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31

32 Effect of larval diet on adult immune function is contingent upon selection 33 history and host sex in *Drosophila melanogaster*

34 35 ABSTRACT

36 Mounting an immune response requires a considerable energy investment by the host. This
37 makes expression of immune phenotypes susceptible to changes in availability of resources.
38 There is ample evidence in scientific literature to suggest that hosts become more vulnerable
39 to infection by pathogens and parasites when access to nutrition is limited. Using populations
40 of *Drosophila melanogaster* experimentally evolved to better survive bacterial infections, we
41 explore if host selection history influences host response to resource deprivation in terms of
42 immune function. We find that when reared on a suboptimal diet (both in larval and adult
43 stages), adult flies from evolved populations are still more immune to infections compared to
44 flies from control populations. Furthermore, we observe a sex-dependent effect of interaction
45 between selection history and diet on immune function. We thus conclude that immune
46 function of hosts adapted to pathogen challenge is less affected by resource limitation
47 compared to non-adapted hosts.

48

49 Key-words: *Drosophila melanogaster*, experimental evolution, bacterial pathogens, poor
50 nutrition, life-history trade-offs.

51

52 1. INTRODUCTION

53 Expression of immune phenotypes is governed by trade-offs, both evolutionary and
 54 physiological (Sheldon and Verhulst 1996). Evolutionary trade-offs stem from antagonistic
 55 pleiotropy or linkage disequilibrium, with more immune-competent genotypes having sub-
 56 optimal fitness in terms of other organismal traits such as reproduction (Schmid-Hempel
 57 2003). Physiological trade-offs are driven by differential resource allocation between immune
 58 function and other organismal traits; increased investment towards immune defense
 59 compromises other life history traits of the individual organism, and vice versa (Lochmiller
 60 and Deerenberg 2000). The resource allocation towards immune function can be plastic
 61 (depending on environmental factors like exposure to pathogens, availability of resources,
 62 etc.) or developmentally pre-determined. Additionally, the cost of investment towards
 63 immune function in terms of its negative effects on other traits often manifest only when the
 64 individual organism is subjected to infection (McKean et al. 2008, Lazzaro and Little 2009).

65 Studying correlated responses to selection in controlled evolution set-ups is an easy method
 66 to elucidate trade-offs between different organismal traits. Such set-ups have repeatedly been
 67 used to study evolution of defense against parasites and pathogens, and correlated evolution
 68 of other life-history traits. *Drosophila melanogaster* populations selected for increased
 69 defense against larval parasitoid show reduced capacity of intra-specific competition
 70 (Kraaijeveld and Godfray 1997, Fellowes et al. 1998). Similarly, *Drosophila* flies selected for
 71 better resistance against bacterial pathogen *Pseudomonas aeruginosa* have reduced egg
 72 viability and adult life-span (Ye et al. 2009). Populations of Indian meal moth, *Plodia*
 73 *interpunctella*, selected for increased resistance to granulosis virus exhibit increased
 74 development time and reduced egg viability (Boots and Begon 1993). Red flour beetles,
 75 *Tribolium castaneum*, populations selected for increased immune defense against bacterium
 76 *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. 2010). 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 (*Vavraia 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 *Photobacterium 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 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 tryoni* (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, we 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. We experimentally evolved two sets of replicate *Drosophila melanogaster* populations to better survive infection with pathogenic bacteria. One set of four replicate populations (with ancestrally paired controls) were selected against a Gram-negative bacterium, *Pseudomonas entomophila* (Gupta et al. 2016), while the another set of four replicate populations (with ancestrally paired controls) were selected against a Gram-positive bacterium, *Enterococcus faecalis* (Singh et al. 2021, preprint). Both selection regimes were derived off the same ancestral baseline population (Figure 1, see METHODS for more details). Using these selection regimes, we 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, we tested if poor larval diet intensifies the trade-off between immune function and life-history traits in the selected populations. Our 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.

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 Blue Ridge Baseline (BRB) populations were used to derive both selection regimes.

2.1 Ancestral populations:

Blue Ridge Baseline (BRB) is a set of four independent, large, outbred populations of *Drosophila melanogaster*, referred to as BRB 1-4 (Singh et al 2015). Each replicate population has a census size of 2800 adults and is maintained on a 14-day discrete generation cycle on standard banana-jaggery-yeast food, at 25 °C on a 12:12 light-dark (LD) cycle at 50-60% relative humidity (RH). Larvae are reared at a density of ~70 eggs per glass vial (25 mm diameter × 90 mm height) containing 6-8 mL of standard banana-jaggery-yeast medium (banana 205g, barley flour 25g, jaggery 35g, yeast 36g, agar 12.4g, ethanol 45ml, water 1180ml, and p-hydroxymethyl benzoate 2.4g per litre of standard banana-jaggery-yeast food). There are 40 such vials per population. Almost all flies eclose by the 12th day post egg laying (PEL) and are transferred to plexiglass cages (25 cm length x 20 cm width x 15 cm height) provided with a 90 mm diameter Petri plate containing standard banana-jaggery-yeast food. On the 14th day PEL, a fresh food plate is provided for 18 hours for oviposition. These eggs are used to start the next generation.

