Investigations into the eco-immunological interactions between *Drosophila melanogaster* and its bacterial pathogens using experimental evolution

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Dedicated to

Mummy and Papa,

for their unconditional love, faith, and support.

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.

Aparajita

(Ph14034)

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

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2. Singh, A., Basu, A.K., Bansal, N., Shit, B., Hegde, T., Prasad, N.G., 2022. Effect of larval diet on adult immune function is contingent upon selection history and host sex in *Drosophila melanogaster* (**biorXiv**). https://doi.org/10.1101/2022.03.03.482770
3. Singh, A., Basu, A., Hegde, T., Shit, B., Bansal, N., Chauhan, A., Prasad, N.G., 2022. Correlated responses to experimental evolution of increased post-infection survival in *Drosophila melanogaster*: Life-history trade-offs and reaction to novel stressors (**biorXiv**). https://doi.org/10.1101/2022.06.25.497416
4. Singh, A., Basu, A., Hegde, T., Chauhan, A., Das, P. N., Bansal, N., Prasad, N.G., 2022. Bacterial co-infection of *Drosophila melanogaster*: Role of selection history, pathogen identity, and host sex in determining infection outcome (**under preparation**).

CONTENTS

SYNOPSIS

Investigations into the eco-immunological interactions between *Drosophila melanogaster* and its bacterial pathogens using experimental evolution

Introduction

Living organisms regularly face various biotic and abiotic threats to their survival, and must evolve mechanisms to counter such threats. Pathogens and parasites are an ever-present threat to the fitness of living organisms. The field of eco-immunology studies how hosts and pathogens/parasites interact in the backdrop of their environment (Schulenberg et al 2009). One of the central questions in eco-immunology is why does variation for disease susceptibility exist in host populations despite a continued, omnipresent selection for better immune hosts (Schmid-Hempel 2003, Lazzaro and Little 2009). Various non-mutually exclusive hypotheses have been put forward to answer this question.

One, defense against pathogens and parasites is costly (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Rolf and Siva-Jothy 2003, Schmid-Hempel 2005). The cost of defense can manifest in various shape and form (Schmid-Hempel 2003, McKean et al 2008, McKean and Lazzaro 2011). Costs may be physiological, also known as inducible costs, costs of maintenance, etc., in which case an infected hosts fails to invest towards various organismal functions (viz. reproduction) due to the high energy/resource requirement of an active immune system (Schmid-Hempel2003, McKean et al 2008, McKean and Lazzaro 2011). Costs may also be evolutionary, also known as constitutive costs, costs of immune deployment, etc., in which case more immune host genotypes allocates excess investment towards developing an immune system and thereby compromises other organismal faculties (Schmid-Hempel 2003, McKean et al 2008, McKean and Lazzaro 2011). Furthermore, host individuals with a high functioning immune system run the risk of self-harm brought about by immunopathology and

auto-immunity (Sadd and Siva-Jothy 2006). These costs set the upper limit for the immune phenotypes of the hosts, both in terms of evolutionary potential and phenotypic expression.

Two, outcomes of host-pathogen interactions are subject to environmental fluctuations (Sandland and Minchella 2003, Lazzaro and Little 2009). The most important environmental factor that affects immune function is the availability of resources. Availability of resources can affect both the host's capacity to mount an immune response and the pathogen's ability to proliferate within host body (Pike et al 2019). Availability of resources also determines the extent of trade-offs between immune function and other organismal faculties (Reznick 1985, Stearns 1989, Schmid-Hempel 2005).

Three, host genotypes immune to one pathogen are often more susceptible to another, a phenomenon also referred to as multiple fronts cost of immunity (Schmid-Hempel 2005, McKean and Lazzaro 2011). This phenomenon is primarily driven by specificity of immune defense mechanisms and negative correlations between the same (Schmid-Hempel 2005, McKean and Lazzaro 2011).

And four, host sex can be a major determinant of host immunocompetence (Belmonte et al 2020). Sexual dimorphism in immune function may be driven by differential expression of immunosuppressive hormones in the two sexes (Zuk and McKean 1996), or because of differences in life-history investment strategies opted for by the two sexes (Sheldon and Verhulst 1996, Rolf 2002).

Experimental evolution studies have been instrumental in answering various important evolutionary questions (Harshman and Hoffman 2000, Prasad and Joshi 2003, Kawecki et al 2012). Experimental evolution of increased immune defense has been successfully executed previously using various model organisms, such as *Drosophila melanogaster* (viz. Kraaijeveld and Godfray 1997, Fellowes et al 1998, Ye et al 2009, Faria et al 2015, Gupta et al 2016,

Ahlawat et al 2022), *Tribolium casteneum* (viz. Khan et al 2017, Prakash et al 2022), *Anopheles gambiae* (viz. Collins et al 1986, Hurd et al 2005), *Aedes aegyptis* (viz. Yan et al 1997), *Plodia interpunctella* (viz. Boots and Begon 1993), *Caenorhabditis elegans* (viz. Penley et al 2018), among others. In this thesis, using two sets of experimentally evolved *D. melanogaster* populations, both selected for increased post-infection survival for a bacterial pathogen, I explore the following questions:

(a) What life-history and reproductive trade-offs are associated with evolution of increased defense against bacterial pathogens?
(b) Is the immune function of the selected populations affected by availability of resources?
(c) Are the evolved immune defenses specific to the native pathogens used for selection?
(d) How do evolved hosts respond when subjected to infection with more than one pathogen?

Study system

In this thesis I report results from two selection regimes, the EPN selection regime which was created by me (Singh et al 2021) and the IUS selection which was created by my predecessor (Gupta et al 2016). I have worked on the IUS populations since around 145 generations of forward selection.

The EPN selection regime consists of three groups of populations:

(a) $E_{1\text{-}4}$: Populations selected for increased post infection survival when infected with a Gram-positive bacteria *Enterococcus faecalis*,
(b) $P_{1\text{-}4}$: Sham-infected control populations, and
(c) $N_{1\text{-}4}$: Uninfected control populations.

The EPN populations were derived from the Blue Ridge Baseline (BRB_{1-4}) populations; E_1, P_1, and N_1 were derived from BRB_1, and so on. This implies that E_1, P_1, and N_1 had a more recent common ancestor, compared to say E_1 and E_2. The E_1, P_1, and N_1 populations constituted 'block 1', the E_2, P_2, and N_2 populations constituted 'block 2', and so on. Populations within a block were always handled together during maintenance and experiments, and the blocks also served as the unit of replication, both evolutionary and experimental.

IUS selection regime was similarly derived from the BRB_{1-4} populations, and consists of the following groups of populations:

(a) I_{1-4}: Populations selected for increased post infection survival when infected with a Gram-negative bacteria *Pseudomonas entomophila*,

(b) S_{1-4}: Sham-infected control populations, and

(c) U_{1-4}: Uninfected control populations.

Cost of immunity in the EPN populations

Using the EPN populations (after 35 generations of forward selection), I tested if evolving increased defense against a bacterial pathogen (in this case, *E. faecalis*) leads to trade-offs with life-history traits in the hosts, comparing flies from selected and control populations. I further tested if mounting a defense against *E. faecalis* is costly, comparing infected and uninfected flies from the same population. To test for trade-off, I selected life-history traits that have been previously demonstrated to have fitness consequences in *D. melanogaster* (reviewed Prasad and Joshi 2003). I measured larval development time and viability, and adult body weight, fecundity, and longevity. The adult traits (except for body weight) were measured for both infected and uninfected flies from each population. In addition to these I tested the response of evolved and control flies to novel biotic (intra-specific competition) and abiotic (starvation and

desiccation) stressors. For the abiotic stressors too, I studied both infected and uninfected flies from each population.

I found no evidence of evolutionary costs: the selected population and control populations did not differ from one another in terms of trait values of life-history traits, either in the juvenile or in the adult stage. Selected populations also did not exhibit an increased susceptibility to abiotic stress. Put together with previous studies that have experimentally evolved fly populations for increased immunity against bacterial population (Ye et al 2009, Faria et al 2015, Gupta et al 2016, Ahlawat et al 2022), this suggests that whether evolving increased defense comes at the cost of other organismal function depend on the bacterial pathogen used for selection. The cost of mounting an immune defense was specific to the trait under focus, but did not differ across different selection histories. Infected flies exhibited shorter life-span compared to uninfected flies, but there was no effect of infection status on female reproductive output. Resistance to starvation was also compromised in infected flies compared to uninfected flies. This suggests that physiological trade-offs between immune function and other organismal functions is not a universal expectation.

Effect of limiting nutrition on post-infection survival of selected populations

Laboratory populations live in an environment with ample access to resources, and this might be the reason why trade-offs are not always observed in laboratory experimental evolution studies (Harshman and Hoffman 2000). It has been often argued that trade-offs only manifest under stressful conditions (Reznick 1985, Stearns 1989, Marden et al 2003). Therefore, one way to expose immune function associated trade-offs may be to assess immunity and life-history traits under resource limited conditions. Using both EPN (generation 40) and IUS (generation 145) populations, I tested if rearing on a poor larval diet (1:1 dilution of contents

of the standard diet) affected the immune function of adult flies of each selection regime, when infected with their native pathogen. Post-infection survival was used as a proxy of immune function in these experiments. The populations were infected with their respective native pathogens: *E. faecalis* for EPN, and *P. entomophila* for IUS. Additionally, I tested if poor larval diet intensifies the trade-off between immune function and life-history traits in the selected populations.

My results suggest that (a) experimentally evolved populations exhibit better post-infection survival compared to control populations, even when raised on poor diet; (b) host sex and selection history interact to determine the effect of poor diet on adult immune function; (c) poor larval diet reduces females fecundity, but fecundity is not affected by either host selection history or infections status; (d) poor larval diet prolongs egg-to-adult development time; and, (e) cost of evolved immune defense can manifest in form of prolonged egg-to-adult development, depending upon the pathogen used for selection (trade-off only observed in the IUS selection regime). I therefore propose that effect of poor nutrition on host immune function is not uniform, but contingent upon host sex, level of host's resistance to pathogen (selection history), and very importantly, the identity of the pathogen.

Susceptibility of evolved populations to novel pathogens

Increased resistance against one pathogen can produce corelated decrease (cross-resistance) or increase (multiple fronts cost, *sensu* McKean and Lazzaro 2011) in susceptibility towards a second pathogen (Fellowes et al 1999, Kraaijeveld et al 2012). At the phenotypic level, cross-resistance manifests when hosts infected with one pathogen show increased or decreased susceptibility to a second pathogen (I dealt with this question in the following section). At evolutionary level, cross-resistance is determined by how a host evolved to counter a particular

pathogen responds to infection by a novel pathogen. Using the EPN (generation 40) and IUS (generation 160) populations I tested (a) if adapting to one pathogen confers the hosts cross-resistance to novel pathogens; (b) is cross-resistance contingent on the identity of the native pathogen; and (c) is cross-resistance sexually dimorphic? I use the phrase *pathogen resistance* to imply the ability of the host to survive an infection with the pathogen. Populations from each selection regime were infected with six novel pathogens. Pathogens used to infect E/P flies were *Erwinia c. carotovora*, *P. entomophila*, *Providencia rettgeri*, *Bacillus thuringensis*, *Bacillus cereus*, and *Staphylococcus succinus*. The I/S flies were infected with *E. c. carotovora*, *P. rettgeri*, *B. thuringensis*, *E. faecalis*, *B. cereus*, and *S. succinus*.

The E (selected) populations were less susceptible to infections, compared to the P (control) populations, to all the novel bacteria except *P. rettgeri*, for which there was no difference in the post-infection survival of E and P populations. Similarly, the I (selected) populations were less susceptible compared to the S (control) populations to all novel pathogens except for *B. thuringiensis*, for which I and S populations exhibited equal mortality. Therefore, out of twelve total tests for cross-resistance (two selection lines × six novel pathogens) I found evidence for cross-resistance in ten comparisons and no effect of selection in the remaining two. I did not observe a single case of multiple-fronts cost.

For the E and P populations sex had no effect on post-infection survival when the populations were challenged with the six novel pathogens. For I and S populations, females exhibited reduced mortality compared to males for all Gram-negative pathogens, but not in case of the Gram-positive pathogens. Sex-by-population interaction was not observed for any bacteria for either of the two selection regimes.

Therefore, the identity of the native pathogen did not predict the novel pathogens against which the selected populations exhibited cross-resistance but it did predict the novel pathogen against which the selected populations did not show cross-resistance. Furthermore, the pattern of cross-

resistance observed in case of either of the selected populations were not affected by sex of the host; even in cases where sex affected post-infection survival the effects were similar for the selected and the control populations. My results suggest that hosts can become cross-resistant to a variety of pathogens by virtue of evolving to resist a single pathogen, and therefore positive correlations between host's resistance against different pathogens may not be very rare in nature.

Susceptibility of evolved populations to co-infections

Within the body of a single host, a pair of co-infecting pathogens may interact in myriad ways that can determine infection outcome. Co-infecting pathogens can either facilitate each other in colonizing and proliferating within the host, or compete with one another to reduce their fitness. Facilitation may be direct, via cross-feeding set-ups where metabolites necessary for one pathogen is produced by the other (Pederson and Fenton 2006), or indirect, via downregulation of the host immune system (Graham 2008). Similarly, competition may be either direct, via production of toxin by one pathogen that harms or kills the other (Mideo 2009), or indirect, either via scramble competition for host resources and within host niches (Pederson and Fenton 2006, Graham 2008, Mideo 2009), or via eliciting a generic immune response from the host that targets both the co-infecting pathogens (Pederson and Fenton 2006). But despite growing understanding of within host interactions between co-infecting pathogens, our understanding of how co-infection changes host fitness (post-infection survival) is still limited (Hoarau et al 2020, Wait et al 2021).

I infected the selected and control populations from both EPN (generation 51-53) and IUS (generation 170) selection regimes with (a) the respective pathogen used for selection (referred henceforth as the native pathogen), (b) a pair of bacteria including the native pathogen and a

novel pathogen, and, (c) a pair of novel pathogens. For each scenario I measured post-infection survival, and addressed the following questions:

(a) Do selected populations survive better than corresponding control populations when subjected to co-infection?

(b) How does the infection outcome change, in case of both selected and control populations, when flies are subjected to co-infection relative to when they are infected with their native pathogen only?

(c) Does host sex determine outcome of co-infections?

E and P, and I and S, populations were infected with *E. faecalis*, *S. succinus*, *P. entomophila*, and *P. rettgeri*, in pairs (1:1 proportion) in a round-robin design. E flies always survived better compared to P flies, except in case of males co-infected with *E. faecalis* and *P. rettgeri*, and *P. entomophila* and P. *rettgeri*; in both cases E and P males died equally. I flies always survived better compared to S flies, except in case of males co-infected with *E. faecalis* and *S. succinus*, in which case I and S males died equally. Flies from selected populations (E and I), when co-infected with different pathogen pairs, exhibited greater change in mortality relative to when infected with their native pathogen, compared to the change in mortality (native vs. co-infection) exhibited by the control populations (P or S). Additionally, depending on the identity of the co-infecting pathogens, females were either less or equally susceptible to infection compared to males.

Based on these results I propose that when flies evolve to defend against a single pathogen, they also become generally better at defending against novel pathogens, both in a one-on-one encounter (results from the previous section) and during co-infections.

Conclusion

To summarize the overall results, I did not observe any evolutionary cost of immunity in the EPN populations. Furthermore, wherever any physiological cost was observed, the cost was equal in the selected and the control population. Both E and I populations had better post-infection survival compared to their respective control populations even when reared on poor diet. No multiple-fronts cost was detected in either the EPN or the IUS populations. The selected populations were either better at resisting novel pathogens (cross-resistant) or survived equally to the control populations. The selected populations were also able to better resist co-infection challenges compared to control populations. This suggests that both evolved populations (E and I) have evolved a fairly generic defense mechanism.

In conclusion, selection for improved post-infection survival, using a single bacterial pathogen, leads to evolution of a generalized immune defense repertoire, without incurring any evolutionary or a differential maintenance cost.

Chapter 1

Introduction

Omnipresence of pathogens in the natural world imposes a strong, ever-present selection on hosts for evolution of myriad defence mechanisms, which in insects involved both physiological and behavioural defences (Schmid-Hempel 2005, Schulenberg et al 2009, Parker et al 2011). Defence mechanisms often entail costs that manifests in form of trade-offs with other organismal faculties, such as life-history traits (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Rolf and Siva-Jothy 2003, Schmid-Hempel 2005). These costs can be generally categorised into three general types (Schmid-Hempel 2003, Sadd and Siva-Jothy 2006, McKean et al 2008, McKean and Lazzaro 2011):

(a) costs that manifest in absence of infection (also known as evolutionary costs, constitutive costs, or costs of immune maintenance),
(b) costs that manifest while mounting an immune defence (also known as physiological costs, inducible costs, or costs of immune deployment), and
(c) costs that manifest due to the defence mechanisms harming the host soma along with or instead of the pathogens (immunopathology, auto-immunity).

Expression of immune defence mechanisms by an individual organism is governed by these various costs. Furthermore, manifestation of costs, and therefore expression of immune phenotypes, is also under the influence of the environmental (both biotic and abiotic) factors encountered by the host (Sandland and Minchella 2003, Lazzaro and Little 2009) and host sex (Sheldon and Verhulst 1996, Zuk and McKean 1996, Rolf 2002).

Single generation studies and experimental evolution studies have both been instrumental in understanding host-pathogen interactions from the ecological viewpoint (Schulenberg 2009), especially in terms of elucidation of costs, and evolution of immune specificity (Schmid-Hempel 2005, McKean and Lazzaro 2011). To cite a few examples, Boots and Began (1993) evolved Indian meal moth (*Plodia interpunctella*) against granulosis virus, and found

correlated increase in the development time and reduction in egg viability in the evolved lines. Mosquito (*Aedes aegypti*) populations selected for resistance to a malaria parasite (*Plasmodium gallinaceum*) had shorter development time, shorter lifespan, smaller body size, smaller blood meal intake, and lower fecundity (Yan et al 1997). Two strains of snails (*Biomphalaria glabrata*) were selected for either resistance or susceptibility to two different strains of *Schistosoma mansoni* independently (Webster and Woodhouse 1998). The evolved lines only exhibited increased resistance, or susceptibility (depending upon what trait was under selection), specifically when challenged with the strain used for selection, and not against the other strain (Webster and Woodhouse 1998). Additionally, independent of infection status, snails selected for increased susceptibility had lower fecundity compared to controls and snails selected for increased resistance (Webster and Woodhouse 1998).

In *Drosophila melanogaster* the consequences of selection for increased defence against various pathogens and parasites depend upon the type of the pathogen/parasite used. Experimentally evolved *D. melanogaster* populations against larval endoparasitoids (insects whose larvae develop within the hosts body, eventually killing them) *Asobara tabida* (Kraaijveld and Godfray 1997) and *Leptopilina boulardi* (Fellowes et al 1998) had reduced larval competitive ability compared to the control populations. Fellowes et al (1999) found lower feeding rate in both of the above selected lines compared to the controls. Populations selected against *A. tabida* also had higher density of haemocytes (used for encapsulating parasitoid larvae, Kraaijeveld et al 2001) and survived better against *Leptopilina heterotoma* compared to control populations (Fellowes et al 1999). Populations selected against *Leptopilina boulardi* survived better against both *Asobara tabida* and *Leptopilina heterotoma* (Fellowes et al 1999) when compared with controls. This shows that the increased defence against larval parasitoids achieved via experimental evolution is not specific to the parasitoid species used for selection. *D. melanogaster* populations when selected for better resistant

against fungus, *Beauveria bassiana*, did not response to selection after fifteen generations of selection (Kraaijeveld and Godfray 2008). *D. melanogaster* populations selected for better defence against Drosophila C Virus (DCV) were also better in surviving cricket paralysis virus (CrPV) and flock house virus (FHV) but not against bacterial pathogens (*Pseudomonas entomophila* and *Enterococcus faecalis*, Martins et al 2014).

Multiple previous studies have experimentally evolved *D. melanogaster* flies to better resist death due to bacterial infections. Ye et al (2009) evolved flies to survive challenge with *Pseudomonas aeruginosa* and found that the increased defence came at a cost of increased larval development time and shorter adult lifespan. Flies evolved to survive challenge with *Bacillus cereus* also exhibit longer development time (Ma et al 2012). Martins et al (2013) evolved flies to survive infection when challenged with *Pseudomonas entomophila*, either via oral route or via systemic infection. The selected populations were only able to defend against the pathogen when infected via the route of infection used during selection, indicating that the defence mechanisms at work in case of either mode of infection is different from one another (Martins et al 2013). Additionally, selected populations were not resistant to other bacterial pathogens (*Enterococcus faecalis*, *Erwinia carotovora*, and *Serratia marcescens*), and more susceptible to viral pathogens (Drosophila C virus and flock house virus, Martins et al 2013). Put together with results from Martins et al (2014), these results suggests that the defence mechanisms utilised against bacteria and viruses is mutually exclusive, and also that mechanisms of defence used against bacterial pathogens are also bacteria-specific. Using the same populations, Faria et al (2015) reported that defence against *P. entomophila* does not entail any fitness costs in terms of life-history trade-offs. Gupta et al (2016) also selected flies to better survive systemic infection with *P. entomophila* and reported absence of costs in absence of infection, and Ahlawat et al (2022) demonstrated that defence against *P. entomophila* does not entail a cost even when the pathogen is free to co-evolve with the host.

Both studies demonstrated that infected flies have shorter life-span and reduce starvation resistance compared to uninfected/sham-infected control flies (Gupta et al 2016, Ahlawat et al 2022).

In this thesis I used two experimental evolution regimes, each having sets of replicate populations of *Drosophila melanogaster*, sharing close common ancestry. One set of populations were selected for better survival following systemic infection with a Gram-negative bacterial pathogen, *Pseudomonas entomophila*; the IUS selection regime (Gupta et al 2016). The other set of populations were similarly selected with a Gram-positive bacterial pathogen, *Enterococcus faecalis*; the EPN selection regime (Singh et al 2021). In both set of selection regimes, populations quickly responded to selection by surviving better post-infection when infected with their native pathogen compared to paired control populations (Gupta et al 2016, Singh et al 2021; also see Chapter 2). These two selection regimes, IUS and EPN, were further used to answer various questions relevant to eco-immunology.

Chapter 3 of the thesis explores correlated evolution of life-history traits in the EPN selection regime. Life- history traits like longevity, fecundity, egg-to-adult development time and viability, and body weight were studied. Additionally, resistance to abiotic stressors (starvation and desiccation) and biotic stressors (larval competition) was assayed. Adult life-history traits and stress resistance were studied under three conditions, i.e., infected, sham-infected, and uninfected. It was found that the selected populations had similar trait values, compared to control populations, for all life-history traits that were studied, during both larval and adult stage. However, in the longevity assay, infected flies survived less compared to uninfected flies, suggesting infected flies were paying cost of immune response late in life. It was also found that selected flies when infected and starved survived better compared to control populations. This suggest that the selected flies are better able to deal with pathogen challenge

even when starved. Overall infected flies died due to starvation before uninfected and sham-infected flies, again suggesting a cost of mounting an immune defence. When flies were infected and desiccated, it was found that all populations were equally worse in surviving. But infected and sham-infected flies in general died faster which can be due to rapid loss of haemolymph as a result of pricking. Therefore, it can be said that there is no apparent cost of evolving immune defence in flies when infected with *Enterococcus faecalis* and that infected flies in general are showing immunopathology compared to unhandled flies by dying faster.

Chapter 4 of the thesis explores the effect of juvenile nutrition on adult immune function of both the EPN and the IUS selection regimes. Deploying immune components for defence against pathogens demands considerable amount of energy investment by the hosts. Therefore, immune function should get affected by the change in availability of resources required to fuel the energy demand. Here, we tested whether host selection history influences host response when infected with native pathogen in the face of resource deprivation. Selected flies of both selection regimes reared on suboptimal diet survived better compared to their control populations. Wherever there was a negative effect of malnutrition on post-infection survival, it was only observed in case of the flies from control populations. Therefore, this suggests that resource deprivation does not affect immune function of the evolved flies of both selection regimes.

Pathogens are omnipresent and hosts are rarely infected by single pathogen in the wild. Host adaptation to one pathogen can make their immune defence better, worse, or unchanged towards a second pathogen. In **Chapter 5**, change in susceptibility to various novel pathogen were tested in the flies selected to be more resistant towards a particular bacterial pathogen. For both EPN and IUS selection regime, it was observed that barring a few exceptions, the evolved populations in general show better cross-resistant against the range of pathogen tested, compared to control populations. Also, neither pathogen identity of the native pathogen or sex

of the host had any major role in determining the pattern of cross-resistance exhibited by the selected populations.

Hosts in the wild are often simultaneously infected with multiple pathogen species (or multiple strains of the same species). Pathogens co-infecting a single host interact with one another in various ways (resource competition, immune modulation, etc.) which ultimately affect post-infection survival. **Chapter 6**, explores if host survival, when simultaneously infected with pairs of bacterial pathogens, is determined by host evolutionary history. Flies from EPN and IUS selection regimes were tested for survival against four pathogens (including the native pathogen), while being simultaneously infected with two bacteria at a time (six co-infection treatments in total). Results indicated that the evolved population always survived better irrespective of the pathogen pair used for infection, compared to control populations. Host sex was also a major determinant of outcome of co-infection. Overall, results suggest that hosts adapting to only one pathogen can also become better at surviving simultaneous challenges by multiple pathogens. This confirms that, both the selected lines have evolved generalized immune response.

To summaries this thesis has attempted to explore various aspect of eco-immunology like trade-offs, immune function under resource deprived conditions, cross-resistance to novel pathogens (both when the host is challenged with a single pathogen and when challenged with two pathogens simultaneously) in the experimentally evolved EPN and IUS flies. It reports that immune function in experimentally evolved flies (a) do not trade-off with other life-history traits, (b) is not affected under resource deprived conditions, and (c) evolved flies develop generic immune response.

Chapter 2

Experimental System

2.1. Experimental Evolution

In this thesis, host-pathogen interaction has been studied through long-term experimental evolution approach. Experimental evolution has proved to be one of the most powerful approaches to understand both the overall patterns and several underlying mechanisms of how organisms adapt to various selection pressures (Prasad and Joshi 2003, Burke and Rose 2009, Garland and Rose 2009, Kawaecki et al 2012, Hoang et al 2016). It offers several obvious advantages such as control over the environment, ability to impose a specific selection pressure, the ability to assay traits in conditions that are meaningful to the regime, and the power of replication (Prasad and Joshi 2003, Burke and Rose 2009, Garland and Rose 2009, Kawaecki et al 2012, Hoang et al 2016).

In any experimental evolution experiment, a series of replicated populations are exposed to a novel environment for many generations, along with a parallel series of control populations maintained on ancestral environmental conditions (Prasad and Joshi 2003, Burke and Rose 2009, Garland and Rose 2009, Kawaecki et al 2012, Hoang et al 2016). The choice of novel environment depends on experimenter and can include various abiotic, biotic, or demographic conditions (Burke and Rose 2009, Garland and Rose 2009, Kawaecki et al 2012). Usually, a single ancestral environmental condition is altered for simplicity of the experimental design and easiness of interpretation of the experimental results (Garland and Rose 2009).

For experimental evolution, choice of model organism is based on characteristics like short generation time, easiness in handling and propagation, and easy maintenance of enough replicates (Prasad and Joshi 2003, Burke and Rose 2009, Garland and Rose 2009, Kawaecki et al 2012, Hoang et al 2016). The model organisms should be well characterized and various tools should be available to study them which helps experimenter while designing and interpreting results (Fox and Wolf 2006, Garland and Rose 2009, Kawaecki et al 2012). The

genetic makeup, population size, experimentally defined environmental conditions, adequate control populations, and control over confounding variables are the points that should be kept in mind while starting and maintaining experimental evolution lines (Fox and Wolf 2006, Garland and Rose 2009).

In this thesis, host-pathogen interaction has been studied through long-term experimental evolution approach. *Drosophila melanogaster* was used as host and two bacteria, *Enterococcus faecalis* and *Pseudomonas entomophila*, were used as pathogens.

2.2. Host system

Drosophila melanogaster is one of the most extensively used model system for experimental evolution (Fox and Wolf 2006, Burke and Rose 2009, Garland and Rose 2009, Kawaecki et al 2012, and Hoang et al 2016). Features like outbreeding with short generation time, easy maintenance and manipulation in the laboratory, well characterized traits (like genetics, physiology, anatomy) and various tools available for studying them, makes it an excellent system for experimental evolution studies (Fox and Wolf 2006, Burke and Rose 2009, Garland and Rose 2009, Kawaecki et al 2012, and Hoang et al 2016).

D. melanogaster is a holometabolous insect belonging to the Phylum Arthropoda, Class Insecta, Order Diptera, Family Drosophilidae, Genus *Drosophila*, and Species *D. melanogaster*. It is normally found on the rotten and fermented fruits and hence also called 'fruit fly' (Hafen 1997). It has four distinct stages in its life cycle: egg, larvae (first instar, second instar, and third instar), pupae, and adult (Hafen 1997, Gilbert 1997, Prasad and Joshi 2003, Wolpert et al 2015). Eggs hatch into larvae, which is an important stage as most of the resource are acquired during these life stage, followed by pupal development (Hafen 1997, Gilbert 1997, Wolpert et al 2015). Pupa further hatch (eclose) into adults (Hafen 1997, Gilbert 1997, Wolpert et al 2015). By 10^{th}-11^{th} days post egg laying, all pupa eclose into adult. After

eclosion, adult fly's outer cuticle hardens and wings develop (Hafen 1997, Prasad and Joshi 2003). They become sexually mature within 8-10 hours of eclosion and can mate (Hafen 1997, Prasad and Joshi 2003). Adult males and females can be distinguished phenotypically. Males are smaller in size and have sex- comb on their first pair of legs and last three segments of the abdomen are fused to give distinct black colour (Hafen 1997). Male abdomen is round and much shorter with fewer stripes while female abdomen curve to a point with longer abdomen having more stripes (Hafen 1997). Female can lay eggs within few hours of mating to start next generation.

2.3. Pathogen system

Two bacteria, *Enterococcus faecalis* and *Pseudomonas entomophila*, were used in this study to select two separate experimental selection regimes.

2.3.1. *Enterococcus faecalis* (Ef)

E. faecalis is a nosocomial, opportunistic human pathogen and are reported to infect flies in the wild (Huycke et al 1991, Lazzaro et al 2006). It is extracellular in nature having Lysine-type peptidoglycan on the outer wall. It is Gram-positive bacteria. Systemic infection with *E. faecalis* can induce phagocytosis (Nehme et al 2011), melanization (Ayres and Schneider 2008), and can strongly induce Toll (Gobert et al 2003, Nehme et al 2011, Hanson et al 2019) but not Imd pathway during systemic infection (Troha and Buchon 2019). *E. faecalis* also produces variety of antioxidative enzymes which are involved in the oxidative stress response (Szemes et al 2010). *Drosophila* hosts that survive infection have persistent infection present in their body (chronic infection; Troha et al 2018, Chambers et al 2019).

E. faecalis (Lazzaro et al 2006) was cultured at 37 ˚C in lysogeny broth (Luria-Bertani-Miller, HiMedia). It causes ~50% mortality in Blue Ridge Baseline (BRB, described in details below in this chapter) population at an infection dose of OD_{600}=0.8-1.0.

2.3.1. *Pseudomonas entomophila* (Pe)

P. entomophila is a motile, rod shaped, obligatory aerobic bacteria found in soil, aquatic or rhizosphere environments. It can infect both larvae and adult flies through oral or systemic infection (Vodovar et al 2005). It is having DAP-type peptidoglycan and are Gram-negative in character. Infection with *P. entomophila* induces Imd pathway having role of AMPs like diptericin, diptericin B, cecropin A1, attacin A, attacin C, cecropin C, drosomysin and drosopterin (Vodovar et al 2005). These AMPs are required for defence against both oral and systemic infection by *P. entomophila*.

P. entomophila strain L48 used in the study was isolated from *Drosophila* itself (Dieppois et al 2014). It was cultured at 27 ˚C in lysogeny broth (Luria-Bertani-Miller, HiMedia) for the study (Mulet et al 2012, Vodovar et al 2005). It kills ~50% of BRB population at OD_{600}=1.

2.4. Experimentally evolved selection regimes

For the present study we used two sets of selected populations of *Drosophila melanogaster*, each selected for improved post-infection survival when infected with a different entomopathogenic bacteria. Both sets of selected populations were derived from a common ancestor, the Blue Ridge Baseline (BRB).

2.4.1. Ancestral populations: BRB populations

The Blue Ridge Baseline (BRB), a wild-type, outbred population, with four evolutionary replicates, BRB1-4. The derivation and maintenance protocol of the BRB populations are described in detail in Singh et al (2015). Briefly, each replicate is maintained at a census size of 2800 adults, on a 14-day discrete generation cycle, at 25 ˚C on a 12:12 light-dark cycle and 50-60% relative humidity on standard banana-yeast-jaggery food (refer table 2.2 for food composition). Juveniles of these populations are reared in 40 glass vials (25mm diameter ×

90mm height) at a density of ~70 eggs per vial, with 6-8 mL of standard banana-jaggery-yeast medium. By 12th day post egg laying (PEL), almost all flies eclose and the adults are transferred to plexiglass cages (25 cm length x 20 cm width x 15 cm height) having food in Petri plate (90 mm diameter) supplemented with live yeast paste. On the 14th day PEL, fresh food plates (cut into halves to expose the vertical surfaces where the flies seem to prefer to lay eggs, hereafter called as cut-plate) are provided in the cages for 18 hours of egg laying. Eggs are collected from these cut-plates at above mentioned densities and dispensed into glass vials to start a new generation.

2.4.2. Selection for increased survival against systemic infection with *Enterococcus faecalis* (*Ef*): EPN populations

Three populations were derived from each replicate population of BRB after 150 generations: (a) E1, infected with *Enterococcus faecalis*, (b) P1, pricking control, and (c) N1, normal control, were derived from BRB1. Similarly, we derived E2, P2 and N2 from BRB2, and so on. Therefore, there were totally 12 populations in this selection regime: E1-4, P1-4, and N1-4. Populations with the same numeral shared a more recent common ancestor. For example, E1, P1 and N1 are more closely related to each other than any of them are to E2, P2, N2, etc. Additionally, populations bearing the same numeral were always handled together, during selection and during experimentation. Therefore, populations with the same numeral were treated as both evolutionary and statistical blocks. Consequently, we had four blocks (Block 1-4) in the EPN selection regime (E1, P1, N1 forming block 1 and so on).

For all populations, eggs were collected at a density of 60-80 eggs per vial (25 mm diameter × 90 mm height) containing 6-8 ml of food (similar to the ancestral population) in 10 such vials and were incubated at standard laboratory conditions as mentioned above. By 10th-11th day 95% of the flies eclose. Further handling depended on the type of selection being imposed.

In the E1-4 populations, on day 12 PEL, when the flies are 2-3 days old as adults, from each of the 10 juvenile development vials, we randomly chose 20 females and 20 males flies, and infected them with the pathogen by septic injury on the thorax with a Minutien pin (0.1 mm, Fine Science Tools, USA) dipped in a bacterial suspension (in $MgSO_4$ saline buffer) at optical density (OD_{600}) of 0.8, under light CO_2 anaesthesia. Therefore, a total of 200 females and 200 males are infected every generation for each E population. After infections the flies were shifted to a plexiglass cage (14 cm length x 16 cm width x 13 cm height) provided with a food plate (60 mm Petri plate in diameter); fresh food plates were provided every alternate day. For flies infected with *Enterococcus faecalis* majority of the mortality is observed between 18- and 48-hours post-infection with very few flies dying before 18 or after 48 hours. After 96 hours post-infection, fifty percent of the infected flies in each E populations would survive to contribute to the next generation. Throughout the selection history of these populations, the pathogen infection dose was modulated to induce fifty percent mortality: this ensured a constant, directional selection process. Therefore, flies of zeroth generation were infected with OD_{600}=0.8, and the infection dose was increased to OD_{600}=1.0 at generation 21, and again increased to OD_{600}=1.2 at generation 41. 96 hours after infection (day 16 PEL) the population cages are provided with fresh oviposition plates (cut-plate) and 18 hours later eggs were collected of these plates to start the next generation.

Flies of the P1-4 populations are maintained identically to the E populations, except that (a) on day 12 PEL, when the flies are 2-3 days old as adults, they are pricked with a Minutien pin (0.1mm, Fine Science Tools, USA) dipped in sterile $MgSO_4$ buffer under light CO_2 anaesthesia, before being placed in cages; (b) From each of the 10 juvenile development vials, we randomly chose 10 females and 10 males such that 100 females and 100 males are sham-infected every generation for each population. There is negligible mortality (1-2%) in these cages between the time of infection and oviposition.

Flies of the N1-4 are maintained identical to P populations except that on day 12 PEL we randomly chose 10 females and 10 males from each of the 10 juvenile development vials under CO_2 anaesthesia such that 100 females and 100 males are subjected to uninfected treatment every generation for each population. There is negligible mortality in these cages.

Each block was handled on a different day, i.e., E1, P1, and N1 were handled together on one day; E2, P2, and N2 were handled together on the next day, and so on. Every generation, in each population the number of eclosing flies is the same (about 700). The flies eclose in the vials by day 10 and would have mated by day 12. On day 12, we subsample 400 flies (200 of each sex) in each of the E populations and subject them to infections. Of these about 100-120 flies per sex survive till day 16 and contribute to the next generation. From the P and N populations, on day 12, we subsample 200 flies (100 of each sex). There is negligible mortality (1-2%) in the P and N regimes. Therefore, on day 16, when we collect eggs to start the next generation, close to 100 flies of each sex are present in these populations. Thus, our protocol ensures that the number of adults at the time of egg collection are similar across populations. The EPN selection regime is thus maintained on a 16-day discrete generation cycle.

2.4.3. Selection for increased survival against systemic infection with *Pseudomonas entomophila* (*Pe*): IUS populations

The IUS populations were similarly derived from the BRB populations after 22 generations of establishment of the base populations, and have been previously described in Gupta et al (2016). Briefly, three selection regimes were derived from each replicate population of BRB: (a) I1-4, infected with *Pseudomonas entomophila*, (b) S1-4, sham-infected control, and (c) U1-4, uninfected controls were derived from BRB1, and so on. The maintenance of these lines is identical to that of the EPN lines, except that (a) I,U,S populations were started from BRB populations after 22 generations of lab adaptation while E,P,N populations were started from

BRB populations after 150 generations of lab adaptation, (b) in the I1-4 populations 150 females and 150 males are infected every generation for each population whereas in E1-4 200 females and males, (c) in I,U,S Gram-negative bacteria *Pseudomonas entomophila* and E,P,N Gram-positive *Enterococcus faecalis* is used, (d) peak mortality window for I is 20 hours to 60 hours and for E is 18 hours to 48 hours. The derivation of both selection regime is summarized in figure 2.1 and table 2.1.

