity ($F_{1,318}$ = 428.8211, p-value < 2.2e-16), but parental infection treatment did not have a significant effect ($F_{1,318}$ = 1.4913, p-value = 0.223), and neither did the interaction between age × parental treatment ($F_{1,320}$ = 1.9333, p-value = 0.165). Parental infection treatment did not have a significant effect on F1 male CFS ($F_{1,190}$ = 0.0181, p-value = 0.893).

Figure 5.5. Reproductive output of offspring (F1) generation flies when their parental (P) generation flies are infected with *Erwinia c. carotovora*. Data pooled from both replicates. Y-axis error bars represent 95% confidence intervals around the respective means. **(A)** Female early-life fecundity. **(B)** Male competitive fertilization success. Reproductive output of offspring measured in absence of any infection.

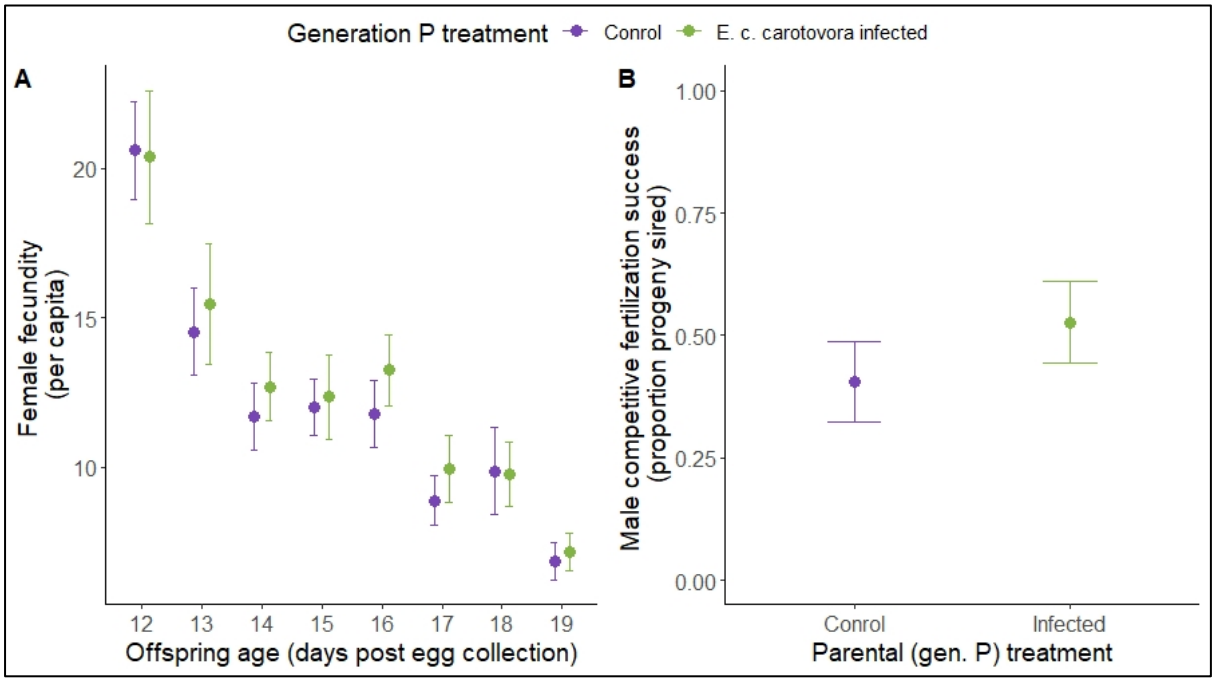
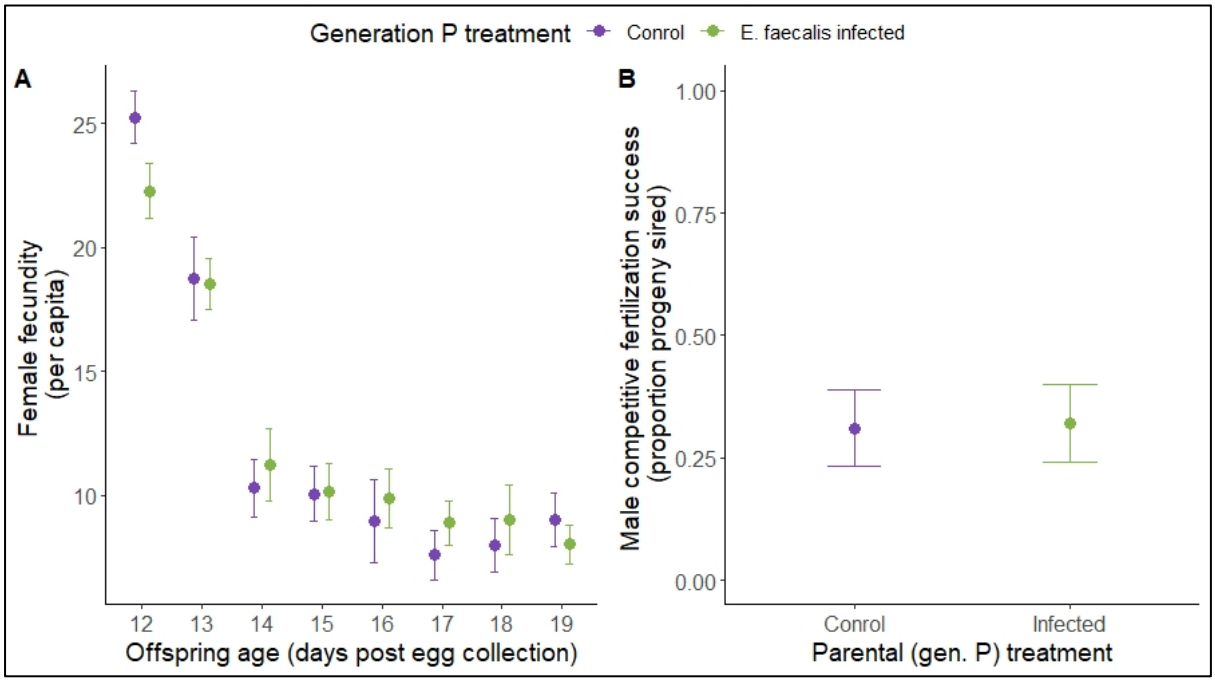


Figure 5.6. Reproductive output of offspring (F1) generation flies when their parental (P) generation flies are infected with *Enterococcus faecalis*. Data pooled from both replicates. Y-axis error bars represent 95% confidence intervals around the respective means. **(A)** Female early-life fecundity. **(B)** Male competitive fertilization success. Reproductive output of offspring measured in absence of any infection.



Discussion

In this part of the chapter, I explored the effect of subjecting flies to systemic pathogenic infections on the immune function of their offspring. I measured post-infection survival of offspring flies, both when infected with the same pathogen to which their parents were exposed (homologous infection challenge) and when infected with other pathogens (heterologous infection challenge). Additionally, I tested for any effect of parental infection on offspring reproductive function, measured as early-life fecundity for females and competitive fertilization success (CFS) for males.

The key observations from these experiments are as follows.

a. *Subjecting parents to pathogenic bacterial infections with make the offspring more immune to the same pathogen.* In the F1 generation, the offspring of parents infected with either *E. c. carotovora* or *E. faecalis* were better at surviving homologous infections compared to the offspring of control parents (figure 5.1). Increased survival was observed in case of both female and male F1 offspring.
b. *Improvement in offspring immune function decays after one generation.* In the F2 generation, both female and male offspring of *E. c. carotovora* infected parents did not differ in survival, following a homologous challenge, from the offspring of control parents (figure 5.2.A). In case of *E. faecalis* infected parents, only the female F2 offspring were better at surviving a homologous challenge; survival of male F2 offspring was similar for both infected and control parents (figure 5.2.C).
c. *Infected parents do not produce more immune offspring throughout the rest of their lives.* In case of *E. faecalis* infected parents, F1 offspring derived from eggs collected at 48 hours-post infection exhibited increased survival following a homologous challenge (figure 5.1.B). F1 offspring derived from eggs collected at 96 hours post-

infection did not exhibit any change in survival following a homologous challenge (figure 5.1.C).

d. *Subjecting parents to infection with one particular pathogen can make the offspring flies less susceptible to other pathogens.*

e. *Subjecting parents to infection either does not affect, or improves, offspring reproductive capacity.* In the F1 generation, female offspring of infected parents did not differ in terms of early-life fecundity compared to female offspring of control parents, for both *E. c. carotovora* infected (figure 5.5.A) and *E. faecalis* infected (figure 5.6.A) parents. Male offspring of *E. faecalis* infected parents had similar CFS compared to male offspring of control parents (figure 5.5.B), while male offspring of *E. c. carotovora* infected parents exhibited an increase in CFS (figure 5.6.B).

These results have important implications for eco-immunology of host-pathogen interactions, both in terms of study design and in context of how host defense traits might evolve in the wild.

Plastic parental effects vs. selection bias

In this study, I focused on exploring the parental effects of pathogenic infections in flies. Therefore, in my experiments, parent flies were infected with alive, pathogenic bacteria and eggs for offspring generations were collected from only the individuals that survived, after the acute phase of mortality had passed. This can lead to selection of more immune individuals in the parental generation. The observed results can therefore be driven by either plastic (without genetic changes) parental effects or the above-described selection bias that leads to over-representation of immune genotypes in the offspring generation (Tetreau et al 2019).

Two lines of evidence suggests that the results are in fact consequences of plastic parental effects. One, improvement in offspring post-infection survival decays after one generation in case of both pathogens used in these experiments. Baring one instance, grand-offspring (F2

generation) of infected flies did not differ in post-infection survival compared to grand-offspring of control flies (figures 5.2.A-C). And two, improvement in offspring post-infection survival is observed when eggs are collected just after the acute phase of infection, but vanishes when eggs are collected at a later time point (figure 5.1.B vs. 5.1.C). At both time points only survivors of the infection challenge contribute to the next generation, but improvement in offspring post-infection survival is only seen for eggs collected at the time point just after the acute phase of infection. Based on these two observations, it is safe to reason that the experimental results are driven by plastic parental effects and not by selection imposed in the parental generation.

Specificity due to parental effects

An immune response is considered to be specific if it can discriminate between two pathogens (Schmid-Hempel and Ebert 2003, Ferro et al 2019). A host, by virtue of being well-defended against one pathogen, can also be better at surviving a challenge with a different pathogen, thereby exhibiting cross-resistance (Lazzaro et al 2006). Alternatively, different arms of the immune system may trade-off against one another, and therefore a host by virtue of being well-defended against one pathogen can be more susceptible to a second pathogen (McKean and Lazzaro 2011). Specificity of immune responses in invertebrates, especially insects, is a debated issue (Ferro et al 2019), especially in context of immune priming (Cooper and Eleftherianos 2017). Previous studies exploring specificity of trans-generational immune priming in invertebrates have yielded equivocal results in favour of presence (viz. Kurtz and Franz 2003, Little et al 2003, Tate et al 2017) and lack (viz. Ben-Ami et al 2020) of specificity, often depending upon the host-pathogen pair being studied (Tetreau et al 2019). In fact, specificity has been strongly recommended as a pre-requisite for any improvement of offspring defense, by virtue of the parents being infected, to be termed as trans-generational immune priming (Contreras-Garduno 2016).

Here I tested if improvement in offspring post-infection survival due to parental exposure to pathogenic infection is specific to only the pathogen used for infecting the parents. In my experiments I observe that infecting the parents with a particular pathogenic bacterium can improve offspring post-infection survival when infected with another pathogenic bacterium. This non-specific improvement in offspring survival is contingent on the identity of both the pathogen used to challenge the parents and the pathogen used to challenge the offspring (figures 5.3 and 5.4). In my experiments, the non-specific improvement in offspring post-infection survival is also contingent on the offspring sex and is asymmetric. For example, parents challenged with *E. c. carotovora* produce males that are better at surviving a challenge with *E. faecalis*, but no such survival advantage is observed in the female offspring (5.3.B). On the other hand, both female and male offspring of *E. faecalis*-challenged parents are better at surviving a challenge with *E. c. carotovora* (5.4.B).

This observed lack of specificity has important eco-immunological implications. In the wild, non-specific parental effects can help otherwise susceptible host populations to survive novel infections, thereby offering an opportunity to adapt to novel, invading pathogens. Non-specific parental effects can also contribute towards the high phenotypic variability in host susceptibility to pathogens, which is commonly observed in wild populations.

Costs of parental effects

When offspring individuals encounter a benign environment, after their parents have been subjected to a stressful one, the offspring often pay a fitness cost due to environmental mismatch (Yin et al 2019). This is true for a wide variety of stressors (reviewed by Yin et al 2019) and can in principle be relevant in context of pathogenic infections too. Possibility of costs have been discussed in context of trans-generational immune-priming in previous publications, but with rare demonstration of said costs.

Here I tested if exposing parents to pathogenic infection affects the reproductive capacity of the offspring. In my experiments, I observe that subjecting parents to pathogenic infections does not lead to a reduction in reproductive capacity of offspring flies. Female fecundity and male competitive fertilization success is not compromised when the parents are subjected to infections, in case of both pathogens tested here. This observation suggests that improvement of offspring post-infection survival, by virtue of parental effects, exact no fitness cost in *D. melanogaster* flies.

The cost of parental effects induced by a pathogenic infection (in the parental generation) can also manifest in form of increased susceptibility to a *novel* pathogen, a pathogen not encountered by the parent flies. In my experiments, I do not observe any instance where subjecting parents to infection with any particular bacterium increased the susceptibility of the offspring to infection with another bacterium. Therefore, I propose that parental effects of pathogenic infection have no associated costs, which manifest in the offspring generation, in *D. melanogaster* flies.

The cost of parental effects can also manifest in the parental generation, with parents paying a cost of transmitting information to the offspring and modifying their phenotype. In an experiment where parents are subjected to a pathogenic infection, it is difficult to measure such costs. This is because the infection on its own changes the parents' fecundity, survival, and various other features which are generally used for measuring costs. Therefore, in my experiments, I was unable to directly investigate costs associated with parental effects in the parental generation flies. Despite this handicap, one observation from my experiments suggests that modifying offspring phenotype might indeed be costly to the parents. I observed that when parents are infected with *E. faecalis*, only eggs produced just after the acute phase of infection yielded progeny with improved post-infection survival. Eggs produced 48 hours down the line yielded progeny whose survival did not differ from that of the control offspring when subjected

to infections. This observation, I propose, suggests that parents pay a cost of modifying offspring phenotype, and therefore do not continue to produce *more immune* offspring for the rest of their lives after being infected.

