Mechanisms of damage prevention, signalling, and repair impact the ability of *Drosophila* to tolerate enteric bacterial infection

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Abstract

Many insects thrive on decomposing and decaying organic matter containing a large diversity of both commensal and pathogenic microorganisms. The insect gut is therefore frequently exposed to pathogenic threats and must be able not only to detect and clear these potential infections, but also be able to repair the resulting damage to gut tissues in order to tolerate relatively high microbe loads. In contrast to the mechanisms that eliminate pathogens, we currently know less about the mechanisms of disease tolerance, and most of this knowledge stems from systemic infections. Here we investigated how well-described mechanisms that either prevent, signal, control, or repair tissue damage during infection contribute to the phenotype of disease tolerance during gut infection. We orally infected adult *Drosophila melanogaster* flies with the bacterial pathogen *Pseudomonas entomophila* in several loss-of-function mutants lacking epithelial responses including damage preventing *dcy* (drosocrystallin - a major component of the peritrophic matrix), damage signalling *upd3* (unpaired protein, a cytokine-like molecule), damage controlling *irc* (immune-regulated catalase, a negative regulator of reactive oxygen species) and tissue damage repairing *egfr*^{1} (epidermal growth factor receptor). Overall, we detect effects of all these mechanisms on disease tolerance. The deterioration of the peritrophic matrix in *dcy* mutants resulted in the highest loss of tolerance, while loss of function of either *irc* or *upd3* also reduced tolerance in both sexes. The absence of tissue damage repair signalling (*egfr*^{1}) resulted in a severe loss in tolerance in male flies but had no substantial effect on the ability of female flies to tolerate *P. entomophila* infection, despite carrying greater microbe loads than males. Together, our findings provide empirical evidence for the role of damage limitation mechanisms in disease tolerance and highlight how sex differences in these mechanisms could generate sexual dimorphism in immunity.

Introduction

Animals have evolved diverse responses to infection, including behavioural avoidance of infection, physical barriers to pathogen entry and a variety of humoral and cellular immune responses (Buchon, Silverman, and Cherry 2014; Kuraishi, Hori, and Kurata 2013; Vale et al. 2018). These responses have been particularly well described in the fruit fly *Drosophila*, where signalling pathways such as *IMD* and *Toll* are recognised as major contributors to pathogen clearance during infection (Apidianakis and Rahme 2011; Buchon, Silverman, and Cherry 2014; Hoffmann 2003; Hultmark 2003; Kuraishi, Hori, and Kurata 2013; Lemaitre and Hoffmann 2007). In addition to mechanisms that clear or otherwise quantitatively reduce pathogen burdens, there is an increasing appreciation that mechanisms that promote disease tolerance are at least equally as important during infection and in recovery to a healthy state (Raberg, Sim, and Read 2007; Ayres and Schneider 2012; Medzhitov, Schneider, and Soares 2012; Soares, Teixeira, and Moita 2017; McCarville and Ayres 2018). Disease tolerance is defined as the ability of hosts to maintain health despite harbouring relatively high pathogen loads, a phenotype that has been observed in several species, including insects (Howick and Lazzaro 2014; Lissner and Schneider 2018; Oliveira, Bahia, and Vale 2020) rodents (Palaferri Schieber et al. 2015; Raberg, Sim, and Read 2007; Cumnock et al. 2018); birds (Adelman et al. 2013; Bonneaud et al. 2019); and humans (Regoes et al. 2014; Nahrendorf, Ivens, and Spence 2021; Soares, Teixeira, and Moita 2017).

The mechanisms of pathogen clearance are well-described in many animal species (Cooper 2018), but we currently know less about the mechanisms underlying disease tolerance. Given that tolerance reflects a distinct ability to maintain health independently of pathogen clearance, we might expect these mechanisms to be related to processes such as detoxification, reduction of inflammation, or tissue damage control and cellular renewal (Ayres and Schneider 2012; Soares, Teixeira, and Moita 2017; Soares, Gozzelino, and Weis 2014). Genome-wide association or transcriptomic studies in *Drosophila* have highlighted potential candidate genes underlying variation in disease tolerance, reviewed in (Lissner and Schneider 2018), but it remains unclear how these genes interact with known mechanisms of immunity and recovery.

Furthermore, almost all candidate genes for disease tolerance in *Drosophila* arise from systemic infections(Howick and Lazzaro 2017; Troha et al. 2018; Lissner and Schneider 2018), leaving a gap in our knowledge about disease tolerance during oral infections. Orally acquired infections are especially relevant given the pervasive nature of oral-faecal transmission routes, and in the context of the ecology of most insects, like *Drosophila*, that thrive on decomposing and decaying organic

85 matter containing a large diversity of microorganisms, both commensal and pathogenic (Chandler
86 et al. 2011; Corby-Harris et al. 2007). For example, recent genomic analyses of *Drosophila* samples
87 collected across Europe revealed sequences belonging to multiple species of bacteria, fungi and
88 viruses, many of which had not been previously described (Kapun et al. 2020; Wallace et al. 2021).
89 The insect gut is therefore frequently exposed to pathogenic threats and must be able not only to
90 detect and clear these potential infections, but also be able to repair the resulting damage to gut
91 tissues in order to tolerate relatively high numbers of ingested pathogens.
92
93 Here, we aimed to specifically test how known mechanisms that either prevent, reduce, or repair
94 tissue damage in the fly gut contribute to the phenotype of disease tolerance. The *Drosophila* gut is
95 a compartmentalized tubular organ which is structurally and functionally similar to the vertebrate
96 intestinal tract (Buchon, Broderick, and Lemaitre 2013; Buchon and Osman 2015; Kuraishi, Hori,
97 and Kurata 2013; Lemaitre and Miguel-Aliaga 2013). We can consider several stages comprising
98 gut defence in *Drosophila*. The first involves the physical barrier of the gut epithelia and the
99 peritrophic matrix (*PM*), which is a layer of chitin and glycoproteins that lines the insect midgut
100 lumen. The *PM* is functionally analogous to mammalian mucus membrane in the digestive tract
101 and acts as the first line of defence against invading pathogens (Hegedus et al. 2009; Kuraishi,
102 Hori, and Kurata 2013). A major component of the *PM* is *drosocrystallin* (*dcy*). Loss-of-function
103 mutations in *dcy* increase the width of the peritrophic matrix, increasing its permeability to larger
104 molecules and to leakage of microbial cells, including pathogens, in to the haemolymph (Kuraishi,
105 Hori, and Kurata 2013). *Dcy* deficient flies therefore exhibit increased susceptibility to oral bacterial
106 infections.
107
108 As second mode of defence during gut infections is the production of reactive oxygen species
109 (*ROS*) by the gut epithelia. For example, oral infection with *P. entomophila* in *D. melanogaster* causes
110 massive destruction of the gut epithelium (Vodovar et al. 2005; Dieppois et al. 2015). In response
111 to ingested *P. entomophila*, *ROS* production is induced by two NADPH enzymes- *nox* (NADPH
112 oxidase) and *duox* (dual oxidase), while *irc* (immune-reactive catalase) negatively regulates *ROS*
113 production once the infection threat is controlled, which otherwise, would lead to cytotoxic effects
114 (Buchon et al. 2013; 2009; Kuraishi, Hori, and Kurata 2013). *ROS* production not only targets
115 pathogens directly, but also plays additional roles in triggering signalling pathways that lead to the
116 production of *IMD* or *Toll* responsive antimicrobial peptides (Buchon et al. 2009; Lemaitre and
117 Hoffmann 2007; Myllymäki, Valanne, and Rämet 2014; Myllymäki and Rämet 2014; Valanne,
118 Wang, and Rämet 2011).