2.2 Populations selected for better survival post systemic infection with *Enterococcus faecalis* (*E. faecalis*): EPN regime

Selection regime for better survival following systemic infection with entomopathogenic bacteria *Enterococcus faecalis* was started after 150 generations of laboratory adaptation of BRB populations (for details refer Singh et al. 2021, preprint). Briefly, from each replicate population of BRB1-4, 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 $\times$ 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 9-10th 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 CO₂ 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 MgSO₄ buffer under light CO₂ 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 CO₂ 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.

2.3 Populations selected for better survival post systemic infection with *Pseudomonas entomophila* (*P. entomophila*): IUS 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.

2.4 Bacterial culture and infection procedure

The bacteria used in the study are *Enterococcus faecalis* (grown at 37 °C) and *Pseudomonas entomophila* (grown at 27 °C). 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 ul 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 $MgSO_4$.

2.5 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 ($14 \times 16 \times 13 \text{ cm}^3$) with food plates (Petri plates, 60 mm diameter). Eggs for experimental flies were collected from these 'standardised' population cages.

2.6 Effect of standard and poor diet on post-infection survival

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

262 On day 12 PEL, flies from each population, reared on either standard or poor diet, were
 263 randomly assigned to one of the following treatments: (a) infected with pathogen: 100
 264 females and 100 males divided into two cages with equal density and sex ratio; and (b) sham-
 265 infected: 100 females and 100 males divided into two cages with equal density and sex ratio.
 266 Post-treatment the flies were housed in plexiglass cages (14 cm x 16 cm x 13 cm) provided
 267 with *ad libitum* access to either standard or poor diet (depending on the diet they were raised
 268 in as larvae). Hence, flies remained on the same diet throughout their life: as larvae, before
 269 infection, and after infection. Mortality of the flies were recorded every 4-6 hours for 96
 270 hours after infection. Therefore, total 96 cages [2 cages × 2 treatments (infected or sham) × 2
 271 diet × 3 populations × 4 blocks] was observed for each selection regime.

272

273 This experiment was carried out using the EPN selection regime after 40 generations of
 274 forward selection, and with the IUS selection regime after 145 generations of forward
 275 selection. Additionally, flies from EPN selection regime were infected with *E. faecalis*
 276 (infection dose: OD₆₀₀ = 1.0) and flies from IUS selection regime were infected with *P.*
 277 *entomophila* (infection dose: OD₆₀₀ = 1.5). For logistic ease, the experiment was carried out
 278 one block on each day, i.e., E1, P1, and N1 (or I1, U1, and S1) were handled together of one
 279 day, and so on.

280 **2.7 Effect of standard and poor diet on female fecundity**

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

289 **2.8 Effect of standard and poor diet on egg-to-adult development time and viability**

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

292 Two days prior to the egg collection, fresh food plates (normal food composition), smeared
 293 with yeast paste, were provided to the standardized fly cages. On the day of egg collection,
 294 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.

2.9 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.

318 **2.10 Statistical analysis**

319 All statistical analysis was done in R statistical software (version 4.1, R Core Team 2021).

320 Post-infection survival of flies was modelled as

321 $\text{Survival} \sim \text{Selection} + \text{Diet} + \text{Selection:Diet} + (1|\text{Block}),$

322 using mixed-effect Cox proportional hazards model (*coxme* function from ‘coxme’ package,

323 Therneau 2020). Test for significant effects of different factors in the model was carried out

324 using analysis of deviance (*Anova* function from ‘car’ package). Data from each sex was

325 analyzed separately.

326 Life-history traits were analyzed using mixed-effect general linear models (*lmer* function

327 from ‘lmerTest’ package) and subjected to type III ANOVA (*anova* function from base R) for

328 significance tests. The mixed-effect linear models used were as follows:

329 $\text{Development time} \sim \text{Diet} + \text{Selection} + \text{Diet:Selection} + (1|\text{Block})$

330 $\text{Viability} \sim \text{Diet} + \text{Selection} + \text{Diet:Selection} + (1|\text{Block})$

331 $\text{Body weight} \sim \text{Diet} + \text{Selection} + \text{Diet:Selection} + (1|\text{Block})$

332 $\text{Fecundity} \sim \text{Selection} + \text{Diet} + \text{Infection_treatment} + \text{Selection:Diet} +$

333 $\text{Selection:Infection_treatment} + \text{Diet:Infection_treatment} +$

334 $\text{Selection:Diet:Infection_treatment}$

335

336

3. RESULTS

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. We 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.