To summarize, I have demonstrated in this part of the chapter that subjecting *Drosophila melanogaster* parents to pathogenic bacterial infections leads to an increase in offspring post-infection survival. This improvement in offspring survival is considerably a plastic change, and not caused by selection in the parental generation. Furthermore, the improvement in offspring survival is not limited to only the pathogen used to infect the parents, but is also not a universal improvement of survival against all pathogenic challenges either. Various factors, such as identity of the pathogen used to challenge the parents, identity of the pathogen used to challenge the offspring, and offspring sex, interact to determine if an increase in offspring post-infection survival is observed following parental exposure to pathogenic infections. Additionally, improvement of survival is not accompanied with any reduction in reproductive capacity in the offspring flies. Therefore, I conclude that exposing parents to pathogenic infections leads to a non-specific increase in offspring post-infection survival, without any measurable costs.

Materials and methods

Experiment 3(a): Effect of parental exposure to pathogenic infections on within-host pathogen growth in infected F1 offspring.

Parental (P) generation. Parental (P) generation. Eggs for the parental generation were collected from the BRB2 population cages, at a density of 6o-80 eggs per vial with 6-8 ml of standard food medium. These were maintained under the regular rearing environment for the BRB2 flies, and the eggs developed into adults by the 10th day post-egg collection. 2–3-day old adult flies were randomly assigned to two treatments: 'infected parents' and 'control parents'. The flies in the 'infected parents' treatment (N = 200 females and 200 males) were subjected to infection with live bacterial pathogens following the protocol described in part A of this chapter, while the flies in the 'control parents' treatment were subjected to sham-infections. After infections the flies of each treatment were housed in separate plexiglass cages (dimensions) with ad libitum access to food. The experiment was set-up with only one pathogen, *Enterococcus faecalis*, and was replicated four times. Parental generation common with experiment 3(b).

Offspring (F1) generation. Eggs for the F1 generation were collected from the flies of the parental generation, after the acute phase of infection was over, i.e., after 48 hours post-infection (HPI). Eggs were collected at a density of 60-80 eggs per vial with 6-8 ml of standard food medium and maintained under the regular rearing environment of the BRB2 flies; the eggs developed into adults by 10th day post-egg collection. 4–5-day old adult F1 flies, from both parental treatments, were either subjected to infections with *E. faecalis* (N = 30 females and 30 males) or were subjected to sham-infections (N = 30 females and 30 males). Flies from each

'offspring treatment', for each 'parental treatment', were housed in separate plexiglass cages. At 4- and 10-hours post-infection (HPI), 12 infected females and 12 infected males from each 'parental treatment' were randomly sampled and their systemic bacterial load was measured. Systemic bacterial load for 12 sham-infected females and 12 sham-infected males from each 'parental treatment' was also measured.

Flies were transferred individually to 1.5 ml vials (micro-centrifuge tubes) containing 50 microliters of sterile $MgSO_4$ (10 mM) buffer. Flies were homogenised in these vials using a motorised pestle for 50-60 seconds. This homogenate was serially diluted (1:10 dilutions) 8 times in sterile $MgSO_4$ (10 mM) buffer. 10 microliters from each dilution, and the original homogenate, were spotted onto a lysogeny agar plate (2% agar, Luria Bertani Broth, Miler, HiMedia). The plates were incubated at 37 ^{O}C, for 8-12 hours, and the number of colony-forming units (CFUs) in each dilution is counted. The number of CFUs in the *countable* dilution ($30 \leq CFUs \geq 300$) was multiplied by appropriate dilution factor to obtain the bacterial load for each individual fly.

Experiment 3(b): Effect of parental exposure to pathogenic infection on post-infection fecundity of F1 females.

Parental (P) generation. Parental generation common with experiment 2(a).

Offspring (F1) generation. Eggs for the F1 generation were collected from the flies of the parental generation, after the acute phase of infection was over, i.e., after 48 hours post-infection (HPI). Eggs were collected at a density of 60-80 eggs per vial with 6-8 ml of standard food medium and maintained under the regular rearing environment of the BRB2 flies; the eggs developed into adults by 10th day post-egg collection. 4–5-day old adult F1 females from both parental treatments were either subjected to infection with E. faecalis (N = 80 females) or were subjected to sham-infections (N = 40 females). Thereafter these females were housed

individually in food vials (with 6 ml standard food medium), where they laid eggs for the next 48 hours, during which their mortality was also monitored every 2 hours. At the end of this period, all the alive females were discarded and the carcasses of the dead females were removed from their vials. The vials were then incubated under regular population maintenance conditions, and 12 days later, the number of adult progeny in each vial was enumerated. Since survival of infected females varied considerably amongst themselves and from that of sham-infected females (for which no death occurred within the observation window), the number of progeny produced by each was divided by the hours survived by the female to obtain the final measure of fecundity, which was subjected to statistical analysis.

Statistical analyses

Log (base 2) transformed bacterial load data was analyzed using type-III analysis of variance (ANOVA), separately for female and male F1 offspring. Parental treatment, time of sampling (hours post-infection), and their interaction were included as fixed factors in the ANOVA, while replicate identity and its interactions with the aforementioned factors were included as random factors. Post-hoc pairwise comparisons were carried out using Tukey's HSD method. Post-infection survival of infected F1 females was compared using mixed-effect Cox proportional-hazards analysis, with parental treatment included as a fixed factor and replicate identity as a random factor. Since no mortality was recorded in sham-infected F1 females, data from these females was excluded from survival analysis. Fecundity data of F1 females was analyzed using ANOVA, with parental treatment, offspring treatment, and their interaction included as fixed factors, while replicate identity and its interactions with the aforementioned factors were included as random factors.

Results

Effect of parental exposure to pathogenic infections on within-host pathogen growth in infected F1 offspring.

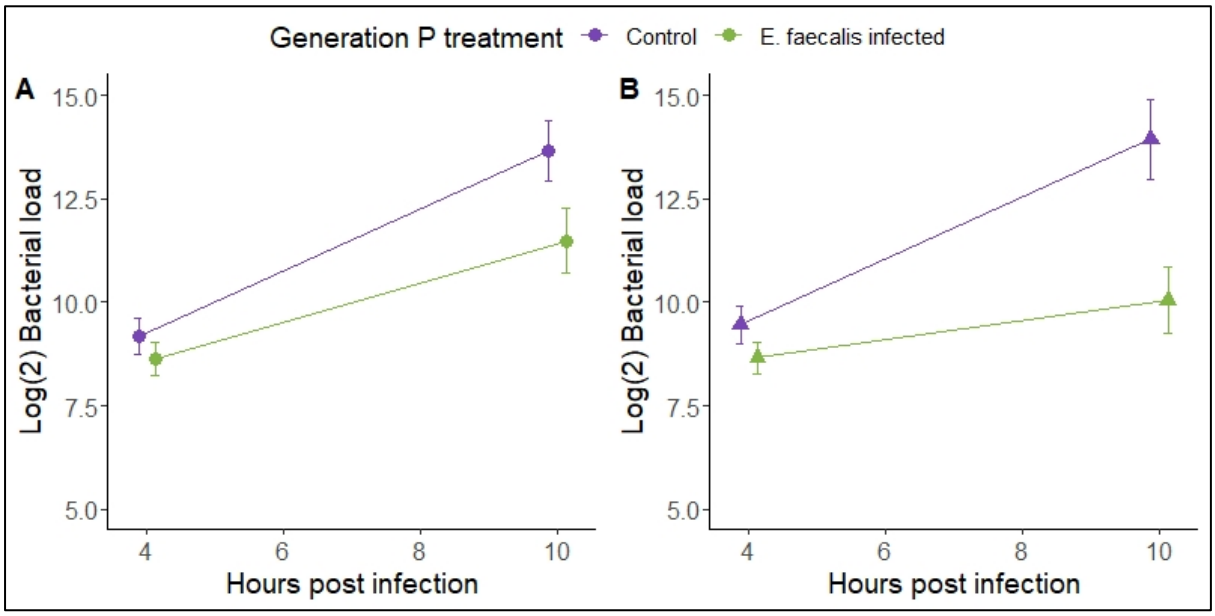
I infected generation P flies with live *Enterococcus faecalis* (along with sham-infected controls), and tested if parental infection treatment determined within-host pathogen growth in females and males from F1 generation upon being infected with *E. faecalis*. Systemic pathogen levels in infected F1 offspring were measured at two time points, 4- and 10-hours post-infection (HPI). Systemic pathogen levels in sham-infected flies were measured only at 4 HPI. None of the sham-infected flies yielded any bacterial colony forming units (CFU).

In infected F1 females, parental infection treatment ($F_{1,192}$ = 20.9157, p-value = 8.59 e-06), HPI ($F_{1,192}$ = 150.7678, p-value < 2.2 e-16), and their interaction ($F_{1,192}$ = 7.4957, p-value = 0.0068) had a significant effect on within-host pathogen levels (figure 5.7.A). At 4 HPI, female offspring of infected parents (least-square mean, 95% confidence interval = 8.62, 8.01-9.23) carried similar bacterial loads as that of female offspring of control (sham-infected) parents (LS mean, 95% CI = 9.17, 8.56-9.78). At 10 HPI, female offspring of infected parents (LS mean, 95% CI = 11.48, 10.87-12.09) carried a significant lower bacterial load compared to female offspring of control parents (LS mean, 95% CI = 13.67, 13.06-14.28).

In infected F1 males, parental infection treatment ($F_{1,4}$ = 39.649, p-value = 0.0032), HPI ($F_{1,4}$ = 28.290, p-value = 0.0060), and their interaction ($F_{1,180}$ = 23.079, p-value = 3.263 e-06) had a significant effect on within-host pathogen levels (figure 5.7.B). At 4 HPI, male offspring of infected parents (LS mean, 95% CI = 8.65, 7.30-10.0) carried similar bacterial loads as that of male offspring of control (sham-infected) parents (LS mean, 95% CI = 9.46, 8.11-10.8). At 10 HPI, male offspring of infected parents (LS mean, 95% CI = 10.05, 8.70-11.4) carried a

significant lower bacterial load compared to male offspring of control parents (LS mean, 95% CI = 13.94, 12.59-15.3).

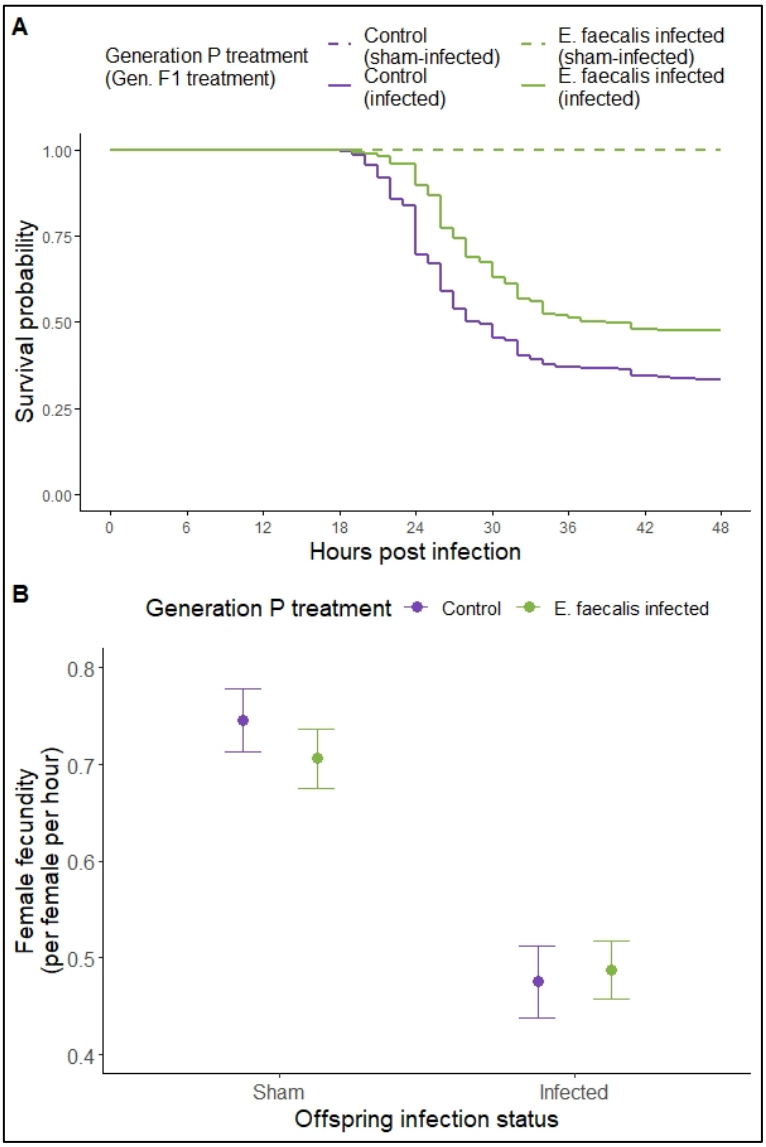
Figure 5.7. Within-host pathogen load in offspring (F1) generation flies after being subjected to infection with the same bacterial pathogen as the corresponding parental (P) generation flies, *Enterococcus faecalis*. Data pooled from all flour replicates. Y-axis error bars represent 95% confidence intervals around the respective means. **(A)** Females. **(B)** Males.



Effect of parental exposure to pathogenic infection on post-infection fecundity of F1 females.

I infected generation P flies with live *E. faecalis* (along with sham-infected controls), and tested if parental infection treatment determined the fecundity of females from F1 generation, with and without being infected with *E. faecalis*. When infected, female offspring of infected parents exhibited reduced mortality compared to female offspring of sham-infected parents (hazard ratio, 95% confidence interval: 0.612, 0.499-0.751). No death was recorded in sham-infected F1 females from either parental treatments (figure 5.8.A). Offspring infection status ($F_{1,4}$ = 97.9970, p-value = 0.0006) had a significant effect on female fecundity, with infected females producing less progeny compared to sham-infected females (figure 5.8.B). Parental infection treatment ($F_{1,938}$ = 0.6072, p-value = 0.4360) and interaction between parental and offspring infection treatments ($F_{1,938}$ = 1.9811, p-value = 0.1596) had no effect on female fecundity.