The final stage in gut defence is to repair the tissue damage caused during the infection. Damage-signalling cytokine-like molecules *upd3* are released from damaged cells which trigger the *Jak/Stat*-pathway, stimulating the proliferation of intestinal stem cells (*ISCs*) and their differentiation into enterocytes (*ECs*) via *egfr*[1] (epidermal growth factor receptor) signalling (Buchon et al. 2010; Chakrabarti et al. 2016; Jiang et al. 2011; Weng et al. 2018). Flies lacking *Jak/Stat* or *Egfr* are therefore highly susceptible to bacterial infections due to their inability to repair and renew damaged tissue (Buchon et al. 2010; Jiang et al. 2011; 2009; Marianes and Spradling 2013; Myllymäki and Rämet 2014).

To investigate how these mechanisms of damage prevention (*dcy*), signalling (*upd3*) control (*irc*) and renewal (*egfr*[1]) contribute to disease tolerance during gut infections we employed oral infections in *Drosophila* lines carrying loss-of-function mutations in each of these genes on a common genetic background (w^{1118}). We orally challenged these flies with a range of infection doses of *Pseudomonas entomophila* and then quantified their effects on survival, pathogen loads and disease tolerance responses during period of peak infection burden.

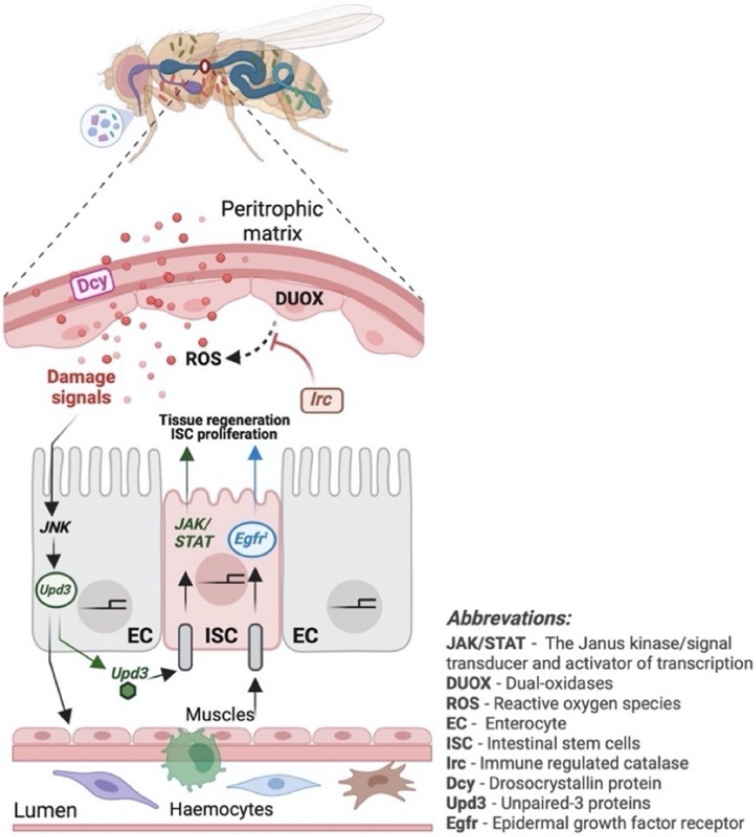

Figure 1: Schematic representation of *Drosophila* epithelial immune response following enteric infection. Several stages of tissue damage prevention and repair epithelial response in the *Drosophila* can be found. ***Stage-I***: involves the physical barrier the peritrophic matrix (*PM* - layer of chitin and

glycoproteins) that lines the *Drosophila* midgut lumen. A major component of the *PM* is ***dcy*** (*drosocrystallin*) and loss-of-function mutations in *dcy* increase the width of the *PM*, increasing permeability to pathogens. ***II***: production of reactive oxygen species (*ROS*) by the gut epithelia - enteric infections cause massive destruction of the gut epithelium and in response to ingested pathogens, *ROS* production is induced by *nox* (NADPH oxidase) and *duox* (dual oxidase), while ***irc*** (immune-reactive catalase) negatively regulates ⊣ *ROS* production by suppressing the cytotoxic effects of *ROS* once the infection threat is lessened, avoiding immunopathology. In addition, *ROS* production is known to trigger signalling pathways the *IMD* or *Toll* that leads to the production of antimicrobial peptides (*AMPs*) ⊥ critical for pathogen clearance in the gut. ***III***: tissue renewal process after infection-induced damage - The final stage in gut defence is to repair the damage caused during enteric infections. Damage-signalling cytokine-like molecules ***upd3***, released from damaged cells trigger the *Jak/Stat*-pathway, stimulating the proliferation of intestinal stem cells (*ISCs*) and their differentiation into enterocytes (*ECs*) via ***egfr***^{1} (epidermal growth factor receptor) signalling which are indispensable for maintaining homeostasis and renewing the damaged cells/tissues. Figure created using Biorender.

Materials and methods

Fly strains: The following fly stocks were obtained from VDRC (Vienna *Drosophila* Resource Centre) and Bloomington Stock Centre, Indiana: *dcy* (*BL26106*), *irc* (*BL29191*), *egfr*^{1} (*BL2079*), *upd3* (*BL19355*). All the mutants were subsequently backcrossed into the wild type *w*^{1118} (*VDRC stock# 60000*) for at least 10 generations. All fly lines were maintained in plastic vials (12 ml) on a standard cornmeal diet (Lewis's medium) at a constant temperature of 25°C (±2°C).

Bacterial culture preparation: To test the impact of bacterial infection on fly survival we used the gram-negative bacteria *Pseudomonas entomophila*, that commonly infects a broad range of insects and other invertebrates. In flies, *P. entomophila* infection mainly occurs in the intestinal epithelium and eventually causes death (Buchon et al. 2009; Vodovar et al. 2005). To obtain bacterial cultures for oral exposure, we inoculated frozen bacterial stock cultures stored at -80℃ onto fresh 15ml LB broth (media composition) and incubated overnight at 37°C with shaking at 120rpm (revolutions per minute). The overnight cultures were diluted 1: 100 into 500ml of fresh LB broth and incubated again at 30°C with shaking at 120rpm. At the mid-log phase (OD_{600}=0.75), we harvested the bacterial cells by centrifugation at 5000rpm for 15 min and re-suspended the bacterial pellet in 5% sucrose (Siva-Jothy et al. 2018). The final inoculum was adjusted to three different bacterial concentrations or infection dose $OD_{600}=10$ (low dose), $OD_{600}=25$ (medium dose) and $OD_{600}=45$ (high dose).