Figure 2.1. The ancestry of the populations used in this study: inter-relatedness of BRB populations and the two selection lineages (EPN and IUS populations).

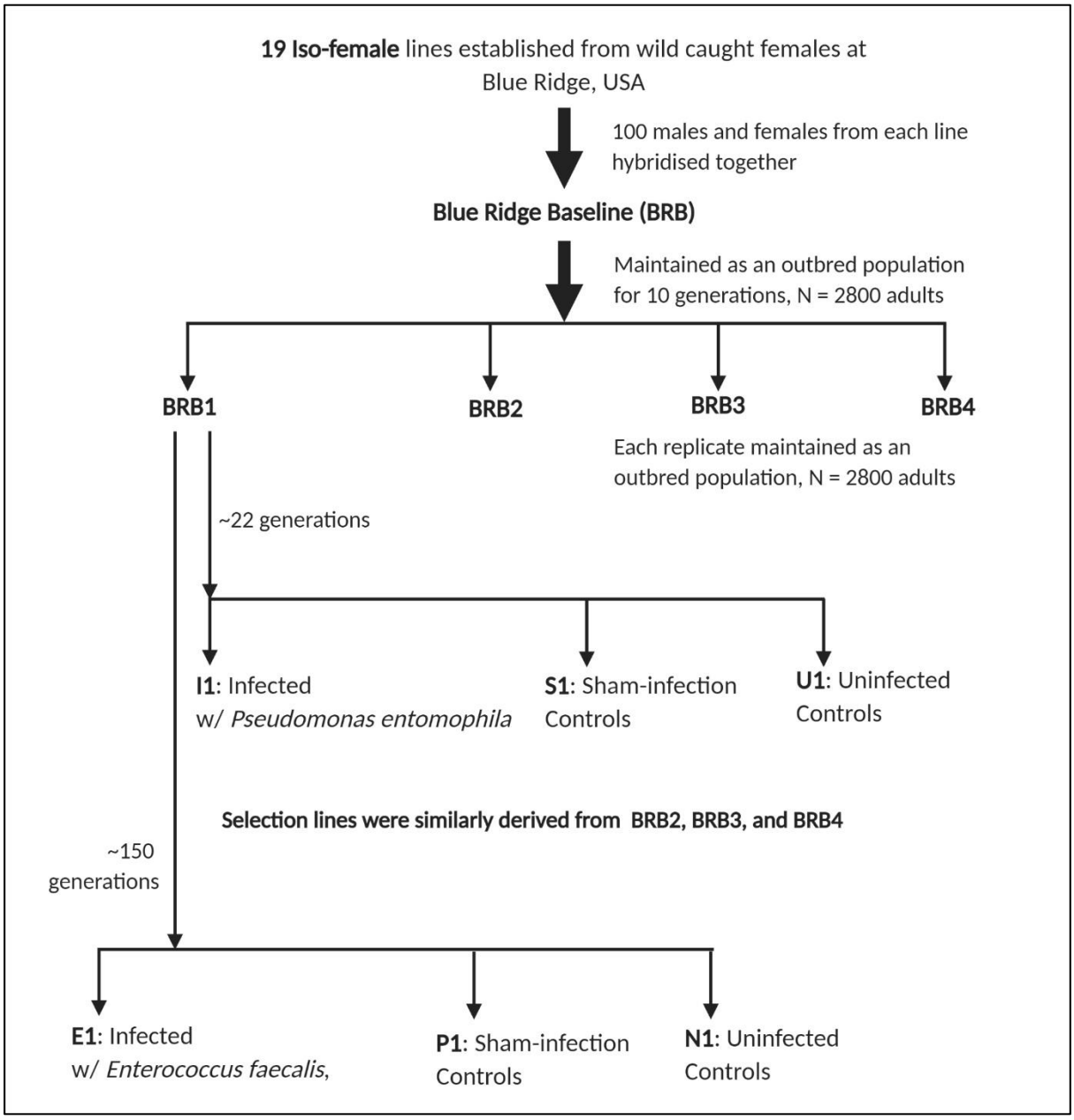


Table 2.1. Comparison of the maintenance regime of the ancestral and the selected populations.

	BRB	**EPN**	**IUS**
Generation cycle	14-days	16-days	16-days
Food medium	Banana-jaggery-yeast medium	Banana-jaggery-yeast medium	Banana-jaggery-yeast medium
Egg density per vial	60-80 eggs	60-80 eggs	60-80 eggs
Number of vials per population	40	10 for E, 10 for P, and 10 for N	10 for I, 10 for S, and 10 for U
Number of replicate populations	4 (BRB1-4)	12 (E1-4, P1-4, and N1-4)	12 (I1-4, S1-4, and U1-4)
Yeasted prior to egg collection	Yes	No	No
Pathogen used	NA	*Enterococcus faecalis* (*Ef*), Gram-positive bacteria	*Pseudomonas entomophila* (*Pe*), Gram-negative bacteria
Ancestral population	NA	BRB1-4	BRB1-4
Number of generations after which populations were started from ancestral population	NA	150 generations	22 generations
Number of adults in each generation (during the window of reproduction)	2800 adults, sex ratio not maintained artificially	Approximately 100 females and 100 males per population	Approximately 100 females and 100 males per population
Peak mortality window	NA	Between 18 hours and 48 hours for E populations	Between 20 hours and 60 hours for I populations

2.5. Bacterial stocks and infection procedure

The two primary bacteria used for selection and experiments in this study are *Enterococcus faecalis* (grown at 37℃, Lazzaro et al 2006) and *Pseudomonas entomophila* (grown at 27℃, strain L48, Vodovar et al 2005, Mulet et al 2012). E populations are infected with *E. faecalis*, and I populations with *P. entomophila*. The bacterial stocks are maintained as 17% glycerol stocks frozen at -80 ˚C. Primary culture of the bacteria is obtained by inoculating a stab of glycerol stock in 10 ml lysogeny broth (Luria-Bertani-Miller, HiMedia) and incubating it

overnight at appropriate temperature with continuous mixing at 150 RPM. To establish secondary culture, fresh 10 ml lysogeny broth is inoculated with 100 µl of the overnight culture; incubated as mentioned above till desired turbidity (OD_{600} =1.0-1.2) is reached. This secondary culture is centrifuged to obtain bacterial pellets which in turn is resuspended in sterile $MgSO_4$ buffer (10 mM) to obtain the required optical density (OD_{600}). Flies are infected (either during selection protocol or experimental infections) by pricking them on the thorax with a 0.1 mm Minutien pin (Fine Scientific Tools, USA) dipped in the bacterial suspension under light CO_2 anaesthesia. Sham-infections are carried out similarly, except with a pin dipped in sterile $MgSO_4$

2.6. Pre-experiment standardization

To account for any non-genetic parental effects, experimental eggs were collected from flies which were grown in common garden conditions for one generation (Rose 1984). Eggs were collected from all the populations at a density of 60-80 eggs per vial: 10 such vials were established per population. The eggs completed their development into adults in these vials, and on day 12 PEL, the adults were transferred to plexiglass cages (14x16x13 cm^3) with food plates (Petri plates, 60 mm diameter). Eggs for experimental flies were collected from these population cages.

2.7. Rearing of the Experimental Flies

Three days prior to the egg collection, food plates supplemented with live yeast were provided to the standardised flies (protocol for pre-experiment standardization of flies is detailed above) in the cages. After 2 days, yeast plate was replaced with cut-plate for the next 18 hours for egg laying. From these cut-plates eggs were transferred into glass vials (25 vials per population to test for response to selection and 50 vials per population to test for cross-resistance), at the

density of 60-80 eggs per vial (90 mm x 25 mm), each vial having 6-8ml of standard banana-jaggery food. The vials were incubated under conditions identical to the maintenance of the selection regime. Eggs developed into adults in these vials within 10 days after egg collection, and the adults remained in these vials till day 12 PEL, wherefrom they were used for experiments.

2.8. Test of response to selection

2.8.1. Test of response to selection in EPN populations

This experiment was carried out after 35 generations of forward selection. On 12th day PEL, we sampled 400 females and 400 males from each of the E, P, and N populations. These were then randomly assigned to one of the three treatments: (a) Infection treatment: 200 females and 200 males were infected with *Enterococcus faecalis* (*Ef)* at OD_{600} = 0.8; (b) Sham-infection treatment: 100 females and 100 males were sham-infected with sterile $MgSO_4$ solution; and (c) Uninfected treatment: 100 females and 100 males were subjected to light CO_2 anaesthesia only. Post treatment, the flies were placed inside plexiglass cages (14x16x13 cm^3) containing food in Petri plates (60 mm diameter). Mortality was noted every 4-6 hours until 96 hours post infection for each cage. Fresh food plates were provided to the cages on every alternate day. Individual blocks were handled on separate days. Altogether, 200 flies/sex/population/block were infected with *Ef*, 100 flies/sex/population/block were sham-infected, and 100 flies/sex/population/block were kept as uninfected control.

2.8.2. Test of response to selection in IUS populations

This experiment was carried out after 160 generations of forward selection. On 12th day PEL, we sampled 100 females and 100 males from each of the I, U, and S populations. These were then randomly assigned to one of the two treatments: (a) Infection treatment: 50 females and

50 males were infected with *Pseudomonas entomophila* (*Pe*) at OD_{600} = 1.5; and (b) Sham-infection treatment: 50 females and 50 males were sham-infected with sterile $MgSO_4$ solution. Post treatment, the flies were placed inside plexiglass cages (14x16x13 cm^3) containing food in Petri plates (60 mm diameter). Mortality was noted every 4-6 hours until 96 hours post infection for each cage. Fresh food plates were provided to the cages on every alternate day. Individual blocks were handled on separate days. Altogether, 50 flies/sex/population/block were infected with *Pe*, and 50 flies/sex/population/block were used as sham-infected controls.

2.8.3. Statistical analysis

All analyses were performed using R statistical software, version 4.1.0 (R Core Team 2021). Mixed-effect cox-proportional hazard models were fitted to the data using the *coxme* function of the "coxme" package (Therneau 2020), and the confidence intervals for these models were calculated using *confint* function of the base R package. Survival curves were plotted using the *ggsurvplot* function of the "survminer" (Kassambara et al 2021) package after modelling the data using *survfit* function from the "survival" (Therneau 2021) package.

For the analysis of data from the response to selection experiments we first modelled the total data as:

survival ~ infection treatment + (1|block),

where infection treatment was considered as a fixed factor and block as a random factor. Next, we modelled the data from only the infected individuals as:

survival ~ selection regime + sex + selection regime : sex + (1|block),

where selection regime, sex and their interaction were considered as fixed factors and block as random factor. This was done separately for the EPN and the IUS selection regimes.

2.8.4. Results

2.8.4.1. Test of response to selection in EPN populations, selected for resistance against *Enterococcus faecalis*

The EPN selection regime consists of three types of populations: (a) E1-4: selected for increased resistance against *Enterococcus faecalis*; (b) P1-4: pricking (sham-infected) controls; and (c) N1-4: normal (uninfected) controls (See 'Materials and Methods' for more details). To test for the primary response to selection, we infected the E, P, and N populations with *Enterococcus faecalis* (*Ef*) at OD_{600} = 0.8 after 35 generations of forward selection.

Both the infected and the uninfected treatments are significantly different from sham-infected treatment (table 2.3), infected treatment surviving less (hazard ratio 14.6184, 95% CI 11.8732, 17.9980) and uninfected treatment surviving more (hazard ratio 0.0851, 95% CI 0.0413, 0.1753) than sham-control treatment. Comparing within the infected treatment, E populations, survived significantly better than flies from the P control populations (hazard ratio 0.636, 95% CIs 0.5454, 0.7417; figure 2.2); the N and P populations were similar in post-infection survival (hazard ratio 1.0464, 95% CI 0.9103, 1.2028). Males were not significantly different from females in terms of post-infection survival (hazard ratio 0.9203, 95% CI 0.7980, 1.0614).

2.8.4.2. Test of response to selection in IUS populations, selected for resistance against *Pseudomonas entomophila*

The IUS selection regime consists of three types of populations: (a) I1-4: selected for increased resistance against *Pseudomonas entomophila*; (b) S1-4: sham-infected controls; and (c) U1-4: uninfected controls (See 'Materials and Methods' for more details). To test for the primary response to selection, we infected the IUS populations with *Pseudomonas entomophila* (*Pe*) at OD_{600} = 1.5 after 160 generations of forward selection.

Infected flies (hazard ratio 14.7962, 95% CI 11.1879, 19.5685) survive significantly less compared to flies from sham-infected treatment (table 2.3). I populations, when infected, survived significantly better than flies from the S control populations (hazard ratio 0.0944, 95% CIs 0.0568, 0.1568; figure 2.3). However, the two control populations, U and S, were similar in terms of post-infection survival (hazard ratio 0.8942, 95% CIs 0.6919, 1.1556). Male survived significantly less when infected compared to females (hazard ratio 2.1630, 95% CIs 1.7063, 2.7420).

2.8.5. Discussion

Evolution of increased immunity: The E populations, that were selected for resistance against *Enterococcus faecalis* showed rapid evolution to the selection pressure. After 35 generations of selection, post-infection survival of the E (selected) populations was better than both P (sham-infected control) and N (un-infected control) populations (figure 2.2, table 2.3) when infected with *E. faecalis*. Host sex didn't have a significant effect on post-infection survival (table 2.3). Similarly, the I populations, selected for resistance against *Pseudomonas entomophila* (Gupta et al 2016), were better at surviving a challenge with *P. entomophila* compared to both S (sham-infected control) and U (un-infected control) populations (figure 2.3, table 2.3), after 160 generations of selection. Post-infection survival of females was better than males for all three populations (I, U, and S; table 2.3). Therefore, even after 160 generations of forward selection of IUS, I populations were better at surviving *P. entomophila* systemic infection when compared to control populations.

2.9. Figures

Figure 2.2. Survival curves for (A) females and (B) males of EPN selection regime tested for response to selection after 35 generations of forward selection, infected with their primary pathogen *Enterococcus faecalis*.

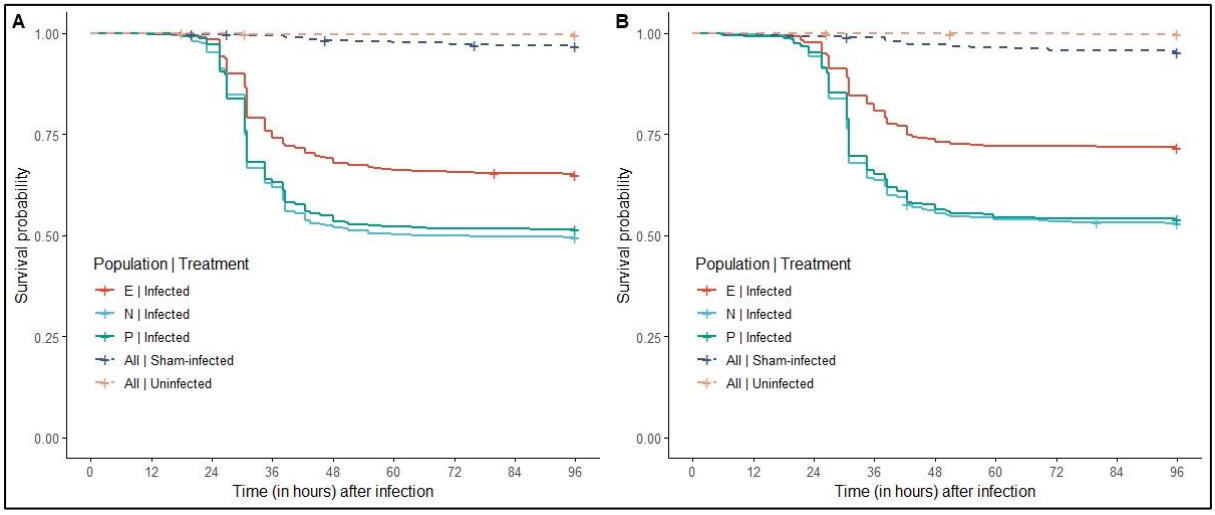
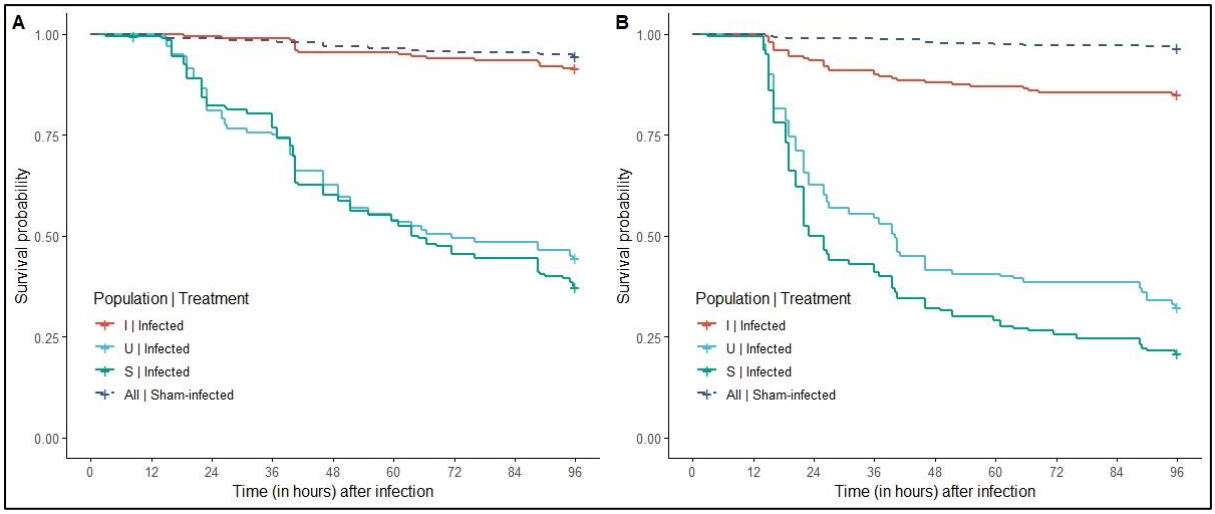


Figure 2.3. Survival curves for (A) females and (B) males of IUS selection regime tested for response to selection after 160 generations of forward selection, infected with their primary pathogen *Pseudomonas entomophila*.



2.10. Tables

Table 2.2: Composition of 1 litre of standard banana-jaggery fly media

Ingredient	**Amount**
Banana (g)	205
Barley flour (g)	25
Jaggery (unrefined cane sugar) (g)	35
Yeast (g)	36
Agar (g)	12.4
Ethanol (ml)	45
Water (ml)	1800
p-Hydroxymethyl Benzoate (g)	2.4

Table 2.3. Output of mixed-effects Cox proportional hazards analysis of flies of EPN (generation 35) and IUS (generation 160) selection regimes tested for response to selection by being infected with their respective primary pathogens, *Enterococcus faecalis* and *Pseudomonas entomophila*. Hazard ratios are relative to the default level for each factor, which is set at 1. The default level for "Treatment" is 'Sham-infected', the default level for "Selection" is 'P' or 'S' depending upon the regime under analysis, and the default level for "Sex" is 'Females'. Hazard ratio greater than 1 implies reduced survival compared to the default level. Significant effects are marked in bold.

	Hazards Ratio	Lower CI (95%)	Upper CI (95%)	Z	p-value	Variance (for random factor)
A. Effect of infection treatments on overall survival in EPN selection regime.						
Treatment *Uninfected*	**0.0851**	**0.0413**	**0.1753**	**-6.69**	**2.3 e-11**	
Treatment *Infected*	**14.6184**	**11.8732**	**17.9980**	**25.28**	**0.0 e+00**	
Block						0.1008
B. Effect of selection history and sex on survival of infected flies in EPN selection regime.						
Selection *E*	**0.6360**	**0.5454**	**0.7417**	**-5.77**	**7.9 e-09**	
Selection *N*	1.0464	0.9103	1.2028	0.64	5.2 e-01	
Sex *Males*	0.9203	0.7980	1.0614	-1.14	2.5 e-01	
Selection *E* : Sex *Males*	0.8337	0.6651	1.0450	-1.58	1.1 e-01	
Selection *N* : Sex *Males*	0.9935	0.8133	1.2137	-0.06	9.5 e-01	
Block						0.1142
C. Effect of infection treatments on overall survival in IUS selection regime.						
Treatment *Infected*	**14.7962**	**11.1879**	**19.5685**	**18.89**	**0.0 e+00**	
Block						0.1060
D. Effect of selection history and sex on survival of infected flies in IUS selection regime.						
Selection *I*	**0.0944**	**0.0568**	**0.1568**	**-9.12**	**0.0 e+00**	
Selection *U*	0.8942	0.6919	1.1556	-0.85	3.9 e-01	
Sex *Males*	**2.1630**	**1.7063**	**2.7420**	**6.38**	**1.8 e-10**	
Selection *I* : Sex *Males*	0.8899	0.4691	1.6883	-0.36	7.2 e-01	
Selection *U* : Sex *Males*	0.7332	0.5192	1.0354	-1.76	7.8 e-02	
Block						0.2160

Chapter 3

Life-history traits of EPN populations

3.1. Introduction

One of the central tenets of eco-immunology is that immune defense comes at a cost to the host (Sheldon and Verhulst 1996, Rolf and Siva-Jothy 2003, Schulenberg et al 2009). Previous authors have classified costs associated with immune function in diverse ways (viz., Schmid-Hempel 2003, McKean et al 2008, McKean and Lazzaro 2011, etc.). The cost of immune defense is expected to manifest in form of trade-offs with other organismal functions, such as reproductive output, life-history traits, and resistance to stressors (Lochmiller and Deerenberg 2000, Schmid-Hempel 2005).

I experimentally evolved replicate *Drosophila melanogaster* populations, selecting flies every generation for increased survival after being infected with a Gram-positive bacterial entomopathogen, *Enterococcus faecalis* (Singh et al, Chapter 2). Using these populations (and their ancestrally paired control populations; see Methods for details of selection design), I explored if evolving increased defense against a bacterial pathogen leads to trade-offs with life-history traits in the hosts, comparing flies from selected and control populations. I further explored if mounting a defense against the same bacterial pathogen is costly, comparing infected and uninfected flies from the same population. To test for trade-off, I selected life-history traits that have been previously demonstrated to have fitness consequences in *D. melanogaster* (reviewed Prasad and Joshi 2003). I measured larval development time and viability, and adult body weight, fecundity, and longevity. The adult traits (except body weight) were measured for both infected and uninfected flies from each population. In addition to these I tested the response of evolved and control flies to novel biotic (intra-specific competition) and abiotic (starvation and desiccation) stressors. For the abiotic stressors too, I studied both infected and uninfected flies from each population.

Previous studies exploring cost of evolving increased immune defense in *D. malenogaster* have been equivocal on the matter. For example, both Kraaijeveld and Godfray (1997) and Fellowes et al (1998) demonstrated reduced larval competitive ability to be a cost of evolving defense against parasitoid infections. Similarly, flies evolved to better defend against the bacteria *Pseudomonas aeruginosa* have reduced egg viability and adult life-span (Ye et al 2009). On the other hand, no life-history costs were reported in two separate experimental evolution studies where flies were selected for increased defense against the bacteria *Pseudomonas entomophila* (Faria et al 2015, Gupta et al 2016). Flies experimentally co-evolved with *P. entomophila* also do not incur any life-history costs (Ahlawat et al 2022). There may be a few possible reasons for the inconsistency in results obtained in these studies, such as the pathogen/parasite used for selection, genetic architecture of the host population, specific life-history trait tested, and the amount of resource available to the host for allocation into different traits.

Studies exploring the cost of mounting an immune defense against pathogens and parasites have also similarly come up with discordant results. For example, trade-off between reproduction and immunity is a common expectation (Lawniczak et al 2007, Schwenke et al 2016), where infected hosts are expected to exhibit reduced reproductive effort (Lochmiller and Deerenberg 2000, Schmid-Hempel 2003, McKean et al 2008). But *D. melanogaster* females when infected with bacterial or viral pathogens are known to increase (Hudson et al 2020), decrease (Brandt and Schneider 2007, Linder and Promislow 2009, Gupta et al 2017), or show no change in their reproductive effort (Kutzer and Armitage 2016, Kutzer et al 2018).

As a final test for costs of increased immune defence, I relaxed the selection pressure on the selected (E) populations. After 40 generations of forward selection, one E Reverse Selection populations was derived from each E populations. The ERS populations were maintained under conditions identical to that of the N populations, i.e., they had the same maintenance regime as

the E populations with similar effect population size but without any pathogen presence. After 15 generations of relaxation of selection, I compared the post-infection survival of the ERS populations to that of the N populations to test if their trait value had reverted to that of the ancestors (represented by the ERS).

My results show that evolution of increased defense against *E. faecalis*, in response to selection for increased post-infection survival, is not accompanied with life-history trade-offs. Selection history of the flies did not have an effect on any of the life-history traits measured. There was a sex-specific effect of selection history on resistance to abiotic stressors: males of the selected populations were more resistant to both starvation and desiccation compared to males from the control population. Intra-specific competitive ability was affected by selection history of the host, but the difference between the selected and the control populations was determined by the intensity of competition. Additionally, relaxation of selection did not lead to reversion of the trait value to ancestral levels.

3.2. Materials and methods

Life-history traits, and resistance to biotic and abiotic stressors, were measured for *Drosophila melanogaster* flies selected for improved post-infection survival against systemic infection with an entomopathogenic, Gram-positive bacteria, *Enterococcus faecalis*. The EPN populations was derived from ancestral BRB populations as detailed in Chapter 2.

Briefly, from each replicate population of BRB_{1-4} three populations were derived: (i) E_{1-4}, infected with *Enterococcus faecalis*; (ii) P_{1-4}, pricking control; and (iii) N_{1-4}, normal control. Populations having same numeral subscript shared a common recent ancestry and were treated as independent blocks (block 1, block 2 and so forth). Individual blocks were always handled together during selection and during experiments. Eggs were collected at a density of ~70 eggs per vial (25mm diameter × 90 mm height) containing 6-8 ml of standard banana-jaggery-yeast food. Ten 10 such vials were collected for each population. These vials were reared under standard laboratory (12:12 light: dark cycle, 25 °C, 60% relative humidity) conditions until 12^{th} day post egg collection. By 10^{th}-11^{th} day all flies eclose and had mated at least once by 12^{th} day (day of infection). Further handling depended on the type of population.

For E populations, on 12^{th} day post egg collection, every generation 200 females and 200 males were randomly picked out of total 700 flies. These flies were pricked on the dorsolateral surface of the thorax with Minutien pin (0.1mm Fine Scientific Tools, USA) dipped in bacterial suspension (Refer Chapter 2 for details). After infection flies were placed inside plexiglass cage (14 cm length x 16 cm width x 13 cm height) with food in 90 mm Petri plate. Fifty percent of the infected flies die within 96 hours of infection. Post 96 hours, fly cages were provided with oviposition food plates for 18 hours. Eggs were collected from these oviposition plates at a density of ~70 eggs per vial (as mentioned above) to start next generation.

Similarly, for P populations, 100 females and 100 males out of total 700 flies are pricked every generation with Minutien pin dipped in sterile 10 mM $MgSO_4$. For N populations, 100 females and 100 males out of total 700 flies are sorted every generation under light CO_2 anaesthesia and transferred to the plexiglass cage. Rest all the protocol are similar to E populations. There is negligible mortality in P and N populations. Post 96 hours, eggs are collected in similar way as for E populations to start the next generation.

3.2.1. Bacterial culture

The bacteria used in this study were *Enterococcus faecalis* (*Ef*, Gram-positive, grown at 37 °C, Lazzaro et al 2006). The bacterial stocks are maintained as 17% glycerol stocks frozen at -80 °C. An overnight primary culture of bacteria was set by inoculating a stab of bacterial glycerol stock in 10 ml lysogeny broth (Luria-Bertani-Miller, HiMedia) and incubating it at appropriate temperature with continuous mixing at 150 RPM (revolution per minute). Once this primary culture turned confluent (OD_{600} = 1.0), it was further diluted 100 times to set a secondary culture, and maintained at their respective conditions until it turned confluent again. This secondary culture was centrifuged and bacterial pellets were resuspended in sterile 10 mM $MgSO_4$ buffer to obtain desired OD_{600} for infection. This bacterial suspension was used to infect flies. Infection was done by dipping needle in the bacterial suspension or sterile 10mM $MgSO_4$ buffer and pricking flies on the thorax.

For stock maintenance, E populations of EPN regime were infected with *E. faecalis*. Throughout the selection history of EPN, the pathogen infection dose was modulated to induce fifty percent mortality in E populations. This ensured a constant, directional selection process. Therefore, flies of zeroth generation of E were infected with *E. faecalis* at OD_{600}=0.8 and when this experiment was done at generations 35-40 dose was increased to OD_{600}=1.0.

3.2.2. Fly standardization

To account for any non-genetic parental effects, experimental eggs were collected from flies which were grown in common garden conditions for one generation (Rose 1984). Flies thus generated were called standardized flies (for details of the standardization protocol refer Chapter 2).

3.2.3. Post-infection survival and longevity

In this experiment, I measured the survival of flies from the EPN populations, under infected, sham-infected, and uninfected conditions, during the selection window (first 96 hours following infection), plus the remaining life-span of the flies that successfully survive the first 96 hours following treatment. This experiment was done after 35 generations of forward selection.

Standardized fly cages for each population (E_i, P_i, and N_i, where 'i' represents blocks 1-4) were provided with *ad libitum* yeast paste smeared on the top of the standard banana-jaggery-yeast food plate, three days prior to the egg collection. After two days, these yeasted food plates were replaced with oviposition plates for 18 hours. Eggs were collected from these oviposition plates at the density of approximately 70 eggs per vial, into 25 vials per population (E_i, P_i, and N_i), with each vial containing 8 ml of banana-jaggery-yeast food. These *rearing* vials were incubated at standard maintenance conditions for next 12 days. Flies generally eclose by 10^{th}-11^{th} day post-egg-laying (PEL) in the rearing vials, and mate at least once by 12^{th} day PEL.

On 12^{th} day PEL, flies from each of the E_i, P_i, and N_i populations were randomly assigned to one of the three treatments:

(a) *infected* treatment: 200 females and 200 males were randomly sampled from each population, and were infected with *E. faecalis* at OD_{600} = 0.8 under light CO_2 anaesthesia;

(b) *sham-infected* treatment: 100 females and 100 males were randomly sampled from each population, and sham-infected with sterile needle dipped in sterile 10 mM $MgSO_4$ solution; and,

(c) *uninfected* treatment: 100 females and 100 males were just randomly sampled under light CO_2 anaesthesia, and not subjected to any further manipulation.

After being subjected to different treatments, the flies were housed in plexiglass cages (14 cm × 16 cm × 13 cm) having *ad libitum* access to banana-jaggery-yeast food provided in Petri plates (60 mm diameter). Individual blocks were experimented upon on separate days.

Survivorship of the flies was monitored every 4-6 hours for the first 96 hours after infection, and after this period, mortality in the cages was recorded once a day until the last fly died in all cages. About fifty percent of the infected fly and almost all sham-infected and uninfected flies survived post 96-hours window. Fresh food plates were provided on alternate days. Altogether, 200 flies/sex/population/block were infected with *E. faecalis*, 100 flies/sex/population/block were sham-infected, and 100 flies/sex/population/block were maintained as uninfected controls.

3.2.4. Fecundity and hatchability

I measured the number of eggs produced by the females (fecundity) from the EPN populations, and what proportion of these eggs produced viable larva (hatchability), to test for the effect of selection history and infection status on fecundity and hatchability. The same experimental cages that were used for assaying post-infection survival was used here for measuring fecundity and hatchability.

Fecundity was assayed after 96 hours of infection treatment (identical to the time when eggs are collected for the next generation during maintenance of the selection regime). Each of the population cages (E_i, P_i, and N_i flies either infected, sham-infected, or uninfected) were provided with an oviposition plate (60 mm diameter), containing standard banana-jaggery-yeast food, for the female flies to lay eggs on for 18 hours. After 18 hours, these plates were withdrawn, labelled with the cage identity, and stored at -20˚C for eggs to be counted later. Eggs on the surface of the food plates were counted visually using a light stereo microscope (Zeiss Stemi 2000) under 2.5X × 10X magnification. Per-female fecundity was calculated by dividing the number of eggs laid by the females during 18-hour window by the number of females alive in the respective cages at the start of the oviposition period.

Hatchability was assayed after 114 hours of infection. Following withdrawal of the oviposition plates, each of the above cages were provided with a fresh food plate (60 mm diameter) for 8 hours. From each of these plates (each coming from a single cage), three samples of 100 eggs each was picked using a moist paint brush and arranged onto the surface of three separate agar plates (90 mm Petri plates, 1.5% agar). These plates were incubated under standard laboratory maintenance conditions, and 48 hours later, I counted the number of eggs on each agar plate that had hatched visually using a light stereo microscope (Zeiss Stemi 2000) under 2.5X × 10X magnification. Hatchability was determined for each agar plate by dividing the number of eggs that hatched by the total number of eggs that were placed on the surface of that plate, and was used as unit of replication. Altogether from each cage 3 × 100 eggs/treatment/population/block were scanned for hatchability.

3.2.5. Egg-to-adult development time and viability, and dry body weight

After 40 generations of forward selection, I tested for the effect of selection history on egg-to-adult development time and viability, and dry body weight at eclosion, of the flies from the EPN populations.

Fresh food plates with excess live yeast paste were provided to the standardised population cages (E_i, P_i, an N_i) for 48 hours. Following this, a fresh food plate with live yeast paste (yeast paste placed at the centre of the plate and with space around the circumference for females to lay eggs) was provided to each cage for 6 hours. This plate was followed up with two more similarly yeasted food plates, each for a 1-hour window. This was done to encourage the females to lay any stored eggs. After this a fresh food plate was provided to each cage for females to lay eggs on for an hour, and eggs were collected using a light stereo microscope (Zeiss Stemi 2000) under 2.5X × 10X magnification and distributed into food vials (with 8 ml of banana-jaggery-yeast food) at an exact density of 70 eggs per vial. 10 vials were set up for each population (E_i, P_i, an N_i), and blocks were handled on separate days. These vials were incubated under standard maintenance conditions, and when flies started eclosing, freshly eclosed flies were transferred to empty glass vials every 4 hours, and frozen at -20 °C for further processing; this was done till the very last pupae had eclosed. The storage vials were labelled so as to preserve parent vial, population, and block identities. The flies eclosed at each time window from each vial was later scored visually using a light stereo microscope (Zeiss Stemi 2000) under 2.5X × 10X magnification to enumerate the total number and sex of the flies eclosed in that time window. Therefore, for each vial, the data was available for the number of males and females that eclosed at each time window, and the total number of flies that eclosed out of the vial. The median development time was calculated as the time taken by half of flies of each sex to eclose (starting for the time of oviposition), and viability was calculated by dividing the total number of flies eclosed by the number of eggs seeded in the vial (which was 70 eggs). After enumeration, the flies were put back into -20 ˚C storage for further use.

Flies preserved from the development time assay were used for measuring dry body weight at eclosion. All flies eclosing out of a single parent vial were pooled together; hence there were 10 pools of flies per population per block. From each pool, 5 females and 5 males were randomly sampled and placed in 1.5 ml micro-centrifuge tubes (MCTs); females and males were placed in separate MCTs. Therefore, each vial used in the development time assay yielded one MCT with 5 males and one MCT with 5 females. These MCTs were dry heated in a hot air oven for 48 hours at 60 ˚C to eliminate all moisture. The flies were then weighed using Sartorius weighing balance (model CPA225D, least count 0.01mg). Individual blocks were handled on separate days. Dry body weight was measured for only 3 blocks because samples of block 2 was lost in handling.

3.2.6. Starvation resistance

After 37-38 generations of forward selection, I measured resistance to starvation of the EPN flies, and tested if selection history and infection status had an effect on starvation resistance.

Eggs were collected from standardised population cages at an approximate density of 70 eggs per vial. 20 such vials were collected for each population (E_i, P_i, an N_i), and reared under standard maintenance conditions. Adults were housed in the rearing vials until 12^{th} day PEL. By this time all flies were sexually mature and have mated at least once inside the rearing vials itself. On 12^{th} day PEL, flies from each population (E_i, P_i, an N_i) were randomly assigned to three treatments:

(a) *infected* treatment: 50 females and 50 males were randomly sampled from each population, and were infected with *E. faecalis* at OD_{600} = 0.8 under light CO_2 anaesthesia;

(b) *sham-infected* treatment: 50 females and 50 males were randomly sampled from each population, and sham-infected with sterile needle dipped in sterile 10 mM $MgSO_4$ solution; and,

(c) *uninfected* treatment: 50 females and 50 males were just randomly sampled under light CO_2 anaesthesia, and not subjected to any further manipulation.

After being subjected to treatments, the flies were housed in vials containing 2 ml 1.5% non-nutritive agar gel, at a density of 10 females (or, males) per vial. The sexes were housed separately. The presence of agar gel in the vials ensured that the flies had *ad libitum* access to water during the course of the starvation assay. Individual blocks were assayed upon on separate days. In total, 5 vials/sex/treatment/population/block were set up for this assay. The vials were monitored every 6-8 hours to record the number of dead flies, until the last fly perished. Surviving flies were transferred to fresh agar vials every 48 hours.

3.2.7. Desiccation resistance

After 38-39 generations of forward selection, I measured resistance to starvation of the EPN flies, and tested if selection history and infection status had an effect on starvation resistance.

A set-up identical to the starvation resistance assay was utilised to test for desiccation resistance; the only difference was that the flies, after being subjected to their respective treatments, were housed in empty vials (no food or agar gel). Additionally, 5 gm silica beads were placed in each vial (above the cotton plug; no direct contact between flies and silica), and the mouth of the vial was sealed off with parafilm tape, to eliminate moisture from the vials. Individual blocks were assayed upon on separate days. In total, 5 vials/sex/treatment/population/block were set up for this assay. The vials were monitored every 1.5 hours to record the number of dead flies, until the very last fly perished.