Figure 5.8. Fecundity offspring (F1) generation females after being subjected to infection with the same bacterial pathogen as the corresponding parental (P) generation flies, *Enterococcus faecalis*. **(A)** Survival of females during the observation window. Survival curves plotted using Kaplan-Meier method after pooling data from all four replicates. **(B)** Female fecundity. Data pooled from all flour replicates. Y-axis error bars represent 95% confidence intervals around the respective means.



Discussion

In the previous part of this chapter, I had observed that *Enterococcus faecalis* infected parents produce offspring which, when themselves subjected to infection with *E. faecalis*, are able to survive better compared to offspring of control parents. Host resistance (i.e., the ability to prevent systemic pathogen proliferation) and tolerance (i.e., ability to ameliorate infection induced somatic damages) complementarily help increase post-infection survival of a host (Raberg et al 2009). Previous studies have questioned the relative contributions of resistance and tolerance mechanisms towards increased host survival brought about by immune priming in insects (Prakash and Khan 2022). Therefore, here we tested if our previous observation was driven by increased resistance in offspring of infected parents.

The results indicate that F1 offspring of *E. faecalis* infected parents, upon being infected, are better at slowing down within-host proliferation of the bacterium compared to offspring of control (sham-infected) parents. This was true in case of both female and male offspring. Although I cannot rule out the role of tolerance mechanisms in explaining increased post-infection survival of offspring of infected parents, since I did not directly measure any canonical proxies of tolerance (viz. BLUD, Duneau et al 2017), I can confirm that increased resistance to infection definitely contributes towards increased post-infection survival in offspring of infected parents.

Increased host-resistance brought about by parental effects can have major implications with relation to host pathogen co-evolution. Increase in host resistance, whether due to evolutionary changes or plastic effects, inadvertently selects for increased virulence in pathogens under natural settings (Miller et al 2006). Therefore, if parental effects make hosts more resistant to pathogens, as in demonstrated in our results, evolution of increased virulence in pathogens will be an expected consequence whenever hosts and pathogens have an opportunity to co-evolve.

The negative effects of pathogen infection on host fitness are not limited to only host mortality, but can also extend to reduction of fecundity (Abbate et al 2015). Hence, here I tested if *E. faecalis* infected parents produce offspring that are better able to withstand infection-induced reduction in their fecundity. The results indicate that in F1 females, infection with *E. faecalis* leads to a reduction in fecundity, but this reduction is uniform in female offspring of infected and control (sham-infected) parents. Female offspring of infected parents survived better after being infected, compared to female offspring of control parents, thus replicating the observation from earlier experiments (experiment 1, chapter 5). Additionally, when sham-infected, all F1 females had similar fecundity irrespective of what treatment their parents were subjected to, thereby reproducing the previous results that parental infection status has no effect on female early-life fecundity in absence of infection (experiment 2(b), chapter 5).

The results thus suggest that hosts reap fitness benefits only in terms of post-infection survival, but not in terms of any effect of infection on fecundity, by virtue of their parents being infected. Post-infection reduction in host fecundity is canonically interpreted either as cost of mounting an immune defense (McKean and Lazzaro 2011) or as being caused by damage to host soma, including reproductive tissue (Brandt and Schneider 2006). Depending on what physiological process underlies reduction in fecundity following infection with *E. faecalis*, my results therefore suggest that parental exposure to pathogenic infections has no impact on immune-deployment costs paid by infected females or the tolerance of infected females to infection-induced tissue damage. If the later is true, results from the fecundity assay rules out the possibility that parental exposure to a pathogenic infection has an effect on hosts' tolerance to infections.

In conclusion, I have demonstrated in this part of the chapter that increased resistance to infection underlies increased offspring post-infection survival brought about by exposure of the parents to pathogenic infection. Additionally, although parental exposure pathogens improve offspring post-infection survival, such exposure does not help the offspring to better deal with negative effects of infection on reproductive effort.

Tables

Table 5.1. Pairwise comparisons using Tukey's HSD accompanying type III ANOVA for the effect of parental treatment and hours post-infection (HPI) on within-host pathogen load in generation F1 flies. Significant effects ($p < 0.05$) are marked in bold font.

Comparison	Estimate	SE	DF	t ratio	p-value
A. Bacterial load in females					
Control 4 - Infected 4	0.55	0.428	193	1.284	0.5740
Control 4 - Control 10	**-4.50**	**0.428**	**193**	**-10.507**	**<.0001**
Control 4 - Infected 10	**-2.31**	**0.428**	**193**	**-5.391**	**<.0001**
Infected 4 - Control 10	**-5.05**	**0.428**	**193**	**-11.791**	**<.0001**
Infected 4 - Infected 10	**-2.86**	**0.428**	**193**	**-6.676**	**<.0001**
Control 10 - Infected 10	**2.19**	**0.428**	**193**	**5.116**	**<.0001**
B. Bacterial load in males					
Control 4 - Infected 4	0.808	0.537	15.86	1.506	0.4573
Control 4 - Control 10	**-4.480**	**0.715**	**9.47**	**-6.267**	**0.0006**
Control 4 - Infected 10	-0.596	0.770	9.35	-0.774	0.8644
Infected 4 - Control 10	**-5.288**	**0.770**	**9.35**	**-6.867**	**0.0003**
Infected 4 - Infected 10	-1.404	0.715	9.47	-1.964	0.2663
Control 10 - Infected 10	**3.884**	**0.537**	**15.86**	**7.235**	**<.0001**

Supplementary figures

Figure S5.1. Survival of parental (P) generation flies after being subjected to infection with a bacterial pathogen or sham-infected. Survival curves plotted using Kaplan-Meier method after pooling data from all replicates.

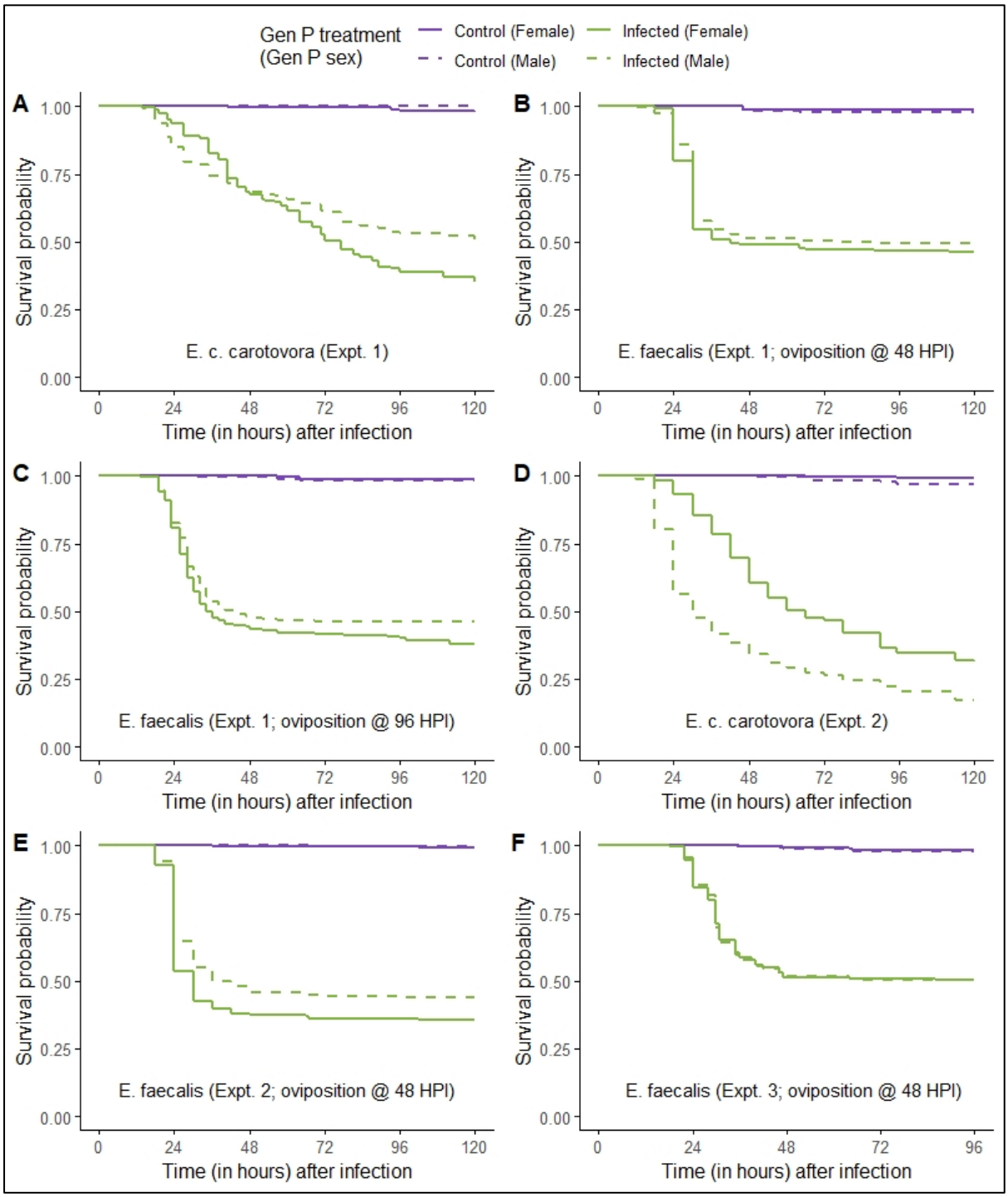


Figure S5.2. Comparison of survival of infected and sham-infected flies in offspring (F1) and grand-offspring (F2) generation in experiment 1. Survival curves plotted using Kaplan-Meier method after pooling data from all replicates. **[Key: Pathogen used to infect generation F1 or F2 / Pathogen used to infect generation P.]**

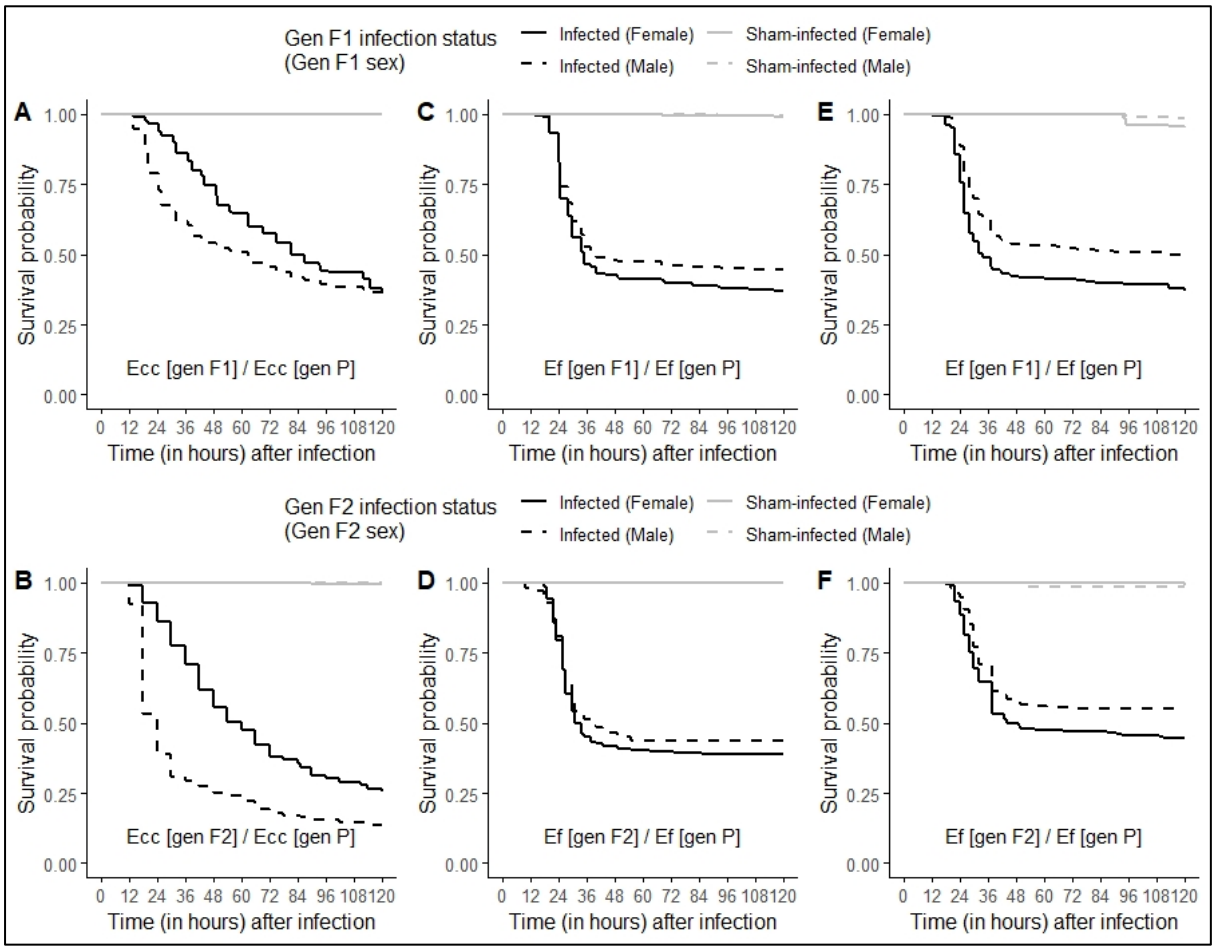
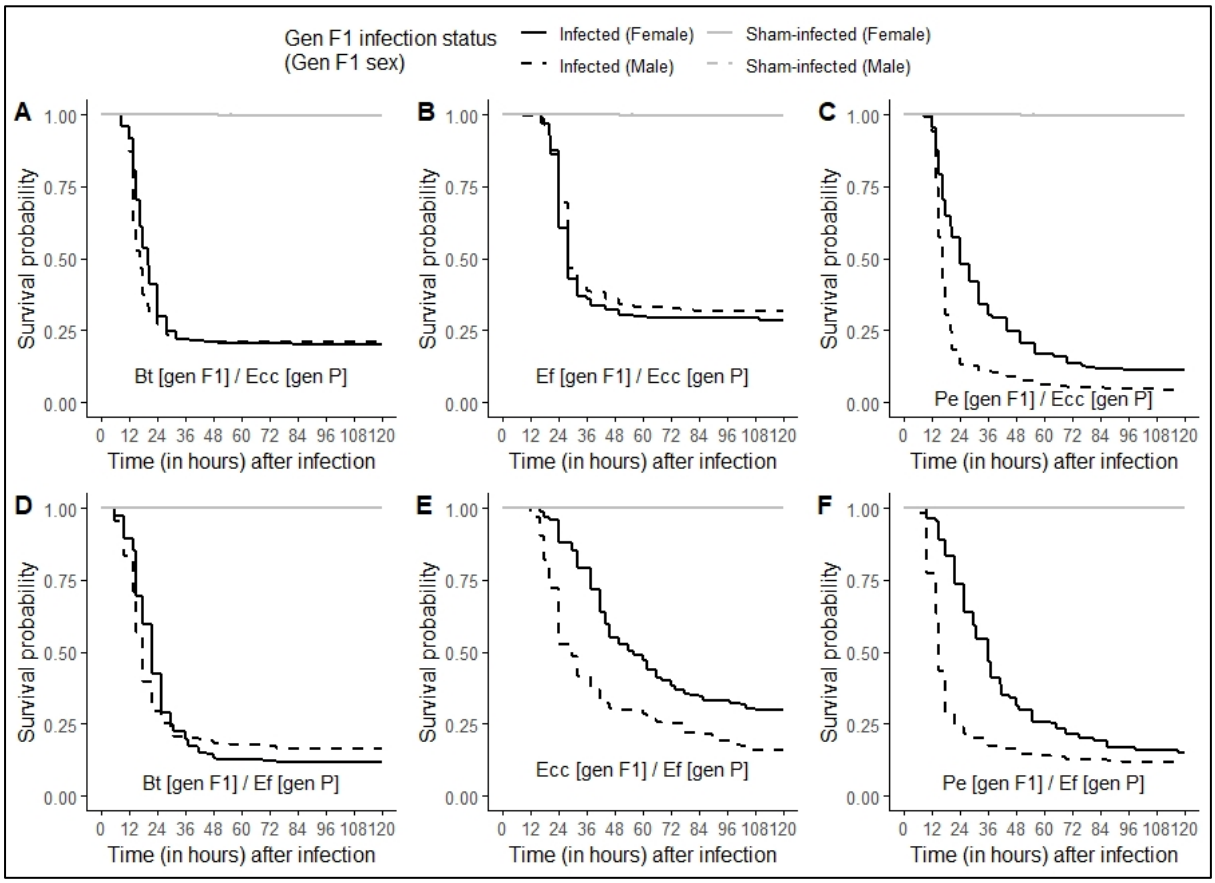