Oral infection assay: Before infecting flies we initially prepared infection vials by pipetting around 350µl of standard agar (1L triple distilled H_2O, 20g agar, 84g brown sugar, 7ml Tegosept anti-fungal agent) onto lid of a 7ml tubes (bijou vials) and allowed it to dry. Simultaneously, we starved

178 the experimental flies on 12ml agar vials for 4-5 hours. Once the agar in the bijou lids dried, we
179 placed a filter disc (Whattmann-10) in the lid and pipetted 80µl of bacterial culture directly onto
180 the filter disc. For control (mock) infections, we replaced bacterial culture with 5% sucrose
181 solution. We then orally exposed flies inside the bijou vials for about 18-hours and then transferred
182 the flies onto fresh vials containing standard cornmeal (Siva-Jothy et al. 2018).
183
184 *Experimental design*: To test how mechanisms of damage repair contribute to disease tolerance we
185 employed a split vial design (see ***Fig***. 2). Here, after oral bacterial exposure each vial containing
186 about 25 flies of each infection treatment, sex and fly line combination were divided into 2 vials
187 for measuring (1) survival following infection (15 flies/combination) and (2) internal bacterial load
188 (10 flies/combination) see ***Fig***. *2*. With this split-vial design we were able to use replicate- matched
189 data for both survival and bacterial load in order to estimate (3) disease tolerance for each fly line
190 that is, the linear relationship between fly survival and internal bacterial load.
191
192 *Bacterial load measurement:* To test whether variation in mortality of experimental flies after *P.*
193 *entomophila* infection is explained by the ability to clear infection, we measured bacterial load at
194 three timepoints (immediately after oral exposure 0-15 minutes, 24-hours, and 96-hours) by
195 exposing 3–5-day old flies with $OD_{600}=25$ *P. entomophila* cells. To confirm oral bacterial infection,
196 we thoroughly surface-sterilised flies (group of 3) with 70% ethanol for 30-60 seconds and then
197 rinsed twice with sterile distilled water. We plated the second wash on LB agar plates and incubated
198 overnight at 30°C to confirm that surface bacteria were successfully removed after alcohol
199 sterilization. We transferred flies onto 1.5ml micro centrifuge tubes and homogenized using a
200 motorized pestle for approximately 30-60 seconds in 100µl LB broth (n=30
201 homogenates/sex/infection treatment/fly line). We performed serial dilution of each homogenate
202 up to 10^{-6}-fold and added 4µl aliquot on a LB agar plate. After this, we incubated the plate
203 overnight for about 18-hours at 30°C and counted the resultant bacterial colonies manually. We
204 note that mock-infected control fly homogenates did not produce any colonies on LB agar plates
205 (Siva-Jothy et al. 2018). We also measured bacterial load at 24-hour following oral *P. entomophila*
206 infection for low dose ($OD_{600}=10$) and high ($OD_{600}=45$) infection dose (See Supplementary
207 Information ***Figs***. *S1* and *S2*).
208
209 **Statistics**
210 1. *Survival following oral infection:* We analysed survival data using a mixed effects Cox model
211 using the R package 'coxme' (Therneau 2015). We specified the model as: survival ~ fly

212 line * treatment * sex * (1|vials/block), with 'fly line', 'treatment' and 'sex' and their
213 interactions as fixed effects, and 'vials' nested in 'block' as a random effect for wild type
214 w^{1118} and mutant flies.
215
216 2. *Internal bacterial load:* We found that residuals of bacterial load data were non-normally
217 distributed when tested using Shapiro–Wilks's test. Hence, we first log-transformed the
218 data and then confirmed that the log-transformed residuals were still non-normally
219 distributed. Subsequently, we used a non-parametric one-way ANOVA (Kruskal-Wallis
220 test) to test the effects of each fly line, that is, wild-type w^{1118} and mutant flies on males and
221 females separately following oral *P. entomophila* infection.
222
223 **3.** *Measuring disease tolerance:* Finally, to understand how tissue damage signalling and repair
224 mechanisms affect disease tolerance in males and females during oral *P. entomophila*
225 infection, we analysed the linear relationship between fly survival against bacterial load by
226 fitting linear models (Raberg, Sim, and Read 2007; Schneider and Ayres 2008; Medzhitov,
227 Schneider, and Soares 2012; Louie et al. 2016; Gupta and Vale 2017; Oliveira, Bahia, and
228 Vale 2020). We assessed differences in disease tolerance (fly survival with increasing
229 bacterial load) using ANCOVA by fitting 'fly line' and 'sex' as categorical fixed effects,
230 'average bacterial load' as a continuous covariate and their interactions as fixed effects.
231 Significant interaction effects between fly line and bacterial load would indicate that the
232 slope of the relationship between fly survival and load varies between fly lines, that is, the
233 tolerance response differs between lines. Because our interest was to find out to what
234 extent loss-of damage prevention and tissue repair mechanisms differed from wildtype
235 w^{1118} in tolerating enteric bacterial infections we compared the estimates of slope for wild
236 type w^{1118} line with each of the mutant line for both males and females separately using a
237 pairwise comparison (f-test).
238

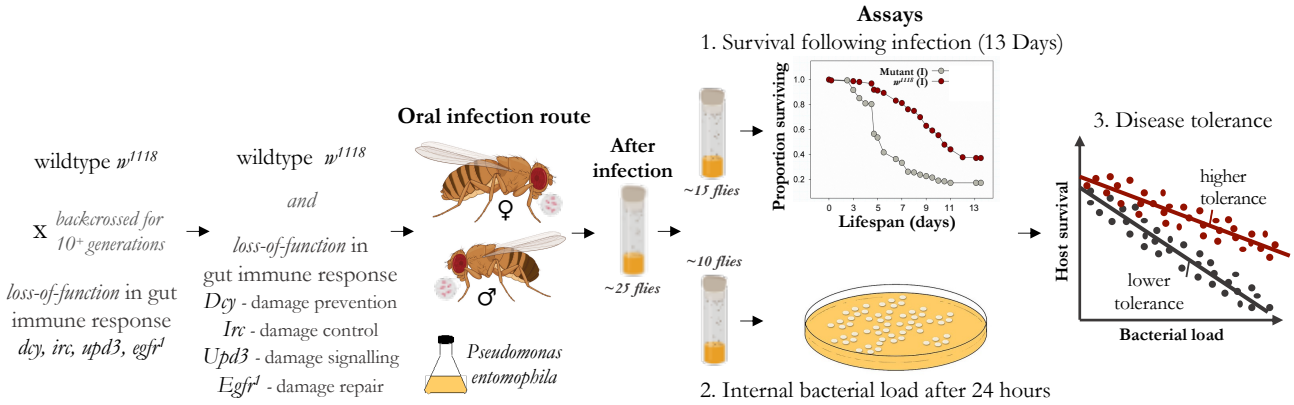

Figure 2: Split-vial experimental design to assay **(1)** survival (~15 flies/combination in a vial) and **(2)** internal bacterial load (~10 flies/combination in a vial) following oral bacterial infection with *Pseudomonas entomophila* to test how epithelial immune response including damage prevention and tissue repair mechanisms in *Drosophila* gut contribute to **(3).** disease tolerance. For the split-vial design, after oral exposure with each vial containing about 25 flies (of each infection treatment, sex, fly line combination) were divided into 2 vials, each for survival (around 15 flies) and bacterial load (around 10 flies). Each point in disease tolerance panel (3) represents replicate-matched data [n=30 vials/infection treatment/sex/fly line – with each vial containing around 25 flies] from survival (1) and bacterial load (2).