3.2.8. Larval competitive assay

After 40 generations of forward selection, I assayed larval competitive ability as a proxy of intra-specific competition. The larval competitive assay between focal (E_i, P_i, and N_i) and competitor (PJB_w) population was measured under two ratios. First, where focal and competitor were present at equal density (1 focal : 1 competitor), and second, where focal was present in one-third density to the competitor (1 focal : 3 competitor). The focal populations had wild-type, red eye colour and competitor had a homozygous-recessive white eye colour marker. The total density of eggs and available food volume in each vial was kept constant, that is, 100 eggs in 8ml of standard banana-jaggery-yeast food. For 1 focal : 1 competitor set-up, 50 eggs of focal population and 50 eggs of competitor population was placed in standard vials (25mm diameter × 90 mm height). Ten such vials were set for each focal population. Similarly, for 1 focal: 3 competitor set-up, 25 eggs of focal population were placed along with 75 eggs of competitor population in standard vial. Ten such replicate vials for each focal population were set up.

To set-up the larval competition assay, fresh food plates smeared with excess live yeast paste were provided to the standardised population cages (E_i, P_i, an N_i) for 48 hours. Post 48 hours, a fresh food plate with live yeast paste (yeast paste placed at the centre of the plate and with space around the circumference for females to lay eggs) was provided to each cage for 6 hours. This plate was followed up with two more similarly yeasted food plates, each for a 1-hour window. This was done to encourage the females to lay any stored eggs. After this a fresh food plate was provided to each cage for females to lay eggs on for two hours, and eggs were collected at required densities using a light stereo microscope (Zeiss Stemi 2000) under 2.5X × 10X magnification and placed into food vials. 10 vials were set up for each population (E_i, P_i, an N_i) and each competitive ratio (1focal: 1competitor, and 1focal: 3competitor). Blocks

were handled on separate days. These bunches were reared under standard laboratory conditions until 12th day. On 13th day, culture vials having adult flies were frozen in -20˚C and later scored according to the eye colour. I scored adult survivors, as proxy for fitness of larvae to reach adulthood. Altogether 10 vials/population/ratio/block were assayed. Competitive index for the focal populations for each vial was calculated as,

$$Competition\ index = \frac{Number\ of\ focal\ adults\ recovered}{Number\ of\ focal\ eggs\ seeded} \times \frac{Number\ of\ total\ adults\ recovered}{Number\ of\ total\ eggs\ seeded}$$

This competitive index for each vial was used as the unit of analysis.

3.2.9. Relaxation of selected populations

After 40 generations of forward selection $E_{1\text{-}4}$RS (E Reverse Selection) populations were derived from the $E_{1\text{-}4}$ populations, with E_iRS population being created off the E_i population. The ERS populations were maintained like the N populations, which are representative of the ancestral populations. To initiate the ERS populations, from the 40th generation E cages, 10 vials of eggs were collected at a density of 70 eggs per vial in 6-8 ml of standard food medium. These vials were housed under regular maintenance conditions, and on day 12 post-egg collections, 100 females and 100 males were sorted under light CO_2 anaesthesia and transferred to plexiglass cages and were provided with a fresh food plate. Another fresh food plate was provided on day 14, and on day 16 fresh oviposition food plates were provided to each cage. This coincided with when oviposition plates were provided to E, P, and N populations too. On the next day, 18 hours after providing the oviposition plate, eggs were collected off this plate to start the next generation of the ERS populations. Therefore, the 1st generation of the ERS populations coincided with the 41st generation of E populations.

After 15 generations of relaxation of selection, when ERS populations were at generation 15 and E populations were at generation 55, I measured post infection survival of all populations:

E, P, N, and ERS, when infected with *E. faecalis* at two infection doses (OD_{600} = 1.0 and OD_{600} = 2.0). For each experimental population, standardized fly cages were provided with *ad libitum* yeast paste smeared on the top of the banana-jaggery-yeast food plate. After two days, these plates were replaced with oviposition food plates for 18 hours. From these oviposition plates, eggs were collected and distributed randomly into 20 vials containing standard diet (6-8 ml per vial) at a density of 60-80 eggs per vial. These vials were incubated under standard laboratory conditions for 12 days PEL. Peak eclosion happens on 10^{th} day PEL and by 12^{th} day PEL, flies would have matured and mated at least once in the rearing vial itself. Please note that the eclosing adults stayed in the rearing vials till the day of infection.

On day 12 PEL, flies from each population were randomly assigned to one of the following treatments: (a) infected with *E. faecalis* at OD_{600} = 1.0: 100 females and 100 males divided into two cages with equal density and sex ratio; (b) infected with *E. faecalis* at OD_{600} = 2.0: 100 females and 100 males divided into two cages with equal density and sex ratio; and (c) sham-infected: 100 females and 100 males divided into two cages with equal density and sex ratio. Post-treatment the flies were housed in plexiglass cages (14 cm x 16 cm x 13 cm) provided with ad libitum access to standard food. Mortality of the flies were recorded every 4-6 hours for 96 hours after infection. Therefore, total 96 cages [2 cages × 3 treatments (infected at OD_{600} = 1.0, infected at OD_{600} = 2.0, and sham infected) × 4 populations × 4 blocks] were observed.

3.2.10. Statistical analysis

All analyses were performed using R statistical software, version 4.1.0 (R Core Team 2021). Mixed-effect cox-proportional hazard models were fitted to the survival data (from post-infection survival, starvation resistance, and desiccation resistance assays) using the *coxme* function of the "coxme" package (Therneau 2020), and the confidence intervals for these models were calculated using *confint* function of the base R package. Survival curves were

plotted using the *ggsurvplot* function of the "survminer" package (Kassambara et al 2021) after modelling the data using *survfit* function from the "survival" package (Therneau 2021).

For the analysis of data from the post-infection survival assay (first 96 hours following infection) we first modelled the total data as:

survival ~ infection treatment + (1|block),

to test for the effect of infection treatment on survival, where 'infection treatment' was considered as a fixed factor and block as a random factor. Next, we modelled the data from infected flies only to test for the effect of selection history and sex on post-infection survival:

survival ~ selection + sex + selection:sex + (1|block),

where 'selection' and 'sex' were considered as fixed factors and 'block' as a random factor.

Survival data from longevity assay (survival from 96 hours post-infection and onwards), starvation resistance assay, desiccation resistance assay, and reverse selection assay were analysed using the following model:

survival ~ selection + infection treatment + selection:infection treatment + (1|block),

where 'selection regime' and 'infection treatment' were considered as fixed factors and 'block' as random factor. The data for each sex was analysed separately.

Data from life-history traits were modelled using mixed-effects general linear models (*lmer* function from "lmerTest" package; Kuznetsova et al 2017) and then subjected to type-III analysis of variance (ANOVA; *anova* function from base R package) for significance tests. Pairwise comparisons wherever necessary was done using Tukey's HSD (*lsmeans* function from "emmeans" package; Lenth 2021). The mixed-effects general linear models used were:

fecundity ~ selection + infection treatment + selection:infection treatment + (1|block),

hatchability ~ selection + infection treatment + selection:infection treatment + (1|block),

development time ~ selection + sex + selection:sex + (1|block),

viability ~ selection + (1|block),

body weight ~ selection + sex + selection:sex + (1|block), and

larval competition index ~ selection + (1|block).

3.3. Results

3.3.1. Response to selection, longevity, and fecundity

Survival during the pre-selection window (first 96 hours following infection) was affected by infection treatment. Both sham-infected (hazard ratio, 95% confidence interval: 11.744, 5.704-24.179) and infected (HR, 95% CI: 171.679, 85.735-343.776) flies perished more following handling compared to uninfected flies. Among infected flies, E (HR, 95% CI: 0.608, 0.522-0.708) flies died less compared to N flies, while P (HR, 95% CI: 0.956, 0.831-1.098) and N flies had similar mortality. Sex of the hosts or interaction between host sex and selection history had no effect on survival of infected flies (table 3.1 B).

Longevity of flies from the selection window onwards (day 5 following infection and onwards) was significantly affected by infection treatment in case of both female and male flies. Both sham-infected (HR, 95% CI: 1.437, 1.237-1.670) and infected (HR, 95% CI: 7.790, 6.654-9.120) females died faster compared to uninfected females. Similarly, both sham-infected (HR, 95% CI: 1.219, 1.050-1.415) and infected (HR, 95% CI: 4.767, 4.109-5.530) males died significantly faster compared to uninfected males. In case of both females (table 3.2 A) and males (table 3.2 B), selection history had no effect on longevity of flies.

Fecundity (per-female) during the selection window (between 96^{th} and 114^{th} hour following infection) was not affected by either selection history or infection treatment of the flies (table 3.3 A). Hatchability of the eggs laid during this same period was also not affected by either selection history or infection treatment of the flies (table 3.3 B).

3.3.2. Development time, egg-to-adult viability, and body weight at eclosion

Sex had a significant effect on egg-to-adult development time ($F_{1,232}$: 1656.81, p = 1.219 e-05), with females eclosing earlier than males. Selection history or selection history × sex interaction

had no effect on development time (table 3.4 A). Selection history also had no effect on egg-to-adult viability (table 3.4 B).

Dry body weight at eclosion was significantly affected by sex ($F_{1,177}$: 9.294, $p < 2$ e-16), with females having greater weight than males. Selection history or selection history × sex interaction had no effect on development time (table 3.4 C).

3.3.3. Starvation and desiccation resistance

Survival of female flies subjected to starvation was affected by infection treatment: infected females (HR, 95% CI: 2.418, 1.975-2.959) died faster compared to uninfected females, while sham-infected (HR, 95% CI: 1.080, 0.881-1.325) females and uninfected females died at a similar rate. Starvation resistance of female flies was not affected by selection history (table 3.5 A). Survival of male flies subjected to starvation was also affected by infection treatment: infected males (HR, 95% CI: 2.429, 1.995-2.958) died faster compared to uninfected males, while sham-infected (HR, 95% CI: 1.021, 0.837-1.246) males and uninfected males died at a similar rate. Starvation resistance of male flies was also affected by selection history: pooling all infection treatments together, E males (HR, 95%CI: 0.814, 0.666-0.993) perished due to starvation significantly later than N males; there was no significant difference between P and N males (table 3.5 B).

Survival of female flies subjected to desiccation was affected by infection treatment, with both sham-infected (HR, 95% CI: 2.242, 1.838-2.734) and infected (HR, 95% CI: 2.117, 1.736-2.583) females dying earlier than uninfected females. Selection history also had a significant effect on female desiccation resistance, with P females (HR, 95%CI: 1.242, 1.020-1.512) dying earlier than N females; there was no difference between mortality rate of E and N females (table 3.6 A). Survival of male flies subjected to desiccation was also affected by infection treatment, with both sham-infected (HR, 95% CI: 2.138, 1.754-2.606) and infected (HR, 95% CI: 2.463,

2.013-3.014) males dying earlier than uninfected males. Selection history also had a significant effect on male desiccation resistance, with E males (HR, 95%CI: 0.779, 0.640-0.948) dying later than N females; there was no difference between mortality rate of P and N males (table 3.6 A).

3.3.4. Intra-specific (larval) competition

Selection history had a significant effect on competitive index (see Methods for calculation of competitive index) irrespective of the infection intensity (ratio of focal and competitor eggs at the beginning of the assay; table 3.7). At low competition intensity (1 focal:1 competitor), P populations had a significantly higher competitive index than both N (t-ratio: -3.769, df: 109, p = 0.0008) and E (t-ratio: 3.685, df: 109, p = 0.0010) populations (post-hoc pairwise comparison using Tukey's HSD). At high competition intensity (1 focal:3 competitor), E populations exhibited a significantly higher competitive index than both N (t-ratio: -2.405, df: 104, p = 0.0468) and P (t-ratio: -2.591, df: 104, p = 0.0292) populations (post-hoc pairwise comparison using Tukey's HSD).

3.3.5. Relaxation of selection

Survival of male flies subjected to infection with *E. faecalis* was significantly affected by the selection history. Male flies when infected at infection dose of OD_{600}=1, survival of ERS populations (HR, 95% CI: 0.582, 0.475-0.714) and E populations (HR, 95% CI: 0.517, 0.419-0.637) were significantly more than survival of N populations. But survival of E populations was not significantly different from survival of ERS populations. Similarly, male flies when infected at infection dose of OD_{600}=2, survival of ERS populations (HR, 95% CI: 0.539, 0.440-0.660) and E populations (HR, 95% CI: 0.335, 0.266-0.421) were significantly more than survival of N populations. Here also, survival of E populations was not significantly different from survival of ERS populations.

Survival of female flies subjected to infection with *E. faecalis* was also significantly affected by the selection history. Female flies when infected at infection dose of OD_{600}=1, survival of ERS populations (HR, 95% CI: 0.527, 0.431-0.645) and E populations (HR, 95% CI: 0.410, 0.333-0.506) were significantly more than survival of N populations. Survival of E populations was similar to that of survival of ERS populations. Similarly, female flies when infected at infection dose of OD_{600}=2, survival of ERS populations (HR, 95% CI: 0.500, 0.407-0.614) and E populations (HR, 95% CI: 0.381, 0.306-0.474) were significantly more than survival of N populations. Here also, survival of E populations was not significantly different from survival of females of ERS populations.

3.4. Discussion

3.4.1. Response to selection

I selected *Drosophila melanogaster* populations for increased post-infection survival when adults are infected with *Enterococcus faecalis*. After 35 generations of forward selection, flies of the selected populations (E populations) exhibited a marked reduction in post-infection mortality compared to flies from the control populations (P and N populations), indicating a successful response to selection (figure 3.1). Susceptibility to infection by *E. faecalis* was not determined by sex of the host in either the control or the selected populations (figure 3.1).

3.4.2. Juvenile life-history traits

Egg-to-adult development time and viability (along with adult dry body weight at eclosion) was measured after 40 generations of forward selection. Egg-to-adult development time in the EPN populations was not affected by selection history (figure 3.4 A). My results are similar to those obtained by Faria et al (2015) and Gupta et al (2016), both of whom selected flies to better survive following infection with *Pseudomonas entomophila* and found no effect of selection history on development time. Flies evolved to better survive infection with *Pseudomonas aeruginosa* have a shorter development time compared to their controls (Ye et al 2009). Egg-to-adult viability in the EPN populations was also not affected by selection history (figure 3.4 B), similar to reports from flies selected using *P. entomophila* (Faria et al 2015, Gupta et al 2016). Flies selected using *P. aeruginosa* exhibit reduced egg-to-adult viability (Ye et al 2009); it must be noted that Ye et al (2009) referred to their assay as *egg viability*, but their protocol suggests that what was measured was indeed egg-to-adult viability, and not the viability of eggs only. Put together this suggests that the effect of evolving increased defense against bacterial pathogens (in the adult stage) on juvenile life history traits is determined by the identity of the pathogen used for selection. In the EPN populations, females

developed faster than males (figure 3.4 A), which is common in *D. melanogaster* studies (reviewed in Prasad and Joshi 2003).

3.4.3. Adult life-history traits

Dry body weight at eclosion for adults was influenced by sex of the flies, with females being heavier than males, but within each sex there was no observable effect of selection history (figure 3.4 C). All three previous experimental evolution studies using bacterial pathogens have reported similar results (Ye et al 2009, Faria et al 2015, Gupta et al 2016).

Longevity and fecundity, with and without infection, was measured after 35 generations of forward selection. Selection history did not have any effect on female fecundity (figure 3.3 A) and hatchability of the eggs laid (figure 3.3 B) in the EPN populations. I define hatchability as the proportion of eggs that produced a living larva within forty hours of being laid. All three previous experimental evolution studies using bacterial pathogens have reported similar results (Ye et al 2009, Faria et al 2015, Gupta et al 2016). This suggests that evolution of increased defense against bacterial pathogens does not come at a cost of female reproductive capacity in *D. melanogaster* hosts. Interestingly, female fecundity (and, hatchability of the eggs laid) was also unaffected by their infection status: females of all populations had comparable fecundity irrespective whether they were subjected to infection, sham-infection, or left uninfected (figures 3.3 A and 3.3 B). Although this goes against the theoretical expectations (Lochmiller and Deerenberg 2000, Schmid-Hempel 2003, McKean et al 2008), my results are in line with some of the previous studies that have demonstrated an apparent lack of change in fecundity when females are infected with bacteria *P. entomophila*, *Lactococcus lactis*, and *Escherichia coli* (Kutzer and Armitage 2016, Kutzer et al 2018).

Selection history had no overall effect on longevity of either females or males (figure 3.1). Previous studies have shown that flies evolved to defend against *P. aeruginosa* have shorter

life-span compared to control flies (Ye et al 2009), but that is not the case for flies evolved to defend against *P. entomophila* (Gupta et al 2016), suggesting that the consequences of evolving increased immunity on host life-span is pathogen specific. In case of both sexes, adult life-span was determined by the infection status of the flies: infected flies of both sexes survived less compared to their sham-/uninfected counterparts. Please note that in this context the infected flies represent such individuals who have survived the acute phase of infection. A few possible hypotheses, individually or together, can explain why survivors of acute infection die early compared to control flies. One possibility is that surviving the acute phase of infection implies mounting a successful immune defense, and the early death (reduced life-span) is an associated cost, probably because of exhaustion of resources or permanent damage to the soma caused by the pathogen. Additionally, flies that have survived acute infection continue to harbor very low dose of pathogens in their system (chronic infection), and it takes a continuous and costly investment towards immune function to ensure that the pathogen load does not re-increase (Chambers et al 2019). A third possibility is that survivors of acute infection die early because of the damage to their organs (immunopathology) caused by their own immune response (Khan et al 2017).

3.4.4. Response to abiotic stressors

Resistance to abiotic stressors was measured between 35-38 generations of forward selection. Similar to what was reported by Faria et al (2015) and Gupta et al (2016), I do not observe an increase in susceptibility to either starvation (figure 3.5) or desiccation (figure 3.6) in the selected populations (E populations) compared to the control populations (P and N populations). In fact, E population males are more resistant to both starvation and desiccation (all infection treatments pooled together) compared to males from N populations.

Infection status of the host had a significant effect on susceptibility to both biotic stressors. Infected flies, from all three selection regimes and both sexes, succumbed to starvation earlier compared to both sham-infected and uninfected flies, both of which perished at a comparable rate (figure 3.5). Early mortality of infected flies when starved might also be a manifestation of costs of mounting an immune response, similar to the case of adult life-span. Previous authors have suggested a correlation between resistance to starvation and adult longevity in *Drosophila melanogaster* (reviewed in Prasad and Joshi 2003, Rion and Kawecki 2007), so similarity in observations is not surprising.

The effect of infection status on desiccation resistance were very different. Both infected and sham-infected flies (irrespective of sex and selection history) succumbed to desiccation before the uninfected flies; there was no discernable difference between the mortality rate of the infected and sham-infected flies (figure 3.6). One possible explanation for this observation is that the procedure for both infection and sham-infection involve pricking the flies with a fine needle, leading to a breach of the cuticle, which can lead to loss of haemolymph, and moisture in general. Rate of losing moisture is a major determinant of desiccation resistance in *Drosophila melanogaster* (reviewed in Prasad and Joshi 2003). Also, desiccation is a much faster acting stress compared to starvation, working at a time scale shorter than even the time taken by the pathogen to kill the flies (flies start dying of infection around 18-20 hours post-infection, while even the most long-lived fly under desiccation stress doesn't live till 24 hours). This might explain the lack of difference between mortality rate between the infected and sham-infected flies when subjected to desiccation.

3.4.5. Response to biotic stressor

I measured larval competitive ability, as a proxy of intra-specific competition, after 40 generations of forward selection. Larval competitive ability across both competition

environment was affected by selection history (figure 3.7), but the difference between selected and control populations was not consistent across different competition environments. When the competition assay was run starting with equal numbers of eggs from focal and competitor populations, the E populations had a competitive index comparable to N populations, while a lower competitive index compared to P populations. When the assay was run starting with focal and competitor eggs in 1:3 ratio, E populations had a higher competitive index compared to both N and P populations. I do not, as of yet, have an explanation for this discrepancy. Previous studies have shown that flies selected for resistance against larval parasitoids have reduced larval competitive ability, especially when resources are scarce (Kraaijeveld and Godfray 1997, Fellowes et al 1998). My results might differ from theirs because of two possible differences: one, the pathogen/parasite used for selection are different (bacteria vs. parasitoid), and two, the life stage at which selection is applied are is different (adults vs. larva).

3.4.5. Response to Relaxation of selection

Costs associated with evolution of increased trait values can remain hidden, not being apparent via changes in life-history traits and stress resistance assays. Under such scenarios, relaxation of selection can help elucidate these hidden costs. When a trait is costly, in absence of the selection pressure necessary to maintain that trait, the population trait value reverts to that of the ancestral levels (Teotonio and Rose 2000, 2001, 2002). Therefore, to test for presence of any hidden costs, I relaxed the selection pressure on E populations, creating the ERS populations. After 15 generations of relaxation of selection, the ERS populations exhibited a significantly better post-infection survival compared to the N populations. Also, the survival of ERS populations was similar to that of the E populations. These results thus suggest that relaxation of selection on the E populations did not lead to loss of the evolved increased post-infection survival. A simple conclusion for this can be that there are no hidden costs of improved survival in the E populations, be it because of cheap immune defense of abundance

of resources in the environment. Alternatively, it is possible that relaxation of selection did not lead to reversal of trait value to ancestral levels because the selection had not been relaxed for a sufficient number of generations or that genetic variation was lacking in the E populations thereby limiting the scope of de-evolution of increased post-infection survival (Tetonio and Rose 2001). My result was similar to the results of Gupta et al (2016).

3.5. Conclusion

I tested for different types of costs of immunity: evolutionary costs of evolving an increased defense and physiological costs of mounting an immune defense, using replicate populations of *Drosophila melanogaster* selected for increased post-infection survival following infection with a Gram-positive bacterium, *Enterococcus faecalis*. I found no evidence of evolutionary costs: the selected population and control populations did not differ from one another in terms of trait values of life-history traits, either in the juvenile or in the adult stage. Selected populations also did not exhibit an increased susceptibility to abiotic stress. Put together with previous studies that have experimentally evolved fly populations for increased immunity against bacterial population (Ye et al 2009, Faria et al 2015, Gupta et al 2016, Ahlawat et al 2022), I propose that whether evolving increased defense comes at the cost of other organismal function depend on the bacterial pathogen used for selection. The cost of mounting an immune defense was specific to the trait under focus, but did not differ across different selection histories. Infected flies exhibited shorter life-span compared to uninfected flies, but there was no effect of infection status on female reproductive output. Resistance to starvation was also compromised in infected flies compared to uninfected flies. This suggests that physiological trade-offs between immune function and other organismal functions is not a universal expectation.

3.6. Figures

Figure 3.1. Post-infection survival of flies from EPN populations for the first 96 hours following infection: (A) females, and (B) males. Note: Same figure as presented before as figure 2.2

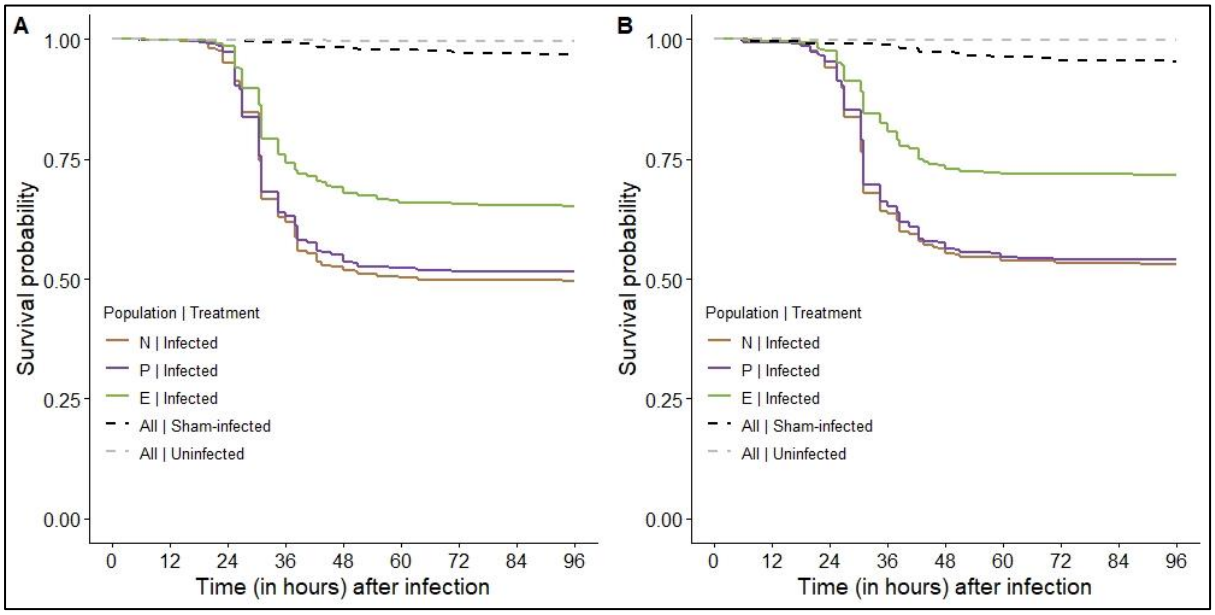


Figure 3.2. Life-time post-infection survival (longevity) of flies from EPN populations from day 5 post-infection onwards: (A) uninfected females, (B) sham-infected females, (C) infected females, (D) uninfected males, (E) sham-infected males, and (F) infected males.

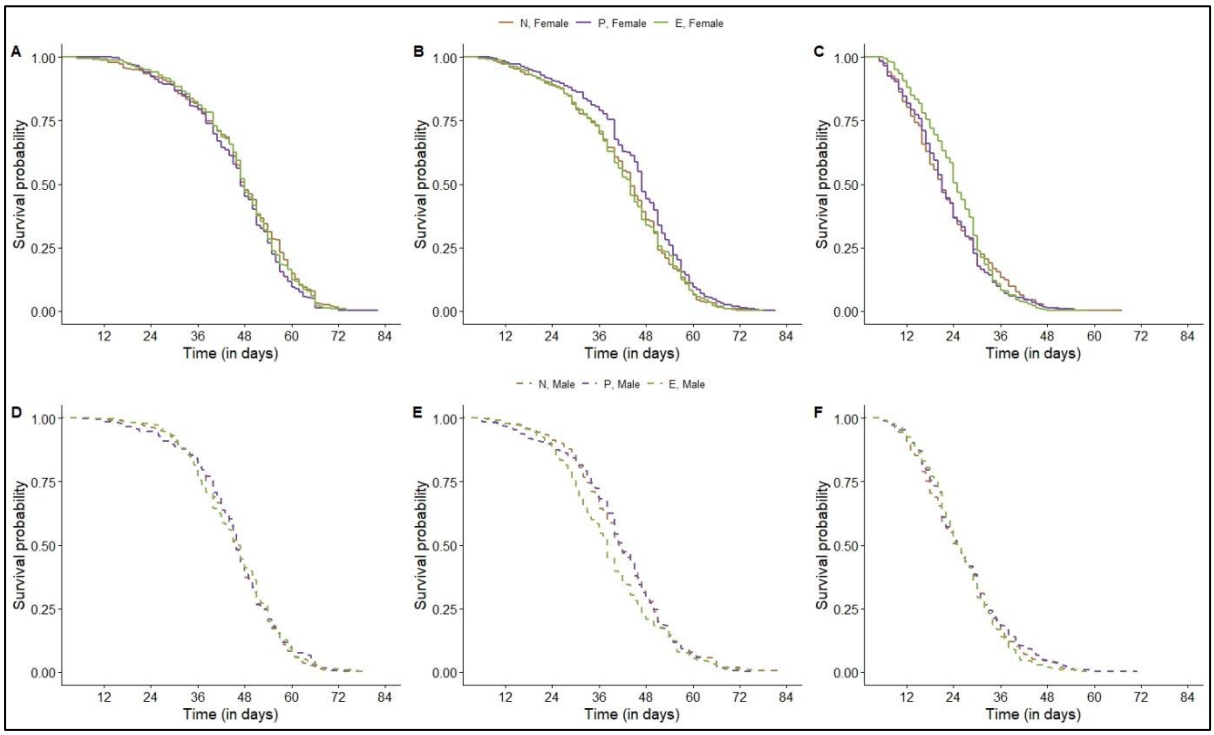


Figure 3.3. Reproductive output of females from EPN populations: (A) fecundity, and (B) egg hatchability. The boxes represent the interquartile range (IQR), with median also demarcated. The whiskers represent 1.5 × IQR below and above the first and the third quartiles, respectively.

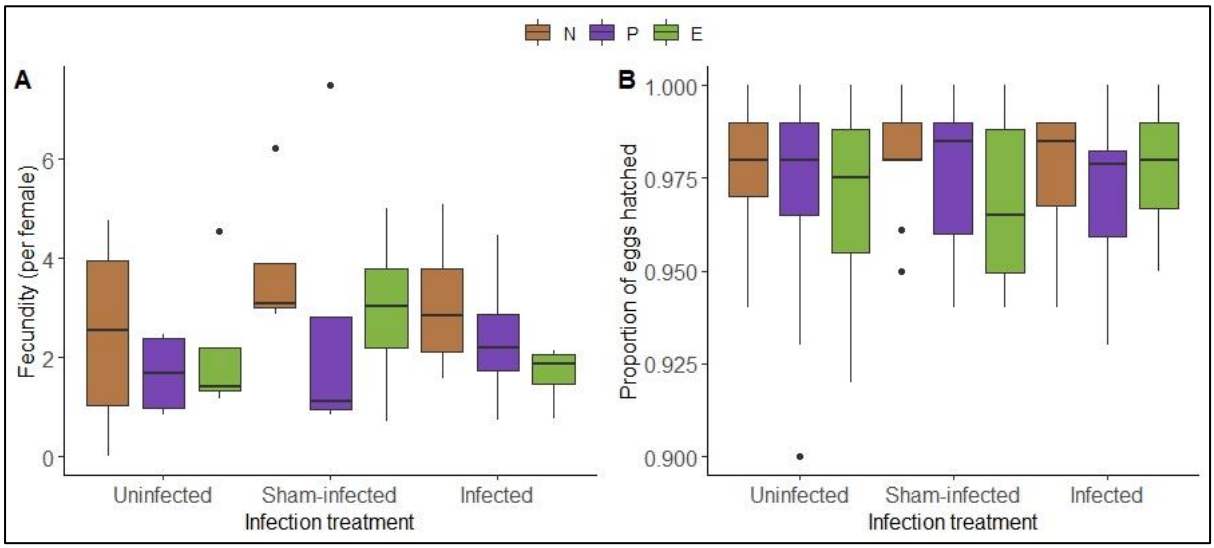


Figure 3.4. Juvenile life-history traits of flies from EPN populations: (A) egg-to-adult development time, (B) egg-to-adult survival, and (C) dry body weight at eclosion.

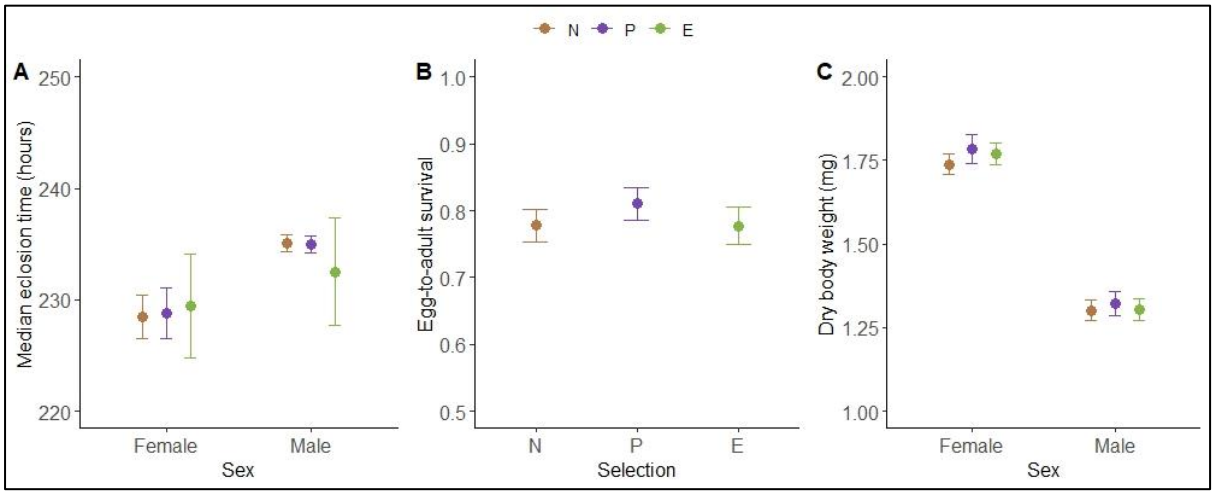


Figure 3.5. Starvation resistance (survival under starved conditions) of adult flies from EPN populations: (A) uninfected females, (B) sham-infected females, (C) infected females, (D) uninfected males, (E) sham-infected males, and (F) infected males.

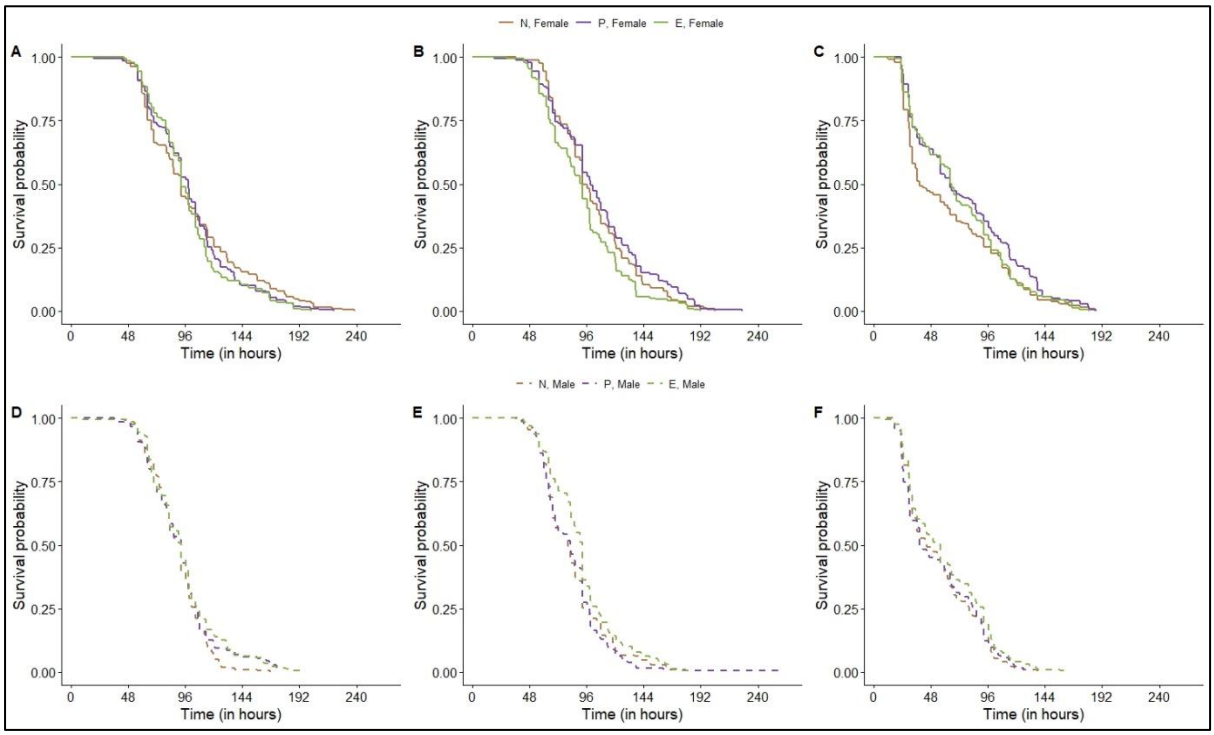


Figure 3.6. Desiccation resistance (survival under desiccated conditions) of adult flies from EPN populations: (A) uninfected females, (B) sham-infected females, (C) infected females, (D) uninfected males, (E) sham-infected males, and (F) infected males.

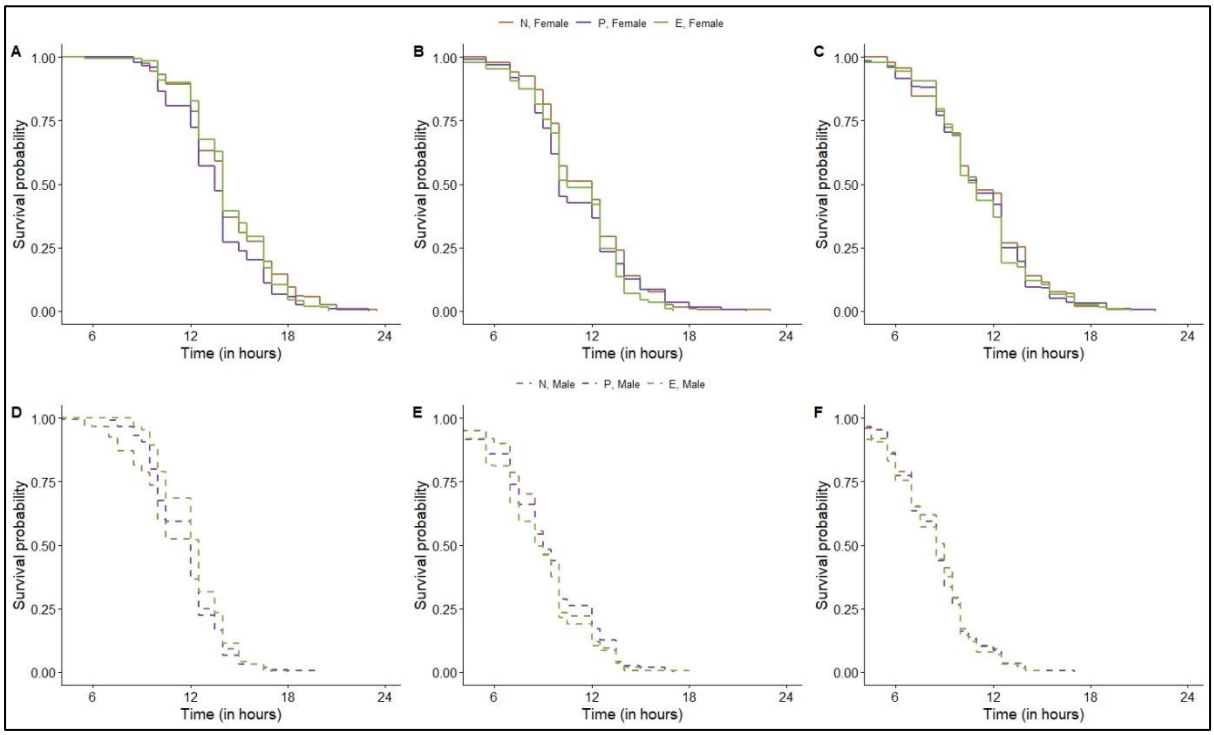


Figure 3.7. Larval competitive ability of EPN populations.