Figure S5.3. Comparison of survival of infected and sham-infected flies in offspring (F1) generation in experiment 2. Survival curves plotted using Kaplan-Meier method after pooling data from all replicates. **[Key: Pathogen used to infect generation F1 / Pathogen used to infect generation P.]**



6. Evolution of increased host resistance in response to selection for increased post-infection survival

Introduction

In order to survive a virulent systemic infection, the infected host must prevent proliferation of the invading pathogen and minimize systemic pathogen loads. Simultaneously, the infected host must ameliorate any damages to its system brought about by the infection process. Therefore, both disease resistance (i.e., the host ability to minimize systemic pathogen burden) and disease tolerance (i.e., the host ability to minimize infection induced damages) are the available alternate, and somewhat complementary, defense strategies an infected host utilizes to maximize its survival (Schneider and Ayres 2008, Read et al 2008, Raberg et al 2009, Raberg and Stjernman 2012, Raberg 2014, Kutzer and Armitage 2016, Ayres and Schneider 2012, Medzhitov et al 2008, Lissner and Schneider 2018). The eco-evolutionary dynamics for these two host defense strategies are distinct from one another and has been elucidated in detail in various theoretical studies (Roy and Kirchner 2000, Restif and Koella 2004, Miller et al 2006, Miller et al 2007, Best et al 2008, Carval and Ferriere 2010, Best et al 2014, Singh and Best 2021).

The genetic architecture of both disease resistance and tolerance has also been investigated in various animal model systems (Hansen and Koella 2003, Raberg et al 2007, Lefevre et al 2011, Parker et al 2014). In *Drosophila melanogaster*, genetic variation for both traits have been documented in both laboratory and wild populations, and single-gene mutations are known that

can modify either or both defense strategies (Lazzaro et al 2004, Lazzaro et al 2006, Corby-Harris et al 2007, Ayres and Schneider 2008, Ayres et al 2008, Dionne and Schneider 2008, Howick and Lazzaro 2014, Vincent and Sharp 2014, Howick and Lazzaro 2017, Hotson and Schneider 2015, Duneau et al 2017). Additionally, environmental parameters that can affect these strategies have also been identified (Lambrechts et al 2006, Corby-Harris and Promislow 2008, Ayres and Schneider 2009, Zeller and Koella 2017, Cumnock et al 2018). Despite these empirical investigations, and a variety of theoretical studies describing the eco-evolutionary dynamics of resistance and tolerance, demonstration of real-time evolution of either of these strategies – either in the wild (Behrman et al 2017) or in the laboratory (Zeller and Koella 2017, Silva 2021) – has been rare.

In the present study I explore if resistance or tolerance evolves when *Drosophila melanogaster* populations are subjected to long-term directional selection for increased post-infection survival in a laboratory experimental evolution set-up. Although resistance or tolerance can evolve in response to direct selection on these traits, such specific direct selection is unlikely in nature. In a natural setting, hosts are under selection to maximize their post-infection fitness, and in such conditions either or both strategies can be selected for, since both strategies offer the solution to the problem of maximizing post-infection survival. Therefore, it is pertinent to test the evolution of resistance and tolerance in response to selection for increased post-infection survival.

I experimentally evolved replicate *Drosophila melanogaster* populations to better survive systemic infection with a Gram-positive bacterial pathogen, *Enterococcus faecalis*. Within 35 generations of forward selection, the selected populations exhibited significant increase in post-infection survival compared to the control populations (Singh et al 2021). Thereafter, I tested if this increase is survival in the selected populations was due to increased resistance, increased tolerance, or both. The results suggest that the selected populations are better at restricting

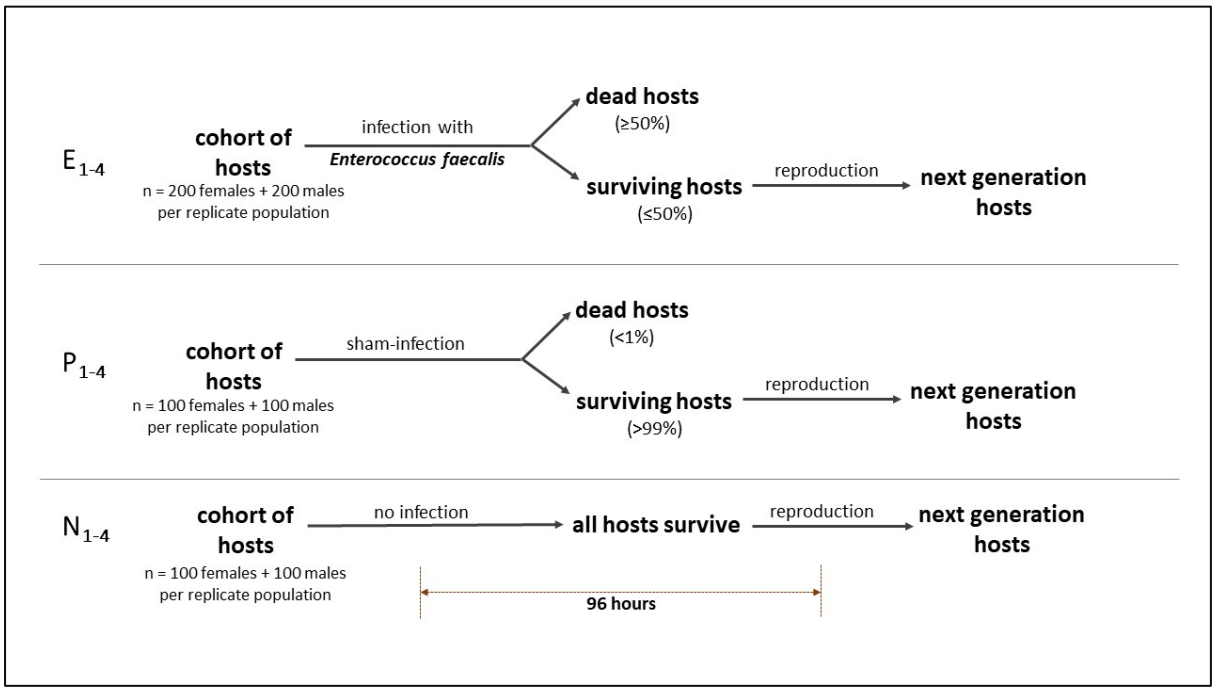
systemic pathogen proliferation after being infected but carry similar bacterial loads at the point of mortality, compared to the control populations. I propose that these observations suggest that the selected populations have evolved to become more resistant to infection, without any observable change in their tolerance levels.

Material and methods

Study system

Flies from the EPN selection regime were used for the experiments reported in this chapter. The EPN selection regime consists of parallelly evolved replicate *D. melanogaster* populations, some selected for increased post-infection survival (E_{1-4}), while others are maintained as either procedural (P_{1-4}) or uninfected (N_{1-4}) controls (Singh et al 2021). Refer to chapter 2 for detailed description of founding and maintenance of these populations.

Figure 6.0. Schematic depicting the maintenance of the EPN selection regime.



Standardization and derivation of experimental flies

To account for any potential parental effects, flies from all populations are maintained in a common environment for one generation prior to any experiment. Flies from the E_{1-4}, P_{1-4}, and N_{1-4} populations are all handled like the ancestral populations. This process is referred to as standardization (Rose 1984), and eggs for experimental flies are collected from these standardized flies.

Infection protocol

Enterococcus faecalis (Lazzaro et al 2006), a Gram-positive bacterium, was used in this study, both for imposing selection on the E_{1-4} populations and for experimental infections.

Systemic pathogen load estimation

In this study, systemic pathogen load was estimated for both dead and alive flies. For estimation of bacterial load upon death (BLUD), the dead flies were homogenized in 200 microliters of sterile $MgSO_4$ buffer within 30-45 minutes of their death. This homogenate was then serially 1:10 diluted eight times; 10 microliters of each dilution, along with 10 microliters of the original homogenate, was spotted onto Luria Bertani (Miller) agar plates. The plates were incubated for 8 hours at 37 ^{O}C. Colony forming units (CFU) were counted for the dilution where CFUs ranged between 25 to 250, and the count was multiplied by the appropriate dilution factor to calculate the systemic pathogen load. For estimation of bacterial load of alive flies, a protocol similar to BLUD estimation was followed, except that the living flies were homogenised in 50 microliters sterile $MgSO_4$ buffer to begin with.

Post-infection survival assay

For assaying post-infection survival, 2–3-day old adult flies from N_{1-4}, P_{1-4}, and E_{1-4} populations were either infected with *Enterococcus faecalis* (n = 100 males and 100 females

per population per block) or sham-infected (n = 50 males and 50 females per population per block), and housed in individual cages. The survival of these flies were monitored every hour for the first 48 hours and thereafter every 4-6 hours till 96 hours post-infection (HPI). Each block was handled separately on separate days. Survival assay was conducted twice, once after 65 generations of forward selection and again after 75 generations of forward selection.

Female fecundity assay (0—48 hours post-infection)

Fecundity of females during 0-48 HPI was assayed after 70 generations of forward selection. For this assay, 4–5-day old, inseminated females were from N_{1-4} and E_{1-4} populations were either infected with *E. faecalis* (n = 80 females per population per block) or sham-infected (n = 40 females per population per block), and thereafter housed individually in food vials where they could oviposit. The survival these females was monitored every 2 hours till 48 HPI. At the end of this window, all surviving females were discarded, and the vials were incubated under standard maintenance conditions, and the number of progenies eclosing out of these vials were counted 12 days after end of oviposition.

Female fecundity assay (96—120 hours post-infection)

Fecundity of females during 96-120 HPI was assayed along with the post-infection survival assay conducted after 75 generations of forward selection. At 96 HPI, alive females from N_{1-4} and E_{1-4} populations, of both infected and sham-infected treatments, were aspirated out of their respective cages and housed individually in food vials (n = 30 females per population per treatment per block). These females were allowed to oviposit for 24 hours, after which they were discarded. The vials with laid eggs were incubated under standard maintenance conditions, and the number of progenies eclosing out of these vials were counted 12 days after oviposition. The assay design closely matches the regular maintenance regime, where 96 hours

after infection the flies are allowed to oviposit on fresh food medium, and these eggs are used to start the next generation.

Systemic bacterial load of dead flies

Systemic bacterial load of dead flies (Bacterial Load Upon Death) was assayed along with the post-infection survival assay conducted after 75 generations of forward selection. Dead females and males of N_{1-4} and E_{1-4} populations were aspirated out of the cages within an hour of their death, and their systemic bacterial load was estimated using the above-described protocol. The sample size varied between 15-30 flies per sex per population per block, depending upon how many flies perished due to infection. Since no death was recorded in sham-infected flies, systemic load of sham-infected flies was not monitored during this assay.

Systemic bacterial load of alive flies

To assay for the time dependent changes in systemic bacterial load in living, infected females, 700 N_{1-4} females and 350 E_{1-4} females (per block) were infected, and housed in separate cages. Starting sample size of N females was double of that of the E females to ensure enough surviving flies were available for bacterial load measurements at later time points, given that N females are expected to have much greater mortality compared to E females. Starting from 3 hours post-infection (HPI), every 3 hours, the systemic bacterial load of the females were assayed for the first 48 hours, after which the sampling frequency was reduced to 6-12 hours. The assay continued till 96 HPI. At every sampling time point, 10 alive females of each population were aspirated out of their respective cages, and their systemic bacterial load was estimated using the above-described protocol. Individual blocks were assayed on separate days. To assay for the time dependent changes in systemic bacterial load in living, infected males, we followed an identical experimental design, except that the starting sample sizes for N_{1-4} and E_{1-4} males were 240 and 120 (per block), respectively, and measurement of systemic bacterial

load was carried out only at 4-, 10-, 48-, and 96-HPI. This assay was done for females after 78 generations of froward selection and for males after 80 generations of forward selection.