Results

1. Flies lacking damage-preventing *dcy* are more susceptible to oral *P. entomophila* infections than those lacking components that minimise, signal or repair tissue damage

Following oral infection with three different doses of *Pseudomonas entomophila*, we found that flies lacking major components of gut immunity such as tissue damage - preventing *dcy*, signalling *upd3*, repairing *egfr*[1] and controlling *irc*, were all significantly more susceptible to oral *P. entomophila* infections than the wild type w^{1118} flies (***Fig.*** *3B* and *1A*, ***Table*** *1*; infection dose $OD_{600}=25$; see Supplementary ***Figs.*** *S1 and S2,* ***Table*** *S1* for low $OD_{600}=10$ and high $OD_{600}=45$ infection doses). Among these mutants, *dcy* mutants were particularly susceptible to infection ***Fig.*** *3B* and *1A* for survival, ***Table*** *1* for hazard ratio; infection dose $OD_{600}=25$). The effect of each mutation on the survival of flies following infection was similar in males and females (fly line x sex x treatment interaction = non-significant, ***Table*** *S1*), and was consistent at both lower ($OD_{600}=10$) and higher infection ($OD_{600}=45$) doses (see Supplementary ***Figs.*** *S1 and S2,* ***Table*** *S1* for low $OD_{600}=10$ and high $OD_{600}=45$ infection dose).

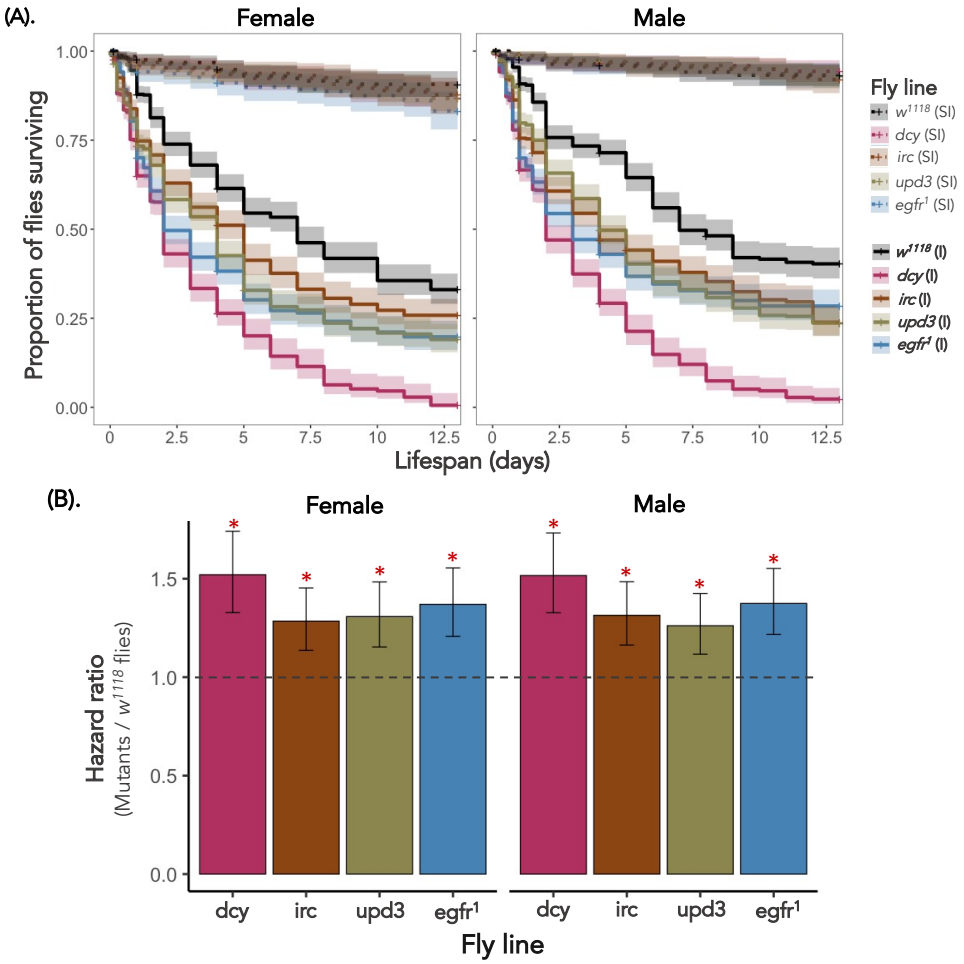

Figure 3. Survival curves for **(*A*)** females and **(*B*)** males of wildtype w^{1118} and mutant flies exposed to oral *P. entomophila* of $OD_{600}=25$ (medium dose, n=30 vials of ~30 flies/treatment/sex/fly line). **(*C*)** Estimated hazard ratios for males and female flies (wild type w^{1118} and mutant flies) calculated from the survival curves. A greater hazard ratio (>1) indicates higher susceptibility to infection. ['*' indicates that the mutant flies are significantly different from wild type w^{1118} flies for males and females respectively]. See ***Figs***. *S1* and *S2* for similar results at lower dose ($OD_{600}=10$) and higher dose ($OD_{600}=45$).

Fly line	*Sex*	*Estimate*	*p*	*lower 95%*	*upper 95%*
dcy	Female	1.520	<0.01	1.32	1.74
	Male	1.516	<0.01	1.32	1.73
Egfr[1]	Female	1.370	<0.01	1.20	1.55
	Male	1.374	<0.01	1.21	1.55
irc	Female	1.284	<0.01	1.13	1.45
	Male	1.314	<0.01	1.16	1.48
upd3	Female	1.307	<0.01	1.15	1.48
	Male	1.261	<0.01	1.11	1.42

Table 1: Summary of estimated hazard ratio from the cox proportional model. A greater hazard ratio (>1) indicates that mutant flies are more susceptible to oral *P. entomophila* infection than w^{1118} wildtype flies.

2. Both wild-type and flies with disrupted tissue damage prevention and repair mechanisms show sex differences in bacterial load during oral infections

The higher susceptibility of mutants to oral bacterial exposure could either be caused by their inability to supress the bacterial growth or due to their inability to tolerate the damage inflicted during oral infection. To distinguish between these mechanisms, we first quantified internal bacterial loads across several time points, that is, 0-15 minutes, 24-hours and 96-hours following oral exposure to *P. entomophila*. All fly lines showed sex-differences at 24-hours peak load, though by 96-hours this sex difference was no longer present in flies lacking damage sensing (*upd3*) and tissue renewal mechanisms (*egfr*) (***Fig***. *4,* ***Table*** *S2*). Flies lacking the negative regulator of ROS *irc* always exhibited lower levels of bacterial load compared to wild type flies (***Fig***. *4B,* ***Table*** *S2*).