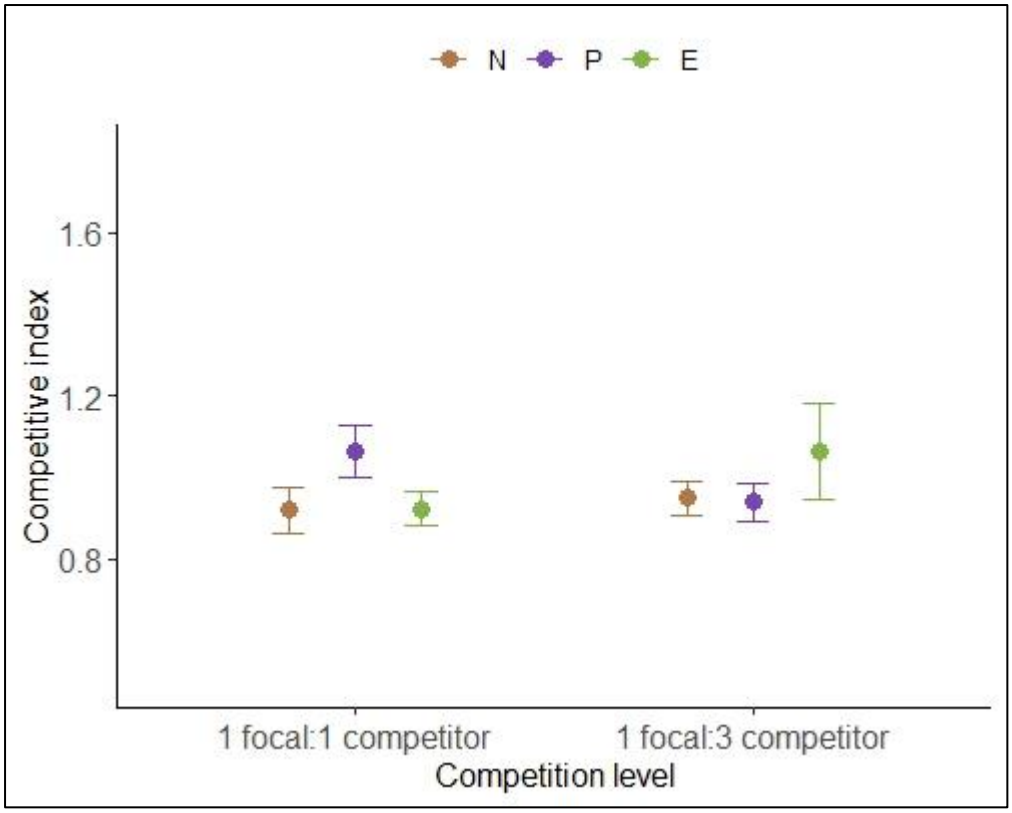
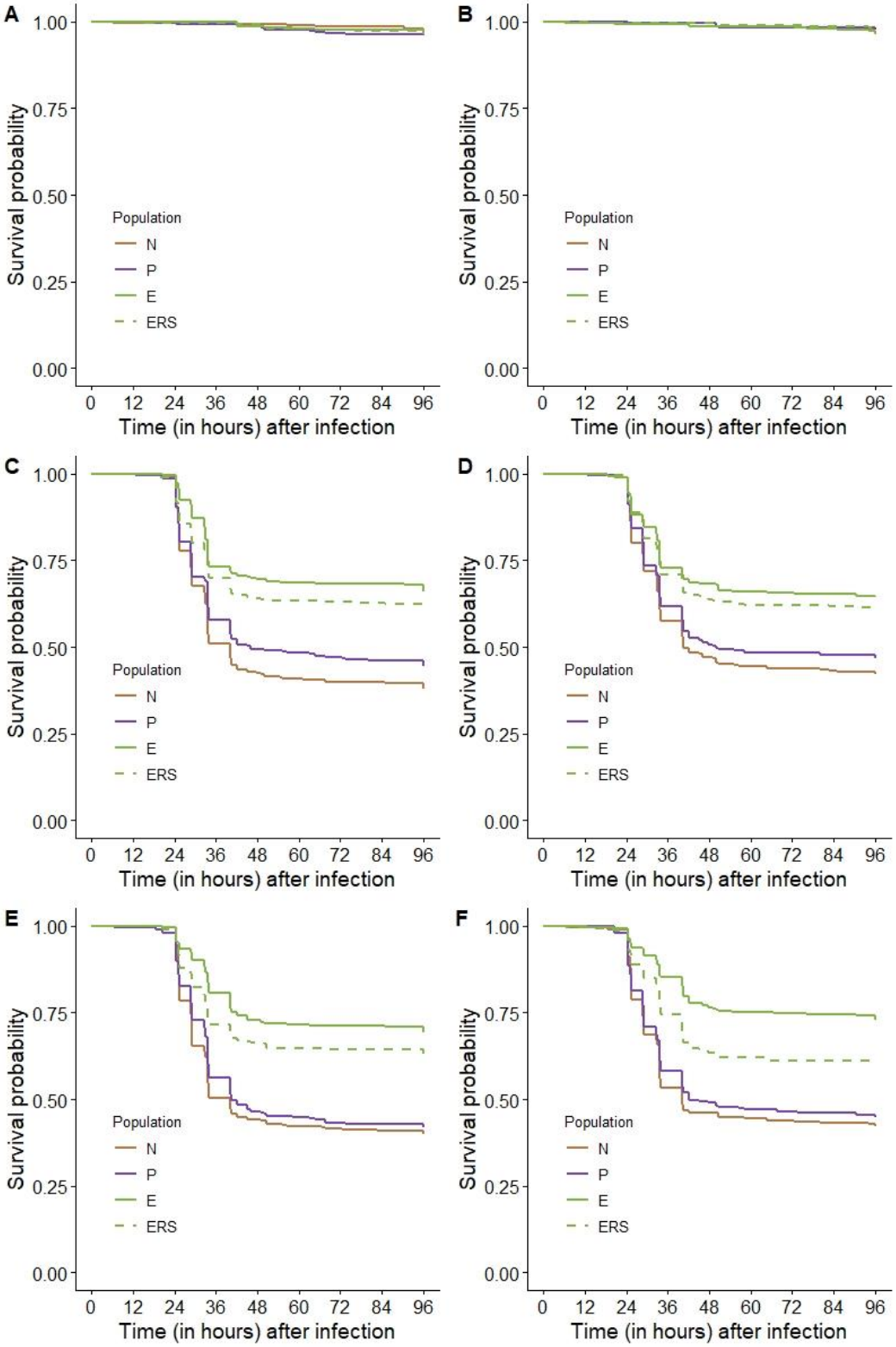


Figure 3.8. Post-infection survival of flies of the EPN and ERS populations for the 96 hours following infection: (A) Sham-infected females, (B) Sham-infected males, (C) Infected females with *Ef* at OD_{600}=1.0, (D) Infected males with *Ef* at OD_{600}=1.0, (E) Infected females with *Ef* at OD_{600}=2.0, and (F) Infected males with *Ef* at OD_{600}=2.0



3.7. Tables

Table 3.1. Output of mixed-effects Cox proportional hazards model for analysis of post-infection survival (data from first 96 hours following infection). Hazard ratios are relative to the default level for each factor which is set to 1. The default level for "Treatment" is 'Uninfected', the default level for "Selection" is 'N', and the default level for "Sex" is 'Females'. Significant effects are marked in bold.

	Hazards Ratio	Lower CI (95%)	Upper CI (95%)	Z	p-value	Variance (for random factor)
(a) Effect of infection treatments on overall survival						
Treatment *Sham-infected*	**11.7441**	**5.704236**	**24.17919**	**6.69**	**2.3e-11**	
Treatment *Infected*	**171.6795**	**85.735467**	**343.77657**	**14.52**	**0.0e+00**	
Block						0.1007759
(b) Effect of selection history and sex on post-infection survival						
Selection *P*	0.9556693	0.8313593	1.0985669	-0.64	5.2e-01	
Selection *E*	**0.6078106**	**0.5218545**	**0.7079248**	**-6.40**	**1.6e-10**	
Sex *Males*	0.9143132	0.7944967	1.0521990	-1.25	2.1e-01	
Selection *P* : Sex *Males*	1.0065507	0.8239498	1.2296190	0.06	9.5e-01	
Selection *E* : Sex *Males*	0.8391703	0.6703517	1.0505036	-1.53	1.3e-01	
Block						0.1141784

Table 3.2. Output of mixed-effects Cox proportional hazards model for analysis of longevity (data from day 5 post-infection onwards). Hazard ratios are relative to the default level for each factor which is set to 1. The default level for "Treatment" is 'Uninfected', and the default level for "Selection" is 'N'. Significant effects are marked in bold.

Factors	Hazards Ratio	Lower CI (95%)	Upper CI (95%)	Z	p-value	Variance (for random factor)
(a) Females						
Selection *P*	1.1551639	0.9969687	1.3384609	1.92	5.5e-02	
Selection *E*	1.0767328	0.9308278	1.2455080	1.00	3.2e-01	
Treatment *Sham*	**1.4373292**	**1.2368143**	**1.6703519**	**4.73**	**2.2e-06**	
Treatment *Infected*	**7.7901587**	**6.6542407**	**9.1199847**	**25.53**	**0.0e+00**	
Selection *P* : Treatment *Sham*	**0.6824614**	**0.5519484**	**0.8438355**	**-3.53**	**4.2e-04**	
Selection *E* : Treatment *Sham*	0.9452391	0.7664630	1.1657144	-0.53	6.0e-01	
Selection *P* : Treatment *Infected*	0.9865656	0.8009505	1.2151957	-0.13	9.0e-01	
Selection *E* : Treatment *Infected*	0.9557911	0.7814021	1.1690993	-0.44	6.6e-01	
Block (Random)						0.01164269
(b) Males						
Selection *P*	1.0021483	0.8663334	1.159255	0.03	0.9800	
Selection *E*	1.0374557	0.8957662	1.201557	0.49	0.6200	
Treatment *Sham*	**1.2189010**	**1.0496558**	**1.415435**	**2.60**	**0.0094**	
Treatment *Infected*	**4.7670378**	**4.1092087**	**5.530176**	**20.61**	**0.0000**	
Selection *P* : Treatment *Sham*	1.0102395	0.8205032	1.243851	0.10	0.9200	
Selection *E* : Treatment *Sham*	1.1893644	0.9644225	1.466772	1.62	0.1000	
Selection *P* : Treatment *Infected*	0.9119939	0.7448162	1.116695	-0.89	0.3700	
Selection *E* : Treatment *Infected*	1.0874873	0.8922925	1.325382	0.83	0.4100	
Block (Random)						0.03896682

Table 3.3. Type III analysis of variance (ANOVA) output for adult life-history traits. Significant effects are marked in bold.

Factors	SS	MS	df	Residual df	F value	p value
(a) Female fecundity						
Selection	6.2074	3.1037	2	36	1.2708	0.2929
Treatment	7.0083	3.5041	2	36	1.4347	0.2515
Selection × Treatment	2.1134	0.5284	4	36	0.2163	0.9276
(b) Hatchability						
Selection	0.00111321	0.00055661	2	104	1.5011	0.2277
Treatment	0.00017956	0.00008978	2	104	0.2421	0.7854
Selection × Treatment	0.00089324	0.00022331	4	104	0.6022	0.6619

Table 3.4. Type III analysis of variance (ANOVA) output for juvenile life-history traits. Significant effects are marked in bold.

Factors	SS	MS	df	Residual df	F value	p value
(a) Development time						
Selection	35.14	17.57	2	232.01	0.2120	0.8091
Sex	**1656.81**	**1656.81**	**1**	**231.97**	**19.9899**	**1.219 e-05**
Selection × Sex	140.51	70.26	2	231.97	0.8477	0.4297
(b) Egg-to-adult viability						
Selection	0.027448	0.013724	2	114.04	2.4787	0.08836
(c) Dry body weight						
Selection	0.0276	0.0138	2	177.04	2.0633	0.1301
Sex	**9.2937**	**9.2937**	**1**	**177.01**	**1388.8270**	**<2e-16**
Selection × Sex	0.0090	0.0045	2	177.01	0.6690	0.5135

Table 3.5. Output of mixed-effects Cox proportional hazards model for analysis of starvation resistance data (survival under starved conditions). Hazard ratios are relative to the default level for each factor which is set to 1. The default level for "Treatment" is 'Uninfected', and the default level for "Selection" is 'N'. Significant effects are marked in bold.

Factors	Hazards Ratio	Lower CI (95%)	Upper CI (95%)	Z	p-value	Variance (for random factor)
(a) Females						
Selection *P*	1.1751116	0.9609941	1.4369362	1.57	0.12000	
Selection *E*	1.1577567	0.9451722	1.4181550	1.42	0.16000	
Treatment *Sham*	1.0804146	0.8811486	1.3247433	0.74	0.46000	
Treatment *Infected*	**2.4178591**	**1.9753521**	**2.9594940**	**8.56**	**0.00000**	
Selection *P* : Treatment *Sham*	0.8232719	0.6196202	1.0938582	-1.34	0.18000	
Selection *E* : Treatment *Sham*	1.1580500	0.8705147	1.5405595	1.01	0.31000	
Selection *P* : Treatment *Infected*	**0.6032435**	**0.4551948**	**0.7994440**	**-3.52**	**0.00043**	
Selection *E* : Treatment *Infected*	**0.7322360**	**0.5524562**	**0.9705195**	**-2.17**	**0.03000**	
Block (Random)						0.1433253
(b) Males						
Selection *P*	0.8863760	0.7276611	1.0797092	-1.20	0.230	
Selection *E*	**0.8135374**	**0.6664097**	**0.9931474**	**-2.03**	**0.043**	
Treatment *Sham*	1.0212526	0.8369739	1.2461043	0.21	0.840	
Treatment *Infected*	**2.4291004**	**1.9948018**	**2.9579523**	**8.83**	**0.000**	
Selection *P* : Treatment *Sham*	1.2696626	0.9583504	1.6821018	1.66	0.096	
Selection *E* : Treatment *Sham*	1.0708688	0.8071027	1.4208353	0.47	0.640	
Selection *P* : Treatment *Infected*	1.0807332	0.8175647	1.4286139	0.55	0.590	
Selection *E* : Treatment *Infected*	0.9888991	0.7474084	1.3084165	-0.08	0.940	
Block (Random)						0.02744811

Table 3.6. Output of mixed-effects Cox proportional hazards model for analysis of desiccation resistance data (survival under desiccated conditions). Hazard ratios are relative to the default level for each factor which is set to 1. The default level for "Treatment" is 'Uninfected', and the default level for "Selection" is 'N'. Significant effects are marked in bold.

Factors	Hazards Ratio	Lower CI (95%)	Upper CI (95%)	Z	p-value	Variance (for random factor)
(a) Females						
Selection *P*	**1.2417770**	**1.0198177**	**1.512045**	**2.16**	**3.1e-02**	
Selection *E*	1.0793754	0.8863367	1.314457	0.76	4.5e-01	
Treatment *Sham*	**2.2417328**	**1.8382754**	**2.733739**	**7.97**	**1.6e-15**	
Treatment *Infected*	**2.1173594**	**1.7358896**	**2.582659**	**7.40**	**1.3e-13**	
Selection *P* : Treatment *Sham*	0.9152636	0.6925459	1.209606	-0.62	5.3e-01	
Selection *E* : Treatment *Sham*	1.1927347	0.9035888	1.574406	1.24	2.1e-01	
Selection *P* : Treatment *Infected*	0.9075353	0.6872511	1.198427	-0.68	4.9e-01	
Selection *E* : Treatment *Infected*	1.0839458	0.8211026	1.430928	0.57	5.7e-01	
Block (Random)						0.03321335
(b) Males						
Selection *P*	0.9058525	0.7439713	1.1029574	-0.98	3.2e-01	
Selection *E*	**0.7786396**	**0.6397107**	**0.9477403**	**-2.50**	**1.3e-02**	
Treatment *Sham*	**2.1379795**	**1.7538748**	**2.6062045**	**7.52**	**5.5e-14**	
Treatment *Infected*	**2.4630441**	**2.0126317**	**3.0142556**	**8.75**	**0.0e+00**	
Selection *P* : Treatment *Sham*	1.0156934	0.7682580	1.3428210	0.11	9.1e-01	
Selection *E* : Treatment *Sham*	**1.4482300**	**1.0960712**	**1.9135345**	**2.61**	**9.2e-03**	
Selection *P* : Treatment *Infected*	1.2051625	0.9124174	1.5918336	1.31	1.9e-01	
Selection *E* : Treatment *Infected*	**1.5812626**	**1.1967567**	**2.0893064**	**3.22**	**1.3e-03**	
Block (Random)						0.08556725

Table 3.7. Type III analysis of variance (ANOVA) output for larval competitive ability. Significant effects are marked in bold.

Factors	SS	MS	df	Residual df	F value	p value
(a) 1 focal : 1 competitor						
Selection	**0.51269**	**0.25634**	**2**	**108**	**9.7583**	**0.0001271**
(b) 1 focal : 3 competitor						
Selection	**0.36332**	**0.18166**	**2**	**102.06**	**4.2513**	**0.01685**

Table 3.8. Output of mixed-effects Cox proportional hazards model for analysis of post-infection survival of flies of EPN and ERS populations. Hazard ratios are relative to the default level which is set to 1, the default level for "Selection" is 'N'. Significant effects are marked in bold.

	Hazard ratio	Lower CI (95%)	Upper CI (95%)	Z	p-value	Variance (for random factor only)
Sex: Male, Infection dose: 1.0 OD						
Selection P	0.8871849	0.7364320	1.0687979	-1.26	2.1e-01	
Selection E	**0.5168113**	**0.4192428**	**0.6370865**	**-6.18**	**6.3e-10**	
Selection ERS	**0.5824344**	**0.4750973**	**0.7140218**	**-5.20**	**2.0e-07**	
Block (random)						0.06390731
Sex: Female, Infection dose: 1.0 OD						
Selection P	0.8345324	0.6962597	1.0002653	-1.96	5.0e-02	
Selection E	**0.4104220**	**0.3327809**	**0.5061774**	**-8.32**	**1.1e-16**	
Selection ERS	**0.5270883**	**0.4314333**	**0.6439515**	**-6.27**	**3.7e-10**	
Block (random)						0.1174080
Sex: Male, Infection dose: 2.0 OD						
Selection P	0.9062908	0.7535715	1.0899602	-1.05	3.0e-01	
Selection E	**0.3346940**	**0.2662981**	**0.4206567**	**-9.38**	**0.0e+00**	
Selection ERS	**0.5386367**	**0.4395951**	**0.6599926**	**-5.97**	**2.4e-09**	
Block (random)						0.1356039
Sex: Female, Infection dose: 2.0 OD						
Selection P	0.9385971	0.7833399	1.1246261	-0.69	4.9e-01	
Selection E	**0.3811543**	**0.3064998**	**0.4739926**	**-8.67**	**0.0e+00**	
Selection ERS	**0.5001913**	**0.4073293**	**0.6142239**	**-6.61**	**3.8e-11**	
Block (random)						0.05285409

Chapter 4

Effect of larval diet on adult immune function and life-history traits of EPN, and IUS populations

4.1. Introduction

Expression of immune phenotypes is governed by trade-offs, both evolutionary and physiological (Sheldon and Verhulst 1996). Evolutionary trade-offs stem from antagonistic pleiotropy or linkage disequilibrium, with more immune-competent genotypes having sub-optimal fitness in terms of other organismal traits such as reproduction (Schmid-Hempel 2003). Physiological trade-offs are driven by differential resource allocation between immune function and other organismal traits; increased investment towards immune defense compromises other life history traits of the individual organism, and vice versa (Lochmiller and Deerenberg 2000). The resource allocation towards immune function can be plastic (depending on environmental factors like exposure to pathogens, availability of resources, etc.) or developmentally pre-determined. Additionally, the cost of investment towards immune function in terms of its negative effects on other traits often manifest only when the individual organism is subjected to infection (McKean et al 2008, Lazzaro and Little 2009).

Studying correlated responses to selection in controlled evolution set-ups is an easy method to elucidate trade-offs between different organismal traits. Such set-ups have repeatedly been used to study evolution of defense against parasites and pathogens, and correlated evolution of other life-history traits. *Drosophila melanogaster* populations selected for increased defense against larval parasitoid show reduced capacity of intra-specific competition (Kraaijeveld and Godfray 1997, Fellowes et al 1998). Similarly, *Drosophila* flies selected for better resistance against bacterial pathogen *Pseudomonas aeruginosa* have reduced egg viability and adult life-span (Ye et al 2009). Populations of Indian meal moth, *Plodia interpunctella*, selected for increased resistance to granulosis virus exhibit increased development time and reduced egg viability (Boots and Begon 1993). Red flour beetles, *Tribolium casteneum*, populations selected for increased immune defense against bacterium *Bacillus thuringiensis* exhibit reduced egg and juvenile viability (Prakash et al 2022).

Contrary to expectations, associated life-history trade-offs are not always observed in experimental evolution studies. For example, both Faria et al (2015) and Gupta et al (2016) selected adult *Drosophila melanogaster* flies for resistance against the same pathogenic bacteria, *Pseudomonas entomophila,* and did not find observable trade-offs with any of the measured life-history traits. Additionally, in *Drosophila melanogaster* populations where larval competitive ability trade-offs with parasitoid defense, no trade-off is observed with respect to fecundity, egg viability, and starvation resistance (Fellowes et al 1998).

Laboratory populations live in an environment with ample access to resources, and this might be the reason why trade-offs are not always observed in laboratory experimental evolution studies (Harshman and Hoffman 2000). It has been often argued that trade-offs only manifest under stressful conditions (Reznick 1985, Stearns 1989, Marden et al 2003). In fact, the trade-off between parasitoid defense and intra-specific competitive ability in *Drosophila melanogaster* is only observed when resources are scarce (Kraaijeveld and Godfray 1997, Fellowes et al 1998). Excess resources are also known to help ameliorate reproduction immunity trade-off in *Drosophila melanogaster* (McKean and Nunney 2005) and *Tenebrio molitor* (Ponton et al 2011). Therefore, one way to identify immune function associated trade-offs may be to assess immunity and life-history traits under resource limited conditions.

There is ample evidence that host organisms exposed to poor nutrition suffer from reduced immune defense and increased susceptibility to pathogens. Starvation reduces phenoloxidase activity in mealworm beetle, *Tenebrio molitor* (Siva-Jothy and Thompson 2002). Probability of survival till adulthood for mosquito (*Aedes aegypti*) larvae infected with microsporidian parasite (*Vavria culicis)* increases with increase in food availability (Bedhomme et al 2004). Tobacco hornworm, *Manduca sexta*, when raised on non-native host plants have reduced melanization and encapsulation capacity (Diamond and Kingsolver 2011). Limiting access to nutrition can alter the functionality of different components of the host immune system, instead

of universal downregulation, making the effect of malnutrition on host immunity mechanism and pathogen specific (Adamo et al 2016). Reducing yeast content in adult diet increases the susceptibility of *Drosophila melanogaster* females to infection with *Pseudomonas entomophila* (Kutzer et al 2018), but not to infection with *Escherichia coli* or *Lactococcus lactis* (Kutzer and Armitage 2016). In addition to total nutrition availability, host immune function is also affected by changes in specific components of the diet (Cotter et al 2011). For example, low protein/high carbohydrate diets enhance survival of *Drosophila melanogaster* when infected with *Micrococcus luteus* (Ponton et al 2020) and of fruit fly *Bactrocera tryoni* when infected with *Serratia marcescens* (Dinh et al 2019). Similarly, burying beetle *Nicrophorus vespilloides* can survive infection with bacterium *Photorhabdus luminiscens* when fed a high fat/low protein diet (Miller and Cotter 2018). Nutrition can also affect immune function indirectly by affecting the physiological state of the host organism (Diamond and Kingsolver 2011).

Reduction in resources (in the form of reduced access to nutrition), instead of unmasking trade-offs, can also lead to improvement of immune function, in a pathogen specific manner. Ayres and Schneider (2009) raised *Drosophila melanogaster* flies on poor diet as larvae and found the adults to be more resistant to infection with *Salmonella typhimurium*, while being more susceptible to *Listeria monocytogenes*; resistance to *Enterococcus faecalis* remained unchanged. Fly mutants (gr28b) that feed less also exhibit identical patterns of resistance (Ayres and Schneider 2009). Reduced feeding in response to infection is observed in many animal species, but it is unclear if this is an adaptive strategy on the part of the host to defend against infections (Hite et al 2020). In response to infection, hosts may also modify their choice of food substrate in order to accommodate their immediate dietary requirements (Abbott 2014). For example, bacterial infection in *Drosophila melanogaster* (Ponton et al 2020) and *Bactrocera tryonni* (Dinh et al 2019) has also been shown to shift diet choice of flies towards

more carbohydrate rich food; general reduction of feeding in *Drosophila* infected with both bacteria and fungi has also been reported (Bashir-Tanoli and Tinsley 2014). Contrary to this, *Sodoptera littoralis* when infected with nucleopolyhedrovirus (Lee et al 2006) and *Sodoptera exempta* when infected with *Bacillus subtilis* (Povey et al 2009) prefer high protein diets. An additional source of complexity is that, since pathogens and parasites are dependent on host to acquire resources for their own proliferation, limiting host's access to nutrition can negatively impact within-host pathogen growth and thereby bias infection outcome in favour of the host (Cressler et al 2014, Pike et al 2019).

In this study, I explore how hosts evolved to be more immune to bacterial pathogens respond to scarcity of resources, in terms of immune function and life-history traits. Using EPN, and IUS selection regimes, I tested if rearing on a poor larval diet affected the immune function of adult flies of each selection regime, when infected with their native pathogen. Post-infection survival was used as a proxy of immune function in these experiments. Additionally, I tested if poor larval diet intensifies the trade-off between immune function and life-history traits in the selected populations. Since, EPN (Chapter 3) and IUS (Gupta et al 2016) populations did not show any life-history trade-offs or cost of immune maintenance under normal maintenance conditions, hence I explored the effect of reduced nutrition on post infection survival and life-history traits of the host. My results indicate that diet and selection history interact to determine post-infection survival of hosts. Additionally, the interactive effect of diet and selection history is not consistent across both sexes.

4.2. Materials and methods

Two selection regimes of *Drosophila melanogaster*, each selected for better survivorship post-infection with a different entomopathogenic bacteria, were used in this study. The two selection regimes used were EPN populations, selected for better survivorship post infection with Gram-positive bacteria *Enterococcus faecalis* (*Ef*); and IUS populations, selected for better survivorship post infection with Gram-negative bacteria *Pseudomonas entomophila* (*Pe*), described in detail in Chapter 2. Both selection regimes were derived from the Blue Ridge Baseline (BRB) populations (described in Chapter 2). The bacteria used in the study are *Enterococcus faecalis* (grown at 37℃) and *Pseudomonas entomophila* (grown at 27℃). E populations are infected with *E. faecalis*, and I populations with *P. entomophila*.

4.2.1. EPN selection regime

Briefly, from each replicate population of BRB1-4 (after 150 generations of laboratory adaptation), three populations were derived: (a) E1-4, infected with *Enterococcus faecalis*, (b) P1-4, pricking control, and (c) N1-4, normal control. Altogether, there were 12 populations in the EPN selection regime: E1-4, P1-4, and N1-4. Populations bearing the same numeral had a more recent common ancestor. For example, E1, P1, and N1 (derived from BRB1) were more closely related to each other than any of them is to E2, P2, and N2 (derived from BRB2) etc. Populations belonging to each block (E1, P1, and N1 constitute block 1, and so on) were handled together on the same day, during both population maintenance and experiments, and were treated as statistical blocks.

At start of each generation, eggs are collected for each population, at a density of 70±10 per vial (25 mm diameter × 90 mm height) containing 6-8 ml of standard banana-jaggery-yeast food; 10 vials for each population. These vials are incubated at standard laboratory conditions as described in Section 2.1. By 10-11th day PEL 95% of the flies eclose. By the 12th day PEL,

all of them were mature and had mated at least once. Till this point, all populations are handled in an identical manner.

On the 12th day PEL, E1-4 populations are infected with *E. faecalis*. From each rearing vial, randomly 20 females and 20 males were infected with the help of Minutien pin (0.1 mm, Fine Science Tools, USA) dipped in *E. faecalis* bacterial suspension (see section 2.4) and pricked on the thorax under light CO_2 anaesthesia. From 10 such vials, total of 200 females and 200 males were infected. After infection, flies were transferred to a plexiglass cage (14 cm length x 16 cm width x 13 cm height) having food plate (60 mm Petri plate in diameter). Fresh food plate was provided every alternate day. Fifty percent of the infected flies would die within 96 hours of infection with *E. faecalis*. Post 96 hours, day 16 PEL, oviposition plates were provided for 18 hours to the population cage to collect eggs for the next generation.

Similarly, on the 12th day PEL, P1-4 populations were pricked with Minutien pin dipped in sterile 10 mM $MgSO_4$ buffer under light CO_2 anaesthesia. From each rearing vial, randomly 10 females and 10 males were pricked. So, total of 100 females and 100 males were sham infected per block. There was negligible mortality (1-2%) post sham infection. Rest of the handling was identical to E1-4.

Handling of N1-4 populations were also similar to E1-4, except, here 10 females and 10 males per vial (total 100 females and 100 males per block) were randomly sorted under light CO_2 anaesthesia. There was no mortality in N1-4 populations.

Therefore, on day 16, about 100 females and males were present in each population which contributed to the next generation. Thus, EPN selection regime is maintained on a 16-day discrete generation cycle.

4.2.2. IUS selection regime

Similar to the EPN regime, the IUS regime was derived from BRB1-4 populations after 22 generations of laboratory adaptation as previously described in Gupta et al. (2016). From each replicate population of BRB three populations were derived: (a) I1-4, infected with *Pseudomonas entomophila*, (b) S1-4, sham-infected control, and (c) U1-4, uninfected.

The maintenance of IUS regime is identical to that of the EPN regime, except that (a) IUS regime was started from BRB populations after 22 generations of lab adaptation while EPN regime after 150 generations, (b) I flies are infected with Gram-negative bacteria *Pseudomonas entomophila* and E flies with Gram-positive bacteria *Enterococcus faecalis*, (c) for each block of the I1-4 150 females and 150 males whereas for E1-4 200 females and 200 males are infected every generation, (d) peak mortality window, for I is 20 hours to 60 hours and for E is 18 hours to 48 hours, (e) for I1-4 ~33% and for E1-4 50% of the infected flies would die within 96 hours of infection.

4.2.3. Bacterial culture and infection procedure

The bacteria used in the study are *Enterococcus faecalis* (grown at 37℃, Lazzaro et al 2006) and *Pseudomonas entomophila* (grown at 27℃, strain L48, Vodovar et al 2005, Mulet et al 2012). E populations are infected with *E. faecalis*, and I populations with *P. entomophila*. The bacterial stocks are maintained as 17% glycerol stocks frozen at -80 ˚C. Primary culture of the bacteria is obtained by inoculating a stab of glycerol stock in 10 ml lysogeny broth (Luria-Bertani-Miller, HiMedia) and incubating it overnight at appropriate temperature with continuous mixing at 150 RPM. To establish secondary culture, fresh 10 ml lysogeny broth is inoculated with 100 µl of the overnight culture; incubated as mentioned above till desired turbidity (OD_{600} =1.0-1.2) is reached. This secondary culture is centrifuged to obtain bacterial pellets which in turn is resuspended in sterile $MgSO_4$ buffer (10 mM) to obtain the required

optical density (OD_{600}). Flies are infected (either during selection protocol or experimental infections) by pricking them on the thorax with a 0.1 mm Minutien pin (Fine Scientific Tools, USA) dipped in the bacterial suspension under light CO_2 anaesthesia. Sham-infections are carried out similarly, except with a pin dipped in sterile $MgSO_4$.

For stock maintenance, E populations of EPN regime were infected with *E. faecalis*. Throughout the selection history of EPN, the pathogen infection dose was modulated to induce fifty percent mortality in E populations. This ensured a constant, directional selection process. Therefore, flies of zeroth generation of E were infected with *E. faecalis* at OD_{600}=0.8 and when this experiment was done after generation 40 dose was increased to OD_{600}=1.2. I populations of IUS regime were infected with *P. entomophila*. Similar to the EPN regime, throughout the selection history of IUS, the pathogen infection dose was modulated to induce ~33% mortality in I populations which ensured a constant, directional selection process. Therefore, I flies were infected at generation zero with *P. entomophila* at OD_{600}=1.0 and at generation 145 when this experiment was done infection dose was increased to OD_{600}=2.5.

4.2.4. Pre-experiment standardization

Prior to any experiment, flies of the selection regimes are reared for a generation under common laboratory conditions. This is done to account for any non-genetic parental effects (Rose 1984), and flies thus generated are called standardized flies. To generate standardized flies, eggs were collected from flies of all the populations at a density of 60-80 eggs per vial; 10 such vials were established per population. The vials were incubated under standard laboratory conditions. On day 12 post egg laying (PEL), by which time almost all the flies would have eclosed, the adults were transferred to plexiglass cages (14x16x13 cm^3) with food plates (Petri plates, 60 mm diameter). Eggs for experimental flies were collected from these ‘standardised’ population cages.

4.2.5. Effect of standard and poor diet on post-infection survival

This experiment tested the effect of poor diet on the post-infection survival of the flies, when compared to the standard food, for both EPN and IUS selection regimes. For each experimental population, standardized fly cages were provided with *ad libitum* yeast paste smeared on the top of the banana-jaggery-yeast food plate. After two days, these plates were replaced with oviposition food plates for 18 hours. From these oviposition plates, eggs were collected and distributed randomly into 20 vials containing standard diet (100% of standard food composition, 6-8 ml per vial) and 20 vials containing poor diet (50% diluted standard food; every component of the standard food composition was reduced to half of the original except water, agar, and preservatives; 6-8 ml per vial) at a density of 60-80 eggs per vial. These vials were incubated under standard laboratory conditions for 12 days PEL. Peak eclosion happens on 10^{th} day PEL and by 12^{th} day PEL, flies would have matured and mated at least once in the rearing vial itself. Please note that the eclosing adults stayed in the rearing vials till the day of infection and hence continued to be on same standard or poor diet in which they were reared as larvae.

On day 12 PEL, flies from each population, reared on either standard or poor diet, were randomly assigned to one of the following treatments: (a) infected with pathogen: 100 females and 100 males divided into two cages with equal density and sex ratio; and (b) sham-infected: 100 females and 100 males divided into two cages with equal density and sex ratio. Post-treatment the flies were housed in plexiglass cages (14 cm x 16 cm x 13 cm) provided with ad libitum access to either standard or poor diet (depending on the diet they were raised in as larvae). Hence, flies remained on the same diet throughout their life: as larvae, before infection, and after infection. Mortality of the flies were recorded every 4-6 hours for 96 hours after

infection. Therefore, total 96 cages [2 cages × 2 treatments (infected or sham) × 2 diet × 3 populations × 4 blocks] was observed for each selection regime.

This experiment was carried out using the EPN selection regime after 40 generations of forward selection, and with the IUS selection regime after 145 generations of forward selection. Additionally, flies from EPN selection regime were infected with *E. faecalis* (infection dose: OD_{600} = 1.0) and flies from IUS selection regime were infected with *P. entomophila* (infection dose: OD_{600} = 1.5). For logistic ease, the experiment was carried out one block on each day, i.e., E1, P1, and N1 (or I1, U1, and S1) were handled together of one day, and so on.

4.2.6. Effect of standard and poor diet on female fecundity

Along with the assay for differences in post-infection survival, I assayed for the effect of diet, infection status, and selection history on female fecundity. 96 hours after infection (or, sham-infection), the above fly cages were provided with oviposition food-plates for the flies to lay eggs on for 18 hours. After 18 hours, these plates were withdrawn, labelled and stored at -20˚C and eggs were counted later. Per-female fecundity was calculated by dividing the number of eggs laid during the 18-hour window by the number of females alive in that cage at the start of the oviposition period. The oviposition food plates were of the same diet (standard or poor) the flies were being held on till that point.

4.2.7. Effect of standard and poor diet on egg-to-adult development time and viability

In this experiment I tested if rearing on standard vs. poor diet affected the egg-to-adult development time and viability of flies from both EPN and IUS selection regime.

Two days prior to the egg collection, fresh food plates (normal food composition), smeared with yeast paste, were provided to the standardized fly cages. On the day of egg collection, similarly yeasted food-plate was provided for 6 hours and withdrawn. This was followed by a

second and a third round of yeasted food-plate, each for an hour only. This was done to encourage the females to lay the stored eggs. Following these, a fresh food plate was provided to the cages for 1 hour, and eggs were collected from this plate to start the assay.

From each population, 20 vials with exactly 70 eggs each were set up: 10 with standard diet and 10 with poor diet. These vials were incubated under standard laboratory conditions. Once flies started eclosing, flies were transferred into fresh empty vials, every 4 hours, and labelled according to vial, population, and diet identity. This was done until the very last fly eclosed. These freshly eclosed flies were immediately frozen in -20˚C, and later sexed and counted.

This experiment was carried out using the EPN selection regime after 38 generations of forward selection, and with the IUS selection regime after 85 generations of forward selection. For logistic ease, the experiment was carried out one block on each day, i.e., E1, P1, and N1 (or I1, U1, and S1) were handled together of one day, and so on.

4.2.8. Effect of standard and poor diet on dry body weight (at eclosion)

Measurement of dry body weight at eclosion was done using flies stored at the end of the development time assay. For each population of the IUS selection regime, flies eclosing from the same vial were pooled together according to vial identity. For each population within each diet treatment, 5 flies of each sex were randomly picked from the pooled sample, and transferred to 1.5 ml micro-centrifuge tubes. The sexes were kept in separate tubes. Therefore, each vial from the development time assay produced one tube of each sex. These tubes were dry heated in hot air oven for 48 hours at 60˚C before being weighed. Weight was measured using Sartorius weighing balance (model CPA225D). Dry body weight of the flies of EPN populations were not measured as there was no difference in the development time.

4.2.9. Statistical analysis

All statistical analyses were done in R statistical software (version 4.1, R Core Team 2021). Post-infection survival of flies was modelled as

Survival ~ Selection + Diet + Selection:Diet + (1|Block),

using mixed-effect Cox proportional hazards model (*coxme* function from 'coxme' package, Therneau 2020). Test for significant effects of different factors in the model was carried out using analysis of deviance (*Anova* function from 'car' package). Data from each sex was analyzed separately.