Statistical analysis

Post-infection survival of infected flies was analyzed using a mixed-effects Cox proportional hazards model, which included host evolutionary history, host sex, and their interaction as fixed factors, and block as a random factor. The model was subjected to Analysis of Deviance (type II) for significance testing. Analysis was done for the survival assays carried out in generations 65 and 75 separately, and only the infected flies were considered since there was very little mortality in the sham-infected flies. Female fecundity was analyzed using Analysis of Variance (type III ANOVA), with infection treatment, host evolutionary history, and their interaction as fixed factors, and block as a random factor. Bacterial Load Upon Death (BLUD, $\log_2$ transformed systemic bacterial load) was analyzed using ANOVA (type III), with time of death (HPI), host evolutionary history, host sex, and their interaction as fixed factors, and block and its interaction with the fixed factors as random factors. Post-hoc pairwise comparisons for ANOVA were carried out using Tukey's HSD method. Bacterial load in living flies ($\log_2$ transformed systemic bacterial load) was analyzed using ANOVA (type III), with hours post-infection (HPI), host evolutionary history, and their interaction as fixed factors, and block and its interaction with the fixed factors as random factors. Data for each sex was analyzed separately. Pairwise comparison between bacterial load carried by E and N flies at each HPI was carried out using Holm-Sidak method for p-value correction.

Results

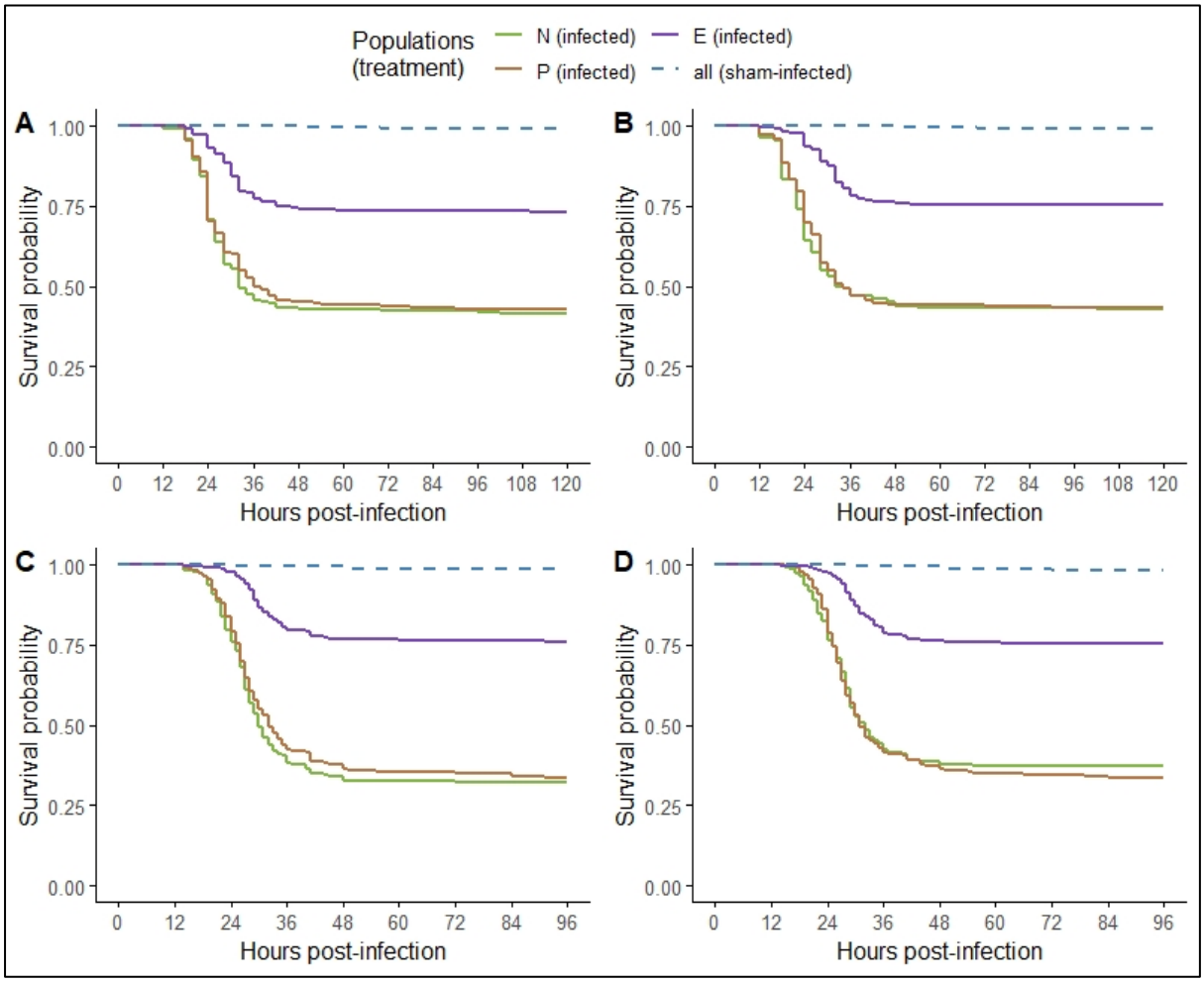
Post-infection survival

To test for response to selection, flies from the N_{1-4}, P_{1-4}, and E_{1-4} populations were infected with *Enterococcus faecalis*, and their post-infection survival was measured. Post-infection survival assay was conducted twice: once after 65 generations of forward selection and again after 75 generations of forward selection.

When assayed for after 65 generations of forward selection, selection history had a significant effect on post-infection survival (table 6.1.A). Host sex, and selection history × host sex interaction did not have any effect on post-infection survival (table 6.1.A). E females (hazard ratio, 95% confidence interval: 0.330, 0.263-0.415) exhibited better post-infection survival compared to N females; P females (HR, 95% CI: 0.949, 0.791-1.139) did not differ from N females in terms of post-infection survival (figure 6.1.A). Similarly, E males (HR, 95% CI: 0.305, 0.241-0.386) exhibited better post-infection survival compared to N males; P males (HR, 95% CI: 0.943, 0.785-1.134) did not differ from N males in terms of post-infection survival (figure 6.1.B).

When assayed for after 75 generations of forward selection, selection history had a significant effect on post-infection survival (table 6.1.B). Host sex, and selection history x host sex interaction did not have any effect on post-infection survival (table 6.1.B). E females (HR, 95% CI: 0.220, 0.174-0.278) exhibited better post-infection survival compared to N females; P females (HR, 95% CI: 0.908, 0.767-1.076) did not differ from N females in terms of post-infection survival (figure 6.1.C). Similarly, E males (HR, 95% CI: 0.250, 0.198-0.317) exhibited better post-infection survival compared to N males; P males (HR, 95% CI: 1.026, 0.862-1.221) did not differ from N males in terms of post-infection survival (figure 6.1.D).

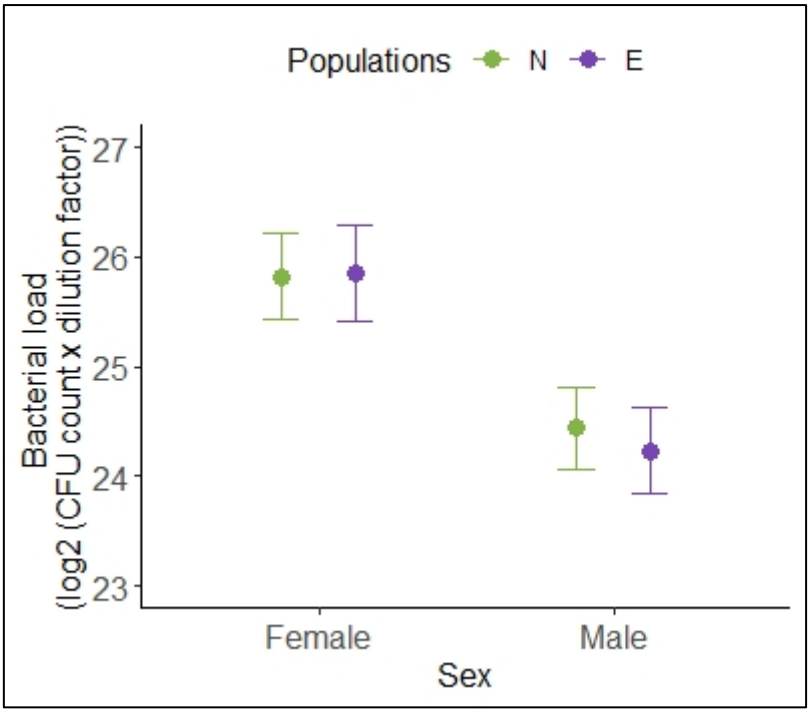
Figure 6.1. Survival of flies from the N_{1-4}, P_{1-4}, and E_{1-4} populations after being infected with *Enterococcus faecalis* or sham-infected. Survival curves plotted using Kaplan-Meier method after pooling data across all four replicates for each selection regime. **(A)** Survival of females, generation 65. **(B)** Survival of males, generation 65. **(C)** Survival of females, generation 75. **(D)** Survival of males, generation 75.



Systemic bacterial load in dead flies

Bacterial load upon death (BLUD) of infected females and males from N_{1-4} and E_{1-4} populations was measured along with the post-infection survival assay conducted after 75 generations of forward selection. Only host sex had a significant effect on BLUD (table 6.2.C); time of death, selection history, and selection history × host sex did not have a significant effect on BLUD (figure 6.2). Pair-wise comparison using Tukey's HSD (table 6.3.C) indicated that N males (Least-square mean, 95% CI: 24.5, 23.8-25.2) and E males (LS mean, 95% CI: 24.2, 23.4-25.0) carried similar bacterial loads at the time of their death, and so did N females (LS mean, 95% CI: 25.9, 25.2-26.6) and E females (LS mean, 95% CI: 25.8, 25.1-26.6) females. Pooled across both populations, the males carried much lower bacterial loads at the time of their death compared to the females.

Figure 6.2. Bacterial load upon death (BLUD) of flies from the N_{1-4} and E_{1-4} populations infected with *Enterococcus faecalis*. Data pooled across all four replicates for each selection regime. Y-axis error bars represent 95% confidence intervals around the respective means.

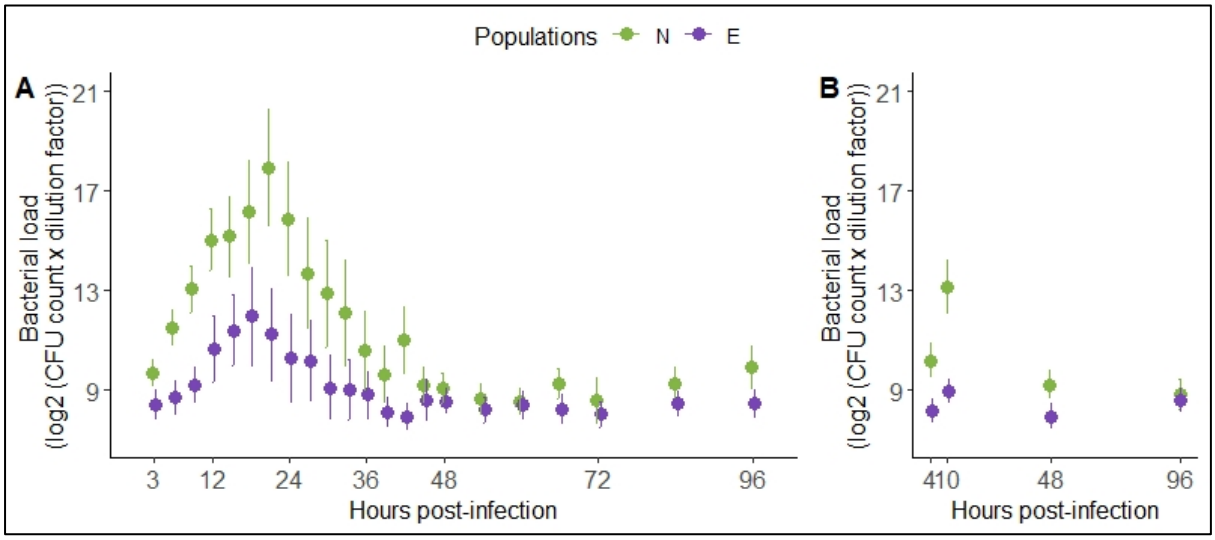


Systemic bacterial load in living flies

Systemic bacterial load of living, infected females from N_{1-4} and E_{1-4} populations was measured after 78 generations of forward selection. Time of sampling (hours post-infection), selection history, and the interaction between the two had a significant effect on the bacterial load carried by living females (table 6.2.D), with E females having either less or equal bacterial load compared to N females at every sampling point (figure 6.3.A. Using Holm-Sidak correction of p-values (table 6.4.A) I identified that E females carried significantly less bacterial load compared to N females at 3- to 24-, 30-, and 42-hours post-infection (HPI).

Systemic bacterial load of living, infected males from N_{1-4} and E_{1-4} populations was measured after 80 generations of forward selection. Time of sampling (hours post-infection), selection history, and the interaction between the two had a significant effect on the bacterial load carried by living males (table 6.2.E), with E males having either less or equal bacterial load compared to N males at every sampling point (figure 6.3.B. Using Holm-Sidak correction of p-values (table 6.4.B) I identified that E males carried significantly less bacterial load compared to N males at 4-, 10-, and 48-hours post-infection (HPI).

Figure 6.3. Systemic bacterial load carried by alive flies from the N_{1-4} and E_{1-4} populations at different time-points (hours post-infection) after being infected with *Enterococcus faecalis*. Data pooled across all four replicates for each selection regime. Y-axis error bars represent 95% confidence intervals around the respective means. **(A)** Systemic bacterial load of females. **(B)** Systemic bacterial load of males.