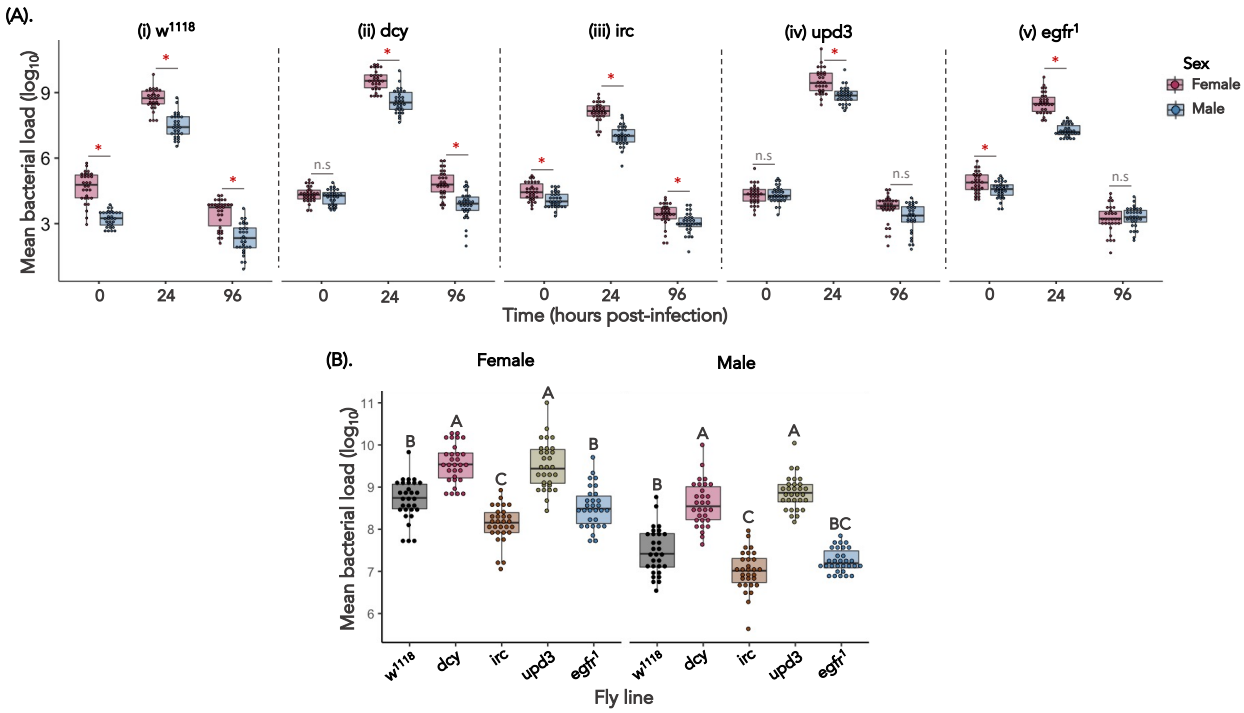

Figure 4. **(A)** Bacterial load measured as CFUs- colony forming units using infection dose (medium OD_{600}=*25*) after different time points 0-15 minutes (immediately after the oral bacterial exposure), 24-hours and 96-hours following infection with *P. entomophila* for wildtype w^{1118} flies and mutants [n=30 vials of (~30 flies/treatment/sex/fly line)]. **(B)** bacterial load at 24-hours following infection [significantly different fly lines are connected with different alphabets using Tukey's HSD as a post hoc analysis of pairwise comparisons]. See ***Figs***. *S1* and *S2* for similar results at lower dose (OD_{600}=*10*) and higher dose (OD_{600}=*45*).

3. Tissue damage repair mechanisms in gut-epithelia mediate sex differences in disease tolerance to oral bacterial *P. entomophila* infections

While some of the variation in survival between mutants (***Fig***. *3*) may be explained by variation in resistance [that is, their ability to clear infection (***Fig***. *4*)], some of that variation may also arise due to differences in tolerance. We were therefore interested in measuring disease tolerance in these lines using the reaction norm of survival relative to bacterial loads, where the slope of the linear relationship reflects the degree of tolerance: steep negative slopes indicate a rapid mortality with increases in pathogen loads (low tolerance), while less steep or flat slopes reflect relatively more tolerant host (Raberg, Sim, and Read 2007; Schneider and Ayres 2008; Louie et al. 2016; Kutzer and Armitage 2016). In statistical terms, the differences in tolerance between lines are indicated by a significant interaction between the bacterial load and the fly line for survival, which reflects the overall rate at which fly health (survival) changes with bacterial load (tolerance) between fly lines (Howick and Lazzaro 2014; Kutzer and Armitage 2016; Louie et al. 2016).

Overall, we found that all mutant lines lacking major components of tissue damage limitation showed reduced tolerance to oral bacterial *P. entomophila* infections compared to wild type w^{1118} flies (***Fig***. *5,* ***Table*** *3*). Here, the differences in tolerance between wild type w^{1118} and mutant flies are indicated by a significant interaction between the fly line bacterial load for survival, which reflects the overall rate at which fly health (survival) changes with bacterial load (tolerance) between fly lines in both males and females (***Fig.*** *5.* ***Table*** *2*). Males lacking $egfr^{1}$ show lower tolerance to bacterial infection than females (***Fig***. *5,* ***Table*** *2*), suggesting sexual dimorphism in gut cell renewal. However, both males and females lacking major component of the peritrophic matrix that is, *dcy* showed significantly reduced tolerance (***Fig***. *5*), indicating the general importance of tissue damage preventing epithelial barrier in defence against oral infections (Kuraishi, Hori, and Kurata 2013).

One notable finding was that $egfr^{1}$ flies, lacking tissue repair and renewal signalling, showed sex-differences in tolerance during oral *P. entomophila* bacterial infections (***Fig***. *5,* ***Table*** *2 and 3*). Previous studies suggest that *D. melanogaster* males are more susceptible to intestinal infections possibly due to males having fewer *ISCs* (intestinal stem cells, a major part of *egfr*-meidated tissue repair process) (Regan et al. 2016; Rera, Clark, and Walker 2012). Males also have lower basal rate of division, overall, making them more susceptible to intestinal oral infection, compared to females. Instead, we found that loss of function of tissue renewal mechanisms makes females more susceptible to *P. entomophila* oral infections across wide range of infection doses (see *Supplementary* ***Figs***. *S1 and S2,* ***Table*** *S1* for low $OD_{600}=10$ and high dose $OD_{600}=45$ survival data).