Life-history traits were analyzed using mixed-effect general linear models (*lmer* function from 'lmerTest' package) and subjected to type III ANOVA (*anova* function from base R) for significance tests. The mixed-effect linear models used were as follows:

Development time ~ Diet + Selection + Diet:Selection + (1|Block)

Viability ~ Diet + Selection + Diet:Selection + (1|Block)

Body weight ~ Diet + Selection + Diet:Selection + (1|Block)

Fecundity ~ Selection + Diet + Infection_treatment + Selection:Diet +
Selection:Infection_treatment + Diet:Infection_treatment +
Selection:Diet:Infection_treatment + (1|Block)

4.3. Results

4.3.1 Effect of poor larval diet on immune function and life-history traits of IUS selection regime

Flies from I (selected against *Pseudomonas entomophila*), S (sham-infected controls), and U (uninfected controls) populations were raised as larvae on two different diets: standard diet and poor diet (50 percent reduction in nutritious diet components). Adult flies were hosted on the same diet as larva during the course of the experiment. I tested the effect of rearing on different diets on (a) immune function and (b) fecundity of adult flies, (c) egg-to-adult development time and survival, and (d) adult dry body weight (figure 4.1).

To test for the effect of diet on immune function, adult flies of both sexes from all populations were infected with *Pseudomonas entomophila*, along with sham-infected controls, and their mortality was recorded for 96 hours post-infection. There was negligible mortality in sham-infected flies of all populations (figures 4.2 A and 4.2 B), hence data from only the infected flies was analyzed for effect of selection, diet, and selection × diet interaction; sexes were analyzed separately. Selection history had a significant effect on post-infection survival in females (figure 4.2 A, $\chi^2_{(df=2)} = 383.1930$, $p < 2.2e\text{-}16$): I females survived better than S and U females. There was a significant effect of selection × diet interaction (figure 4.2 A, $\chi^2_{(df=2)} = 6.2361$, $p = 0.04424$): U and S females raised on poor diet survived less compared to flies raised on standard diet; I females survived equally well irrespective of the diet they were raised on. Selection history also had a significant effect on post-infection survival in males (figure 4.2 B, $\chi^2_{(df=2)} = 441.6823$, $p < 2.2e\text{-}16$): I males survived better than S and U males. Males raised on poor larval diet were more susceptible to infection compared to males raised on standard diet ($\chi^2_{(df=1)} = 7.8464$, $p = 0.005092$), but no significant effect of selection × diet ($\chi^2_{(df=2)} = 4.6308$, $p = 0.098727$) was observed on survival of infected males (figure 4.2 B).

Along with immune function assay, I tested for differences in fecundity of flies of all three populations, raised on both diets, when infected or sham-infected (see METHODS, section 4.2.6, for more details). Diet had a significant effect on female fecundity ($F_{1,92}$ = 17.6550, p = 6.122e-05): females raised on poor diet had less per-capita fecundity compared to females raised on standard diet. Selection history and infection status (and interaction between these and with diet) had no effect on female fecundity (table 4.2 A, figure 4.3 A). Block (random factor) had no significant effect on female fecundity (log-likelihood = -303.29, p = 0.2186).

Egg-to-adult development time of flies reared on poor diet was significantly longer than flies reared on standard diet ($F_{1,230}$ = 37.7641, p = 3.486e-09). There was also a significant effect of diet × selection history interaction on development time ($F_{2,230}$ = 5.6562, p = 0.004, figure 4.3 B): among flies reared in standard diet, those from I populations took significantly longer to develop compared to both S (Tukey's HSD, p = 0.0021) and U (Tukey's HSD, p < 0.0001) flies. No such effect of selection history was observed among flies reared on poor diet. Egg-to-adult survival was also affected by diet × selection history interaction (table 4.2 C, figure 4.3 C): among flies reared in poor diet, survival was reduced in case of I flies compared to U flies (Tukey's HSD, p = 0.0199). Block (random factor) had significant effect on both development time and egg-to-adult survival (development time: log-likelihood = -904.39, p = 8445e-09; survival: log-likelihood = -895.83, p = 4.674e-08).

Dry body weight at eclosion for females was significantly affected by larval diet, selection history and the interaction between the two (table 4.2 D, figure 4.3 D). Females reared on poor diet overall had lower body weight at eclosion ($F_{1,231}$ = 11.2784, p = 0.000917). Within the females reared on standard diet, I females had lower body weight compared to both U (Tukey's HSD, p < 0.0001) and S females (Tukey's HSD, p = 0.0002). No such effect of selection history was apparent among females raised on poor diet. In fact, I females raised on standard diet did not differ in body weight from I females raised on poor diet (Tukey's HSD, p = 0.0536). Dry

body weight at eclosion for males was significantly affected by larval diet and selection history, but not their interaction (table 4.2 E, figure 4.3 D). Males reared on poor diet had lower body weight at eclosion ($F_{1,231}$ = 66.7791, p = 1.997e-14). Across both diets, males of I and S populations had lower body weight compared to those of U population (Tukey's HSD, p = 0.0055 and 0.0056 respectively); weights of males of I and S populations were not different from one another (Tukey's HSD, p = 0.9997). Block (random factor) had significant effect on dry body weight of both females and males (females: log-likelihood = 48.817, p = <2.2e-16; males: log-likelihood = 165.20, p = <2.2e-16).

4.3.2 Effect of poor larval diet on immune function and life-history traits of EPN selection regime

Flies from E (selected against *Enterococcus faecalis*), P (sham-infected controls), and N (uninfected controls) populations were raised as larvae on two different diets: standard diet and poor diet (50 percent reduction in nutritious diet components). Adult flies were hosted on the same diet as larva during the course of the experiment. I tested the effect of rearing on different diets on (a) immune function and (b) fecundity of adult flies, and (c) egg-to-adult development time and survival.

To test for effect of diet on immune function, adult flies of both sexes from all populations were infected with *Enterococcus faecalis*, along with sham-infected controls, and their mortality was recorded for 96 hours post-infection. There was negligible mortality in sham-infected flies of all populations (figures 4.2 C and 4.2 D), hence data from only the infected flies was analyzed for effect of selection, diet, and selection × diet interaction; sexes were analyzed separately. Selection history had a significant effect on post-infection survival in females (figure 4.2 C, $\chi^2_{(df=2)}$ = 56.2652, p = 6.056e-13): E females survived better than P and N females. Overall, across all populations, larval diet had no effect on post-infection survival

of females (figure 4.2 C, $\chi^2_{(df=1)}$ = 3.0068, p = 0.08291). There was a significant effect of selection × diet interaction (figure 4.2 C, $\chi^2_{(df=2)}$ = 8.2023, p = 0.01655): E females raised on poor diet were less susceptible to infection compared to E females raised on standard diet; no such difference was observed in case of P and N females. Selection history also had a significant effect on post-infection survival in males (figure 4.2 D, $\chi^2_{(df=2)}$ = 37.1255, p = 8.767e-09): E males survived better than P and N females. No significant effect of diet ($\chi^2_{(df=1)}$ = 0.1018, p = 0.7496) or selection × diet ($\chi^2_{(df=2)}$ = 2.6123, p = 0.2709) was observed on survival of infected males (figure 4.2 D).

Diet had a significant effect on female fecundity ($F_{1,92}$ = 104.4017, p < 2e-16): females raised on poor diet had less per-capita fecundity compared to females raised on standard diet across all three populations. Selection history and infection status (and interaction between these and with diet) had no effect on female fecundity (table 4.3 A, figure 4.3 E). Block (random factor) had no significant effect on female fecundity (log-likelihood = -256.88, p = 3.015e-07).

Only larval diet had a significant effect on egg-to-adult development time (table 4.3 B, figure 4.3 F), with all three populations taking longer to develop when reared in poor diet ($F_{1,231}$ = 373.7692, p < 2e-16). Flies reared on poor larval diet also had reduced egg-to-adult survival ($F_{1,231}$ = 39.1141, p = 1.916 e-09, figure 4.3 G). Egg-to-adult survival was also significantly affected by selection history, and selection × diet interaction (table 4.3 C, figure 4.3 G): within flies reared on poor diet, flies of N population had reduced survival compared both P (Tukey's HSD, p = 0.0133) and E (Tukey's HSD, p = 0.0.004) population, which did not differ among themselves (Tukey's HSD, p = 0.9327). No such effect of selection was apparent within flies reared on standard diet. Block (random factor) had significant effect on both development time and egg-to-adult survival (development time: log-likelihood = -845.53, p = 7.307e-07; survival: log-likelihood = -845.87, p = 9.72e-07).

4.4. Discussion

4.4.1 Adult immune function

Selection history had a dominating influence on adult immune function. Flies from populations experimentally selected for improved immune function (I and E) exhibited significantly less mortality when infected, compared to their corresponding controls (U and S, and P and N populations, respectively), irrespective of the quality of the diet the flies were reared on (figure 4.2). Improvement of immune function, in response to experimental evolution, therefore, is not lost due to resource scarcity.

Rearing on poor larval diet reduces post-infection survival of flies in a pathogen specific manner. When flies of IUS selection regime are infected with *Pseudomonas entomophila* I see a marked decrease in survival of flies reared on poor larval diet (figures 4.2 A and 4.2 B). On the other hand, when EPN flies are infected with *Enterococcus faecalis* survival of flies was not affected by whether they were raised on standard or poor diet (figures 4.2 C and 4.2 D). These results agree with that of previous studies that have shown that host's access to nutrition determines their resistance against *P. entomophila* (Kutzer et al 2018), but not against *E. faecalis* (Ayres and Schneider 2009). There can be a few possible reasons for this observation. First, defense against *E. faecalis* may be less resource intensive compared to defense against *P. entomophila*. Flies with chronic *E. faecalis* infection do not exhibit any change in starvation resistance, while such change is observable for chronic infection with other pathogens (Chambers et al 2019). Flies housed at high densities, where resources become limiting, show little or no reduction of defense against *E. faecalis*, while reduction in defense against other pathogens is clearly apparent (Das et al 2022, preprint). Second, mechanisms utilized by flies to defend against the two pathogens may be different (Lemaitre and Hoffman 2007), and different mechanisms are known to be affected by resource limitation to different extents

(Adamo et al 2016). In this study, I did not test for the effect of poor diet on defense against *E. faecalis* of I flies (selected for defense against *P. entomophila*) or defense against *P. entomophila* of E flies (selected for defense against *E. faecalis*). Therefore, I am unable to comment on any role of selection history × pathogen identity in determining the effect of poor diet on immune function. My previous results have shown that the I flies (compared to S controls) exhibit cross-resistance against *E. faecalis* and E flies (compared to P controls) exhibit cross-resistance against *P. entomophila* (Singh et al 2021, preprint). Therefore, it will be interesting to explore this avenue further in future experiments.

The effect of poor diet on immune function of the host is dependent upon both the selection history of the host and the sex of the host. Males from I, U, and S populations exhibited greater mortality when reared on poor larval diet, but such increase in mortality was only observable in females of U and S populations (figures 4.2 A and 4.2 B); females of I populations survived equally well when infected irrespective of the larval diet they were reared on. On the other hand, males from E, P, and N populations, and females from P and N populations, do not show any larval diet dependent difference in post-infection survival, but E females exhibit decreased mortality when raised on poor diet (figures 4.2 C and 4.2 D). Therefore, counterintuitively, females of both the selected populations (I and E) exhibit reduced post-infection mortality when raised in poor diet than what would be expected of them if the response of their corresponding controls are considered typical for flies. Sex-specific differential effect of diet quality is also observed in field cricket, *Gryllus texensis*, where high quality diet increases survival of males, but compromises survival of females, when the crickets are infected with *Serratia marcescens* (Kelly and Tawes 2013). Although sexual dimorphism in immune function can stem from various sources (Zuk and McKean 1996, Rolf et al 2002, Nunn et al 2009, Vincent and Sharp 2014, Sharp and Vincent 2015, Belmonte et al 2020), the reason for sex-specific diet × selection history interaction is not very obvious. Resource allocation

priorities can shift depending upon environmental factors and resource levels (Ng'oma et al 2017), and it is possible that adaptation to regular bacterial challenge involves prioritizing investment towards immune function, especially when resources are scarce. Females of the selected populations, therefore, may have evolved to prioritize immune defense over other function, compared to corresponding controls, especially when resources are scarce. Reduced fecundity in females due to poor quality diet may also free up whatever limited resources, which can then be channelized towards immune defense (see section 4.2). Excess resources in form of dietary yeast supplement are known to ameliorate reproduction-immunity trade-off in females, but not in male *Drosophila melanogaster* (McKean and Nunney 2005). Not much is known about the effect of larval malnutrition of male reproductive capacity in flies.

Another reason why any population – E, P, or N – when reared on poor larval diet do not become more susceptible to infection with *E. faecalis* may be due to reduced insulin signalling. Poor larval diet is known to reduce insulin signalling in flies (Rehman and Vargheshe 2021), and inactivated insulin signalling makes flies more resistant to infection with *E. faecalis* (Libert et al 2008). This might explain why in E females I see an increase in post-infection survival when reared on poor larval diet: a combination of evolved increased investment into immune function, reduction in insulin signalling, and reduced investment towards reproduction (later two influenced by poor larval diet) may drive this phenomenon.

4.4.2 Fecundity

In both selection regimes, IUS and EPN, female fecundity was only affected by larval diet, with flies from poor diet producing less eggs than flies from standard diet (figures 4.3 A and 4.3 E). Larval access to nutrition is a major determinant of adult reproductive fitness across many different Dipteran species (Hodin 2009). Poor larval diet is known to reduce adult fecundity in female *Drosophila melanogaster*, either directly due to reduced resources or

indirectly due to reduction in both ovariole number and body size (Hodin and Riddiford 2000, Tu and Tatar 2003, Deas et al 2019, Klepsatel et al 2020). Decreased ovariole count also implies a smaller *resource sink*: less ovarioles equals to less opportunity to allocate resources towards reproduction. This can potentially free up resources, that would otherwise have been invested in oogenesis, and even in times of resource scarcity, help maintain other organismal functions (such as immune function) at their optimal levels. Ablation of germline, and therefore the *resource sink*, has been previously shown to improve immune function in flies (Short et al 2012, Rodrigues et al 2021)

Neither infection status, nor selection history had any effect on female fecundity in my experiments (figures 4.2 A and 4.2 E). Flies experimentally evolved to defend against *P. entomophila* have previously been shown to not pay any fecundity cost of improved resistance (Faria et al 2015, Gupta et al 2015). Here I confirm the results for *P. entomophila*, and also report absence of fecundity costs when flies adapt to defend against *E. faecalis*. Reduced fecundity, either because of increased genetic resistance to pathogens or because of the energetic cost of mounting an immune response, is a typical predicted manifestation of cost of immunity (Lochmiller and Deerenberg 2000, Schmid-Hempel 2003, McKean et al 2008). Despite of this assertion, reduction of fecundity in response to infection is not always observed in experiments with *D. melanogaster*. For example, infection with *P. entomophila*, *L. lactis*, and *E. coli* does not lead to fecundity decline in female flies (Kutzer and Armitage 2016, Kutzer et al 2018), while infection with *P. aeruginosa* is even known to increase fecundity (Hudson et al 2020, but see Linder and Promislow 2009). Various factors, like pathogen identity, host genotype, infection route, and whether the bacteria colonize the ovaries, may potentially underlie the observed variation in experimental outcome (Brandt and Schneider 2007, Linder and Promislow 2009, Gupta et al 2017). An additional determining factor can be the time of fecundity measurement relative to the time of infection. In my study, I measured fecundity after

the period of infection-induced acute mortality had passed. Differences in fecundity due to cost of immune activation may be more apparent if measured during the acute phase of infection.

4.4.3 Egg-to-adult development time and viability

In both selection regimes, IUS and EPN, rearing on poor larval diet negatively affected various larval traits. In IUS, rearing on poor larval diet led to increase in development time and reduction in dry body weight at eclosion, although egg-to-adult viability was unaffected (figure 4.3 B, 4.3 C, 4.3 D). In EPN, poor larval diet increased development time and reduced egg-to-adult viability (figure 4.3 F and 4.3 G). Multiple previous studies have demonstrated similar effects of poor larval diet on larval traits (Kolss et al 2009, Deas et al 2019), with the effects being primarily driven by reduction in protein content in the diet (Tu and Tatar 2003, Klepsatel et al 2020). Interestingly enough, poor or low protein larval diet increases adult lipid reserves, and increases starvation resistance by slowing down metabolic rate, without any effect on adult lifespan (Tu and Tatar 2003, Klepsatel et al 2018, Rehman and Varghese 2021).

4.5. Conclusion

As discussed above (see section 4), developing as larvae in resource poor environments can affect numerous adult traits in *Drosophila melanogaster*, including body size and reproductive capacity. My results show that populations experimentally selected to defend against pathogen challenge can become better at counteracting the effect of poor larval nutrition on adult immune function. For example, poor larval diet had no effect on post-infection survival of I population females (selected for defense against *P. entomophila*), while the corresponding control U and S population females showed reduced post-infection survival upon being reared on poor larval diet (figure 4.2 A). Interestingly, the selected populations exhibited similar depression of female fecundity, induced by poor larval diet, as exhibited by the control population (figure 4.3 A and 4.3 E). This indicates that since adult immune function is under direct selection in the I populations, these flies have evolved to prioritize investment towards immune function even under circumstances where resources are limited. I see no such selection history-dependent differential effect of larval diet in E population females (selected for defense against *E. faecalis*), and the corresponding control P and N population females (figure 4.2 C), because larval diet does not affect immune defense against *E. faecalis*, which is the selective agent in this case.

Adaptation to poor larval diet has been shown to increase susceptibility of selected *Drosophila melanogaster* population to oral infection (Vijendravarma et al 2015); this increase in susceptibility is not driven by resource scarcity, but is due to the evolution of increased gut permeability as an adaptation to malnutrition. These selected populations are also better at counteracting the negative effects of poor larval diet on various other life-history traits (Kolss et al 2009). It would be interesting to test how these populations fair in terms of adult immune function (systemic pathogen challenge) given that immune function is not directly under selection in these populations.

Previous theory and empirical research have suggested that life-history trade-offs associated with increased immune function are expected to be more overt under low resource environment (Lazzaro and Little 2009). Results from my experiments do not agree with this expectation. In my experiments, although most measured life-history traits were negatively affected by poor larval diet, the selected populations were not adversely affected compared to the control populations when reared on poor diet (figure 4.3). In fact, I population flies exhibited prolonged development time and reduced dry body weight at eclosion (only for females) compared to controls (U and S population flies) when reared on standard diet, but not when reared on poor larval diet. Hence trade-offs were observed when resources were abundant, and not when resources were limited. I did not observe any trade-offs in the E, P, and N populations on either diet.

To summarize, in this study I explored if poor larval nutrition has an effect on adult immunity and other life-history traits in *Drosophila melanogaster* populations experimentally evolved to be immune to bacterial infection. My results suggest that (a) effect of poor larval nutrition on adult defense against bacterial infection is pathogen specific; (b) experimentally evolved populations maintain a better functioning immune system, compared to control populations, even when raised on poor diet; (c) host sex and selection history interact to determine the effect of poor diet on adult immune function; (d) poor larval diet reduces females fecundity, but fecundity is not affected by either host selection history or infections status; (e) poor larval diet prolongs egg-to-adult development time; and, (f) cost of evolved immune defense can manifest in form of prolonged egg-to-adult development, depending upon the pathogen used for selection. I therefore conclude that effect of poor nutrition on host immune function is not uniform, but contingent upon host sex, level of host's resistance to pathogen (selection history), and very importantly, the identity of the pathogen.

4.6. Figures

Figure 4.1. Experimental design: outline of experimental design used to study the effect of standard vs. poor larval diet on adult immune function in flies of EPN and IUS populations.

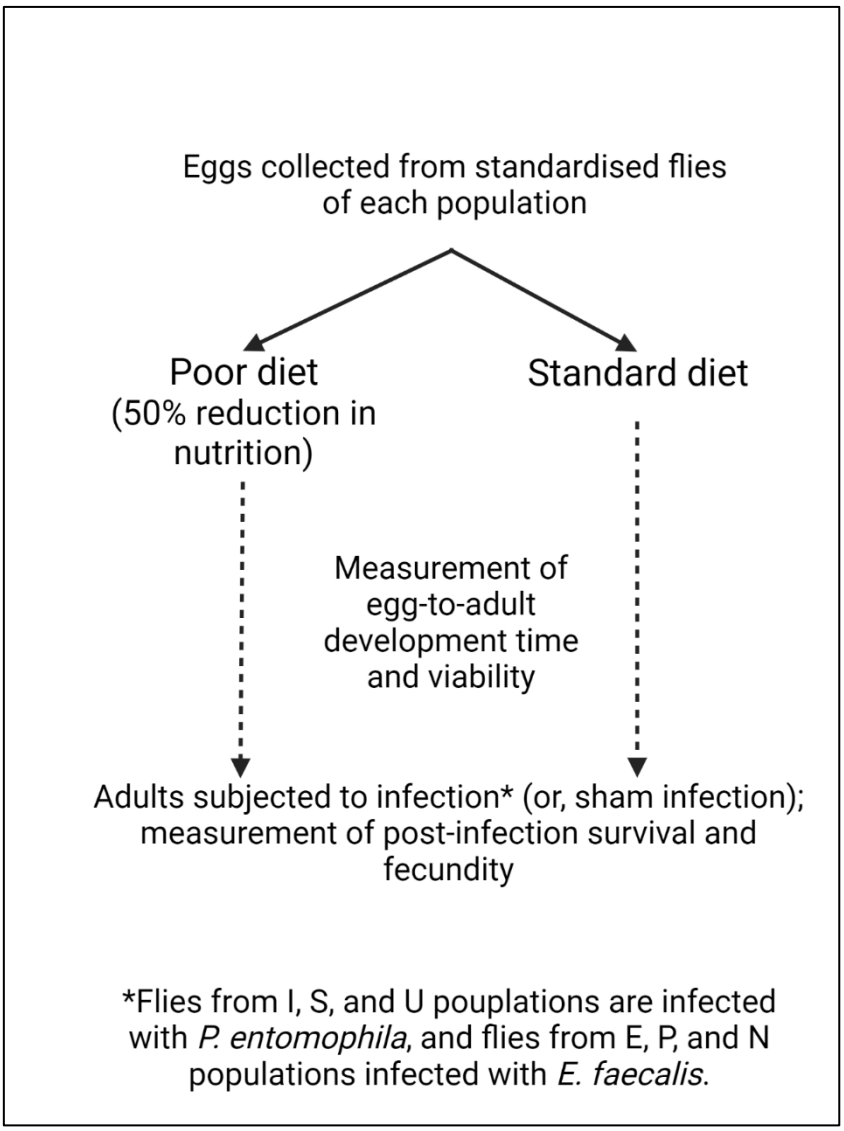


Figure 4.2. Effect of standard vs. poor larval diet on immune function (post-infection survival) of (a) females and (b) males from I, U, and S populations (infected with *Pseudomonas entomophila*), and (c) females and (d) males from E, P, and N populations (infected with *Enterococcus faecalis*).

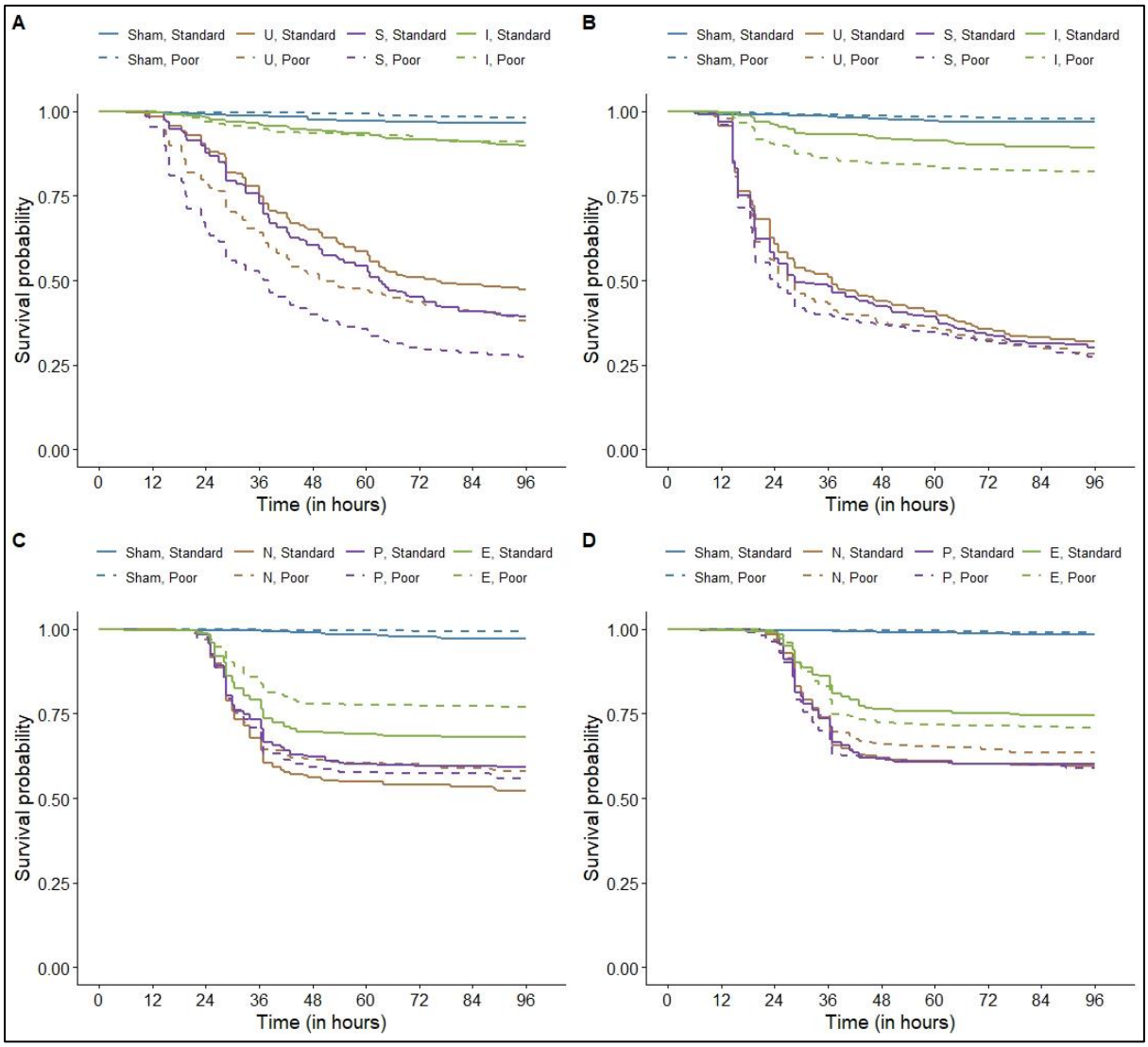
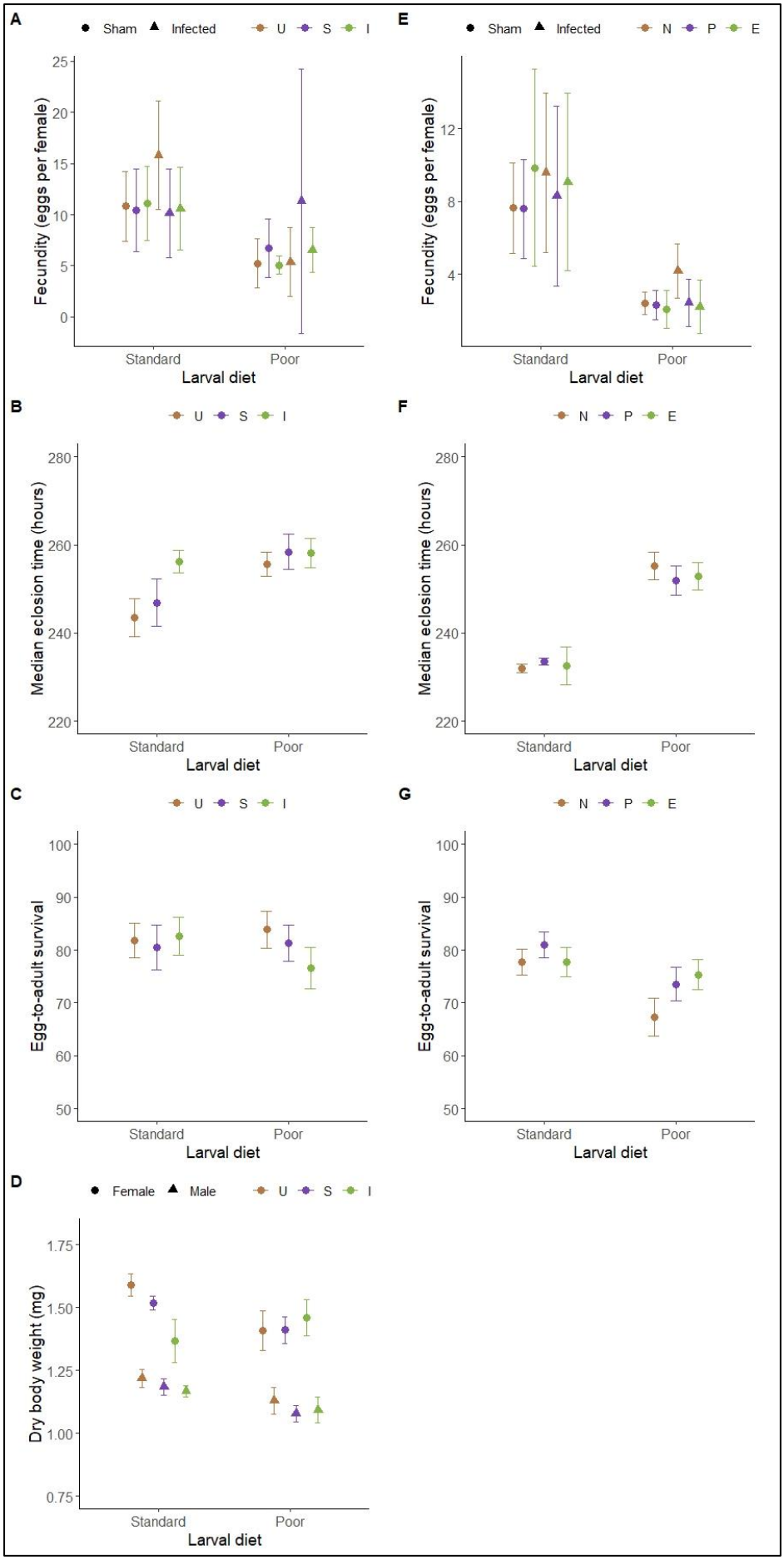


Figure 4.3. Effect of standard vs. poor larval diet on fecundity (a, e), egg-to-adult development time (b, f) and survival (c, g), and dry body weight at eclosion (d), of flies from I, U, and S populations (a-d) and E, P, and N populations (e-g).


A
Sham
Infected
U
S
I
Fecundity (eggs per female)
Standard
Poor
Larval diet
E
N
P
E
B
Median eclosion time (hours)
F
C
Egg-to-adult survival
G
D
Female
Male
Dry body weight (mg)

4.7. Tables

Table 4.1. Analysis of deviance on mixed-effect Cox proportional hazards models for effect of selection history and larval diet on post-infection survival of flies (blocks used as random factors).

Terms of model	χ^2	**df**	**p**
(A) Survival of females from I, U, S populations infected with *Pseudomonas entomophila*			
Selection	383.1930	2	< 2.2 e-16
Diet	34.5336	1	4.19 e-09
Selection:Diet	6.2361	2	0.04424
(B) Survival of males from I, U, S populations infected with *Pseudomonas entomophila*			
Selection	441.6823	2	< 2.2 e-16
Diet	7.8464	1	0.005092
Selection:Diet	4.6308	2	0.098727
(C) Survival of females from E, P, N populations infected with *Enterococcus faecalis*			
Selection	56.2652	2	6.056 e-13
Diet	3.0068	1	0.08291
Selection:Diet	8.2023	2	0.01655
(D) Survival of males from E, P, N populations infected with *Enterococcus faecalis*			
Selection	37.1255	2	8.676 e-09
Diet	0.1018	1	0.7496
Selection:Diet	2.6123	2	0.2709

Table 4.2. Analysis of variance (type III sum of squares) for the effect of selection history and larval diet (and infection status, if applicable) on life-history traits of flies from I, U, and S populations (blocks used as random factors). Significant effects are marked in bold letters.

Factors	SS	MS	df	Residual df	F value	p
(A) Female fecundity						
Selection	30.40	15.20	2	92	0.4913	0.61346
Diet	**546.30**	**546.30**	**1**	**92**	**17.6550**	**6.122e-05**
Treatment	72.03	72.03	1	92	2.3278	0.13051
Selection × Diet	180.23	90.11	2	92	2.9122	0.05938
Selection × Treatment	18.48	9.24	2	92	0.2986	0.74254
Diet × Treatment	2.71	2.71	1	92	0.0876	0.76789
(B) Development time						
Diet	**4178.4**	**4178.4**	**1**	**230**	**37.7641**	**3.486e-09**
Selection	**2422.9**	**1211.4**	**2**	**230**	**10.9487**	**2.871e-05**
Diet × Selection	**1251.7**	**625.8**	**2**	**230**	**5.6562**	**0.004**
(C) Egg-to-adult viability						
Diet	75.67	75.67	1	230	0.7242	0.39566
Selection	450.44	225.22	2	230	2.1555	0.11818
Diet × Selection	**753.89**	**376.94**	**2**	**230**	**3.6075**	**0.02866**
(D) Dry body weight, females						
Diet	**0.25485**	**0.25485**	**1**	**231**	**11.2784**	**0.000917**
Selection	**0.28062**	**0.14031**	**2**	**231**	**6.2094**	**0.002362**
Diet × Selection	**0.83623**	**0.41811**	**2**	**231**	**18.5033**	**3.519e-08**
(E) Dry body weight, males						
Diet	**0.46799**	**0.46799**	**1**	**231**	**66.7791**	**1.997e-14**
Selection	**0.09412**	**0.04706**	**2**	**231**	**6.7184**	**0.001464**
Diet × Selection	0.01222	0.00611	2	231	0.8718	0.419555

Table 4.3. Analysis of variance (type III sum of squares) for the effect of selection history and larval diet (and infection status, if applicable) on life-history traits of flies from E, P, and N populations (blocks used as random factors). Significant effects are marked in bold letters.

Factors	SS	MS	df	Residual df	F value	p
(A) Female fecundity:						
Selection	11.30	5.65	2	92	0.6669	0.5157
Diet	**884.60**	**884.60**	**1**	**92**	**104.4017**	**<2e-16**
Treatment	10.08	10.08	1	92	1.1897	0.2782
Selection × Diet	18.71	9.36	2	92	1.1042	0.3358
Selection × Treatment	19.42	9.71	2	92	1.1461	0.3224
Diet × Treatment	0.02	0.02	1	92	0.0019	0.9651
(b) Development time						
Diet	**25295.0**	**25295.0**	**1**	**231.02**	**373.7692**	**<2e-16**
Selection	39.3	19.6	2	231.02	0.2901	0.7485
Diet × Selection	254.3	127.2	2	231.02	1.8789	0.1551
(c) Egg-to-adult viability						
Diet	**2659.72**	**2659.72**	**1**	**230.98**	**39.1141**	**1.916e-09**
Selection	**1005.41**	**502.70**	**2**	**230.98**	**7.3928**	**0.0007725**
Diet × Selection	**649.59**	**324.79**	**2**	**230.98**	**4.7764**	**0.0092762**

Table 4.4. Test for significance for random effects in ANOVA for the effect of selection history and larval diet (and infection status, if applicable) on life-history traits of flies from I, U, and S populations. Significant effects are marked in bold letters. (Refer to table 4.2 for test of significance for fixed effects.)

Factors	npar	logLik	AIC	LRT	Df	Pr (>Chisq)
(A) Female fecundity						
<none>	12	-302.53	629.06			
(1|Block)	11	-303.29	628.57	1.5136	1	0.2186
(B) Development time						
<none>	8	-887.81	1791.6			
(1|Block)	**7**	**-904.39**	**1822.8**	**33.17**	**1**	**8445e-09**
(C) Egg-to-adult viability						
<none>	8	-880.91	1777.8			
(1|Block)	**7**	**-895.83**	**1805.7**	**29.848**	**1**	**4.674e-08**
(D) Dry body weight, females						
<none>	8	104.349	-192.699			
(1|Block)	**7**	**48.817**	**-83.633**	**111.07**	**1**	**< 2.2e-16**
(E) Dry body weight, males						
<none>	8	241.15	-466.31			
(1|Block)	**7**	**165.20**	**-316.40**	**151.91**	**1**	**< 2.2e-16**

Table 4.5. Test for significance for random effects in ANOVA for the effect of selection history and larval diet (and infection status, if applicable) on life-history traits of flies from E, P, and N populations. Significant effects are marked in bold letters. (Refer to table 4.3 for test of significance for fixed effects.)

	npar	logLik	AIC	LRT	Df	Pr (>Chisq)
(A) Female fecundity						
<none>	12	-243.76	511.51			
(1|Block)	**11**	**-256.88**	**535.75**	**26.24**	**1**	**3.015e-07**
(b) Development time						
<none>	8	-833.26	1682.5			
(1|Block)	**7**	**-845.53**	**1705.1**	**24.532**	**1**	**7.307e-07**
(c) Egg-to-adult viability						
<none>	8	-833.87	1683.8			
(1|Block)	**7**	**-845.87**	**1705.7**	**23.983**	**1**	**9.72e-07**

Chapter 5

Cross-resistance in EPN, and IUS populations

5.1. Introduction

Continuous selection for better defence should erode additive genetic variation for defence related traits, as more and more resistant genotypes are driven to fixation (Schelunburg et al 2009, Lazzaro and Little 2009). Empirical studies have repeatedly found evidence contradicting this theoretical expectation. Genetic variation for anti-pathogen defence have been reported in studies, both in field and lab, across various organisms: (viz. Tinsley et al 2006, Lazzaro et al 2006, Raberg et al 2007). Various factors can contribute towards this difference between the predicted and observed results, including cost and condition-dependence of immune defence, host-pathogen co-evolution and resultant evolution of specific defence, and variation in biotic and abiotic environment (Schmid-Hempel 2003, Lazzaro and Little 2009).

Hosts and pathogens exist as part of a complex network of interactions, and hosts are rarely challenged by a single pathogen in the wild (Betts et al 2016). Under such circumstances where a host must counter multiple pathogens, hosts can evolve a generic defence mechanism to counter all threats, or different host genotypes may specialise against different types of pathogens, leading to evolution of immune specificity (Decaestecker et al 2003, Schmid-Hempel and Ebert, 2003). Increased resistance against one pathogen can produce corelated decrease (positive cross-resistance) or increase (negative cross-resistance) in susceptibility towards a second pathogen (Fellowes et al 1999, Kraaijeveld et al 2012).