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 2A and 2B), hence data from only the infected flies was analyzed for effect of selection, diet, and selection $\times$ diet interaction; sexes were analyzed separately. Selection history had a significant effect on post-infection survival in females (Figure 2A, $\chi^2_{(df=2)} = 383.1930$, $p < 2.2e-16$): I females survived better than S and U females. There was a significant effect of selection $\times$ diet interaction (Figure 2A, $\chi^2_{(df=2)} = 6.2361$, $p = 0.04424$): 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 2B, $\chi^2_{(df=2)} = 441.6823$, $p < 2.2e-16$): 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 $\times$ diet ($\chi^2_{(df=2)} = 4.6308$, $p = 0.098727$) was observed on survival of infected males (Figure 2B).

Along with immune function assay, we tested for differences in fecundity of flies of all three populations, raised on both diets, when infected or sham-infected (see METHODS, section 2.7, 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 2A, Figure 3A). 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 $\times$ selection history interaction on development time ($F_{2,230} = 5.6562$, $p = 0.004$, Figure 3B): 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 $\times$ selection history interaction (Table 2C, Figure 3C): 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, $p = 8.445e-09$; 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 2D, Figure 3D). 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 2E, Figure 3D). 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, $p = <2.2e-16$).

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. We 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 2C and 2D), hence data from only the infected flies was analyzed for effect of selection, diet, and selection $\times$ diet interaction; sexes were

analyzed separately. Selection history had a significant effect on post-infection survival in females (Figure 2C, $\chi^2_{(df=2)} = 56.2652$, $p = 6.056e-13$): 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 2C, $\chi^2_{(df=1)} = 3.0068$, $p = 0.08291$). There was a significant effect of selection $\times$ diet interaction (Figure 2C, $\chi^2_{(df=2)} = 8.2023$, $p = 0.01655$): 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 2D, $\chi^2_{(df=2)} = 37.1255$, $p = 8.767e-09$): 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 $\times$ diet ($\chi^2_{(df=2)} = 2.6123$, $p = 0.2709$) was observed on survival of infected males (Figure 2D).

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 3A, Figure 3E). 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 3B, Figure 3F), 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 3G). Egg-to-adult survival was also significantly affected by selection history, and selection $\times$ diet interaction (Table 3C, Figure 3G): 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, $p = 0.0004$) 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

433 both development time and egg-to-adult survival (development time: log-likelihood = -
434 845.53, $p = 7.307e-07$; survival: log-likelihood = -845.87, $p = 9.72e-07$).

4. DISCUSSION

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 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* we see a marked decrease in survival of flies reared on poor larval diet (Figures 2A and 2B). 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 2C and 2D). 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. 2017), 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. 2021, 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, we 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, we are unable to comment on any role of selection history $\times$ pathogen identity in determining the effect of poor diet on immune function. Our 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 2A and 2B); 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 2C and 2D). 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 $\times$ 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 we 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.

4.2 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 3A and 3E). 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 our experiments (Figures 2A and 2E). 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 we 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 our study, we 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.

4.3 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 3B, 3C, 3D). In EPN, poor larval diet increased development time and reduced egg-to-adult viability (Figure 3F and 3G). 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 2019, Rehman and Varghese 2021).

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. Our 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 2A). Interestingly, the selected populations exhibited similar depression of female fecundity, induced by poor larval diet, as exhibited by the control population (Figure 3A and 3E). 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. We 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 2C), 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 our experiments do not agree with this expectation. In our 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 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. We did not observe any trade-offs in the E, P, and N populations on either diet.

To summarize, in this study we 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. Our 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. We 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.

6. TABLES

Table 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 <i>Pseudomonas entomophila</i>			
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 <i>Pseudomonas entomophila</i>			
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 <i>Enterococcus faecalis</i>			
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 <i>Enterococcus faecalis</i>			
Selection	37.1255	2	8.676 e-09
Diet	0.1018	1	0.7496
Selection:Diet	2.6123	2	0.2709

Table 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 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

616 7. FIGURE LEGENDS

617 **Figure 1.** Population history and experimental design. (a) Ancestry of populations used to
618 study the effect of standard vs. poor larval diet on adult immune function in flies, and (b)
619 outline of experimental design.

620 **Figure 2.** Effect of standard vs. poor larval diet on immune function (post-infection survival)
621 of (a) females and (b) males from I, U, and S populations (infected with *Pseudomonas*
622 *entomophila*), and (c) females and (d) males from E, P, and N populations (infected with
623 *Enterococcus faecalis*).

624 **Figure 3.** Effect of standard vs. poor larval diet on fecundity (a, e), egg-to-adult development
625 time (b, f) and survival (c, g), and dry body weight at eclosion (d), of flies from I, U, and S
626 populations (a-d) and E, P, and N populations (e-g).

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A

Experiments done after 40 generations of
forwards selection

I1, S1, and U1 derived from BRB1, and so on. Similarly, E1, P1, and N1 were derived from BRB1, and so on.

B

*Flies from I, S, and U populations are infected
with *P. entomophila*, and flies from E, P, and N
populations infected with *E. faecalis*.