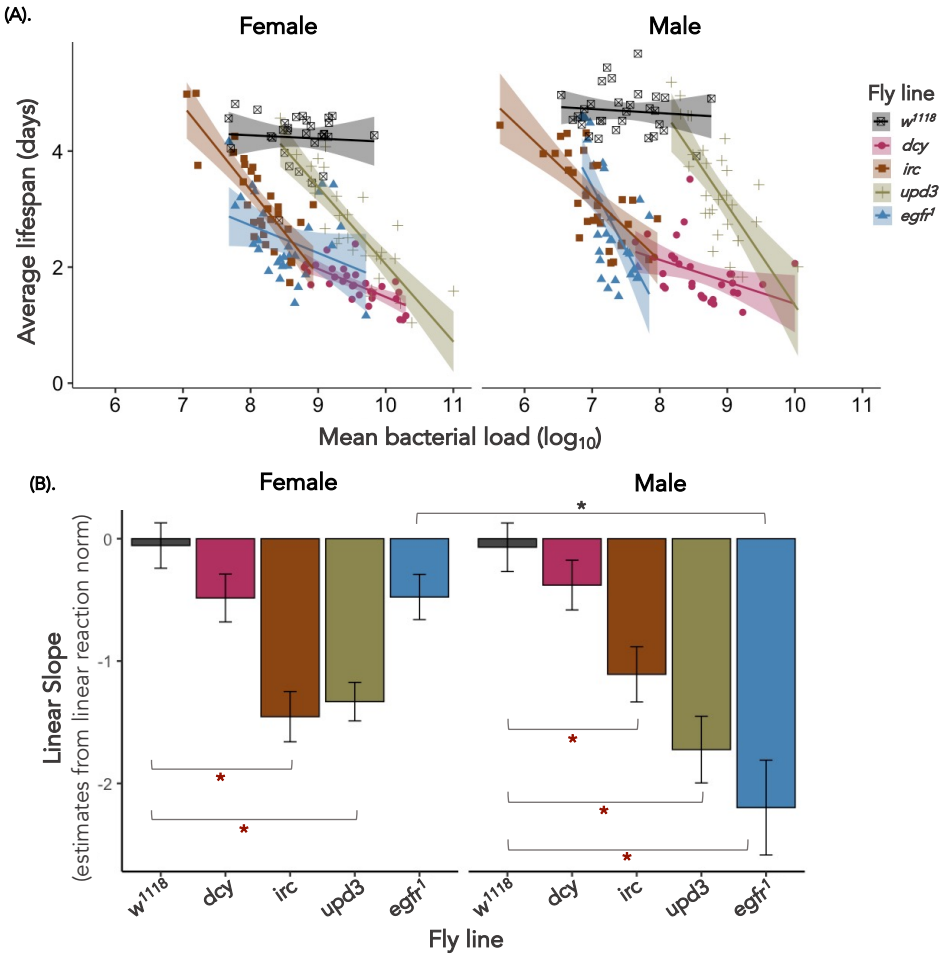

Figure 5. **(A).** The relationship between fly survival (measured as average lifespan) and bacterial load (as mean CFUs – colony forming units), analysed using linear models for female and male flies (w^{1118} and mutant flies). Each point shows data for average lifespan and mean bacterial load (CFUs) of 30 vials (with each vial containing about 25 individual flies/fly line/sex combination) after 24-hours post oral bacterial exposure. The data shown here are for the infection doses (medium $OD_{600}=25$). **(B).** shows the slope of the linear reaction norm extracted from the linear models. ['*' indicates significant difference in tolerance between males and females in tolerance to bacterial infections (interaction between the bacterial load and the sex for each fly line measured using ANCOVA, Table 2),'*' indicates that mutant fly lines are significantly different from wild type w^{1118}, analysed using pairwise f-test from linear norm estimates see Table 3].

Sex	*Source*	*df*	*Sum of Sq.*	*F ratio*	*p*
Female	Fly line	4	66.33	71.6	<0.01
	Bact. load	1	19.27	83.3	<0.01
	Fly line x Bact. load	4	10.02	10.8	<0.01
Male	Fly line	4	84.60	62.8	<0.01
	Bact. load	1	28.39	84.3	<0.01
	Fly line x Bact. load	4	14.88	11.0	<0.01

Table 2. Summary of ANCOVA. To assess differences in infection tolerance (fly survival with increasing bacterial burden) following oral *P. entomophila* infection with $OD_{600}=25$ infection dose, after 24-hours. We analysed ANCOVA and fitted 'sex' as categorical fixed effects, 'average bacterial load' as a continuous covariate and their interactions as fixed effects for each of the fly lines (w^{1118} and mutants).

Sex	*Fly line*	SSE	*F ratio*	*p*
Female	*dcy* vs. w^{1118}	7.32	4.85	0.03
	egfr^{1} vs. w^{1118}	18.32	1.88	0.17
	irc vs. w^{1118}	17.75	28.00	<0.01
	upd3 vs. w^{1118}	18.33	29.79	<0.01
Male	*dcy* vs. w^{1118}	10.66	2.17	0.14
	egfr^{1} vs. w^{1118}	29.07	21.41	<0.01
	irc vs. w^{1118}	16.06	18.72	<0.01
	upd3 vs. w^{1118}	24.87	27.01	<0.01

349 Table 3. Summary of pairwise comparisons (f-test) of linear slope estimates from linear reaction
350 norm for wildtype w^{1118} flies and mutants.
351

352 **Discussion**

353 In the present work we tested how mechanisms of tissue damage prevention (*dcy*), signalling (*upd3*)
354 control (*irc*) and renewal (*egfr*^{1}) contribute to disease tolerance during enteric infection. We present
355 evidence that all of these mechanisms contribute to disease tolerance, and that some of these
356 effects are sexually dimorphic. Previous studies have identified several candidate genes associated
357 with disease tolerance, including - *CrebA, grainyhead and debris buster, dFOXO* (Troha et al. 2018;
358 Howick and Lazzaro 2017; Dionne et al. 2006; Lissner and Schneider 2018). However, these genes
359 arising from transcriptomic, genome-wide association (GWAS) or global profiling of gene
360 expression (microarrays) studies have been screened from flies that are systemically infected to
361 bacterial pathogens and do not seem to be associated with the well described damage prevention
362 and tissue repair processes in the *Drosophila* gut, or with any canonical immune response. Here, we
363 took a more targeted approach to specifically investigate how some of the mechanisms that
364 prevent, sense, control and repair tissue damage affect disease tolerance during enteric bacterial
365 infections.
366
367 Though repairing infection-damage is crucial to fly survival, we found that mutant flies with
368 disrupted tissue damage-preventing (*dcy*) are particularly susceptible to oral infections compared to
369 those lacking components that minimise, signal or repair tissue damage. In other words, preventing
370 tissue damage would appear to be better than repairing from the perspective of fly survival. This
371 result is consistent with previously described *dcy* knockout phenotypes. The loss-of-function in *dcy*
372 increases the peritrophic matrix width by half and increases its permeability to larger molecules,
373 including pathogens (Kuraishi, Hori, and Kurata 2013), making the gut leaky and compromising
374 gut barrier function during oral bacterial infections (Opota et al. 2011; Kuraishi et al. 2011;
375 Chakrabarti et al. 2012; Blemont et al. 2013). We also found increased bacterial loads measured
376 after 24-hours following infection in both male and female *dcy* mutants, This is likely because of