At the phenotypic level, cross-resistance manifests when hosts infected with one pathogen show increased or decreased susceptibility to a second pathogen. For example, mice infected with *Schistosomatium douthitti* are more resistant to subsequent infection by *Schistosoma mansoni* (Hunter et al 1961). Similarly, *Anopheles gambiae* mosquito hosts that are previously exposed to *Vavria culicis* are more resistant to *Plasmodium berghei* (Bargielowski and Koella 2009). In *Drosophila melanogaster* flies, infection with any one of *Providencia rettgeri*,

Enterococcus faecalis and *Serratia marcescens* make flies more resistance towards later infection by the other two pathogens (Chambers et al 2019).

At evolutionary level, cross-resistance is determined by how a host evolved to counter a particular pathogen responds to infection by a novel pathogen. Iso-female lines of *Drosophila melanogaster* show positive correlation for resistance to two parasitoids *Leptopilina boulardi* and *Leptopilina heterotoma* (Boulétreau and Wajnberg 1986; Delpuech et al 1994). *D. melanogaster* populations selected for increased resistance to *L. boulardi* had increased resistance to *Asobara tabida* and *L. heterotoma* where as those populations selected for increased resistance to *A. tabida* had higher resistance to *L. heterotom*a (Fellows et al 1999). Martins et al (2013) selected *D. melanogaster* populations for increased survivorship against infection from *Pseudomonas entomophila* and found that the evolved populations were also better at surviving *Pseudomonas putida*. *D. melanogaster* populations selected against DCV showed positive cross-resistance against Cricket Paralysis Virus (CrPV) and Flock House Virus (FHV) (Martins et al 2014). In contrast to these results of the evolution of positive cross-resistance, many other studies have found no evidence for the evolution of cross-resistance as a result of evolution towards a particular pathogen/parasite. Martins et al (2013) found that their populations selected for increased survivorship against *P. entomophila* was as good as the controls in surviving infections from *Erwinia carotovora* or *Serratia marcescens*. Selection for increased resistance against the parasitoid *A. tabida* did not increase resistance to the parasitoid *L. boulardi* (Fellows et al 1999), the microsporidian *Tubulinosema kingi* or the fungus *Beauveria bassiana* (Kraaijeveld et al 2012). Populations of *D. melanogaster* evolved against *Bacillus cereus* did not evolve cross-resistance to DSV (Bentz et al 2017). Similarly, greater wax moth evolved against *B. bassiana* did not evolve resistance to *Metarhizium anisopliae* (Dubovskiy et al 2013). *Tribolium castaneum* larvae coevolved with *B. bassiana* was cross resistant to *Bacillus thuringiensis* but not *P. entomophila* (Biswas et al 2018). To the best of

my knowledge, only one study, has found the evolution of negative cross-resistance. Martins et al (2013) found that populations of *D. melanogaster* evolved against *P. entomophila* were more susceptible to infections from *Enterococcus faecalis*, DCV and FHV. To summarise, at the evolutionary level, most of the studies have either found evidence for positive cross-resistance or have found no evidence for cross-resistance. There is little empirical evidence for the evolution of negative cross-resistance.

The variation in outcomes of the above described studies may be attributed to numerous factors: (a) the phylogenetic relatedness between the pathogen used for experimental evolution and the pathogens used for testing cross-resistance (Schmid-Hempel and Ebert 2003), (b) common mechanisms of pathogen virulence or host resistance (Vallet-Gely et al 2008, Dubovskiy et al 2013), (c) route of infection (Martins et al 2013, Biswas et al 2018), and (d) the genetic architecture of the host-population in the study. Additionally, host-sex is a major determinant of host immune function. The sexes differ from one another in terms of optimal life-history, environmental infection risk, and physiological modulators of immunity (viz. testosterone in male mammals and juvenile hormone in female insects); these factors together contribute towards sexual dimorphism in immune function (Zuk and McKean 1996 , Rolff 2002, Schmid- Hempel and Ebert 2003, Nunn et al 2009, Vincent and Sharp 2014, Sharp and Vincent 2015), and can potentially be another factor that leads to differential patterns of cross-resistance observed in empirical studies. Studies on immunity often focus on only one sex, and even when both sexes are used in experiments, the statistical analysis does not involve sex as a factor. Hence, sexual dimorphism in cross-resistance has not been explored to a great extent in the existing literature.

Using EPN and IUS populations I tested (a) if adapting to one pathogen confers the hosts cross-resistance to novel pathogens; (b) is the pattern of cross-resistance contingent on the identity of the native pathogen; and (c) is cross-resistance sexually dimorphic? I use the phrase

pathogen resistance to imply the ability of the host to survive a challenge with a pathogen, unless mentioned otherwise.

5.2. Materials and methods

For the present study I used two sets of selected populations of *Drosophila melanogaster*, each selected for improved post-infection survival when infected with a different entomopathogenic bacteria. Both sets of selected populations were derived from a common ancestor, the Blue Ridge Baseline (BRB), a wild-type, outbred population, with four evolutionary replicates, BRB1-4. The derivation and maintenance protocol of the BRB populations are described in detail in Singh et al (2015) and Chapter 2. The two selection regimes used were EPN populations, selected for better survivorship post infection with Gram-positive bacteria *Enterococcus faecalis* (*Ef*); and IUS populations, selected for better survivorship post infection with Gram-negative bacteria *Pseudomonas entomophila* (*Pe*).

5.2.1. EPN populations

Three populations were derived from each replicate population of BRB after 150 generations: (a) E_{1-4}, infected with *Enterococcus faecalis*, (b) P_{1-4}, pricking control, and (c) N_{1-4}, normal control were derived from BRB_{1-4}. Therefore, there were totally 12 populations in this selection regime: E1-4, P1-4, and N1-4. Populations with the same numeral shared a more recent common ancestor. For example, E1, P1 and N1 were more closely related to each other than any of them is to E2, P2, N2 etc. Additionally, populations bearing the same numeral were always handled together, during selection and during experimentation. Therefore, populations with the same numeral were treated as statistical blocks. Consequently, we had four blocks (Block 1-4) in the EPN selection regime (E1, P1, N1 forming block 1 and so on). For all populations, eggs were collected at a density of 60-80 eggs per vial (25 mm diameter × 90 mm height) containing 6-8 ml of food (similar to the ancestral population) in 10 such vials and were incubated at standard laboratory conditions as mentioned above. By 10th-11th day 95% of the flies eclose. Further handling depended on the type of selection being imposed.

In the E1-4 populations, on day 12 PEL, when the flies are 2-3 days old as adults, from each of the 10 juvenile development vials, we randomly chose 20 females and 20 males flies, and infected them with the pathogen by septic injury on the thorax with a Minutien pin (0.1 mm, Fine Science Tools, USA) dipped in a bacterial suspension (in $MgSO_4$ saline buffer) at optical density (OD_{600}) of 0.8, under light CO_2 anaesthesia. Therefore, a total of 200 females and 200 males are infected every generation for each E population. After infections the flies were shifted to a plexiglass cage (14 cm length x 16 cm width x 13 cm height) provided with a food plate (60 mm Petri plate in diameter); fresh food plates were provided every alternate day. For flies infected with *Enterococcus faecalis* majority of the mortality is observed between 18 and 48 hours of post-infection with very few flies dying before 18 or after 48 hours. After 96 hours post-infection, fifty percent of the infected flies in each E populations would survive to contribute to the next generation. 96 hours after infection (day 16 PEL) the population cages are provided with fresh oviposition plates (cut-plate) and 18 hours later eggs were collected of these plates to start the next generation.

Flies of the P1-4 populations are maintained identically to the E populations, except that (a) on day 12 PEL, when the flies are 2-3 days old as adults, they are pricked with a Minutien pin (0.1mm, Fine Science Tools, USA) dipped in sterile $MgSO_4$ buffer under light CO_2 anaesthesia, before being placed in cages; (b) From each of the 10 juvenile development vials, we randomly chose 10 females and 10 males such that 100 females and 100 males are sham-infected every generation for each population. There is negligible mortality (1-2%) in these cages between the time of infection and oviposition.

Flies of the N1-4 are maintained identical to P populations except that on day 12 PEL we randomly chose 10 females and 10 males from each of the 10 juvenile development vials under CO_2 anaesthesia such that 100 females and 100 males are subjected to uninfected treatment

every generation for each population. There is negligible mortality in these cages. The EPN selection regime is thus maintained on a 16-day discrete generation cycle.

5.2.2. IUS populations

The IUS populations were similarly derived from the BRB populations after 22 generations of establishment of the base populations, and have been previously described in Gupta et al. (2016). Briefly, three selection regimes were derived from each replicate population of BRB: (a) I1-4, infected with *Pseudomonas entomophila*, (b) S1-4, sham-infected control, and (c) U1-4, uninfected controls were derived from BRB1, and so on. The maintenance of these lines is identical to that of the EPN lines, except that (a) I,U,S populations were started from BRB populations after 22 generations of lab adaptation while E,P,N populations were started from BRB populations after 150 generations of lab adaptation, (b) in the I1-4 populations 150 females and 150 males are infected every generation for each population whereas in E1-4 200 females and males, (c) in I,U,S Gram-negative bacteria *Pseudomonas entomophila* and E,P,N Gram-positive *Enterococcus faecalis* is used, (d) peak mortality window for I is 20 hours to 60 hours and for E is 18 hours to 48 hours.

5.2.3. Bacterial stocks and infection procedure

The bacterial stocks were maintained as 17% glycerol stocks frozen at -80℃. To obtain fresh bacterial cells for infection (either for regular selection protocol or during experimental infections), 10 ml lysogeny broth (Luria-Bertani-Miller, HiMedia) is inoculated with a stab of bacterial glycerol stock and incubated overnight at appropriate temperature with continuous mixing at 150 RPM. A secondary culture is established by inoculating 10 ml lysogeny broth using 100 µl of the overnight culture; this secondary culture is incubated at appropriate temperature till desired turbidity is reached. The bacterial cells from this culture is pelleted down via centrifugation and resuspended in sterile $MgSO_4$ buffer (10 mM) at the required

optical density (OD_{600}). Flies are infected by pricking them in the thorax under light CO_2 anaesthesia with a 0.1 mm Minutein pin (Fine Scientific Tolls, USA) dipped in the bacterial suspension. Sham infection are carried out similarly except with a pin dipped in sterile $MgSO_4$.

Seven pathogens were used in total in this study. Four Gram-positive bacteria: *Enterococcus faecalis* (hereafter *Ef*, grown at 37℃, Lazzaro et al 2006), *Bacillus thuringiensis* (hereafter *Bt*, grown at 30℃), *Bacillus cereus* (hereafter *Bc*, grown at 37℃), and *Staphylococcus succinus* (hereafter *Ss*, grown at 37℃, Singh et al. 2016) were used. Three Gram-negative bacteria: *Pseudomonas entomophila* (hereafter *Pe*, grown at 27℃, strain L48, Vodovar et al 2015, Mulet et al 2012), *Erwinia c. carotovora* (hereafter *Ecc*, grown at 30℃, Martins et al. 2013), and *Providencia rettgeri* (hereafter *Pr*, grown at 37℃, Short and Lazzaro 2010) were used.

All bacteria used in the experiment are described below:

5.2.3.1. *Enterococcus faecalis* (*Ef*)

E. faecalis is a nosocomial, opportunistic human pathogen and are reported to infect flies in the wild (Huycke et al 1991, Lazzaro et al 2006). It is extracellular in nature having Lysine-type peptidoglycan on the outer wall. It is Gram-positive bacteria. Systemic infection with *E. faecalis* can induce phagocytosis (Nehme et al 2011), melanization (Ayres and Schneider 2008), and can strongly induce Toll (Gobert et al 2003, Nehme et al 2011, Hanson et al 2019) but not Imd pathway during systemic infection (Troha and Buchon 2019). *E. faecalis* also produces variety of antioxidative enzymes which are involved in the oxidative stress response (Szemes et al 2010). *Drosophila* hosts that survive infection have persistent infection present in their body (chronic infection; Troha et al 2018, Chambers et al 2019).

E. faecalis (Lazzaro et al 2006) was cultured at 37˚C in lysogeny broth (Luria-Bertani-Miller, HiMedia) for the study. It causes ~50% mortality in Blue Ridge Baseline (BRB, described in details in Chapter 2) population at OD_{600}=1.

5.2.3.2. *Pseudomonas entomophila* (*Pe*, strain L48)

P. entomophila is a motile, rod shaped, obligatory aerobic bacteria found in soil, aquatic or rhizosphere environments. It can infect both larvae and adult flies through oral or systemic infection. It is having DAP-type peptidoglycan and are Gram-negative in character. Infection with *P. entomophila* induces Imd pathway having role of AMPs like diptericin, diptericin B, cecropin A1, attacin A, attacin C, cecropin C, drosomysin and drosopterin (Vodovar et al 2005). These AMPs are required for defence against both oral and systemic infection by *P. entomophila*.

P. entomophila strain L48 is used in the study, was isolated from *Drosophila* itself (Dieppois et al 2015). It was cultured at 27˚C in lysogeny broth (Luria-Bertani-Miller, HiMedia) for the study. It kills ~50% of BRB population at OD_{600}=1.

5.2.3.3. *Erwinia carotovora carotovora* (*Ecc*, strain *Ecc15*)

E. c. carotovora is extracellular, Gram-negative bacteria having DAP-type peptidoglycan, and are rifampicin resistant (Troha and Buchon 2019). It causes soft rot in plants (Barras et al 1994) and insects are its natural transmitter. It can be used to infect *Drosophila* both orally and systemically (Neyen et al 2014, Troha and Buchon 2019). It causes very strong immune response (Buchon et al 2009, Buchon et al 2013, Troha et al 2018) and infection is cleared from the body after ~5.5 days (Troha et al 2018). Infection with *E. c. carotovora* induces Imd pathway having role of AMPs like diptericin, diptericin B, cecropin A1, attacin A, attacin C, cecropin C, drosomysin and drosopterin (Vodovar et al 2005). These AMPs are required for defence against both oral and systemic infection by *E. c. carotovora* (Vodovar et al 2005).

It was cultured at 30˚C in lysogeny broth (Luria-Bertani-Miller, HiMedia) for the study. It kills ~50% of BRB population at OD_{600}=1.

5.2.3.4. *Providencia rettgeri (Pr)*

Providencia rettgeri is known as opportunistic pathogen of hospitalized patients and causes traveller's diarrhoea (Sagar et al 2017; Sharma et al 2017, Yoh et al 2005). It is extracellular and DAP-type Gram-negative pathogen which also infects *Drosophila* in the wild (Corby-Harris et al, 2007; Juneja and Lazzaro 2009, Galac and Lazzaro 2011). It activates robust immune response via Diptericin and Drosomycin antimicrobial peptides (Troha and Buchon 2019). Fly that survives infection are asymptomatic, have low burden of the pathogen throughout life resulting in chronic infection (Duneau et al 2017, Galac & Lazzaro 2011, Troha et al 2018). It is naturally resistant to antibiotic tetracyclin).

It kills ~30% of BRB population at OD_{600}=1. It was cultured at 37˚C in lysogeny broth (Luria-Bertani-Miller, HiMedia) for the study.

5.2.3.5. *Bacillus thuringiensis* (*Bt*, DSM 2046)

Bacillus thuringiensis is rod-shaped, ubiquitous, spore forming and DAP-type Gram-positive pathogen. It activates both Toll and Imd pathway (Neyen et al 2014). It carries insecticidal crystal (Cry) toxic protein (Bravo 2007, Soberon 2007) and are sprayed on crops as bio-pesticides. It commonly occurs in soils.

It kills ~70% of BRB population at OD_{600}=1. It was cultured at 30˚C in lysogeny broth (Luria-Bertani-Miller, HiMedia) for the study.

5.2.3.6. *Bacillus cereus* (*Bc*)

Bacillus cereus is rod-shaped, ubiquitous, aerobic, facultatively anerobic, motile, beta-hemolytic, spore-forming, and DAP-type Gram-positive pathogen commonly found in soil,

food and marine sponges (Paul et al 2021). It activates both Toll and Imd pathway (Neyen et al 2014).

It is cultured at 37˚C in lysogeny broth (Luria-Bertani-Miller, HiMedia) for the study. It is mild pathogen and kills ~30% of BRB population at OD_{600}=1.

5.2.3.7. *Staphylococcus succinus* (*Ss*, PK-1)

Staphylococcus succinus is coccus, non-motile, Lysine-type Gram-positive bacteria. Colonies are raised, opaque, smooth, circular, off-white in colour, and aerobic. Both males and females have similar bacterial load post 24 hours of infection (Singh et al 2016). It induces mortality only with systemic infection and not with oral infection (Singh et al 2016).

It is cultured at 37˚C in lysogeny broth (Luria-Bertani-Miller, HiMedia) for the study. It is mild pathogen and kills ~20% of BRB population at OD_{600}=1.

5.2.4. Pre-experiment standardization

To account for any non-genetic parental effects, experimental eggs were collected from flies which were grown in common garden conditions for one generation (Rose 1984). Eggs were collected from all the populations at a density of 60-80 eggs per vial: 10 such vials were established per population. The eggs completed their development into adults in these vials, and on day 12 PEL, the adults were transferred to plexiglass cages (14x16x13 cm^3) with food plates (Petri plates, 60 mm diameter). Eggs for experimental flies were collected from these population cages.

5.2.5. Rearing of the Experimental Flies

Three days prior to the egg collection, food plates supplemented with live yeast were provided to the standardised flies in the cages. After 2 days, yeast plate was replaced with cut-plate for the next 18 hours for egg laying. From these cut-plates eggs were transferred into glass vials

(25 vials per population to test for response to selection and 50 vials per population to test for cross-resistance), at the density of 60-80 eggs per vial (90 mm x 25 mm), each vial having 6-8ml of standard banana-jaggery food. The vials were incubated under conditions identical to the maintenance of the selection regime. Eggs developed into adults in these vials within 10 days after egg collection, and the adults remained in these vials till day 12 PEL, wherefrom they were used for experiments.

5.2.6. Test of cross-resistance in EPN populations

Test for cross-resistance in the EPN populations were carried out after 40 generations of forward selection. Throughout the selection history of EPN stock, the pathogen infection dose was modulated to induce fifty percent mortality in E populations. This ensured a constant, directional selection process. Therefore, flies of zeroth generation of E were infected with *E. faecalis* at OD_{600}=0.8 and when this experiment was done after generations 40 dose was increased to OD_{600}=1.2.

For experiment, flies from E and P populations were infected with 6 pathogens: *Bacillus thuringiensis* (*Bt*), *Bacillus cereus* (*Bc*), *Staphylococcus succinus* (*Ss*), *Erwinia c. carotovora* (*Ecc*), *Pseudomonas entomophila* (*Pe*), and *Providencia rettgeri* (*Pr*) along with sham-infected controls. Infection dose for all pathogens was OD_{600} = 1.0. On 12^{th} day PEL (flies were 2-3 day old as adults), 50 flies/sex/pathogen/population were infected and transferred to plexiglass cages (14 x 16 x 13 cm^3) with food plates (60 mm diameter). Mortality was noted every 4-6 hours until 96 hours post infection for each cage. Fresh food plates were provided to the cages on every alternate day. Individual blocks of the selection regime were handled on different days.

5.2.7. Test of cross-resistance in IUS populations

Test for cross-resistance in the IUS populations were carried out after 160 generations of forward selection. Similar to the EPN regime, throughout the selection history of IUS, the pathogen infection dose was modulated to induce ~33% mortality in I populations which ensured a constant, directional selection process. Therefore, I flies were infected at generation zero with *P. entomophila* at OD_{600}=1.0 and at generation 160 when this experiment was done infection dose was increased to OD_{600}=2.9.

For experiment, flies from I and S populations were infected with 6 pathogens: *Bacillus thuringiensis* (*Bt*), *Bacillus cereus* (*Bc*), *Staphylococcus succinus* (*Ss*), *Enterococcus faecalis* (*Ef*), *Erwinia c. carotovora* (*Ecc*), and *Providencia rettgeri* (*Pr*) along with sham-infected controls. Infection dose for all pathogens was OD_{600} = 1.0. On 12th day PEL (flies were 2-3 day old as adults), 50 flies/sex/pathogen/population were infected and transferred to plexiglass cages (14 x 16 x 13 cm^3) with food plates (60 mm diameter). Mortality was noted every 4-6 hours until 96 hours post infection for each cage. Fresh food plates were provided to the cages on every alternate day. Individual blocks of the selection regime were handled on different days.

5.2.8. Statistical analysis

All analyses were performed using R statistical software, version 4.1.0 (R Core Team 2021). Mixed-effect cox-proportional hazard models were fitted to the data using the *coxme* function of the "coxme" package (Therneau 2020), and the confidence intervals for these models were calculated using *confint* function of the base R package. Survival curves were plotted using the *ggsurvplot* function of the "survminer" (Kassambara et al 2021) package after modelling the data using *survfit* function from the "survival" (Therneau 2021) package.

For the analysis of the data from the cross-resistance experiments I modelled the data for each pathogen as:

survival ~ selection regime + sex + selection regime : sex + (1|block),

where selection regime, sex and their interaction were considered as fixed factors and block as random factor. This was done separately for the EPN and the IUS selection regimes.

5.3. Results

5.3.1. Test of cross-resistance against novel pathogens in EPN populations, selected for resistance against *Enterococcus faecalis*

Test for cross-resistance in the EPN populations, after 40 generations of forward selection, flies from E (selected) and P (pricking controls) populations were infected with six novel pathogens: *Bacillus thuringiensis* (*Bt*), *Bacillus cereus* (*Bc*), *Staphylococcus succinus* (*Ss*), *Erwinia c. carotovora* (*Ecc*), *Pseudomonas entomophila* (*Pe*), and *Providencia rettgeri* (*Pr*), (along with sham-infected controls) with infection dose for all pathogens maintained at OD_{600} = 1.0.

E populations are significantly better in post-infection survival from P populations when infected with five out of six novel pathogens: *Bt* (hazard ratio 0.7108, 95% CIs 0.5682, 0.8892), *Bc* (hazard ratio 0.7099, 95% CIs 0.5206, 0.9681), *Ss* (hazard ratio 0.5158, 95% CIs 0.3497, 0.7609), *Ecc* (hazard ratio 0.6409, 95% CIs 0.4802, 0.8556), and *Pe* (hazard ratio 0.4604, 95% CIs 0.3501, 0.6054). The E and P populations were not significantly different in terms of survival when infected with *Pr* (hazard ratio 0.7585, 95% CIs 0.5459, 1.0538). Males and females did not differ from one another in post-infection survival when infected with any of the six pathogens (figure 5.1, table 5.1).

5.3.2. Test of cross-resistance against novel pathogens in IUS populations, selected for resistance against *Pseudomonas entomophila*

Test for cross-resistance in the IUS populations, after 160 generations of forward selection, flies from I (selected) and S (sham-infection controls) populations were infected with six novel pathogens: *Bacillus thuringiensis* (*Bt*), *Bacillus cereus* (*Bc*), *Staphylococcus succinus* (*Ss*), *Enterococcus faecalis* (*Ef*), *Erwinia c. carotovora* (*Ecc*), and *Providencia rettgeri* (*Pr*), (along with sham-infected controls) with infection dose for all pathogens maintained at OD_{600} = 1.0.

I populations are significantly better in post-infection survival from S populations when infected with five out of six novel pathogens: *Ef* (hazard ratio 0.7238, 95% CIs 0.5376, 0.9746), *Bc* (hazard ratio 0.6562, 95% CIs 0.4804, 0.8962), *Ss* (hazard ratio 0.5002, 95% CIs 0.3366, 0.7433), *Ecc* (hazard ratio 0.1072, 95% CIs 0.0661, 0.1738) and *Pr* (hazard ratio 0.2473, 95% CIs 0.1522, 0.4020). The I and S populations were not significantly different in terms of survival when infected with *Bt* (hazard ratio 1.1228, 95% CIs 0.8779, 1.4360). Additionally, males survived significantly less than females when infected with two out of six novel pathogens: *Ecc* (hazard ratio 1.8834, 95% CIs 1.4825, 2.3927) and *Pr* (hazard ratio 1.8229, 95% CIs 1.3492, 2.4630), but males and females were equally susceptible to the rest of the four pathogens (figure 5.2, table 5.2).

5.4. Discussion

Hosts selected to be more immune-competent against one particular pathogen can evolve correlated resistance to other pathogens. I tested for evolution of such cross-resistance in two sets of replicate *Drosophila melanogaster* populations, one selected for resistance against a Gram-negative bacterial pathogen *Pseudomonas entomophila* (Gupta et al 2016) for 160 generations, and another selected for resistance against a Gram-positive pathogen *Enterococcus faecalis* (Singh et al 2021, Chapter 2) for 40 generations. Each selected regime and its corresponding paired controls were infected with six novel bacterial pathogens to test if the selected populations were better at surviving a pathogen challenge compared to the controls. My primary observations from these experiments are as follows:

(a) Evolution of cross-resistance: When challenged with six novel pathogens, the E (selected) populations were less susceptible to infections, compared to the P (control) populations, to all the novel bacteria except *Providencia rettgeri*, for which there was no difference in the post-infection survival of E and P populations (figure 5.1, table 5.1). Similarly, the I (selected) populations survived better compared to the S (control) populations when challenged with six novel pathogens, except for *Bacillus thuringiensis*, for which I and S populations exhibited equal mortality (figure 5.2, table 5.2). Therefore, out of twelve total tests for cross-resistance (two selection lines × six novel pathogens) I found evidence for positive cross-resistance in ten comparisons and no effect of selection in the remaining two. I did not observe a single case of negative cross-resistance. Interestingly the population selected against *E. faecalis* were resistant to *P. entomophila* and the populations selected against *P. entomophila* were resistant to *E. faecalis*.

(b) Sexual dimorphism: For the populations selected against *E. faecalis* sex had no effect on post-infection survival when the populations were challenged either by the native

pathogen or by the six novel pathogens (figure 5.1, table 5.1). For populations selected against *P. entomophila* females exhibited reduced mortality compared to males for all Gram-negative pathogens (native and novel), but not in case of the Gram-positive pathogens (all novel) (figure 5.2, table 5.2). Sex-by-population interaction was not observed for any bacteria for either of the two selection regimes.

In case of both of the selected populations I noted the evolution of cross-resistance against a wide range of pathogens. Previous studies, using similar selection designs have reported evolution of cross-resistance against only a few limited pathogens. For example, Martins et al (2013) reported that flies evolved to be resistant against *P. entomophila* were cross-resistant against *P. putida* only, and exhibited increased or no change in susceptibility when infected with *E. faecalis*, and *S. marcescens* and *E. carotovora*, respectively; the selected flies were also more susceptible than controls when infected with viruses. For neither of my selected populations did I observe a scenario where the selected populations were more susceptible to a novel pathogen compared to controls. More importantly, my populations evolved to resist *P. entomophila* were also resistant to *E. faecalis*; another point of difference between my results and that reported by Martins et al (2013).

There can be two possible explanations for the different outcomes of the two studies. One, the genetic architecture of the starting base-line populations is a major determinant of the outcome of any selection experiment. The genotypic co-variances of susceptibilities to different pathogens in my starting populations might have been different from that of Martin et al (2013). Two, correlated responses to selection observed upon in selection experiments can depend upon the number of generations of forward selection (Chippindale et al 1997, Tetonio and Rose 2000). Martins et al (2013) tested for cross-resistance after 27-30 generations of forward selection whereas I tested for cross-resistance after 160 (IUS populations) generations. It is

possible that populations subjected to sustained to long term directional selection exhibit a broader range of cross-resistance.

With respect to the predictive effect of the identity of the native pathogen (the pathogen used for selection) on the pattern of cross-resistance exhibited by the selected populations, I expected that the selected populations would be more resistant to novel pathogens that are most similar to the native pathogen phylogenetically and mechanistically (in terms of both pathogen virulence and host resistance). In contradiction with my expectation, both of my selected populations (E and I populations) evolved cross-resistance against a wide range of bacterial pathogens, barring a few exceptions: E populations did not exhibit cross-resistance against *P. rettgeri*, while the I populations did not exhibit cross-resistance against *B. thuringiensis*. In both cases the novel pathogen was on the opposite Gram-character to that of the native pathogen, and hence phylogenetic dissimilarity, or more accurately, cellular/morphological dissimilarity, may be invoked as an explanation. I did not test the phylogenetic dissimilarity hypothesis to its full extent given I used only bacterial pathogens as novel challenges. Previous studies that have tested evolved flies with different taxa of pathogens/parasites have reported mixed results. Kraaijeveld et al (2012) selected *D. melanogaster* flies for improved defence against parasitoid *Asobara tabida* and found that selection had no effect on defence against fungal pathogen *Beauveria bassiana* and microsporidian pathogen *Tubulinosema kingi*. In the same study, selection for increased defence against *B. bassiana* had no effect on defence against *A. tabida* (Kraaijeveld et al 2012). Martins et al (2013) found that selecting *D. melanogaster* flies for resistance against bacteria *P. entomophila* increases their susceptibility to Drosophila C Virus and Flock House Virus. Biswas et al (2018) selected *Tribolium castaneum* beetles for resistance against fungus *B. bassiana*, and found that defence against bacteria *B. thuringiensis* increased as a consequence of selection, while defence against bacteria *P. entomophila* was compromised.

Alternatively, results from my experiments may indicate shared pathways of pathogen virulence or hosts defence. Insect immunity is a composite trait with multiple layers of complexity. Post-infection survival is a function of the hosts ability to both control the systemic proliferation of pathogens and to deal with the systemic damage incurred in its interaction with the pathogen (Dionne and Schneider 2008, Raeberg et al 2009). Insects have multiple mechanism of resisting pathogen growth, some specific while some general, with these mechanisms not always acting in a mutually exclusive manner (Lemaitre and Hoffmann 2007). For example, defence against systemic infection by *Enterococcus faecalis* requires two cellular defence mechanisms: phagocytosis (Nehme et al 2011) and melanization (Ayres and Schneider 2008), and also the involvement of genes downstream of the toll signalling pathway (Gobert et al 2003, Nehme et al 2011, Hanson et al 2019). Cross-resistance can be driven by the overlap of either the virulence traits employed by the pathogen (Vallet-Gely et al 2008) or the common mechanism of defence utilized by the host. Previous research has suggested that there is some order of specificity of immune defences at the level of the Gram-character of the bacterial pathogens (Lemaitre and Hoffmann 2007). My results from the EPN selection regime indicate that flies evolved to counter *Enterococcus faecalis* infection were better at defending against novel pathogens independent of the pathogen identity. This points at the fact that the selected populations have evolved a mechanism which can serve as a general defence against a wide variety of novel bacterial pathogen. Phagocytosis or melanization are the most likely candidates given these pathways tend to be more generalist defence mechanisms compared to IMD/Toll regulated anti-microbial peptide (AMP) based humoral defences (Lemaitre and Hoffmann 2007).

There is limited information regarding the mechanism of host defence against systemic infection by *Pseudomonas entomophila*. The primary reason for this is that most studies have focused on elucidating the host's response to oral infection by *Pseudomonas entomophila*

(Vodovar et al 2005, Liehl et al 2006), however there is some indication that response to systemic infection and oral infection share certain common mechanisms (Martin et al 2013). Previous research had shown that flies evolved to fight off systemic infection by *Pseudomonas entomophila* are less susceptible to infection by other Gram-negative bacteria compared to controls while having similar or increased susceptibility to Gram-positive bacteria and viruses (Martin et al 2013). This is congruent with the IMD/Toll dichotomy of defence mechanisms. My results, on the other hand, show that flies evolved to survive systemic challenge with *Pseudomonas entomophila* exhibit reduced susceptibility to a variety of pathogens independent of their identity; with the only exception being *Bacillus thuringiensis*, in the case of which both selected and control populations were equally susceptible. Interestingly, the mechanistic basis of virulence following oral infection by *Pseudomonas entomophila* share certain common features with that of *Bacillus thuringiensis*. Crystal proteins produced by *Bacillus thuringiensis* perforate the gut wall of insects (Bravo et al 2007, Soberon et al 2007); a similar role is played by monolysin produced by *Pseudomonas entomophila* (Opota et al 2011). Resistance against *Pseudomonas entomophila* is driven by genes downstream of the Imd signalling pathway, which includes AMPs such as diptericin, diptericin B, cecropin A1, attacin A, attacin C, cecropin C, drosomysin and drosopterin (Vodovar et al 2005). These same AMPs are required for defence against both oral and systemic infection by *Pseudomonas entomophila*; the same set of genes is also required for defence against *Erwinia carotovora carotovora* (Vodovar et al 2005). Given that infection by *Pseudomonas entomophila* induces the expression of a very wide range of AMPs, evolution of cross-resistance against a wide variety of pathogens is not surprising.

The I and S populations were previously tested for cross-resistance against *S. succinus* and *P. rettgeri* after 35 generations of forward selection. At that time, I populations were found to be more resistant to both novel pathogens (Gupta 2015), same as I find in my experiments.

Previous theoretical development has suggested better post-infection survivor of hosts when infected by novel pathogens (pathogens they have not directly evolved to resist) may be because of pathogens evolving to specialize on narrow range of hosts (Antonovics et al 2013). This phenomenon from hosts view-point mimic cross-resistance. My results as an alternative to this, suggests that hosts can become cross-resistant against novel pathogens by evolving to resist only one pathogen.

Sexual dimorphism in immune function has been theoretically predicted and empirically established by previous studies (Zuk and McKean 1996, Rolff 2002, Schmid- Hempel and Ebert 2003, Nunn et al 2009, Vincent and Sharp 2014, Sharp and Vincent 2015). In populations selected for defence against *E. faecalis* no effect of sex on post-infection survival was observed, when the selected and the paired control populations were challenged with the native or the novel pathogens. Effect of sex was seen in case of populations selected against *P. entomophila*, but only for Gram-negative pathogens; no sex × selection regime interaction was observed in case of any pathogen. Therefore, although I observed evolution of sexual dimorphism in one of my selection regimes, sexual dimorphism had no deterministic contribution towards the pattern of cross- resistance observed in my experiments.

5.5. Conclusion

To summarize, in this paper I report that selecting replicate *D. melanogaster* populations against either *Enterococcus faecalis* or *Pseudomonas entomophila* leads to the correlated evolution of cross-resistance of a wide variety of novel pathogens. The identity of the native pathogen did not predict the novel pathogens against which the selected populations exhibited cross-resistance but it did predict the novel pathogens against which the selected populations did not show cross-resistance. Furthermore, the pattern of cross-resistance observed in case of either of the selected populations were not affected by sex of the host; even in cases where sex affected host infection survival the effects were similar for the selected and the control populations. Differences in susceptibility of a host to different pathogens is one of the common justifications for presence of genetic variation in immune function related traits in natural populations. My results suggest that hosts can become cross-resistant to a variety of pathogens by virtue of evolving to resist a single pathogen, and therefore positive correlations between host's resistance against different pathogens may not be very rare in nature. In this study, I tested the evolution of cross-resistance against novel pathogens one at a time. It would be interesting to study how my selected populations fair in comparison to the controls when co-infected with more than one pathogen, because co-infecting pathogens often interact amongst themselves and with the host in unique ways that are not apparent in single infections.

5.6. Figures

Figure 5.1. Survival curves for flies of EPN selection regime (only E and P populations were used in this experiment) tested for cross-resistance against novel pathogens after 40 generations of forward selection: (A) Sham-infection controls, (B) *Bacillus thuringiensis*, (C) *Erwinia c. carotovora*, (D) *Bacillus cereus*, (E) *Pseudomonas entomophila*, (F) *Providencia rettgeri*, and (G) *Staphylococcus succinus*.

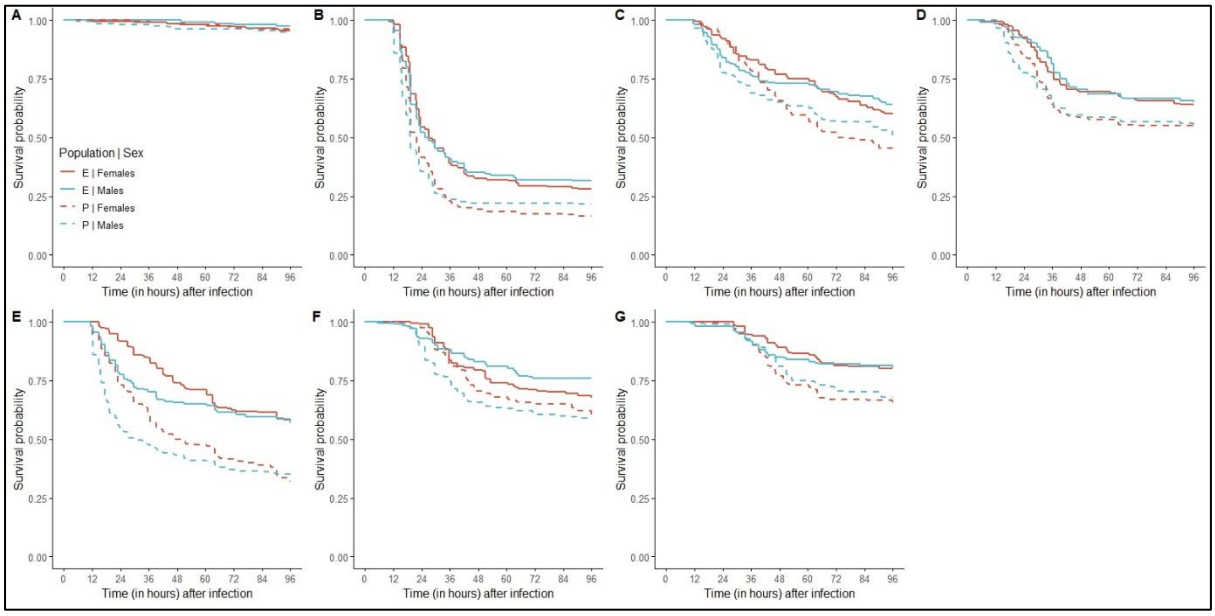
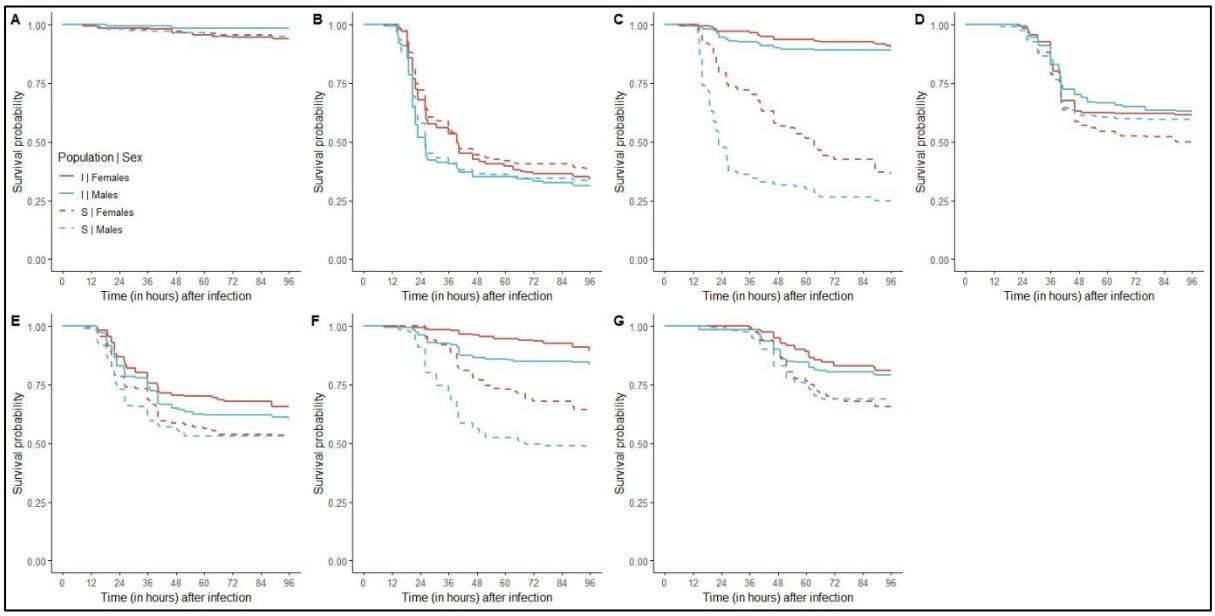


Figure 5.2. Survival curves for flies of IUS selection regime (only I and S populations were used in this experiment) tested for cross-resistance against novel pathogens after 160 generations of forward selection: (A) Sham-infection controls, (B) *Bacillus thuringiensis*, (C) *Erwinia c. carotovora*, (D) *Enterococcus faecalis*, (E) *Bacillus cereus*, (F) *Providencia rettgeri*, and (G) *Staphylococcus succinus*.