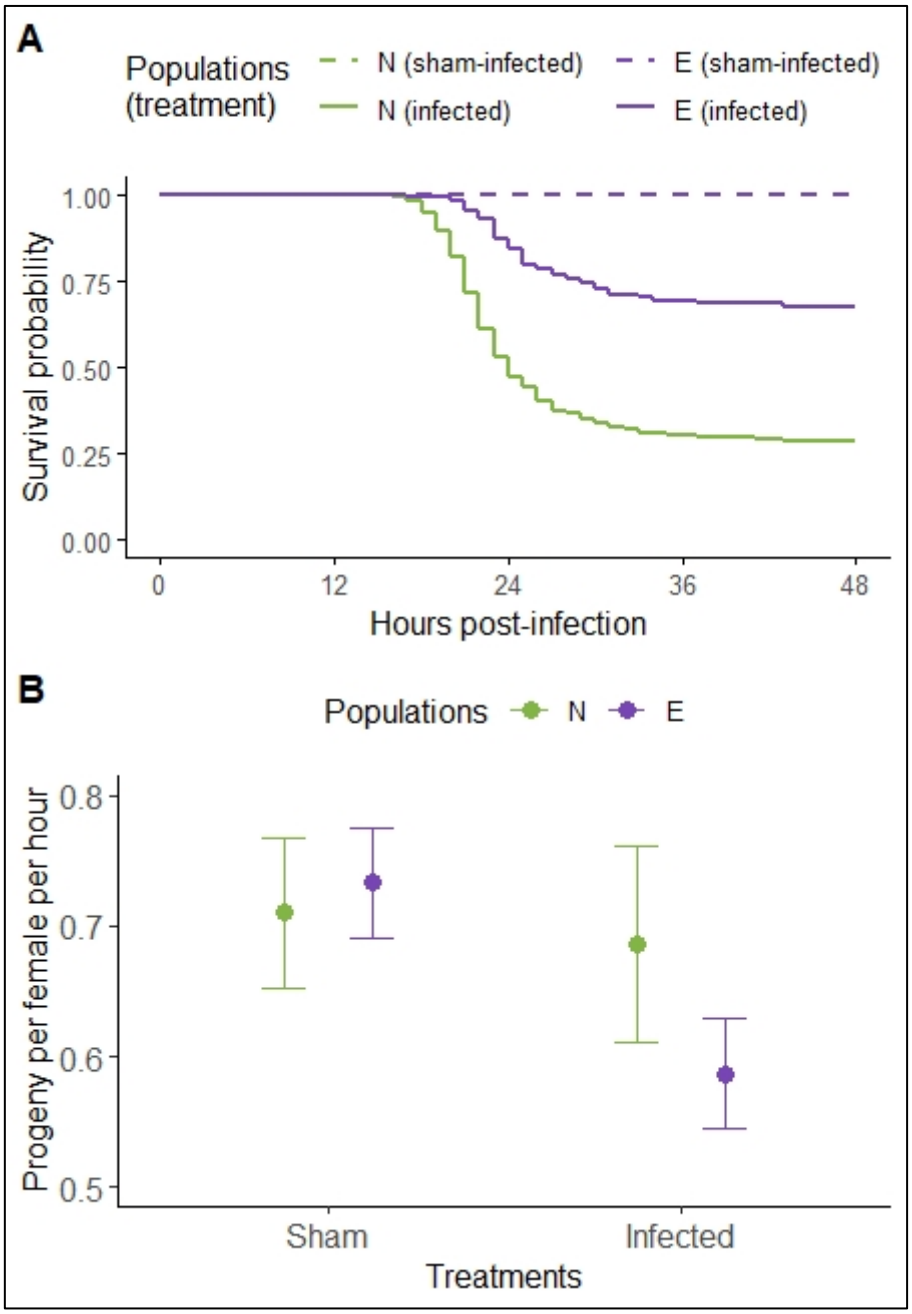


Female fecundity (0-48 hours post-infection)

Female fecundity (number of progenies produced by an individual female per hour) for 0-48 hours post-infection of infected and sham-infected females from $N_{1\text{-}4}$ and $E_{1\text{-}4}$ populations was measured after 70 generations of forward selection. Infected females succumbed to infection during the period when fecundity was being measured in this assay. Hence, to accommodate different periods of survival between infected and sham-infected females, and amongst infected females, the number of progenies produced by an individual female was divided by the number of hours the female survived, and this standardized number of progenies was used as unit of fecundity in this assay.

During the assay duration, no mortality was recorded in the sham-infected females from either $N_{1\text{-}4}$ or $E_{1\text{-}4}$ populations (figure 6.4.A). Among the infected females, females from the $E_{1\text{-}4}$ populations died significantly less compared to females from the $N_{1\text{-}4}$ populations (Hazard ratio, 95% confidence interval: 0.292, 0.231-0.369). Neither selection history nor infection treatment had any significant effect on female fecundity, but selection history × infection treatment had a significant effect (table 6.2.A, figure 6.4.B). Pairwise comparison using Tukey's HSD (table 6.3.A) indicated that E-infected females (least-square mean, 95% CI: 0.587, 0.308-0.867) produced less number of progenies per hour compared to N-infected (LS mean, 95% CI: 0.686, 0.407-0.966), E-sham-infected (LS mean, 95% CI: 0.733, 0.455-1.011), and N-sham-infected (LS mean, 95% CI: 0.712, 0.434-0.990) females. N-infected, E-sham-infected, and N-sham-infected females did not differ from one another in terms of the number of progenies produced per hour.

Figure 6.4. Survival and fecundity of females from the N_{1-4} and E_{1-4} populations during 0 – 48 hours post-infection. **(A)** Survival of the females after being subjected to infection with *Enterococcus faecalis* or to sham-infection. Survival curves plotted using Kaplan-Meier method after pooling data across all four replicates for each selection regime. **(B)** Fecundity of females after being subjected to infection with *Enterococcus faecalis* or to sham-infection. Data pooled across all four replicates for each selection regime. Y-axis error bars represent 95% confidence intervals around the respective means.

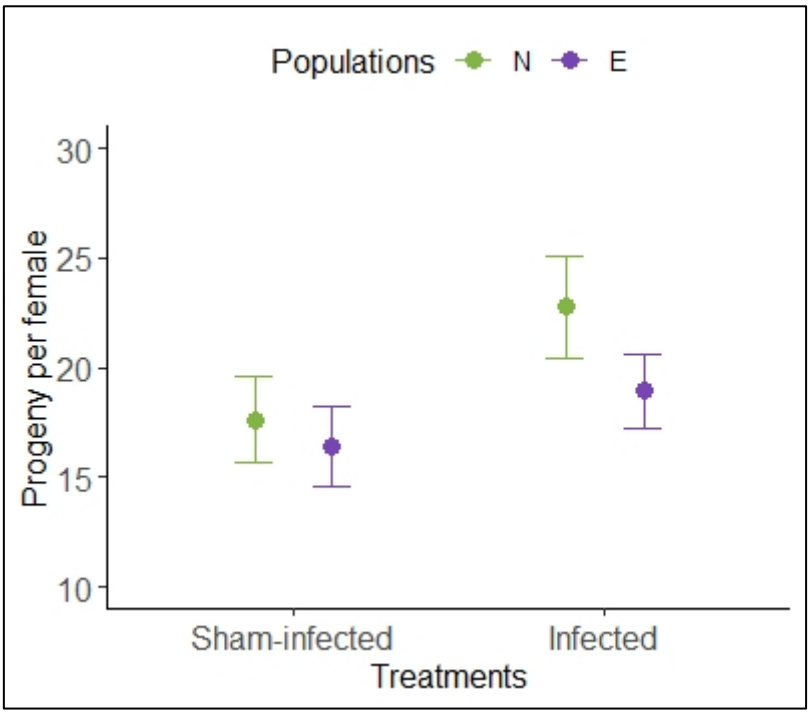


Female fecundity (96-120 hours post-infection)

Female fecundity (number of progenies produced by an individual female) for 96-120 hours post-infection of infected and sham-infected females from N_{1-4} and E_{1-4} populations was measured along with the post-infection survival assay conducted after 75 generations of forward selection. Since no death was recorded during this period in females of either infection treatment, absolute number of progenies produced by an individual female was used as unit of fecundity in this assay.

Selection history and infection treatment had a significant effect on female fecundity (table 6.2.B), but their interaction did not have a significant effect (figure 6.5). Pair-wise comparison using Tukey's HSD (table 6.3.B) indicated that N-infected females (least-square mean, 95% CI: 22.7, 19.4-26.1) produced a greater number of progenies compared to N-sham-infected (LS mean, 95% CI: 17.6, 14.3-20.9), E-infected (LS mean, 95% CI: 18.9, 15.6-22.2), and E-sham-infected (LS mean, 95% CI: 16.4, 13.1-19.7) females. N-sham-infected, E-infected, and E-sham-infected females did not differ from one another in terms of number of progenies produced.

Figure 6.5. Fecundity of females from the N_{1-4} and E_{1-4} populations during 96 – 120 hours post-infection, after being subjected to infection with *Enterococcus faecalis* or to sham-infection. Data pooled across all four replicates for each selection regime. Y-axis error bars represent 95% confidence intervals around the respective means.



Discussion

The fitness of an infected host is contingent upon both its resistance and tolerance to the infecting pathogen. Host resistance determines the intensity of the infection (i.e., the systemic pathogen load carried by the host), while host tolerance determines the effect of a given infection intensity on host fitness (i.e., the level of morbidity or mortality experienced by the host). In this study, I investigated whether hosts evolve increased resistance, tolerance, or both when subjected to selection for increased post-infection survival in a laboratory experimental evolution set-up.

Briefly, I experimentally evolved replicate *Drosophila melanogaster* populations to survive better after being infected with an entomopathogenic Gram-positive bacterium, *Enterococcus faecalis*. The strain of *E. faecalis* used for imposing selection was originally isolated from wild-caught flies (Lazzaro et al 2006), making it suitable for use in a study of experimental evolution of immune function. The selected flies were infected with the bacterium every generation, and only those individuals that survived the infection were allowed to reproduce and contribute to the next generation. Within 35 generations of forward selection, the flies of the selected (E_{1-4}) populations exhibited increased post-infection survival compared to the flies of both the procedural control (P_{1-4}) and the uninfected control (N_{1-4}) populations (Singh et al 2021). The selected populations continued to become significantly better at surviving infection with *E. faecalis* with continued selection, as demonstrated by the results from the survival assays, carried out after 65 generations (figures 6.1.A and 6.1.B) and 75 generations (figures 6.1.C and 6.1.D) of forward selection, reported in this study. Given that the selected populations rapidly evolved increased post-infection survival, I explored if this increase in survival was explained by increase in resistance, tolerance, or both in these populations.

No evidence for evolution of tolerance

To compare the tolerance of flies from the selected and control populations, I compared the Bacterial Load Upon Death (BLUD) of infected females and males from the E_{1-4} and N_{1-4} populations. BLUD has been suggested as a suitable proxy of host tolerance to bacterial infections in *D. melanogaster* in previous studies (Duneau et al 2017, Duneau et al 2017, Vincent et al 2020). The results show that the flies from the selected (E_{1-4}) and the control (N_{1-4}) populations carry similar BLUD (figure 6.2), suggesting that the flies succumb to infection at similar systemic pathogen loads irrespective of their selection history. I propose that this observation indicates that there has been no evolution of host tolerance in the selected populations, even after 75 generations of forward selection.

The results also show that there is sexual dimorphism in BLUD: females of both selected and control populations carry a significantly higher BLUD compared to males from their respective populations (figure 6.2). This observation may represent sexual dimorphism in tolerance to infection with *E. faecalis* in these populations, with the females being more tolerant to *E. faecalis* infection than the males. Sexual dimorphism in disease tolerance is known in *D. melanogaster* (Vincent and Sharp 2014), although a previous study has reported that BLUD for *Providencia rettgeri* is not affected by host sex in certain laboratory lines (Duneau et al 2017). Alternatively, my observation may simply be a manifestation of sexual dimorphism in body size. *D. melanogaster* females tend to be bigger compared to males, and this trend holds true in case of these populations too, as has been demonstrated by previous experiments (Singh et al 2022). It is possible that the females, by virtue of having a greater body size compared to the males, succumb to infection, and die at a greater systemic bacterial load.

Evolution of increased resistance

A more resistant host is better at limiting systemic proliferation of pathogens. Therefore, I measured the systemic bacterial load of living flies from the $E_{1\text{-}4}$ and $N_{1\text{-}4}$ populations at regular intervals after being infected with *E. faecalis*, to study the dynamics of systemic bacterial growth during different stages of infection. The results demonstrate that in the infected females (figure 6.3.A), of both $E_{1\text{-}4}$ and $N_{1\text{-}4}$ populations, systemic bacterial load initially increases (3—18 hours post-infection, HPI) and then decreases (18—48 HPI), and thereafter eventually settles down to a somewhat stagnant bacterial load (48—96 HPI). Mortality due to *E. faecalis* infection in these populations is mostly limited to before 48 HPI, with very few flies dying after 48 HPI (figure 6.1). Therefore, 0—48 HPI represents the *acute phase* of *E. faecalis* infection in these populations, and the period after 48 HPI represents the *chronic phase* (Chambers et al 2019, Hidalgo et al 2022).

Females of the selected ($E_{1\text{-}4}$) populations carry a lower systemic bacterial load compared to females of the control ($N_{1\text{-}4}$) populations during the acute phase of infection (figure 6.3.A); significantly so during 3—30 HPI (table 6.4.A). From 48 HPI onwards, females from both populations carry a similar Set Point Bacterial Load (SPBL, *sensu* Duneau et al 2017) during the chronic phase of infection. Similar dynamics is seen in case of systemic bacterial load in males, with males of the selected ($E_{1\text{-}4}$) populations carrying a lower systemic bacterial load compared to males of the control ($N_{1\text{-}4}$) populations during 4—48 HPI, but having similar SPBL at 96 HPI (figure 6.3.B, table 6.4.A). These observations suggest that the $E_{1\text{-}4}$ flies are better at suppressing the proliferation of bacteria within their body compared to $N_{1\text{-}4}$ flies. Therefore, I propose that the selected populations have evolved increased resistance to *E. faecalis* infection in response to the selection for increased post-infection survival imposed upon them for 78—80 generations.

Additionally, the results indicate that the different between systemic bacterial loads in infected flies from $E_{1\text{-}4}$ and $N_{1\text{-}4}$ populations is evident at the earliest time point for which bacterial load

was measured (3 HPI for females and 4 HPI for males). Previous studies have proposed that systemic bacterial dynamics early on in infection eventually determine infection outcome at later stages (Duneau et al 2017). An early difference in the systemic bacterial loads carried by the flies from the selected and the control populations might additionally suggest that whatever mechanism underlies increased resistance in the selected populations is either a constitutively active defense or a defense that can be rapidly induced following infection.