5.7. Tables

Table 5.1. Output of mixed-effects Cox proportional hazards analysis of flies of EPN selection regime (generation 40; only E and P populations were used in this experiment) infected with novel pathogens to test for cross-resistance. Hazard ratios are relative to the default level for each factor, which is set at 1. The default level for "Selection" is 'P' and the default level for "Sex" is 'Females'. Hazard ratio greater than 1 implies reduced survival compared to the default level. Significant effects are marked in bold. (Pathogens marked with '#' are of the same Gram-character as the primary pathogen used in the selection regime.)

	Hazards Ratio	Lower CI (95%)	Upper CI (95%)	Z	p-value	Variance (for random factor)
A. Bacteria: *Bacillus thuringiensis* (*Bt*)[#].						
Selection *E*	**0.7108**	**0.5682**	**0.8892**	**-2.99**	**0.0028**	
Sex *Males*	1.0593	0.8518	1.3174	0.52	0.6000	
Selection *E* : Sex *Males*	0.9242	0.6713	1.2725	-0.48	0.6300	
Block						0.1360
B. Bacteria: *Erwinia c. carotovora* (*Ecc*).						
Selection *E*	**0.6409**	**0.4802**	**0.8556**	**-0.32**	**0.0025**	
Sex *Males*	0.9258	0.7048	1.2159	-0.55	0.5800	
Selection *E* : Sex *Males*	1.0079	0.6629	1.5326	0.04	0.9700	
Block						0.0572
C. Bacteria: *Baillus cereus* (*Bc*)[#].						
Selection *E*	**0.7099**	**0.5206**	**0.9681**	**-2.16**	**0.03**	
Sex *Males*	1.0152	0.7573	1.3609	0.10	0.92	
Selection *E* : Sex *Males*	0.9387	0.6042	1.4585	-0.28	0.78	
Block						0.0436
D. Bacteria: *Pseudomonas entomophila* (*Pe*).						
Selection *E*	**0.4604**	**0.3501**	**0.6054**	**-5.55**	**2.8 e-08**	
Sex *Males*	1.1667	0.9176	1.4834	1.26	2.1 e-01	
Selection *E* : Sex *Males*	0.9950	0.6769	1.4625	-0.03	9.8 e-01	
Block						0.1214
E. Bacteria: *Providencia rettgeri* (*Pr*).						
Selection *E*	0.7585	0.5459	1.0538	-1.65	0.099	
Sex *Males*	1.1193	0.8215	1.5252	0.71	0.480	
Selection *E* : Sex *Males*	0.6553	0.4036	1.0642	-1.71	0.088	
Block						0.1046
F. Bacteria: *Staphylococcus succinus* (*Ss*)[#].						
Selection *E*	**0.5158**	**0.3497**	**0.7609**	**-3.34**	**0.0008**	
Sex *Males*	0.8880	0.6327	1.2464	-0.69	0.4900	
Selection *E* : Sex *Males*	1.1356	0.6510	1.9808	0.45	0.6500	
Block						0.0417

Table 5.2. Output of mixed-effects Cox proportional hazards analysis of flies of IUS selection regime (generation 160; only I and S populations were used in this experiment) infected with novel pathogens to test for cross-resistance. Hazard ratios are relative to the default level for each factor, which is set at 1. The default level for "Selection" is 'S' and the default level for "Sex" is 'Females'. Hazard ratio greater than 1 implies reduced survival compared to the default level. Significant effects are marked in bold. (Pathogens marked with '#' are of the same Gram-character as the primary pathogen used in the selection regime.)

	Hazards Ratio	Lower CI (95%)	Upper CI (95%)	Z	p-value	Variance (for random factor)
A. Bacteria: *Bacillus thuringiensis* (*Bt*).						
Selection *I*	1.1228	0.8779	1.4360	0.92	0.360	
Sex *Males*	1.2728	0.9953	1.6275	1.92	0.055	
Selection *I* : Sex *Males*	1.0005	0.7101	1.4098	0.00	1.000	
Block						0.1288
B. Bacteria: *Erwinia c. carotovora* (*Ecc*)$^{\#}$.						
Selection *I*	**0.1072**	**0.0661**	**0.1738**	**-9.06**	**0.0 e+00**	
Sex *Males*	**1.8834**	**1.4825**	**2.3927**	**5.18**	**2.2 e-07**	
Selection *I* : Sex *Males*	0.6341	0.3282	1.2254	-1.36	1.8 e-01	
Block						0.1204
C. Bacteria: *Enterococcus faecalis* (*Ef*).						
Selection *I*	**0.7238**	**0.5376**	**0.9746**	**-2.13**	**0.033**	
Sex *Males*	0.8115	0.6054	1.0879	-1.40	0.160	
Selection *I* : Sex *Males*	1.1394	0.7388	1.7573	0.59	0.550	
Block						0.0378
D. Bacteria: *Bacillus cereus* (*Bc*).						
Selection *I*	**0.6562**	**0.4804**	**0.8962**	**-2.65**	**0.0081**	
Sex *Males*	1.0676	0.8014	1.4222	0.45	0.6500	
Selection *I* : Sex *Males*	1.1253	0.7306	1.7334	0.54	0.5900	
Block						0.0891
E. Bacteria: *Providencia rettgeri* (*Pr*)$^{\#}$.						
Selection *I*	**0.2473**	**0.1522**	**0.4020**	**-5.64**	**1.7 e-08**	
Sex *Males*	**1.8229**	**1.3492**	**2.4630**	**3.91**	**9.2 e-05**	
Selection *I* : Sex *Males*	0.9194	0.4924	1.7167	-0.26	7.9 e-01	
Block						0.0452
F. Bacteria: *Staphylococcus succinus* (*Ss*).						
Selection *I*	**0.5002**	**0.3366**	**0.7433**	**-3.43**	**0.0006**	
Sex *Males*	0.9396	0.6677	1.3221	-0.36	0.7200	
Selection *I* : Sex *Males*	1.2306	0.7057	2.1459	0.73	0.4600	
Block						8.399 e-05

Chapter 6

Co-infection in EPN, and IUS populations

6.1. Introduction

Multi-parasite infections are rampant in nature (Read and Taylor 2001, Betts et al 2016), yet a majority of host pathogen studies focus on a single host and a single pathogen, mostly because of logistic ease. Multi-parasite infections can influence various aspects of host pathogen interaction, starting with infection outcome and going up to evolution of virulence and pathogen transmission (Read and Taylor 2001, Alizon et al 2013, Susi et al 2015, Hoarau et al 2020, Seppala and Jokela 2016). Multi-parasite infections, in their simplest form, involves two pathogens co-infecting a single host individual. In the present study I focus on simultaneous co-infection of *Drosophila melanogaster* flies by pairs of bacterial pathogens. Co-infections may also be sequential, where a host pre-colonized by a pathogen is later infected by another (Karvonen et al 2019), but I do not focus on such co-infections in this study.

A majority of my current understanding of co-infections comes from studies on humans and other vertebrate model organisms (Graham 2008, Balmer and Tanner 2011, Mabbot 2018, Hoarau et al 2020), and their vectors (Gomez-Chamorro et al 2021), with relatively limited amount of knowledge available from insects and other invertebrates (Thomas et al 2003, Thompson et al 2017). Within the body of a single host, a pair of co-infecting pathogens may interact in myriad ways that can determine infection outcome (Karvonen et al 2019, Gomez-Chamorro et al 2021). Co-infecting pathogens can either facilitate each other in colonizing and proliferating within the host, or compete with one another to reduce their fitness. Facilitation may be direct, via cross-feeding set-ups where metabolites necessary for one pathogen is produced by the other (Pederson and Fenton 2007), or indirect, via downregulation of the host immune system (Graham 2008). Similarly, competition may be either direct, via production of toxin by one pathogen that harms or kills the other (Mideo 2009), or indirect, either via scramble competition for host resources and within host niches (Pederson and Fenton 2007, Graham 2008, Mideo 2009), or via eliciting a generic immune response from the host that

targets both the co-infecting pathogens (Pederson and Fenton 2007). But despite growing understanding of within host interactions between co-infecting pathogens, my understanding of how co-infection changes host fitness (post-infection survival) is still limited (Hoarau et al 2020).

Previous experiments have demonstrated that the selected populations of both EPN and IUS selection regimes have evolved a generalized defense against multiple bacterial pathogens, barring a few exceptions (Singh et al 2021, Chapter 5). Here I infected the selected and control populations from both EPN and IUS selection regimes with (a) the respective pathogen used for selection (referred henceforth as the native pathogen), (b) a pair of bacteria including the native pathogen and a novel pathogen, and, (c) a pair of novel pathogens. For each scenario I measured post-infection survival, and addressed the following questions:

(1) Do selected populations survive better than corresponding control populations when subjected to co-infection?
(2) How does the infection outcome change, in case of both selected and control populations, when flies are subjected to co-infection relative to when they are infected with their native pathogen only?
(3) Does host sex determine outcome of co-infections?

My results suggest that, barring a few exceptions, the selected populations (E and I) survived better relative to the controlled populations (P and S, respectively) when subjected to co-infection with a pair of bacteria. In the exceptional cases, flies from the selected and control populations survived equally well. There were no instances observed where the control populations survived better than their corresponding selected population. Presence or absence of the native pathogen was not a determining factor in whether the selected population survived better relative to the corresponding control population. In cases where effect of selection

history was not observed on the outcome of co-infection, it was always in case of male flies. Sex was an important factor in determining the outcome of the co-infection: females either survived better, or equally well, relative to the males, depending upon host selection history and identity of the co-infecting pathogen.

6.2. Materials and methods

This study was conducted on two experimental evolution regimes of *Drosophila melanogaster* which were derived from common ancestral baseline population, Blue Ridge Baseline population (BRB, for details refer Chapter 2). The two selection regimes were: (1) selection regime evolved for better survivorship post infection with Gram-positive bacteria, *Enterococcus faecalis*, hereafter referred to as EPN, and (2) selection regime evolved for better survivorship post infection with Gram-negative bacteria, *Pseudomonas entomophila*, hereafter referred to as IUS.

6.2.1. EPN selection regime

From each replicate of BRB_{1-4} population, three selection regimes were derived after 150 generations: (a) E_{1-4}, infected with *Enterococcus faecalis*, (b) P_{1-4}, pricking control, and (c) N_{1-4}, normal control (for details refer Chapter 2). Altogether, 12 populations were there and E1, P1, and N1 were regarded as block 1; E2, P2, and N2 as block 2 and similarly block 3 and block 4. Different blocks were handled on separate days during selection and experiments due to handling logistics. These blocks were regarded as random statistical blocks for analysis. Populations having same numeral subscript (say, block 1) shared more recent ancestry compared to population having different numeral subscript (block 2 or block 3 or block 4).

Eggs were collected at a density of 60-80 eggs per vial (25 mm diameter × 90 mm height) containing 6-8 ml of standard banana-jaggery-yeast food in 10 such vials per population and were incubated under standard laboratory conditions (25 °C, 12:12 light dark cycle, and 60% relative humidity) until 12th day PEL (post egg laying). By 10^{th}–11^{th} day PEL, 95% of the flies eclose. By the 12^{th} day PEL, flies have matured and mated at least once within the rearing vial itself. Further handling depended on the type of selection being imposed on the population.

On day 12 PEL, for E_{1-4} populations, 20 females and 20 males from each of the 10 rearing vials (each originally housing roughly 70 adult flies) were randomly picked and infected. Infection was done with the help of 0.1 mm Minutien pin (Fine Science Tools, USA) dipped in *E. faecalis* bacterial suspension (prepared in 10 mM $MgSO_4$ saline buffer; refer the Bacterial culture section below for more details) and pricked on the thorax, under light CO_2 anaesthesia. Therefore, a total of 200 females and 200 males were infected every generation (for each E_{1-4} population) and were transferred to a plexiglass cage (14 cm length x 16 cm width x 13cm height) having food plate (60mm Petri plate in diameter). Fresh food plate was provided every alternate day. Peak mortality window would be between 18-48 hours of infection with *E. faecalis*. Fifty percent of the infected flies would survive to contribute to the next generation. Post 96 hours, on day 16 PEL, the cages were provided with oviposition food plate for 18 hours to collect eggs for the next generation. The EPN selection regime is thus on a 16-day discrete generation cycle. Each replicate (block) is handled on a separate day.

For P_{1-4} populations, on 12^{th} day PEL, 10 females and 10 males from each of the 10 rearing vials were randomly picked and sham infected with 0.1 mm Minutien pin dipped in sterile, 10 mM $MgSO_4$, under light CO_2 anaesthesia, before being transferred into the cages. For N_{1-4} populations, on 12^{th} day PEL, 10 females and 10 males from each of the 10 rearing vials were randomly sorted under light CO_2 anaesthesia and placed inside cages. There was negligible mortality in P_{1-4} and N_{1-4} populations. Rest of the handling of P and N populations was identical to the E_{1-4} populations. Therefore, on 16^{th} day PEL, when eggs were collected for the next generation, close to 100 flies of each sex were alive in all population cages. This ensured that the number of adults contributing to the next generation are similar across populations

6.2.2. IUS selection regime

IUS selection regime was derived from BRB_{1-4} populations after 22 generations of laboratory adaptation (see Gupta et al 2016 for further details), similar to the EPN regime. Briefly, from each replicate population of BRB, three populations were derived: (a) I_{1-4}, infected with *Pseudomonas entomophila*, (b) S_{1-4}, sham-infected control, and (c) U_{1-4}, uninfected.

The maintenance of the IUS regime differs from that of the EPN regime in following ways: (a) IUS regime was started after 22 generations while EPN regime after 150 generations of lab adaptation of BRB populations, (b) every generation flies of I populations were infected with a Gram-negative bacteria *Pseudomonas entomophila* while flies of E populations with a Gram-positive bacteria *Enterococcus faecalis*, (c) every generation for each block, 150 flies of each sex are infected for I_{1-4} while 200 flies of each sex are infected for E_{1-4}, (d) peak mortality window, for I populations is 20-60 hours while for E populations it is 18-48 hours, (e) within the first 96 hours post-infection, ~33% of the infected flies die in I_{1-4} while ~50% of the infected flies die in E_{1-4}.

6.2.3. Bacterial culture

Multiple bacterial pathogens were used in this study, for both maintenance of the selection regimes (as described above) and assaying resistance to co-infections. The bacteria used in this study were *Enterococcus faecalis* (Ef, Gram-positive, grown at 37 °C, Lazzaro et al 2006), *Staphylococcus succinus* (Ss, Gram-positive, grown at 37 °C; Singh et al 2016), *Providencia rettgeri* (Pr, Gram-negative, grown at 37 °C, Short and Lazzaro 2010), and *Pseudomonas entomophila* (Pe, strain L48, Gram-negative, grown at 27 °C, Mulet et al 2012, Vodovar et al 2005). The bacterial stocks are maintained as 17% glycerol stocks frozen at -80 °C. An overnight primary culture of bacteria was set by inoculating a stab of bacterial glycerol stock in 10 ml lysogeny broth (Luria-Bertani-Miller, HiMedia) and incubating it at appropriate

temperature with continuous mixing at 150 RPM (revolution per minute). Once this primary culture turned confluent (OD_{600} = 1.0), it was further diluted 100 times to set a secondary culture, and maintained at their respective conditions until it turned confluent again. This secondary culture was centrifuged and bacterial pellets were resuspended in sterile 10 mM $MgSO_4$ buffer to obtain desired OD_{600} for infection. This bacterial suspension was used to infect flies (see below for modifications to this protocol during co-infection assays). Infection was done by dipping needle in the bacterial suspension or sterile 10 mM $MgSO_4$ buffer and pricking flies on the thorax.

For stock maintenance, E populations of EPN regime were infected with *E. faecalis*. Throughout the selection history of EPN, the pathogen infection dose was modulated to induce fifty percent mortality in E populations. This ensured a constant, directional selection. Therefore, flies of zeroth generation of E were infected with *E. faecalis* at OD_{600}=0.8, and by the time this experiment was done at generations 51-53 infection dose had been increased to OD_{600}=1.2. For experiments, flies of E and P populations were either sham-infected, infected with *E. faecalis* (Ef, native bacteria) only, or co-infected with a pair of bacteria. All four bacterial pathogens were used for co-infection, in round-robin style which gave rise to six co-infection treatments: Ef+Ss, Ef+Pe, Ef+Pr, Pe+Pr, Pe+Ss, and Pr+Ss. For bacterial suspension, all four bacterial pellets were individually adjusted to obtain OD_{600} = 1.0 and then mixed in 1:1 ratio to obtain various co-infection mixes. For example, Ef+Pe had 1ml of Ef at OD_{600}=1.0 and 1ml of Pe at OD_{600}=1.0, and like-wise. Native pathogen *E. faecalis* for EPN was also used at OD_{600}=1.

For stock maintenance, I populations of IUS regime were infected with *P. entomophila*. Similar to the EPN regime, throughout the selection history of IUS, the pathogen infection dose was modulated to induce ~33% mortality in I populations which ensured a constant, directional selection process. Therefore, I flies were infected at generation zero with *P. entomophila* at

OD_{600}=1.0, and at generation 170 when this experiment was done infection dose had been increased to OD_{600}=2.9. For experiments, flies of I and S populations were either sham-infected, infected with *P. entomophila* (Pe, native bacteria) only, or co-infected with a pair of bacteria. Similar to EPN, all four bacterial pathogens were used for co-infection, in round-robin style which gave rise to six co-infection treatments: Pe+Ss, Pe+Ef, Pe+Pr, Ef+Pr, Ef+Ss, and Pr+Ss. For bacterial suspension, all four bacterial pellets were individually adjusted to obtain OD_{600} = 1.0 and then mixed in 1:1 ratio to obtain various co-infection mixes. For example, Pe+Ef had 1ml of Pe at OD_{600}=1.0 and 1ml of Ef at OD_{600}=1.0, and like-wise. Native pathogen *P. entomophila* for IUS was also used at OD_{600}=1.

6.2.4. Standardization and rearing of the experimental flies

Flies were reared in common garden conditions for one generation before collecting experimental eggs, to account for any non-genetic parental effects (Rose, 1984). These flies are referred to standardized flies. To generate standardized flies, eggs were collected at a density of approximately 60-80 eggs per vial in 10 such vials for each population (E_{1-4} and P_{1-4}, or I_{1-4} and S_{1-4}). They were allowed to grow in these rearing vials until the 12th day. On the 12th day PEL, flies were transferred into plexiglass cages (14x16x13 cm^3) with food in 60mm Petri-plates. From these standardized flies, experimental eggs were collected.

Three days prior to the egg collection, standardized fly cages were provided with *ad libitum* yeast paste smeared on the top of the fresh banana-jaggery-yeast food plate. After 48 hours, these plates were replaced with fresh oviposition food plate for next 18 hours to allow flies to lay eggs. Eggs were collected from these oviposition food plate at a density of ~70eggs per vial (90 mm x 25 mm) into 40 such vials per population (E_{1-4} and P_{1-4}, or I_{1-4} and S_{1-4}) in 6-8ml of food. These vials were incubated for the next 12 days under standard laboratory conditions. By 10^{th}-11^{th} day PEL, all the flies eclose and by 12^{th} day (day of infection) all the flies would have

matured and mated at least once in the rearing vials itself. Please note that the eclosing adults stayed in the rearing vials itself till the day of infection.

6.2.5. Test for co-infection in the selection regimes

This experiment was done to test the effect of co-infecting bacterial pathogen in two separate experimentally evolved populations having different selection history. Co-infection was done by mixing two bacterial suspensions together and infecting the fly with the help of the Minutien pin dipped in this mixed suspension. It was done on both the selection regime, EPN and IUS. Altogether, four bacteria: [a] two gram-positive: *Enterococcus faecalis* (Ef, Lazzaro et al 2006) and *Staphylococcus succinus* (Ss, Singh et al. 2016); [b] two gram- negative: *Pseudomonas entomophila* (Pe, strain L48, Mulet et al 2012, Vodovar et al 2005), *Providencia rettgeri* (Pr, Short and Lazzaro 2010) were used. These bacteria were mixed in 1:1 ratio in all the possible combinations along with primary pathogen of corresponding selection regime (Ef for EPN and Pe for IUS) and sham controls. So, there were 8 treatments for each selection regime (see section 6.2.3 for more details).

On 12^{th} day PEL, 50 flies of each sex of $E_{1\text{-}4}$ and $P_{1\text{-}4}$ (or $I_{1\text{-}4}$ and $S_{1\text{-}4}$) were infected, either with a pair of bacteria (6 pairs or co-infection treatments), or the native pathogen (native infection treatment), or sham-infected (sham-infection treatment), and thereafter transferred to plexiglass cages (14x16x 13 cm^3) with food in 60 mm diameter Petri plates. Fresh food plate was provided every alternate day. After infections, flies were monitored every 4-6 hours for mortality until 96 hours. Individual blocks were handled on different days. For the EPN regime, experiment was carried out after 51-53 generations of forward selection and for the IUS regime after 170 generations of forward selection. Individual blocks of each selection regime were infected on separate days. Total 200 flies/sex/treatment (total 8 treatments)/

population/selection regime (EPN or IUS) were infected. Therefore, there was total 12,800 flies infected for this experiment.

6.2.6. Statistical analysis

Survival data was analyzed using mixed-effect Cox proportional-hazards models (*coxme* function from 'coxme' package, Therneau 2020), after stratifying the data to the highest possible level. The output of these analyses is tabulated in tables 6.1-6.3 and represented as hazard-ratio plots in figures 6.3-6.5. Post-infection survival curves are shown in figures 6.1-6.2.

To test for the effect of selection history on post-infection survival – survival of E flies relative to P flies, or survival of I flies relative to S flies – data from each sex and co-infection treatment was modeled separately as, *Survival ~Selection history + (1|Block)*, where block identities are included as a random factor.

To test for the effect of infection treatments of post-infection survival – survival of co-infected flies relative to the flies infected with the native pathogen only – data from each population and each sex was modelled separately as, *Survival ~Infection treatment + (1|Block)*, where block identities are included as a random factor.

To test for the effect of sex on post-infection survival – survival of females relative to the males – data from each population and co-infection treatment was modelled separately as, *Survival ~ Selection history + (1|Block)*, where block identities are included as a random factor.

All analyses were carried out in R statistical software (version 4.1.0, R Core Team 2021). Cox proportional-hazards models were fitted using *coxme* package (Therneau 2020), and the confidence intervals for these models were calculated using *confint* function of the base R package. The survival plots were created using the *survival* and *survminer* packages

(Kassambara et al. 2021). Hazard-ratio plots were created using *ggplot2* packages (Wickham 2016).

6.3. Results

6.3.1. Effect of host selection (evolutionary) history on post-infection survival of the host

Flies from E and P populations were either infected with *Enterococcus faecalis* (Ef) only, or co-infected with a pair of bacteria. Four bacterial pathogens were used for co-infection, in round-robin style: *E. faecalis* (Ef), *Staphylococcus succinus* (Ss), *Providencia rettgeri* (Pr), and *Pseudomonas entomophila* (Pe). This gave rise to six co-infection treatments: Ef+Ss, Ef+Pe, Ef+Pr, Pe+Pr, Pe+Ss, and Pr+Ss. E flies had better post-infection survival relative to the P flies, that is a hazard ratio (HR) significantly lower than 1, when infected with either Ef only or co-infected with pairs of bacteria (figures 6.1 and 6.3 A; table 6.1 A), except in two instances. E and P males when infected with Ef+Pr (HR 0.9256, 95% CI: 0.7215-1.1873) and Pe+Pr (HR 1.1127, 95% CI:0.8920-1.3880) had similar post-infection survival.

Flies from I and S populations were either infected with *Pseudomonas entomophila* (Pe) only, or co-infected with a pair of bacteria. Four bacterial pathogens were used for co-infection, in round-robin style: *E. faecalis* (Ef), *S. succinus* (Ss), *P. rettgeri* (Pr), and *P. entomophila* (Pe). This gave rise to six co-infection treatments: Pe+Ss, Pe+Ef, Pe+Pr, Ef+Pr, Ef+Ss, and Pr+Ss. I flies had better post-infection survival relative to the S flies, that is a hazard ratio (HR) significantly lower than 1, when infected with either Pe only or co-infected with pairs of bacteria (figures 6.2 and 6.3 B; table 6.1 B), except in one instance. I and S males when infected with Ef+Ss (HR0.8160, 95% CI:0.5925- 1.1237) had similar post-infection survival.

6.3.2. Effect of co-infection treatment on post-infection survival

In case of both E/P (figure 6.4 A; table 6.2 A) and I/S (figure 6.4 B; table 6.2 B) populations, flies co-infected with pairs of bacteria exhibited different levels of mortality – either increased, decreased, or equal – relative to flies infected with the corresponding native pathogen (*E.*

faecalis and *P. entomophila*, respectively). The change in mortality was determined by the co-infection treatment, host selection history, and host sex (figure 6.4; table 6.2).

6.3.3. Effect of host sex on post-infection survival

Females from E/P (figure 6.5 A; table 6.3 A) and I/S (figure 6.5 B; table 6.3 B) populations, relative to the males of the corresponding populations, survived either better or equally when subjected to co-infection. Survival of females relative to males was determined by both the identity of the co-infecting treatment and the selection history of the host (figure 6.5; table 6.3). For example, P females died equally to P males (HR 1.1404, 95% CI: 0.8956-1.4522) when co-infected with Ef+Pr, but for the same treatment E females died less than E males (HR 0.7172, 95% CI: 0.5492-0.9367). Similarly, S females died equally to S males (HR 1.3037, 95% CI: 0.9688-1.7545) when co-infected with Ef+Ss, but I females died less than I males (HR 0.6249, 95% CI: 0.4317-0.9045) for the same treatment. Again, S females died less than S males when co-infected with either Pe+Pr (HR 0.3573, 95% CI: 0.2788-0.4579) or Ef+Pr (HR 0.7390, 95% CI: 0.5850-0.9336), but I females died equally to I males when infected with both Pe+Pr (HR 0.7744, 95% CI: 0.4696-1.2771) or Ef+Pr (HR 0.9925, 95% CI: 0.7668-1.2848).

6.4. Discussion

In the experiments reported here, using *Drosophila melanogaster* populations experimentally evolved to better survive infection with a particular bacterium, I tested if host selection history, host sex, and co-infection treatments influence post-infection survival when hosts are co-infected with various bacterial pathogens in pairs. The E populations are evolved to better survive infection with a Gram-positive bacteria *Enterococcus faecalis* (P populations serve as evolutionary controls; Singh et al 2021), and the I populations are evolved to better survive infection with a Gram-negative bacteria *Pseudomonas entomophila* (S populations serve as evolutionary controls; Gupta et al 2016). Previous results have shown that both the selected populations (E and I) are better at surviving infection with a wide range of *novel* pathogens, relative to the corresponding control populations (P and S, respectively), despite having been selected to survive infection with only one pathogen (*E. faecalis* and *P. entomophila*, respectively), which I refer to as their *native* pathogen (Singh et al 2021). In the previous study (Chapter 5), the test of cross resistance was done against using novel pathogens, one at a time. In this study, I simultaneously co-infected flies from selected and control populations with two bacteria at a time, with the co-infecting pair either being composed of the respective native pathogen and a novel pathogen, or being composed of two novel pathogens (see MATERIALS AND METHODS for more details).

Based on the results obtained in this study, my main observations are as follows:

(a) Barring a few sex-dependent exceptions, the selected populations (E and I) survive better relative to the corresponding control populations (P and S, respectively) when subjected to co-infections (figure 6.3).

(b) The identity of the native pathogen dictates the degree of resistance to co-infection (difference in susceptibility relative to the control populations) exhibited by the selected

population, and also determines the co-infection treatments against which resistance is exhibited by the selected populations (figure 6.3).

(c) Presence of the native pathogen as one of the co-infecting bacteria does not predict whether resistance to co-infection is exhibited by the selected population. For example, E males survive better relative to P males when infected with Ef+Ss and Ef+Pe, but there is no difference between survival of E and P males when infected with Ef+Pr (figure 6.3).

(d) The selected populations exhibit much greater susceptibility to co-infection, relative to when infected with the corresponding native pathogen only, compared to the control populations (figure 6.4). Susceptibility to co-infections was dependent on the identity of the co-infecting pathogens, but presence or absence of the native pathogen among the co-infecting pair was not predictive in any way (figure 6.4).

(e) Females were either less or equally susceptible to co-infection treatments relative to the males (figure 6.5). The survival differences between females and males were determined by both the identity of the host population and the identity of the co-infecting pathogens (figure 6.5).

In the experiments reported here, my primary aim was to study if co-infection has an effect on host fitness, and if this effect is determined by the evolutionary history of the host. I therefore measured post-infection survival of the host after being simultaneously co-infected by a pair of bacterial pathogens. Since I did not quantify within-host pathogen dynamics, it is difficult for us to interpret the data from survival measurements in terms of within-host processes listed in the introduction. Despite this shortcoming, my results suggest that co-infections in general lead to greater mortality relative to single pathogen infections (figure 6.4), of course with certain exceptions (for example, S males die less when co-infected with Pe+Ef, Ef+Pr, Ef+Ss, and Pr+Ss, relative to S males infected with Pe only).

More importantly, I observed here that the selected populations exhibit greater reduction in survival, compared to the control populations, when co-infected versus when infected with their respective native pathogens only (figure 6.4); whether the native pathogen was a member of the co-infecting pair did not affect this observation. This observation is unlikely to be driven by multiple-fronts costs (*sensu* McKean and Lazzaro 2011), because previous experiments using these populations have proved the selected populations to be cross-resistant to almost all the novel pathogens used for co infection (Singh et al 2021), the only exception being that E and P flies are equally susceptible to infection with Pr. One possible explanation for this observed difference between selected and control populations can be that selected populations die less when infected with the native pathogen compared to the control populations (figures 6.1 and 6.2). This leaves a greater *parameter space* for co-infection to manifest its phenotypic effect in case of the selected populations (figures 6.1and 6.2). Outbred populations are usually considered a reservoir of standing genetic variation (Barett and Schluter 2008). Therefore, alternatively, it is possible that selection, and thereby change in genetic composition of the selected populations, brings out the variation in susceptibility to co-infections that is otherwise hidden/cryptic in the control populations.

The host acts as the arena where co-infecting pathogens interact, and therefore the host identity should be a major determinant in infection outcome, as seen in my results. The selected hosts are not physiologically equivalent to the control hosts because of their evolutionary history, and therefore same pair of co-infecting pathogens can lead to different outcome in the selected and the control host. For example, relative to when infected with only Pe, S males suffer less mortality when infected with Pe+Ef, but I males suffer greater mortality (figure 6.4 B). For the same reason my result show that the survival of the selected populations relative to their corresponding control populations is highly contingent on the identity of the co-infecting pathogens (figure 6.3).

Another important observation from my experiments is that the post-infection survival of a co-infected host is dependent on host sex. Sex differences in immune function is frequently theorized (Zuk and McKean 1996, Sheldon and Verhulst 1996, Rolf 2002), but empirical evidences are frequently equivocal (Kelly et al 2018, Belmonte et al 2020). Here I show that survival difference between females and males is affected by host selection history and the identity of the pathogens used for co-infection (figure 6.5). Sex-specific co-infection outcome has also been reported in *Daphnia magna* (Thompson et al 2017). Sex-specific co-infection outcomes also suggest that host-physiology is a major determinant of how co-infections play out.

6.5. Conclusion

To summarize, in the experiments reported in this chapter, I explored if the host's evolutionary history, the host's sex, and the co-infection treatments influence post-infection survival when hosts are co-infected with various bacterial pathogens in pairs. My results indicate that all the three factors tested interact in a complex way to determine host survival following co-infection, and this complex interaction is only partially predicted based on the identity of the native pathogen, that is the pathogen used for selection. Further experiments are required to relate the observed post-infection survival pattern to the within-host dynamics of the co-infecting pathogens.

6.6. Figures

Figure 6.1. Post-infection survival of E and P flies when subjected to various co-infection treatments: (A) Sham, (B) Ef, (C) Ef+Ss, (D) Ef+Pe, (E) Ef+Pr, (F) Pe+Pr, (G) Pe+Ss, and (H) Pr+Ss

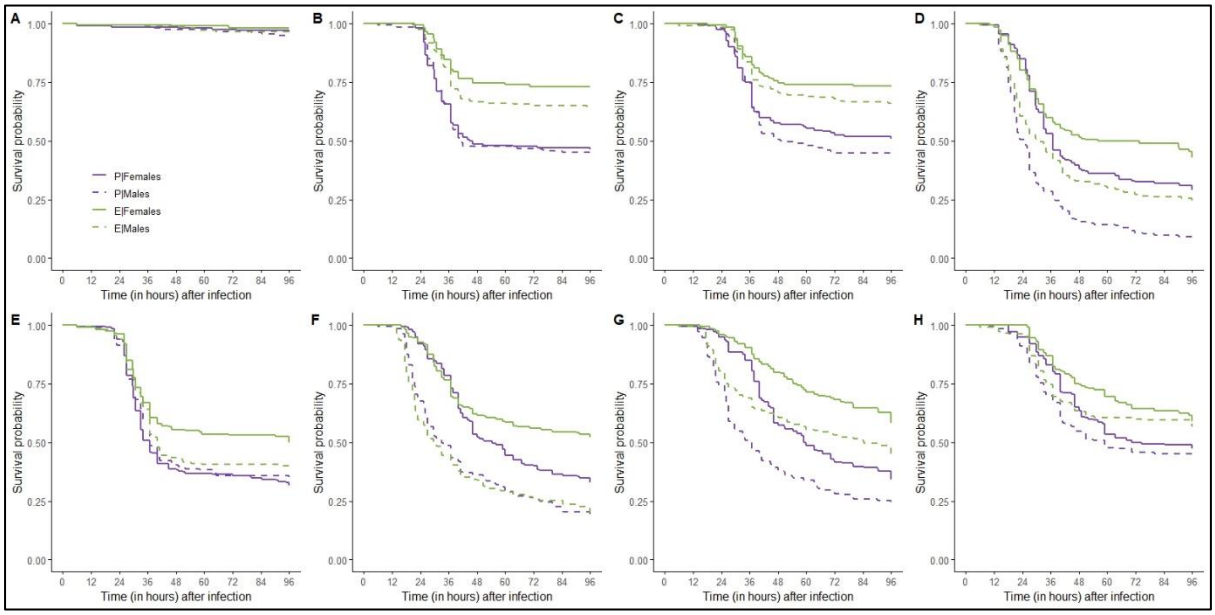


Figure 6.2. Post-infection survival of I and S flies when subjected to various co-infection treatments: (A) Sham, (B) Pe, (C) Pe+Ss, (D) Pe+Ef, (E) Pe+Pr, (F) Ef+Pr, (G) Ef+Ss, and (H) Pr+Ss

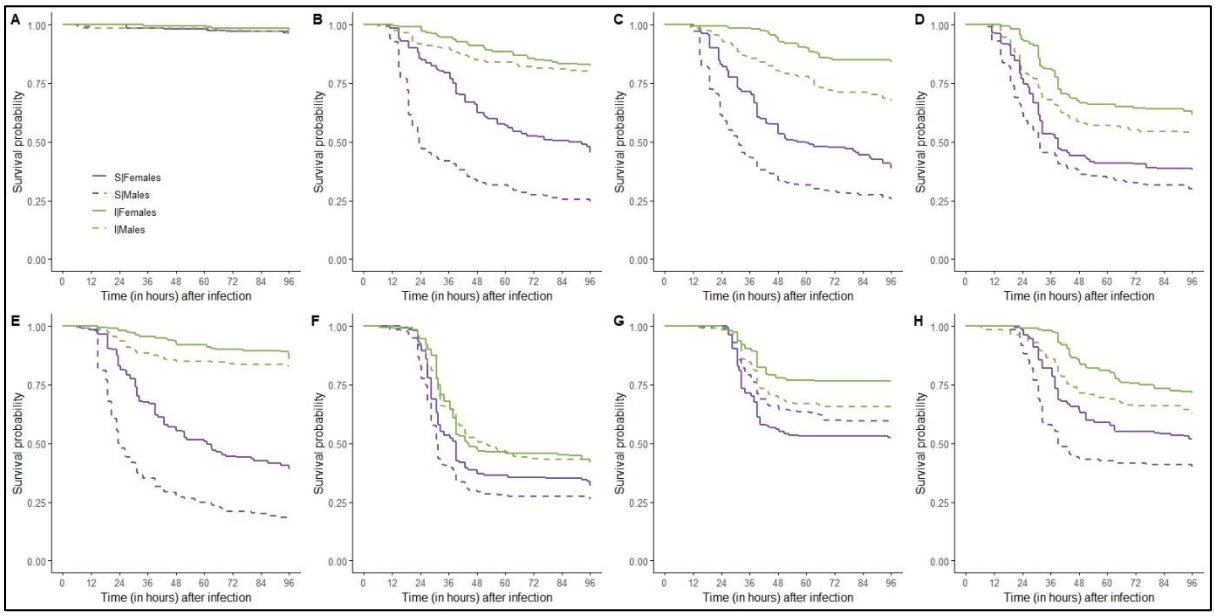


Figure 6.3. Hazard ratio plots for survival of selected populations relative to corresponding control population, when subjected to different co-infection treatments: (A) survival of E relative to P, and (B) survival of I relative to S.