Fecundity cost associated with increased resistance

Previous studies, both theoretical and empirical, have suggested that evolution of increased resistance imposes various costs on the host organism (reviewed in Raberg and Stjernman 2012). Such costs may manifest as physiological trade-offs, such as reduced reproductive capacity, both in presence and in absence of an infection (Schmid-Hempel 2005, McKean and Lazzaro 2011). Therefore, I investigated if evolution of increased resistance in the selected populations is accompanied by a concomitant reduction in female fecundity, with and without being infected with *E. faecalis*. I compared fecundity of infected and sham-infected females from the $E_{1\text{-}4}$ and $N_{1\text{-}4}$ populations, both during the acute phase (0—48 HPI) and chronic phase (96—120 HPI) of infection, in two separate experiments. The effect of bacterial infections on female fecundity changes in *D. melanogaster* depending upon the phase of infection (Howick and Lazzaro 2014). Therefore, examining the presence of costs during both the acute and the chronic phase is relevant here. It should be noted that the window between 96—120 HPI coincides with the breeding window of these populations during their regular maintenance, and therefore is to be considered as their true fitness window. During regular population maintenance, only the flies that reproduce during this period contribute to future generations. Fecundity of females during this period is akin to their lifetime reproductive success.

When female fecundity was measured during the acute phase of infection (figure 6.4.B), females from the selected (E_{1-4}) and the control (N_{1-4}) populations had similar fecundity when sham-infected, i.e., in absence of infection. This observation suggests that the selected populations do not pay any *maintenance cost* (sensu McKean et al 2008, McKean and Lazzaro 2011) of increased resistance. When infected with *E. faecalis*, females from the selected populations exhibited a decline in fecundity compared to sham-infected controls. Such a decline in fecundity was not observed in case of females from the control populations. These observations suggest that the selected populations incur an evolved cost of *immune deployment* (sensu McKean et al 2008, McKean and Lazzaro 2011) associated their increased resistance. This decline in fecundity of females from the selected populations after being infected can be caused either by resource/energy trade-offs between reproduction and defense mechanisms (Schmid-Hempel 2003), or by damage to the reproductive tissue from the infection (Brandt and Schneider 2006).

When female fecundity was measured during the chronic phase of infection (figure 6.5), the females from the selected (E_{1-4}) populations had similar fecundity irrespective of whether they were infected with *E. faecalis* or sham infected. The females from the control (N_{1-4}) populations exhibited a mild, but significant, increase in fecundity after being infected. Again, in absence of infection, the females from the selected and the control populations had similar fecundity. Therefore, during the chronic phase of infection, I do not see any cost of increased resistance, *maintenance* or *deployment*, in the selected populations. Importantly, this window of fecundity measurement also represents the selection window of these populations. Thus, during the selection window, when it matters most, the selected populations incur no fecundity cost of increased resistance.

Absence of any fecundity costs during the chronic phase (also the selection window) has two important implications. First, this shows that flies can recover from suppression of fecundity

induced by *E. faecalis* infection and that chronic infection by this pathogen does not exact a fitness cost on the host. This also suggests that the suppression of fecundity observed during the acute phase is more likely to be driven by resource reallocation trade-off and not damage to the reproductive tissue. And second, absence of costs associated with increased resistance facilitates the evolution or resistance. Physiological costs associated with disease resistance is one of the major factors that limit evolution of increased resistance under natural settings. Absence of physiological costs of resistance to *E. faecalis* therefore has favoured the evolution of increased resistance in the selected populations, and may have also relaxed the selection of evolution of tolerance.

Additionally, presence of a cost of *deployment* in the selected populations only during the acute phase of infection has an interesting implication. In principle, it is possible that the females from the selected populations have evolved fecundity suppression as a defense strategy. They temporally partition their priorities. Early in infection, during the acute phase, the selected females reduce reproductive investment and re-allocate available resources towards resisting systemic pathogen growth, thereby improving resistance. Following this period, they recover from the infection, and thereafter in the selection window (chronic phase of infection) they invest towards reproduction and thereby ensuring their contribution to the next generation. Previous studies have debated if post-infection suppression of fecundity is a consequence of the pathogen manipulating the host physiology or a host defense strategy to better its own fitness (Hurd 2001). It has also been previously questioned if suppression of reproductive effort can help hosts improve their post-infection survival (Javois 2013). Therefore, my result may act as a proof-of-principle that suppression of fecundity can indeed help host survive an infection challenge, and can also evolve as a viable defense strategy.

In addition to absence of costs associated with increased resistance, evolution of tolerance in the selected populations may have been hindered by lack of heritable genetic variation for

tolerance. Previous studies have argued that wild populations are not likely to harbour genetic polymorphism for tolerance, because whenever a more tolerant mutant invades a population, it quickly goes to fixation (Roy and Kirchner 2000). Therefore, although the baseline populations used to initiate the selected populations in our study was derived from wild-caught flies, it is possible that there was a lack of heritable genetic variation for tolerance to *E. faecalis* infection in the starting populations. However, I believe that this is an unlikely scenario. These same baseline populations have been successfully used to initiate three separate experimental evolution set-ups, all selecting for increased post-infection survival, including the present populations (Gupta et al 2015, Ahlawat et al 2022, Singh et al 2021). At every instance, a rapid response to selection was observed, suggesting an abundance of heritable genetic variation for antibacterial defense strategies. However, since partitioning of resistance and tolerance was never carried out in case of these other populations, I cannot confirm if the observed increase in post-infection survival in these populations was due to resistance or tolerance.

To summarize, in this chapter, I have demonstrated that hosts evolve increased resistance to bacterial infection when they are subjected to selection for increase post-infection survival following a systemic pathogen challenge. The same selection pressure does not lead to an evolution of tolerance.

Tables

Table 6.1. Analysis of deviance (type II) on mixed-effect Cox proportional hazards model to test for the effect of selection history (population identity), sex, and their interaction on survival of flies after being infected with *Enterococcus faecalis*. Significant effects ($p < 0.05$) are marked in bold font.

Factor	DF	Chi-sq	Pr(>Chi-sq)
A. Post-infection survival assay after 65 generations of forward selection			
Population	**2**	**210.4453**	**<2e-16**
Sex	1	0.0373	0.8468
Population × Sex	2	0.6623	0.7181
B. Post-infection survival assay after 75 generations of forward selection			
Population	**2**	**332.6102**	**<2e-16**
Sex	1	0.5500	0.4583
Population × Sex	2	1.3849	0.5004

Table 6.2. Analysis of variance (ANOVA, type III) for the effect of selection history (population identity), sex, etc. on various assayed traits. Significant effects ($p < 0.05$) are marked in bold font.

Factors	SS	MS	DF	Residual DF	F value	p-value
A. Female fecundity, during 0—48 hours post-infection						
Treatment	0.01506	0.01506	1	4.04	0.0826	0.78790
Population	0.28966	0.28966	1	3.99	1.5891	0.27607
Treatment × Population	**0.78827**	**0.78827**	**1**	**946.36**	**4.3246**	**0.03783**
B. Female fecundity, during 96—120 hours post-infection						
Treatment	**1697.95**	**1697.95**	**1**	**462**	**15.976**	**7.467e-05**
Population	**720.51**	**720.51**	**1**	**462**	**6.779**	**0.00952**
Treatment × Population	188.66	188.66	1	462	1.775	0.18342
C. Bacterial load upon death (BLUD)						
HPI	0.494	0.494	1	296	0.1311	0.717528
Population	1.037	1.037	1	6	0.2752	0.619233
Sex	**92.595**	**92.595**	**1**	**4**	**24.5787**	**0.007409**
Population × Sex	1.013	1.013	1	4	0.2690	0.632114
D. Within-host bacterial dynamics, females						
HPI	**527.88**	**527.88**	**1**	**84**	**35.611**	**5.549e-08**
Population	**1842.30**	**1842.30**	**1**	**20**	**124.281**	**5.495e-10**
HPI × Population	**562.32**	**562.32**	**1**	**1661**	**37.914**	**9.159e-10**
E. Within-host bacterial dynamics, males						
HPI	**109.52**	**109.52**	**1**	**320**	**23.091**	**2.38e-06**
Population	**373.66**	**373.66**	**1**	**320**	**78.785**	**< 2.2e-16**
HPI × Population	**107.72**	**107.72**	**1**	**320**	**22.712**	**2.86e-06**

Table 6.3. Pairwise comparisons using Tukey's HSD accompanying type III ANOVA for the effect of selection history (population identity), sex, etc. on various assayed traits. Significant effects ($p < 0.05$) are marked in bold font.

Comparison	Estimate	SE	DF	t ratio	p-value
A. Female fecundity, during 0—48 hours post-infection					
N Sham - E Sham	-0.0215	0.0505	957	-0.425	0.9741
N Sham - N Infected	0.0253	0.0438	957	0.579	0.9385
N Sham - E Infected	**0.1246**	**0.0438**	**957**	**2.844**	**0.0235**
E Sham - N Infected	0.0468	0.0437	957	1.072	0.7069
E Sham - E Infected	**0.1461**	**0.0437**	**957**	**3.342**	**0.0048**
N Infected - E Infected	**0.0993**	**0.0357**	**957**	**2.780**	
B. Female fecundity, during 96—120 hours post-infection					
Sham N - Infected N	**-5.10**	**1.38**	**465**	**-3.695**	**0.0014**
Sham N - Sham E	1.22	1.34	465	0.911	0.7989
Sham N - Infected E	-1.33	1.34	465	-0.999	0.7503
Infected N - Sham E	**6.32**	**1.38**	**465**	**4.576**	**<.0001**
Infected N - Infected E	**3.77**	**1.38**	**465**	**2.729**	**0.0333**
Sham E - Infected E	-2.55	1.34	465	-1.910	0.2253

Table 6.3. [continued]

Comparison	Estimate	SE	DF	t ratio	p-value
C. Bacterial load upon death (BLUD)					
N Female - E Female	0.0473	0.428	12.18	0.110	0.9995
N Female - N Male	**1.3954**	**0.405**	**7.81**	**3.443**	**0.0367**
N Female - E Male	**1.6723**	**0.490**	**12.10**	**3.412**	**0.0229**
E Female - N Male	1.3481	0.486	11.64	2.772	0.0715
E Female - E Male	**1.6250**	**0.443**	**10.31**	**3.669**	**0.0182**
N Male - E Male	0.2769	0.427	11.98	0.648	0.9141

Table 6.4. Pairwise comparisons using Holm-Sidak method of p-value correction for comparison between systemic bacterial loads in $E_{1\text{-}4}$ and $N_{1\text{-}4}$ flies at every sampling time point (hours post-infection, HPI). Significant effects ($p < 0.05$) are marked in bold font.

HPI	Sum square	Mean square	$F_{1,76}$ value	p-value	Corrected p-value	Significance
A. Within-host bacterial dynamics, females						
3	**32.584**	**32.584**	**11.017**	**0.001388**	**0.020618925**	*
6	**157.07**	**157.07**	**39.621**	**1.811E-08**	**3.8031E-07**	***
9	**297.91**	**297.91**	**46.193**	**2.117E-09**	**4.6574E-08**	***
12	**391.04**	**391.04**	**30.513**	**4.576E-07**	**9.15196E-06**	***
15	**283.09**	**283.09**	**13.569**	**0.0004313**	**0.006878523**	**
18	**352.73**	**352.73**	**9.7851**	**0.002494**	**0.034355585**	*
21	**889.14**	**889.14**	**25.172**	**0.000003353**	**6.37051E-05**	***
24	**621.56**	**621.56**	**15.266**	**0.0001942**	**0.003296276**	**
27	242.04	242.04	6.6574	0.01173	0.111298075	ns
30	**281.21**	**281.21**	**9.35**	**0.003043**	**0.03884473**	*
33	190.65	190.65	6.8927	0.01037	0.10833571	ns
36	65.477	65.477	4.6115	0.03499	0.220667664	ns
39	45.649	45.649	6.1707	0.01519	0.128691303	ns
42	**181.41**	**181.41**	**20.215**	**0.00002479**	**0.000446126**	**
45	7.3113	7.3113	1.3993	0.2405	0.42315975	ns
48	4.8192	4.8192	1.6401	0.2043	0.681032239	ns
54	3.9686	3.9686	1.4655	0.2298	0.648104221	ns
60	0.44946	0.44946	0.1574	0.6927	0.6927	ns

66	20.081	20.081	6.1654	0.01523	0.115539426	ns
72	6.0684	6.0684	1.4543	0.2316	0.54630701	ns
84	13.39	13.39	4.2547	0.04239	0.228862123	ns
96	39.935	39.935	7.6924	0.006973	0.080540333	ns
B. Within-host bacterial dynamics, males						
4	**81.754**	**81.754**	**25.465**	**0.000002992**	**8.97597E-06**	***
10	**347.83**	**347.83**	**50.812**	**4.018E-10**	**1.6072E-09**	***
48	**32.364**	**32.364**	**13.025**	**0.0005482**	**0.001096099**	**
96	1.1459	1.1459	0.4838	0.4888	0.4888	ns

7. General discussion

A few notable conclusions can be drawn based upon the results obtained across various experiments reported in this thesis. I list these conclusions below and discuss their relevance and significance considering the existing eco-immunology literature.

Relative contribution of resistance and tolerance towards post-infection survival

Host resistance and tolerance complement each other to maintain post-infection fitness of infected hosts (Raberg et al 2009, Raberg & Stjernman 2012). Although historically overlooked, recent years have seen a flurry of papers emphasizing the role of tolerance in eco-immunological phenomenon (Schneider 2021). Tolerance has become a one size fits all solution to all eco-immunological problems: from immune priming (Prakash and Khan 2021) to zoonosis (Seal et al 2021).

One key observation from the experiments reported here is that increase or decrease in post infection survival of flies is frequently associated with a corresponding increase or decrease in host resistance. This is true irrespective of if the change in survival is brought about by plastic, phenotypic manipulations or if the change is due to alteration of genetic architecture of the host population in response to selection. First, in resource limited hosts (chapter 4), decrease in post-infection survival was always accompanied with increased within-host proliferation of the bacterial pathogen, which can be attributed to a decrease in host resistance. Second, in context of increased survival of hosts by virtue of their parents being subjected to pathogenic infections (chapter 5), increase in survival was associated with reduced within-host growth in pathogen numbers, suggesting an increase in host resistance. And third, hosts evolve to be more resistant when subjected to selection for increased survival following bacterial infection in a laboratory

experimental set-up (chapter 6). In the last example, the role of tolerance was explicitly tested but no evidence for evolution of host tolerance was found.