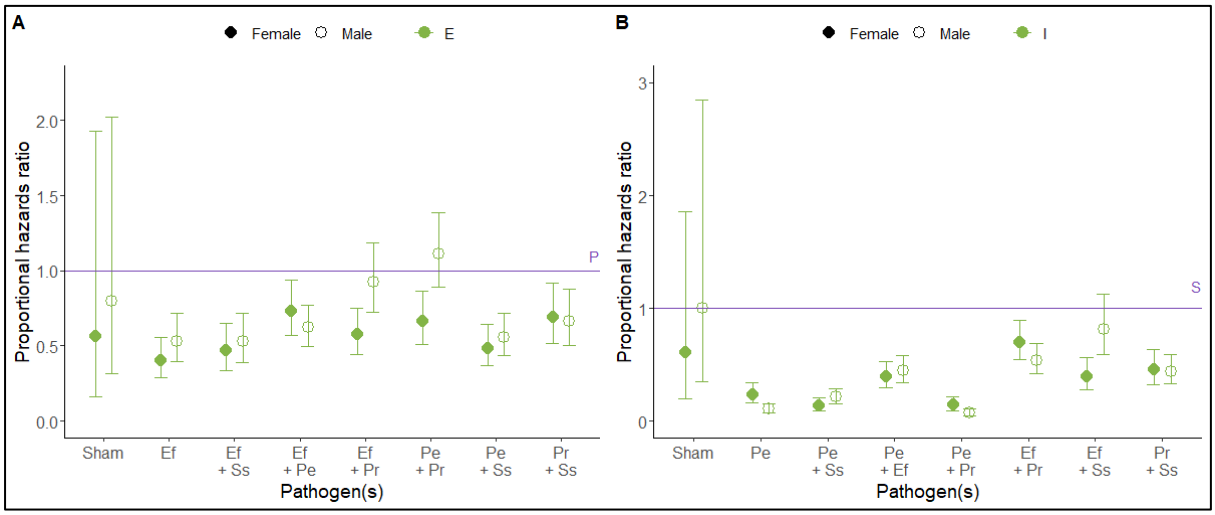


Figure 6.4. Hazard ratio plots for survival of flies infected with various co-infection treatents relative to flies infected with the corresponding native pathogen only: (A) survival of E and P populations, and (B) survival of I and S populations.

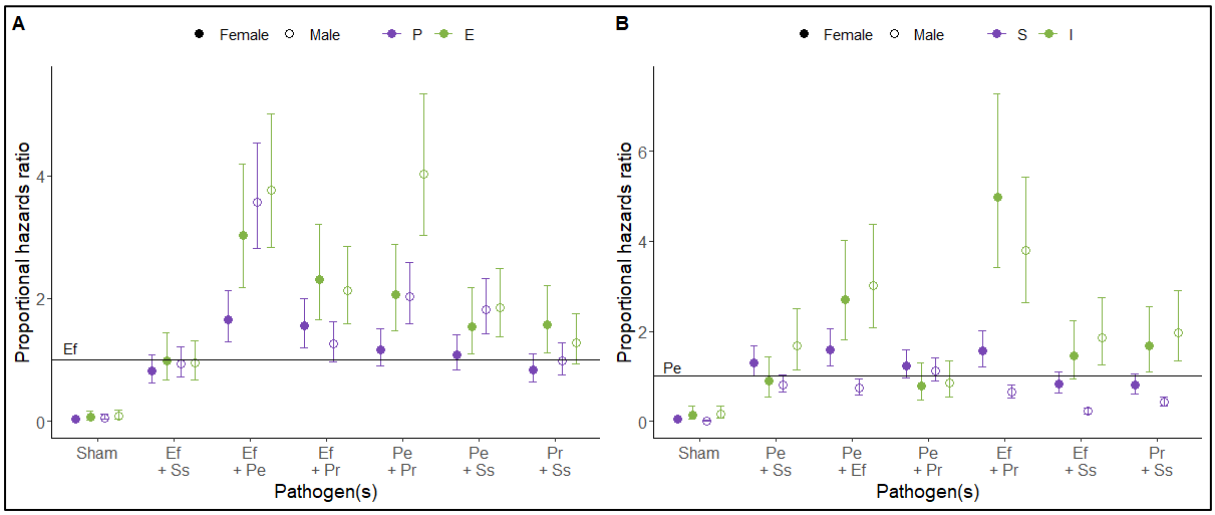
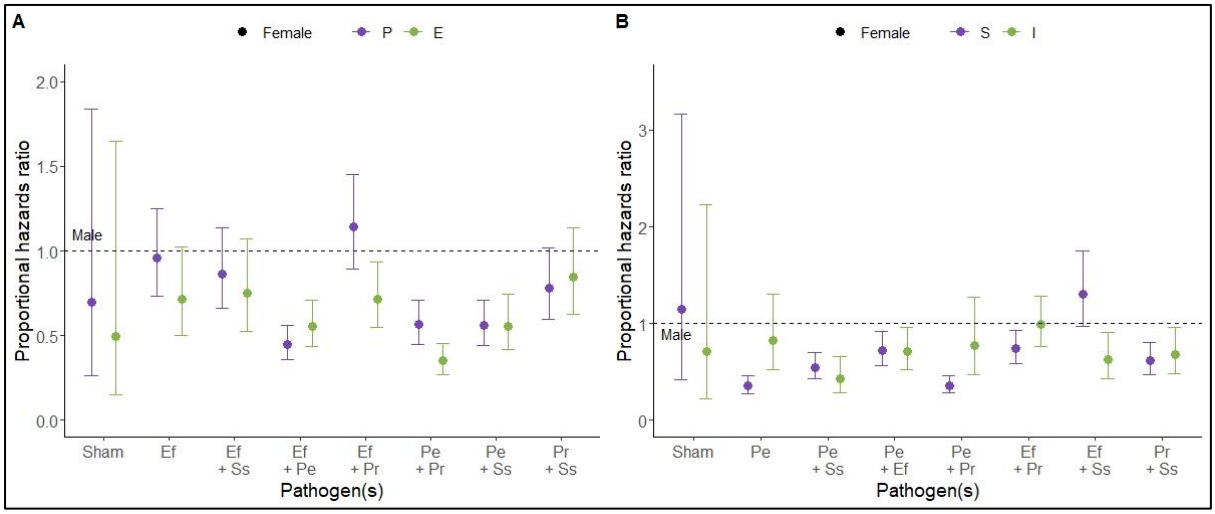


Figure 6.5. Hazard ratio plots for survival of female flies relative to male flies, when subjected to different co-infection treatments: (A) survival of E and P populations, and (B) survival of I and S populations.



6.7. Tables

Table 6.1. Proportional hazard ratio for selected population(s) relative to the control population(s) for each infection treatment, with sexes considered separately. This table accompanies and lists the data used to plot figure 6.3.

Control population	Selected population	Host sex	Treatment (pathogens)	Hazard ratio	Lower CI (95%)	Upper CI (95%)
(A) Proportion hazard ratios for the EPN selection regime. Hazard ratio calculated for the E population flies with P population flies as reference.						
P	E	Female	Sham	0.56386	0.1650577	1.926224
P	E	Female	Ef	0.4031008	0.2904629	0.5594182
P	E	Female	Ef + Ss	0.4667788	0.3347421	0.6508965
P	E	Female	Ef + Pe	0.733061	0.5715608	0.9401948
P	E	Female	Pe + Pr	0.662226	0.509069	0.8614613
P	E	Female	Pe + Ss	0.4862288	0.3685832	0.6414249
P	E	Female	Ef + Pr	0.5787162	0.4462207	0.7505533
P	E	Female	Pr + Ss	0.6875784	0.5144701	0.9189341
P	E	Male	Sham	0.7971753	0.3146222	2.019846
P	E	Male	Ef	0.5307022	0.3937451	0.7152974
P	E	Male	Ef + Ss	0.5290242	0.3914221	0.7149993
P	E	Male	Ef + Pe	0.6216887	0.4992725	0.7741202
P	E	Male	Pe + Pr	1.112728	0.8920263	1.388034
P	E	Male	Pe + Ss	0.5572116	0.4339634	0.7154629
P	E	Male	Ef + Pr	0.9255555	0.721493	1.187334
P	E	Male	Pr + Ss	0.6642773	0.5014815	0.8799213
(B) Proportion hazard ratios for the IUS selection regime. Hazard ratio calculated for the I population flies with S population flies as reference.						
S	I	Female	Sham	0.606541	0.1984024	1.854272
S	I	Female	Pe	0.2347201	0.1599938	0.344348
S	I	Female	Ef + Ss	0.3959784	0.2787347	0.5625382
S	I	Female	Pe + Ef	0.397637	0.2973583	0.531733
S	I	Female	Pe + Pr	0.1438194	0.09500503	0.2177151
S	I	Female	Pe + Ss	0.1397122	0.09374439	0.2082205
S	I	Female	Ef + Pr	0.700012	0.5458749	0.8976724
S	I	Female	Pr + SS	0.4541488	0.326479	0.631744
S	I	Male	Sham	0.9996176	0.3506282	2.849844
S	I	Male	Pe	0.1058542	0.07298254	0.1535313
S	I	Male	Ef + Ss	0.8159608	0.5925098	1.123681
S	I	Male	Pe + Ef	0.4463158	0.3427255	0.5812167
S	I	Male	Pe + Pr	0.0715382	0.04797594	0.1066725
S	I	Male	Pe + Ss	0.2140007	0.1581127	0.2896433
S	I	Male	Ef + Pr	0.5356295	0.4188788	0.6849212
S	I	Male	Pr + SS	0.4419738	0.3302515	0.5914911

Table 6.2. Proportional hazard ratio for various co-infection treatments relative to the native pathogen for each infection population, with sexes considered separately. This table accompanies and lists the data used to plot figure 6.4.

Population	Host sex	Reference treatment	Focal treatments (pathogens)	Hazard ratio	Lower CI (95%)	Upper CI (95%)
(A) Proportion hazard ratios for the EPN selection regime. Hazard ratio calculated for the various co-infection treatment flies with Ef-infected flies as reference.						
E	Female	Ef	Ef + Ss	0.99101976	0.6796092	1.445125
E	Female	Ef	Ef + Pe	3.02539464	2.1867805	4.1856111
E	Female	Ef	Pe + Pr	2.06209789	1.4772185	2.8785504
E	Female	Ef	Pe + Ss	1.54803068	1.0988203	2.1808834
E	Female	Ef	Ef + Pr	2.30643927	1.6560513	3.2122569
E	Female	Ef	Pr + Ss	1.57141708	1.1143953	2.2158668
E	Female	Ef	Sham	0.06252958	0.0226456	0.1726582
E	Male	Ef	Ef + Ss	0.9451735	0.67860189	1.3164611
E	Male	Ef	Ef + Pe	3.7701352	2.8396475	5.0055226
E	Male	Ef	Pe + Pr	4.0218395	3.03666639	5.3266283
E	Male	Ef	Pe + Ss	1.8515981	1.37338854	2.4963187
E	Male	Ef	Ef + Pr	2.1251404	1.5847799	2.8497469
E	Male	Ef	Pr + Ss	1.285292	0.93930061	1.7587294
E	Male	Ef	Sham	0.092022	0.04429945	0.1911547
P	Female	Ef	Ef + Ss	0.823856	0.62634365	1.08365233
P	Female	Ef	Ef + Pe	1.65745	1.28934595	2.13064659
P	Female	Ef	Pe + Pr	1.1675312	0.90530054	1.50571995
P	Female	Ef	Pe + Ss	1.0887277	0.84303453	1.40602538
P	Female	Ef	Ef + Pr	1.5538415	1.20534675	2.00309443
P	Female	Ef	Pr + Ss	0.8441368	0.644769	1.10515084
P	Female	Ef	Sham	0.0416014	0.01936125	0.08938866
P	Male	Ef	Ef + Ss	0.93130551	0.71579958	1.2116939
P	Male	Ef	Ef + Pe	3.57291016	2.8179298	4.530165
P	Male	Ef	Pe + Pr	2.03272255	1.59706939	2.5872144
P	Male	Ef	Pe + Ss	1.82187974	1.42528389	2.3288313
P	Male	Ef	Ef + Pr	1.25767047	0.97546264	1.6215229
P	Male	Ef	Pr + Ss	0.9903529	0.76210853	1.2869543
P	Male	Ef	Sham	0.06079845	0.03182163	0.1161616
(B) Proportion hazard ratios for the IUS selection regime. Hazard ratio calculated for the various co-infection treatment flies with Pe-infected flies as reference.						
I	Female	Pe	Ef + Ss	1.4460456	0.9334636	2.2400959
I	Female	Pe	Pe + Ef	2.6985251	1.80916452	4.0250833
I	Female	Pe	Pe + Pr	0.7864541	0.47845436	1.2927252
I	Female	Pe	Pe + Ss	0.8899941	0.55104401	1.4374341
I	Female	Pe	Ef + Pr	4.9812929	3.40899775	7.2787606
I	Female	Pe	Pr + SS	1.6771925	1.1009022	2.555154

I	Female	Pe	Sham	0.1324786	0.05190213	0.3381475
I	Male	Pe	Ef + Ss	1.8590078	1.26035487	2.742013
I	Male	Pe	Pe + Ef	3.0235281	2.08787672	4.378478
I	Male	Pe	Pe + Pr	0.8434463	0.53393207	1.332382
I	Male	Pe	Pe + Ss	1.6816644	1.13413842	2.493519
I	Male	Pe	Ef + Pr	3.7904826	2.6446049	5.432856
I	Male	Pe	Pr + SS	1.9725145	1.34252486	2.898131
I	Male	Pe	Sham	0.1561169	0.06993479	0.348503
S	Female	Pe	Ef + Ss	0.83050056	0.6302563	1.0943662
S	Female	Pe	Pe + Ef	1.58695935	1.22586833	2.0544131
S	Female	Pe	Pe + Pr	1.23444052	0.9533732	1.5983703
S	Female	Pe	Pe + Ss	1.3032351	1.00667076	1.687167
S	Female	Pe	Ef + Pr	1.56455984	1.2150379	2.0146265
S	Female	Pe	Pr + SS	0.80263888	0.6095691	1.0568599
S	Female	Pe	Sham	0.05065467	0.02469956	0.1038843
S	Male	Pe	Ef + Ss	0.22432885	0.170446398	0.29524491
S	Male	Pe	Pe + Ef	0.74461135	0.591905213	0.93671428
S	Male	Pe	Pe + Pr	1.12505314	0.902667811	1.40222633
S	Male	Pe	Pe + Ss	0.81850958	0.65292358	1.02608936
S	Male	Pe	Ef + Pr	0.64432398	0.511921906	0.81097017
S	Male	Pe	Pr + SS	0.42608324	0.334316017	0.54303987
S	Male	Pe	Sham	0.01623178	0.007592197	0.03470281

Table 6.3. Proportional hazard ratio for females relative to the males for each infection population, with populations considered separately. This table accompanies and lists the data used to plot figure 6.5.

Selection regime	Population	Host sex	Treatment (pathogens)	Hazard ratio	Lower CI (95%)	Upper CI (95%)
(A) Proportion hazard ratios for the EPN selection regime. Hazard ratio calculated for the females with male as reference.						
EPN	E	Female	Sham	0.495968	0.149348	1.647057
EPN	E	Female	Ef	0.717164	0.503382	1.021738
EPN	E	Female	Ef + Ss	0.752295	0.526886	1.074137
EPN	E	Female	Ef + Pe	0.555432	0.434495	0.710031
EPN	E	Female	Ef + Pr	0.71722	0.549181	0.936677
EPN	E	Female	Pe + Pr	0.349834	0.270207	0.452928
EPN	E	Female	Pe + Ss	0.55566	0.416157	0.741929
EPN	E	Female	Pr + Ss	0.842899	0.623312	1.139845
EPN	P	Female	Sham	0.698797	0.265991	1.835839
EPN	P	Female	Ef	0.958699	0.735049	1.250399
EPN	P	Female	Ef + Ss	0.864311	0.658607	1.134263
EPN	P	Female	Ef + Pe	0.448776	0.359533	0.56017
EPN	P	Female	Ef + Pr	1.14041	0.895583	1.452166
EPN	P	Female	Pe + Pr	0.563365	0.448161	0.708183
EPN	P	Female	Pe + Ss	0.559076	0.44155	0.707884
EPN	P	Female	Pr + Ss	0.781168	0.598823	1.019039
(B) Proportion hazard ratios for the IUS selection regime. Hazard ratio calculated for females as males as reference.						
IUS	I	Female	Sham	0.707654	0.224598	2.229647
IUS	I	Female	Pe	0.827969	0.525954	1.303406
IUS	I	Female	Pe + Ss	0.430879	0.282138	0.658034
IUS	I	Female	Pe + Ef	0.710142	0.525138	0.960323
IUS	I	Female	Pe + Pr	0.774393	0.469561	1.277116
IUS	I	Female	Ef + Pr	0.992549	0.766779	1.284794
IUS	I	Female	Ef + Ss	0.624888	0.431729	0.904469
IUS	I	Female	Pr + SS	0.676897	0.479142	0.956271
IUS	S	Female	Sham	1.147006	0.415905	3.16328
IUS	S	Female	Pe	0.359283	0.277712	0.464815
IUS	S	Female	Pe + Ss	0.54693	0.430123	0.695459
IUS	S	Female	Pe + Ef	0.721537	0.566609	0.918827
IUS	S	Female	Pe + Pr	0.357284	0.278769	0.457913
IUS	S	Female	Ef + Pr	0.739028	0.584985	0.933636
IUS	S	Female	Ef + Ss	1.303747	0.968795	1.754505
IUS	S	Female	Pr + SS	0.615443	0.470293	0.805392

Chapter 7

Discussion and Conclusion

Discussion

Pathogen/parasitic infection is one of the biggest sources of mortality in the natural environment, making the study of host-pathogen interactions an important question in both ecology and evolutionary biology. *Drosophila melanogaster* has proved to be a useful model system for investigating host-pathogen interactions, both at a physiological level (Dionne and Schneider 2008) and at the evolutionary level (Kraaijeveld and Godfray 1997, Fellowes et al 1998, Ye et al 2009, Faria et al 2015, Gupta et al 2016, Ahlawat et al 2022).

In this thesis, I did a comparative study using two sets of experimentally evolved *Drosophila melanogaster* populations. I evolved one set of *D. melanogaster* populations to better survive infection with a Gram-positive bacteria *Enterococcus faecalis*, here after referred to as EPN populations (Singh et al 2021, Chapter 2). E (selected) populations rapidly evolved better post-infection survival (by 35 generations of forward selection) when infected with *E. faecalis*, compared to P and N (control) populations. (Singh et al 2021, Chapter 2). The second set of *D. melanogaster* populations were evolved to better survive an infection with a Gram-negative bacteria *Pseudomonas entomophila*, here after referred to as IUS populations (Gupta et al 2016). I used these two sets of experimentally evolved populations to investigate (i) the costs associated with better resistance to pathogenic bacteria at physiological and evolutionary level, (ii) the effect of poor larval diet on immune function and various life-history traits, (iii) the specificity of the evolved immune response, and (iv) the immune function of evolved flies under co-infection challenges.

Cost of immunity in EPN populations

E (selected) populations evolved better resistance to *E. faecalis* infection within 35 generations of forward selection, compared to the P (sham-infected control) and N (uninfected control) populations. I assayed various life-history traits of EPN populations to test for cost of immune

function. There are primarily two types of costs: cost of immune maintenance and cost of immune deployment (McKean and Lazzaro 2011). Cost of immune maintenance, or evolutionary cost, is the cost that is paid by the host while maintaining better level of basal immune function (McKean and Lazzaro 2011). Cost of immune deployment is the cost paid while mounting an immune response in the presence of pathogenic challenge (McKean and Lazzaro 2011). It is also known as physiological cost.

Fecundity, hatchability, development time, egg-to-adult viability, body weight

I found that fecundity and hatchability of the EPN flies are not significantly different for infected, sham-infected, and un-infected treatment (Chapter 3). Therefore, there is no role of selection history or treatments in case of fecundity or hatchability. Females of EPN develop faster than males, while selected and control populations are not significantly different. *D. melanogaster* females in general develop earlier than males so this is a common observation. There is no significant difference in the overall egg to adult survival of the EPN flies. Females of EPN populations are heavier than males; as males are usually smaller in size compared to females in *D. melanogaster*, this is an expected observation.

Longevity, starvation, and desiccation

Life-time survival and starvation resistance (abiotic stress) of the EPN populations were monitored under infected, sham-infected and unhandled conditions. There was no role of selection history; infected flies died earlier compared to sham-infected or uninfected treatment (Chapter 3). This may be due to three reasons. One, surviving infection during early age in life may lead to exhaustion of resources for late life or permanent damage to the somatic tissue of the host. Second, flies that have survived acute infection harbours small dose of bacteria in their system and it take continuous resource investment to ensure that bacterial load do not re-increase (Chambers et al 2019). Third, immunopathology (damage caused to the one's own

organs) caused by own immune defence response (Sadd and Siva-Jothy 2006). Males' lifespan and starvation resistance were less compared to females, probably owing to smaller body size and less fat reserve in the body.

In case of desiccation (abiotic stress), infected and sham-infected flies both died earlier compared to uninfected flies (Chapter 3). Infection and sham-infection both were done by pricking needle. Wounding during the infection procedure can lead to loss of haemolymph and therefore make flies more susceptible to desiccation. Also, fly mortality peaks after 18 hours of infection when infected with *E. faecalis*, while most of the flies died before 18 hours due to desiccation. Hence, lethal effect of bacteria does not have an opportunity to affect fly mortality during desiccation assay.

Larval competitive assay

EPN flies were assayed for biotic stress where they were competed with common competitor for resources acquisition under larval conditions. This assay was done under two competitive environments (Chapter 3). First where focal (E or P or N) flies were present in equal ratio with the common competitor and, second where focal flies were present in one-third numbers to the common competitor. When focal and competitor flies are present in equal density, E flies had similar competitive index to N flies, while E an P both had lower competitive index with P flies. However, when reared under one-third density to the competitor, competitive index of the E flies was higher than P or N flies, but P and N flies were equally competitive. Kraaijeveld and Godfray (1997), and Fellowes et al (1998) competed *Drosophila melanogaster* populations for better resistant to parasitoid under reduced resource conditions and found that selected flies were worse competitor. My result is different from them which can be because of two reasons. One, because of the type of the pathogen used (bacteria vs. parasitoid). Second, life cycle stage at which selection act (adult vs. larva).

Relaxation of selection

For the EPN populations, relaxation of selection in the ERS populations (derived from the E populations; Chapter 3) did not lead to reduction in post-infection survival upon infection with *E. faecalis*. Under relaxed selection pressure costly evolved traits can revert back to their ancestral state. In my experiments, I did not observe any reversal of post-infection mortality patterns of the ERS to that of the ancestral populations, represented here by the N populations. This suggests that whatever defence mechanism the E populations have evolved to help survive *E. faecalis* infection is not costly.

Costly traits can often manifest as apparently free of any cost in a benign and rich environment. This can possibly be a reason why there was no reversal of evolved increased post-infection survival in the ERS populations. I address this issue in Chapter 4 by measuring the post-infection survival of the selected and control populations is resource-poor environment. Two other possibilities exist as to why no reversal of evolved increased post-infection survival was not observed in my experiments. One, due to strong directional selection, E populations might be lacking in genetic variation, and therefore are not able to respond to relaxation of selection. Two, since I only relaxed selection for 15 generations before testing for its effects, sufficient evolutionary time might not have passed to allow for the relaxation of selection to take effect on trait value. Based on the data at hand, I cannot speculate if these two possibilities are relevant to my populations or not.

Therefore, overall, we found no cost of immune maintenance. The cost of immune deployment was present in a trait specific manner. Infected flies were different from uninfected flies in case of longevity and starvation but not for fecundity and hatchability. These results were consistent with study of Faria et al (2015), Gupta et al (2016), and Ahlawat et al (2022). Therefore, EPN

populations were not paying any cost of maintenance, however cost of immune deployment was present for certain trait.

Effect of limiting nutrition on post-infection survival of selected populations

The resource allocation towards immune defence can be plastic and depends on many factors like exposure to pathogens, temperature, availability of resources, etc. Resource allocation priority changes to suit optimal fitness of organism depending upon the availability of resources to invest into different traits (Ng'oma et al 2017). Resource restriction can lead to calibration of the immune networks to the new stable state such that immune fitness can be maximized (Adamo et al 2016, Adamo et al 2021), and can therefore have pathogen specific outcomes. Also, pathogen depends on the host for its own resource requirement and host's reduced access to resources can significantly affect infection outcome (Pike et al 2019). Immune defence can also be developmentally predetermined and juvenile access to food resource actively decides how much investment is made towards somatic tissue and how much for reproductive tissue. It has been reported that trade-offs often manifest under stressful conditions (Reznick 1985, Stearns 1989, Marden et al. 2003). Under stress, organisms can decide to invest more in somatic maintenance including immune defence to increase their lifespan rather than investing into reproduction. Kraaijeveld and Godfray (1998), and Fellowes et al (1999) observed decrease larval competitive index in *Drosophila melanogaster* flies evolved to better resist parasitoid attack, under reduced resource condition. Laboratory organisms have access to excess food and this might be one of the reasons for EPN, and IUS populations for showing no cost of immune maintenance. Therefore, I explored next how EPN, and IUS populations adapted to be more immune to their native pathogens, *E. faecalis* and *P. entomophila* respectively, respond to reduced resources in terms of poor diet when assay for post-infection survival and life-history traits. Poor diet here means fifty percent reduced nutritional content of the standard diet.

I found that improvement of immune function against their native pathogens, in response to experimental evolution, is not lost due to resource scarcity (Chapter 4). EPN populations survival was not affected by poor diet when compared with standard diet while survival of IUS populations decreased under poor diet. EPN did not show decreased survival under resource restricted condition when infected with native pathogen *E. faecalis* may be because defence against *E. faecalis* is less resource intensive. Chambers et al (2019) found that *Drosophila* flies with chronic *E. faecalis* infection do not exhibit any change in starvation resistance. Das et al (2022) when housed adult *Drosophila* flies on high density and looked for immune defence against *E. faecalis*, no change in survival was found, while immune defence against other bacteria was reduced. Effect of poor diet was also affected by the selection history and sex of the host populations. Males of IUS populations has increased susceptibility to *P. entomophila* infection under poor diet. Females of U and S populations also show increased susceptibility to infection under poor diet. But I populations under poor and standard diet were equally susceptible to *P. entomophila* infection. Males of EPN populations and females of P and N populations were equally resistant to *E. faecalis* infection under poor diet when compared to standard diet but resistance of E females increases under poor diet. However, both selected (E and I) populations exhibit increased survival when infected with native pathogen on poor diet when compared to their respective control populations. Resource priorities change according to environmental factors and amount of resource available (Ng'oma et al. 2017). It is quite possible that adaption to regular pathogenic challenge with the native bacteria has prioritized investment toward immune function particularly under reduces resource conditions. Hence selected populations are better at surviving pathogenic challenge with native pathogen under poor diet. E females survived better when on poor diet compared to standard diet because of these reasons: increased investment towards immune function, reduction in insulin signalling (poor diet reduces insulin signalling in flies which in turn makes flies more resistant to infection

with *E. faecalis*, Rehman and Vargheshe 2021, and Libert et al 2008), reduced investment towards reproduction.

I also assayed fecundity of EPN and IUS flies post sham-infection and infection with native pathogens under poor diet. EPN and IUS populations were less fecund under poor diet when compared to standard diet. Poor larval diet reduces adult fecundity in *Drosophila melanogaster* females either due to reduced available resources or reduced ovariole numbers and body size (Hodin and Riddiford 2000, Tu and Tatar 2003, Deas et al 2019, Klepsatel et al 2020). However, infection status or selection history had no effect on fecundity of EPN and IUS. Fecundity of host is affected by the identity of the infecting pathogen (Kutzer and Armitage 2016, Kutzer et al 2018, Hudson et al 2020). Also, time of fecundity measurement relative to the time of infection is an important factor that affects fecundity. I measured fecundity after acute infection phase has passed and this might also lead to the observed, no difference, in fecundity between sham-infected and infected flies or selection history of the flies.

Next, I assayed larval traits like egg-to-adult development time and viability. Both of these traits were negatively affected by poor diet. In IUS, rearing on poor diet had increased development time and reduction in dry body weight but egg-to-adult viability was unaffected. However, in EPN flies poor larval diet increased development time and reduced egg-to-adult viability. Multiple studies have reported similar effect of poor larval diet on larval traits (Kolss et al 2009, Deas et al 2009).

Overall, I found that poor diet does not affect improved survivorship of the experimentally selected flies (E and I) when immune challenged with the native pathogen. Post infection survival of the males was not affected by poor diet in EPN populations but negatively affected IUS populations. Post-infection survival of females was population specific where poor diet leads to decreased mortality in E females, but not in P and N females. However, poor nutrition

leads to increased mortality in U and S females but not in I females. Also, life history traits under poor larval diet depended on the traits being studied. In general, poor diet led to increased development time, lesser adult body weight in females, and reduced fecundity.

Cross-resistance

When a host is more resistant to one pathogen it can show either increased, decreased or equal resistance (no change in resistance) to second novel pathogen. Resistance against one pathogen can be costly and accordingly host response to second novel pathogen depends on the overall cost required to defend itself against second pathogenic challenge. This type of cost is known as multiple front cost (McKean and Lazzaro 2011) and are supposed to be highly pathogen specific. Hence, I next decided to check multiple front cost in EPN and IUS populations: two populations evolved against two completely different pathogen (*E. faecalis* and *P. entomophila*) to six novel pathogens. Pathogens used to infect E/P flies were *Erwinia c. carotovora*, *P. entomophila*, *Providencia rettgeri*, *Bacillus thuringensis*, *Bacillus cereus*, and *Staphylococcus succinus*. The I/S flies were infected with *E. c. carotovora*, *P. rettgeri*, *B. thuringensis*, *E. faecalis*, *B. cereus*, and *S. succinus*. Susceptibility of the both evolved host populations (E and I) against novel pathogens were checked via post infection survival. I addressed following questions using EPN (generation 40) and IUS (generation 160) populations: (i) the response of evolved host in terms of survival (cross-resistant) when challenged with novel pathogens; (ii) whether cross-resistant response is dependent on the selection history of the host populations; and (iii) whether cross-resistance is sexually dimorphic? Populations from each selection regime were infected with six novel pathogens.

The E (selected) populations survived better post-infection than P (control) populations for all novel bacteria except *Providencia rettgeri*, for which there was no difference in survival between selected and control populations (Chapter 5). Similarly, I (selected) populations

showed better post-infection resistance than S (control) populations for all novel pathogen except *Bacillus thuringensis,* for which I and S populations exhibited equal mortality. Overall, both sets of evolved populations showed positive cross-resistance to ten out of twelve total tests (two selection lines × six novel pathogens) of cross resistance comparisons with novel pathogens and no effects in remaining two comparisons. Not even a single case of multiple-fronts costs was observed. Interestingly, E populations were cross-resistant to *Pseudomonas entomophila* and I populations were cross resistant to *Enterococcus faecalis*. Martins et al. (2013) found that populations evolved for better resistance to *P. entomophila* was more susceptible to *E. faecalis*. The difference in my result and Martins et al. (2013) study could be because of difference in the genetic composition of the starting baseline populations, or numbers of generations of forward selection after which cross-resistance was checked. My study checked after 160 generations of forward selection as opposed to 27-30 generations of forward selection by Martins et al (2013).

E and I populations showed cross-resistant to wide variety of pathogen suggesting evolution of generalized immune response. *Drosophila* host use phagocytosis (Nehme et al 2011), melanization (Ayres and Schneider 2008), and genes downstream of toll pathway (Gobert et al. 2003, Nehme et al 2011, Hanson et al 2019) against *E. faecalis*. Phagocytosis and melanization are part of generalized immune defense and hence E populations evolved to better resist *E. faecalis* are better against other novel pathogens. *Drosophila* hosts use IMD pathway to combat *P. entomophila* infection when infected orally. Same set of AMPs are used for defence against *P. entomophila* when infected orally or systemically (Vodovar et al. 2005). IMD pathway act by producing AMPs in response to infection which includes AMPs such as diptericin, diptericin B, cecropin A1, attacin A, attacin C, cecropin C, drosomysin and drosopterin (Vodovar et al. 2005). This wide range of AMPs produced while defending *P.*

entomophila might be the reason for better survival of I flies against wide range of novel pathogens tested.

Co-infection

Hosts are often infected by multiple pathogens. Yet, majority of studies have studied single host and single pathogen system because of logistics ease. Multi-pathogen infection is influenced not only by inter-pathogen interactions within the host but also by environment and genetic architecture of the host body. Multi-pathogen infection here simply means two pathogen co-infecting host simultaneously. Previous studies from cross-resistance (Chapter 5) have shown that both selected (E and I) are better in post-infection survival with wide range of novel pathogens. Also, native pathogen identity predicts the nature of the pathogen against which selected populations are same as control populations in post-infection survival. In cross-resistance evolved hosts were infected with novel pathogens one at a time. Next, I co-infected evolved hosts with pair of pathogens. E/P and I/S populations were co-infected with native pathogen, pair of bacteria having native and novel pathogens, and pair of bacterial having both novel pathogens to address questions like: (i) effect of co-infection on selected populations compared to control populations, (ii) outcome of co-infection compared to infection with native pathogens in both selected and control populations, and (iii) effect of sex on co-infection outcomes.

Selected populations (E/I) are better in surviving against most of the co-infections when compared to control populations (P/S, Chapter 6). E/P populations were less or equally resistant against coinfections when compared to native pathogen (*E. faecalis*). Barring few exceptions (S males are less susceptible to Pe+Ef, Ef+Pr, Ef+Ss, and Pr+Ss when compared to infection with Pe alone), I/S populations were also less or equally resistant against co-infection relative to infection with native pathogen *P. entomophila*. Presence of native pathogen in the co-

infection mix does not affect the resistance of the selected populations. For example, E males were more resistant to Ef+Ss and Ef+Pe compared to P males but equally susceptible when infected with Ef+Pr. Females in general were more or equally resistant to the co-infection when compared to males of the E/P and I/S populations.

Overall, I see that identity of the native pathogen dictates the extent of the susceptibility to co-infection by the selected populations when compared to control populations. It also determines the co-infection mix against which resistant is exhibited by the selected populations. I observed that presence of the native pathogen in the mix is having no major role in determining the overall susceptibility of the populations and hence it is unlikely to be driven by multiple front cost (*sensu* McKean and Lazzaro 2011). My previous result (Chapter 5, also Singh et al 2021), shows that selected populations are better in resisting against all the novel pathogens used in co-infection except Pr in the case of E/P populations, where selected and control populations are equally susceptible.

Co-infection kills more compared to native pathogen. Presence of simultaneously two bacteria in the host system can have completely different response than presence of single pathogen, where interactions between two bacteria within a host system also plays an important role. EPN and IUS were derived from same ancestral outbred populations having large reservoir of genetic variation. Now, selection brings change in the genetic composition of the selected populations when compared to the control populations and therefore different populations are showing difference in response to the co-infection.

Effect of sex

Females and males are physiologically very different and hence have different life-history priorities (Sheldon and Verhulst 1996, Rolff 2002). Females tend to invest more towards surviving longer and hence having extended reproductive phase, whereas males tend to invest

more in mating multiple females and having higher probability of siring progeny (reviewed in Rolff 2002).

EPN populations selected to better survive against *E. faecalis* do not show significant effect of sex on post infection survival where both females and males die equally (Chapter 2). However, for IUS population when infected with *P. entomophila*, females survive significantly more compared to males (Chapter 2). For life history traits females develop faster, have more body weight, survive longer (longevity/starvation/desiccation) owing to bigger body size and more fat reserve than males (Chapter 3), very common observation for female *Drosophila* (reviewed in Prasad and Joshi 2003).

Selected populations of both EPN and IUS shows improved resistant to their native pathogen when compared to control populations under reduced resource conditions (Chapter 4). This result was true for both females and males. In general, males of I, U, and S populations, and females of U and S populations were more susceptible to native pathogen under reduced nutrition when compared to standard diet. But females of I populations were equally susceptible under normal and reduced resource conditions. Males of E, P, and N populations, and females of P and N populations were equally susceptible to the native pathogen (*E. faecalis*) under poor resource conditions compared to normal diet but E females were more resistant when on poor diet. Improved survival of the E females on poor diet may be due to three reasons: (i) selected populations have evolved to prioritize immune defence and hence invest more towards immune function compared to other life-history traits, (ii) poor diet reduces insulin signalling which in turn make flies more resistant to infection with *E. faecalis* (Rehman and Vargheshe 2021, Libert et al 2008), (iii) overall reduction in reproduction makes more resources available for immune function.

There was no role of sex in EPN populations when checked for cross-resistance to novel pathogens (Chapter 5). For IUS, females survived better than males for all Gram-negative novel pathogens (*E. c. carotovora, P. rettgeri*) but males were equally cross-resistant to all Gram-positive novel pathogens (*B. thuringensis, E. faecalis*, *B. cereus*, and *S. succinus*) compared to females. Since, IUS females survive better against its native pathogen, *P. entomophila*, which is Gram- negative bacteria the observed difference in females and males survival for two Gram-negative novel pathogen is understandable. Therefore, role of sex for cross-resistance depends on the selection history and pathogen identity.

The role of sex in EPN and IUS populations for co-infection treatment depended on the selection history and pathogen composition in the co-infection mix (Chapter 6). In general, females survived better than males for all co-infection treatment, barring few exceptions, for both EPN and IUS populations.

Conclusion

To summarize the overall results, EPN populations were selected to survive better post-infection against *E. faecalis* and IUS populations against *P. entomophila*. EPN populations did not show any cost of immune maintenance however it did show cost of immune deployment in trait specific manner. The cost of immune deployment or physiological cost whenever observed was equal for selected and control populations. This result was consistent with the result observed by Gupta et al (2016) for IUS populations. Under reduced resource conditions, both E and I populations survived better post-infection with native pathogen compared to control populations. There was no multiple front cost either in EPN or IUS populations. The selected population (E and I) either survived better (cross-resistant) or equally to the control populations (P/S) against novel pathogens. Selected populations also survived better against co-pathogenic challenges when compared to control populations. Combined results of cross-resistance and co-infection suggests that both selected populations have evolved generalized immune defence.

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