Although these results do not discredit the role of tolerance in maintaining host fitness post-infection, my results do suggest that changes in resistance are often necessary, and occasionally sufficient, to explain changes in host susceptibility, at least in laboratory fly populations challenged with bacterial pathogens. This can be extrapolated to state that variation in host resistance probably contributes to variation in disease susceptibility more than host tolerance. Additionally, existing theoretical studies suggest that host populations are more likely to harbour genetic polymorphisms for resistance compared to tolerance (Roy and Kirchner 2000), which can act as the underlying cause of variation in disease susceptibility. My results further suggest that in contexts where differences in disease susceptibility stems from non-genetic sources, such as change in the abiotic environment or parental effects, resistance is still the best candidate explaining differences in disease susceptibility.

Individual-level variability in response to infection

Heterogeneity among hosts in terms of susceptibility to infectious diseases is one of the key curiosities in eco-immunology (Schmid-Hempel 2003, Lazzaro and Little 2008). Most studies addressing heterogeneity in susceptibility to diseases focus on variability in infection outcome: why certain hosts die while others survive (viz. Duneau et al 2017). But beyond the survivor vs dead dichotomy, there can also be differences in response to the infection within the hosts that either survive or die as a consequence of infection, and this variation is not necessarily restricted to immune function related traits. My results provide one such example. In chapter 3, flies that died of infection exhibited increased individual level variability in their reproductive output compared to control (sham-infected) flies and flies that survived the infection (figure 3.3). The variability in fecundity of flies dying from infection was not

explained by either the identity of the infecting pathogen nor the time of death: two probable factors expected to explain such variability.

A potential source of this variability can be genetic differences between individual hosts. Previous studies have demonstrated that *D. melanogaster* genotypes vary in terms of the change in fecundity they exhibit after being infected with bacterial pathogens (Howick and Lazzaro 2014, Kutzer et al 2019; but see Kutzer et al 2018). Given that I used an outbred, wild-type population for these experiments, which is expected to harbour considerable standing genetic variation for various traits, a part of the observed variability in fecundity of dying females may be derived from genetic variation among hosts. Alternatively, the observed variability may be driven by between-individual differences in physiological conditions which can limit a host's capacity to invest towards both immune function and fecundity (Duffield et al 2017). Given all females used in the experiments were of similar age and had been maintained under identical and resource rich maintenance conditions, between-individual variability in physiological state is unlikely. Furthermore, neither of these seem likely to explain why increased variability in fecundity is only observed if females that succumb to infection. Therefore, further experiments are necessary to elucidate the underlying cause of this variability in post-infection fecundity of *D. melanogaster* females, focusing on the above two potential sources of variation: genetic differences and differences in host physiological state.

Can parental effects drive long-term evolution of immune traits?

Plasticity can both support and hinder adaptation in organisms (Bonduriansky and Day 2009, Prasad et al 2015). Plasticity supports adaptation by helping organisms cope with selection pressure in absence of standing genetic variation and allows opportunity for beneficial genetic elements to emerge, either via mutation or recombination. On the other hand, plasticity can hinder adaptation by reducing the selection pressure. Parental effects, a form of trans-

generational plasticity, therefore can affect how organisms adapt to novel environmental challenges (Yin et al 2019), including response to selection imposed by pathogens. But for parental effects to drive trait evolution, such effects need to be stable across multiple generations (Charlesworth et al 2017). My results from chapter 5 demonstrate that parental effects of pathogenic infection dilute away after one generation: baring one exception, F2 offspring of either *E. c. carotovora* or *E. faecalis* infected parents did not exhibit any difference in post-infection survival compared to offspring of control parents (figure 5.2). This suggests that parental effects induced by a pathogenic bacterial infection in *D. melanogaster* flies are not stably passed on for multiple generations and are therefore not likely to affect long-term evolution of immune traits. What remains to be tested is if this observation is unique to *D. melanogaster*-bacteria interactions or is a general feature of all host-pathogen systems.

No evolution of tolerance in response to selection for post-infection survival

In **chapter 6** of this thesis, I demonstrated that when hosts are subjected to selection for increased post-infection survival (using *E. faecalis* as the pathogen) in a laboratory experimental evolution set-up, host resistance evolves in response to the selection, but not host tolerance. This is a surprising result given that numerous theoretical studies exist that suggest that tolerance as a strategy has multiple associated advantages over resistance, and therefore, is more likely to evolve in response to selection imposed by pathogenic infections (Raberg & Stjernman 2012). There can be three obvious explanations for the result obtained in my study. One, the result is unique to the pathogen strain and the host population used for the experimental evolution set-up, and therefore, is not generalizable to other experimental evolution set-ups or to any natural setting. Two, the ancestral populations used here for experimental evolution lacked heritable variation for tolerance. And three, evolution of increased resistance had no associated costs for this particular pathogen in these populations,

in this experimental evolution set-up. I did find some empirical evidence supporting the third conjecture, although the other two possibilities cannot be completely discounted.

Beyond these three possible explanations for no evolution of host tolerance in my study, I speculate that the experimental evolution set-up might have had certain caveats that could potentially bias the evolutionary trajectory of the host populations towards preferential evolution of resistance. The caveats are discussed below.

Separation of mortality and fecundity windows. Under natural settings the fitness of an organism is determined by its contribution to the next generation, measured as the lifetime reproductive success (Fisher 1930). An infected host can thus maximize its fitness by either surviving the infection, or increasing its immediate reproductive effort, or if it is physiologically feasible, then both. And therefore, under natural settings, strategies like terminal investment (Minchela 1985) and fecundity compensation (Parker et al 2011) can evolve in response to selection due to pathogen pressure. In some sense, both these strategies that help host maximize their reproductive fitness, without affecting host resistance to pathogens directly, and thus should be considered as part of the host tolerance repertoire (Vale and Little 2012, Kutzer and Armitage 2016, Kutzer et al 2019). Contrary to the natural environment, during laboratory experimental evolution, reproduction is made conditional on surviving the infection. By design in most, if not all, experimental evolution studies the reproduction window comes after the first wave of infection-induced mortality (the acute phase of infection) has passed (viz. Ye et al 2009, Martins et al 2013, Faria et al 2015, Gupta et al 2015, Ahlawat et al 2022). The same is true for my populations. Under such a situation resistance becomes the only feasible strategy. Evolving to limit systemic pathogen load, and thereby minimizing the somatic damage caused by pathogen-derived virulence factors, helps the infected host survive the infection and reproduce adequately during the selection window.

In fact, having the reproduction window after the mortality window can even lead to hosts temporally sorting out their priorities - first resist, then reproduce - as demonstrated in my results (figures 6.4 and 6.5).

Artificial infection maintains pathogen prevalence. Under natural settings pathogen presence fluctuates with fluctuations in levels of host resistance. As resistance evolves in the host population, pathogen prevalence falls, thereby also reducing the selection pressure on the host to become more resistant. Under such circumstances, the cost-to-benefit ratio for increasing resistance becomes steep, and hosts evolve to be less resistant. This again increases pathogen prevalence, which brings back the selection pressure on the hosts to become more resistant. This cyclic process maintains polymorphism in host resistance traits, and resultantly resistance strategies never go to fixation in the host population (Roy and Kirchner 2000). But during laboratory experimental evolution, hosts are artificially infected every generation, thereby maintaining pathogen prevalence. This severs the link between host resistance and pathogen prevalence, thereby ensuring that selection for increased resistance is always maintained irrespective of how resistant the host becomes. Under such a scenario, continuously increasing resistance may be the only feasible strategy available to the host, till the point when resistance cannot be increased further because of physiological constraints. I speculate that hosts would evolve tolerance in this type of an experimental evolution set-up only after a prolonged period of selection: when resistance has evolved to its physiological limit or when resistance has evolved to the point that cost of immunopathology outweighs the benefits of pathogen control (Lazzaro and Tate 2022).

Lack of co-evolution, and therefore no ecological cost. A major cost associated with evolution of increased resistance under natural settings is that increased resistance in the hosts selects for more virulent pathogens (Miller et al 2006). But, barring rare exceptions (viz.

Berenos et al 2011, Rafaluk-Mohr et al 2018, Biswas et al 2018, Ahlawat et al 2022), in most laboratory experimental studies, especially those using insects as hosts, the pathogen is not allowed to co-evolve with the host (viz. Ye et al 2009, Martins et al 2013, Faria et al 2015, Gupta et al 2015). In my populations too, the pathogen was not allowed to co-evolve with the hosts. This ensures that the pathogen virulence remains constant irrespective of however resistant the host becomes, thereby eliminating one of the key costs of evolving increased resistance. Under such a scenario, the host is free to evolve increased resistance *ad libitum*, again until the physiological capacity to do so is reached. The few studies that have explored host-pathogen co-evolution using laboratory insect populations did not specifically test for evolution of tolerance in the evolved populations (Berenos et al 2011, Rafaluk-Mohr et al 2018, Biswas et al 2018, Ahlawat et al 2022).

Sexual dimorphism in immune function

Host sex is a major determining factor of differences in immune function in *D. melanogaster* flies (Belmonte et al 2020), but that is not necessarily true across all animal taxa (Kelly et al 2018). Sexual dimorphism, whenever present, can both drive and constrain the evolution of immune function (Rolff 2002, Nunn et al 2008), and therefore is a relevant topic in eco-immunology. None of the chapters in this thesis focused specifically on the issue of sexual dimorphism in immune function or response to infection. In certain cases, this was by design and due to logistic considerations. For example, in chapter 3, while exploring the effect of bacterial infection on host reproductive output, I focused only on the female flies. This is because reproductive fitness of female flies is easily quantified as the total number of eggs (or progeny) produced (Bateman 1948). On the other hand, accurate measurement of reproductive fitness of male flies is rather difficult, since it is determined by the number of eggs a male can fertilize (ideally in a competitive scenario, since *D. melanogaster* is a species where females

mate with and store sperm from multiple males at once) across multiple females (Bateman 1948). Similarly in chapter 4, only female flies were used for the experiments since Juvenile Hormone mediated reallocation of resources towards reproductive effort following mating is well characterized for *D. melanogaster* females (Schwenke and Lazzaro 2017). Additionally, a previously published study had demonstrated that mating status and nutrition status interact to determine host immune function in *D. melanogaster* females, but not in males (McKean and Nunney 2005). But despite not being in direct focus, the issue of sexual dimorphism in immune function comes up in the results from some of my experiments.

Sexual dimorphism in immune function, in form of post-infection survival, can manifest in two manners. First, one of the sexes may be more susceptible to infection and more prone to dying after being infected. Examples of this can be seen in the results of experiments reported in chapter 5. Males tend to be more susceptible to infection with both *E. c. carotovora* and *P. entomophila* (figures 5.1 to 5.4), while females are somewhat more susceptible (although not consistently) to infection with *E. faecalis* (figures 5.1 to 5.4). On the other hand, males and females die equally when infected with *B. thuringiensis* (figures 5.1 to 5.4). Therefore, it is obvious that the differential susceptibility of the sexes to bacterial infections is contingent upon the infecting pathogen, which is not surprising given the existing literature (reviewed Belmonte et al 2020).

Second, the two sexes might be differentially responsive to any phenotypic or genotypic manipulation that affects post-infection mortality. Some examples of this are also present in the results of experiments reported in chapter 5. For example, F2 female offspring of *E. faecalis* infected parents exhibit increased post-infection survival following a homologous challenge (parents and offspring infected with the same pathogen), compared to F2 female offspring of control parents, but no such differences in survival is observed in F2 male offspring (figure

5.2). Similarly, F1 male offspring of *E. c. carotovora* infected parents exhibit increased post-infection survival following certain heterologous challenges (parents and offspring infected with different pathogens), compared to F1 male offspring of control parents, but no differences in mortality was observed in F1 female offspring (figure 5.3). Overall, these results suggest that parental effects of pathogenic infections can act as a cause of sexual dimorphism in immune function in the hosts. Therefore, in addition to the general influence of parental effects in the process of natural selection (discussed above), parental effects can also influence evolution of host immune function via induction of sexual dimorphism. It is therefore imperative that it be tested whether such induction of sexual dimorphism due to parental effects is universally observed across other host organisms and for other pathogens.

Beyond differences in post-infection survival, sexual dimorphism can also manifest in terms of different immune strategies, i.e., resistance and tolerance (Vincent and Sharp 2014). In chapter 6, male flies die of infection with *E. faecalis* while carrying a lower bacterial load upon death (BLUD) compared to females, irrespective of their selection history (figure 6.2). This observation suggests that males are less tolerant to infection with *E. faecalis* compared to females, assuming that BLUD is a suitable measure of tolerance. The males in these populations do not exhibit a greater post-infection mortality compared to the females (figure 6.1). Therefore, we have a curious case, where sexual dimorphism in tolerance does not translate into sexual dimorphism in post-infection survival. I speculate that there may be a potential explanation for this oddity.

Based on the existing literature on within-host pathogen dynamics and the relevance of BLUD to the overall infection process (Duneau et al 2017, Chambers et al 2019, Hidalgo et al 2022, Lafont et al 2022), the number of hosts that die from infection is essentially the number of hosts that are unable to control their systemic infection intensity from reaching the BLUD. Therefore,

if two groups of hosts are equally efficient in preventing their systemic infection intensity from reaching their respective BLUDs, they will die from infection in equal numbers, even when their respective BLUDs are significantly different. BLUD has little bearing on the number of hosts that die; a higher BLUD is beneficial simply because it provides more opportunity for the host resistance mechanisms to act and control systemic pathogen proliferation. This suggests that differences in survival, including sexual dimorphism, in the present model of within-host pathogen dynamics primarily stems from differences in host resistance. I wonder if this implies that BLUD is a useful, but not the best, measure of tolerance, in addition to pre-existing criticisms (Lafont et al 2022). This potentially explains why a sexual dimorphism in tolerance, measured in from of BLUD, may not necessarily lead to a sexual dimorphism in post infection survival.

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