377 the combination of leaky gut and pore-forming toxin produced by *P. entomophila* (Kuraishi, Hori,
378 and Kurata 2013) resulted in higher bacterial growth in the fly haemolymph.
379
380 In the case of *upd3*-deficient flies, we found reduced survival and higher bacterial loads compared
381 to wild type w^{1118} flies. Previous work has shown that in response to *P. entomophila* infections,
382 excessive *ROS* (reactive oxygen species) produced by host cells destroy the gut epithelia and block
383 the gut repair process (Han and Ulevitch 2005; Lambeth 2007; Chakrabarti et al. 2012). The *JNK*
384 and *Hippo* pathways are activated in damaged *ECs* (enterocytes), which produce *upd3*, in turn
385 activating the *Jak/Stat* pathway in *ISCs* (intestinal stem cells). Meanwhile, *upd3* also triggers the
386 activation of *EGFR* signalling in *ISCs* to promote their proliferation and differentiation to renew
387 the damaged cells/tissues (Chakrabarti et al. 2012; Kuraishi, Hori, and Kurata 2013; Buchon,
388 Broderick, and Lemaitre 2013). We also found that loss-of-function in *irc* (immune-regulated
389 catalyse – negative regulator of *ROS*), results in lower bacterial loads, and this is likely because *ROS*
390 is higher leading to greater clearance.
391
392 Regarding the effects of these damage limitation mechanisms on disease tolerance, overall, we
393 found that both male and female wild type w^{1118} flies were quite tolerant of enteric bacterial
394 infections (reflected in their relatively flat tolerance slopes, ***Fig. 5. Table 3***), while disrupting any
395 damage prevention and tissue repair mechanism lowered disease tolerance (steep decline in slopes
396 for all the mutant lines). While we found reduced tolerance in all mutants, disrupting some
397 components of damage limitation had particularly severe effects on disease tolerance. Significant
398 reductions in disease tolerance were observed in flies with loss-of-function in *irc* and *upd3*, and in
399 these mutants the effect was comparable in both sexes. *Irc*-deficient flies are unable to regulate
400 *ROS* levels which would lead to increased cytotoxic effects (Buchon et al. 2009; Kuraishi, Hori,
401 and Kurata 2013) while *upd3* mutants are unable to trigger the activation of the *Jak/Stat* pathway
402 (Jiang et al. 2009; Buchon et al. 2010). Our results suggest that the absence of these mechanisms
403 affects fly health independently of pathogen load, thereby resulting in changes in disease tolerance.
404
405 We observed, the fastest decline in tolerance in male flies lacking $egfr^{1}$, but the disease tolerance of
406 female $egfr^{1}$ mutants appeared unaffected. This sex difference in tolerance may arise as the result of
407 sex differences in gut physiology and repair. Recent work has demonstrated that during oral *Ecc15*
408 infection, males showed significantly lower gut *ISCs* in response to infection, while female had
409 higher *ISCs* and were resistant to infection and other stress (Regan et al. 2016). The differentiation
410 and proliferation of *ISCs* (intestinal stem cells) via *Jak/Stat* signalling into *ECs* (enterocytes) via

411 *egfr* is indispensable for tissue renewal. Loss of *egfr*^{1} signalling might therefore be felt more severely
412 in males than in females, explaining why male but not female *egfr*^{1} mutants showed severe a
413 decrease in disease tolerance. To date, only a small proportion of studies have compared sex-
414 differences in intestinal immunity, with the majority of work focusing on one particular sex, usually
415 females (Ayyaz, Li, and Jasper 2015; Chakrabarti et al. 2016; Regan et al. 2016; Belmonte et al.
416 2020).
417
418 Another possibility for the observed sex-difference in tissue renewal process, could relate to sexual
419 dimorphism in gut-plasticity and remodelling. For instance, females of mammals such as mice
420 extensively remodel their guts, increasing both digestive and absorptive capacity depending on the
421 nutritional demands of lactation (Speakman 2008). The remodelling of the gut might be one of the
422 possible driving factors for dimorphism in gut immunity, since males and females differ in their
423 nutritional needs (Belmonte et al. 2020). Studies using *Drosophila* have shown that males and
424 females can make different diet or nutritional choices in accordance with their reproductive role
425 and demand (Camus, Piper, and Reuter 2019) and that the *Drosophila* midgut plastically resizes in
426 response to changes in dietary sugar and yeast (Bonfini et al. 2021). Whether gut remodelling and
427 nutritional choice-demand causes sex-differences in tissue damage repair process during disease
428 tolerance remains a question for future research.
429
430 Although host mechanisms of immune-mediated clearance are key for pathogen defence and
431 elimination, there is an increasing appreciation that additional defence mechanisms which prevent,
432 signal, repair or renew the extent of tissue damage are also key to infection outcomes by promoting
433 disease tolerance (Soares, Gozzelino, and Weis 2014; Vale, Fenton, and Brown 2014). Tissue repair
434 mechanisms that promote disease tolerance are interesting from a therapeutic perspective (Ayres
435 and Schneider 2012; Medzhitov, Schneider, and Soares 2012; Vale et al. 2016). For instance, in
436 mice, mechanisms that prevent damage or repair tissues have been shown to confer disease
437 tolerance during malarial *Plasmodium* infection and also during co-infections by pneumonia causing
438 bacteria (*Legionella pneumophila*) and influenza virus (Ferreira et al. 2011; Jamieson et al. 2013).
439 Understanding how tissue damage prevention and repair mechanisms contribute to disease
440 tolerance may also help explain how other arthropods are able to vector bacterial and viral
441 infections without substantial health loss (Taracena et al. 2018; Oliveira, Bahia, and Vale 2020;
442 Lambrechts and Saleh 2019). In summary, our results show that the absence of tissue repair
443 processes resulted in severe loss of disease tolerance, and highlight how sex differences in some
444 tissue damage repair mechanisms could generate sexual dimorphism in gut immunity.

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Authors' contribution

A.P and P.F.V conceived and designed the experiments; A.P carried out experiments with assistance from K.M.M; A.P analysed the data; A.P and P.F.V wrote the manuscript.

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Competing interests

None.

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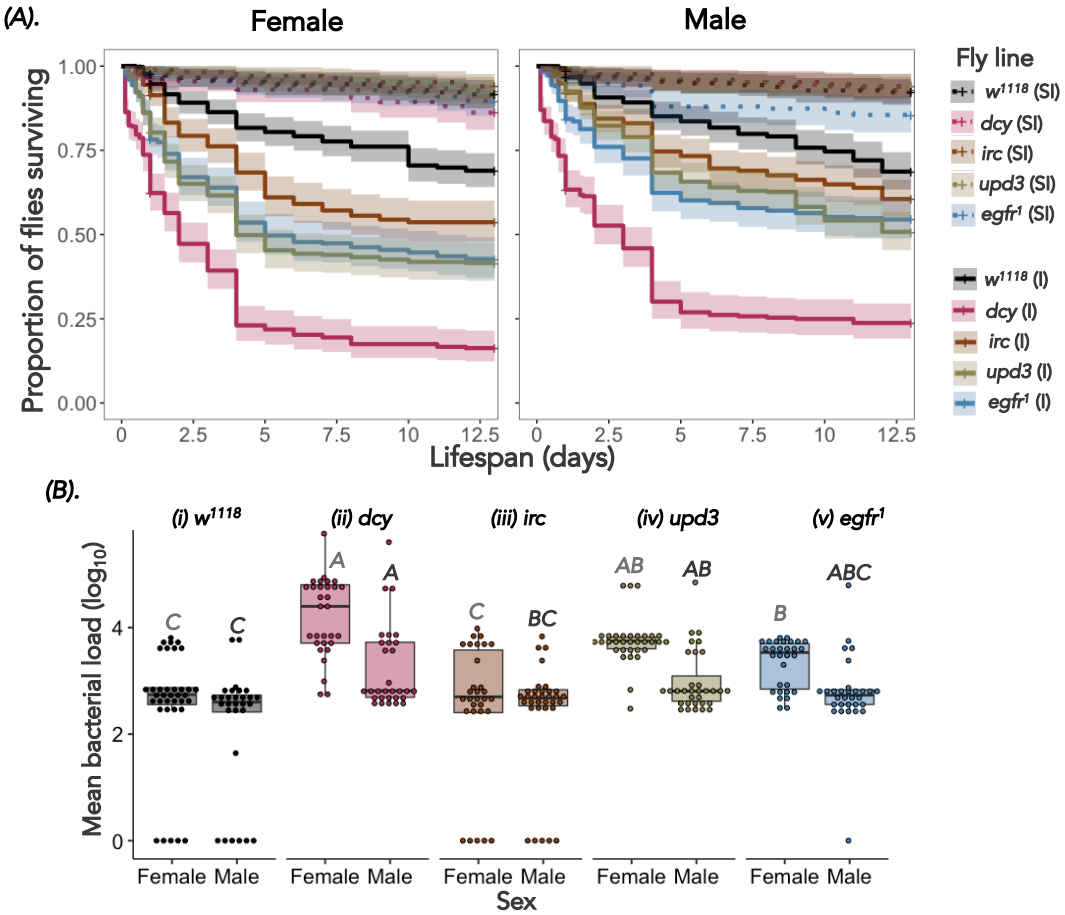
729 **Supplementary information**:

730 **Figure S1. (A)** Survival curves for male and female flies of wild-type *w*^{1118} and mutants with disrupted tissue
731 damage prevention and repair process in adult *Drosophila*, exposed to OD_{600}=10 (low dose) of oral
732 *Pseudomonas entomophila* infection **(B)** Bacterial load measured at 24-hours after OD_{600}=10 of oral *Pseudomonas*
733 *entomophila* infection for male and female flies of wild-type *w*^{1118} and mutant flies. Significantly different fly
734 lines are connected with different alphabets using Tukey's HSD as a post hoc analysis of pairwise
735 comparisons in panel B.
736



737

Figure S2. (A) Survival curves of male and female flies of wild-type w^{1118} and mutant flies exposed to OD_{600}=45 (high dose) of oral *Pseudomonas entomophila* infection **(B)** Bacterial load measured at 24-hours after OD_{600}=45 of oral *Pseudomonas entomophila* infection for male and female flies of wild-type w^{1118} and mutant flies. Significantly different fly lines are connected with different alphabets using Tukey's HSD as a post hoc analysis of pairwise comparisons in panel B.

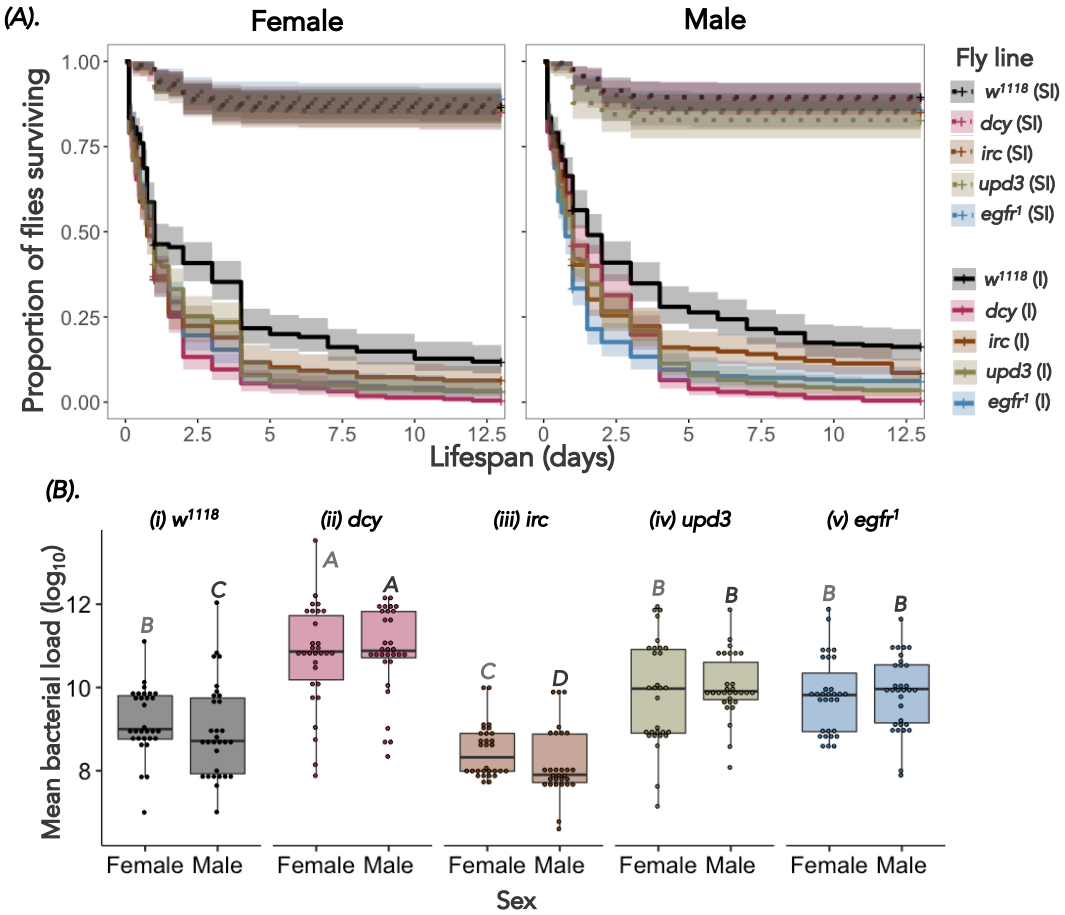


Table S1. Summary of mixed effects Cox model, for male and female wildtype w^{1118} and mutant flies orally infected with 3 different doses of *P. entomophila*. We used 3-5-day old adult males and females of each fly lines (wildtype and mutants) and specified the model as: Survival ~ Treatment x Sex x Fly line (1 | vial/block), with 'Treatment', 'Sex' and 'Fly line' as fixed effects, and 'vials' nested in 'block' as a random effect. The table shows model output (ANOVA) for survival post oral infection for wildtype w^{1118} flies and mutant flies.

Infection dose	***Source***	***Loglik***	***Chisq***	***df***	***p***
OD_{600}=10	Treatment	-12880	968.61	1	< 0.001
	Sex	-12872	17.367	1	< 0.001
	Fly line	-12669	404.70	4	< 0.001
	Treatment x sex	-12669	0.9114	1	0.33
	Treatment x fly line	-12652	33.944	4	< 0.001
	Sex x fly line	-12650	3.9894	4	0.40
	Treatment x sex x fly line	-12647	6.4149	4	0.17
	Random effect	*Std dev*			
	vial/ block	*8.88E-04*			
OD_{600}=25	Treatment	-37499	3058.87	1	< 0.001
	Sex	-37487	25.046	1	< 0.001
	Fly line	-37257	459.75	4	< 0.001
	Treatment x sex	-37250	14.685	1	< 0.001
	Treatment x fly line	-37240	18.773	4	< 0.001
	Sex x fly line	-37236	7.8494	4	0.09
	Treatment x sex x fly line	-37235	2.9252	4	0.57
	Random effect	*Std dev*			
	vial/ block	*1.92E-03*			
OD_{600}=45	Treatment	21144	3256.2	1	< 0.001
	Sex	21141	5.5753	1	< 0.001
	Fly line	21101	80.198	4	< 0.001
	Treatment x sex	21101	1.0625	1	0.30
	Treatment x fly line	21098	5.7909	4	0.21
	Sex x fly line	21094	6.6203	4	0.15
	Treatment x sex x fly line	21094	1.8525	4	0.76
	Random effect	*Std dev*			
	vial/ block	*0.019*			

754 **Table S2.** Summary of $\log_{10}$ transformed bacterial load data after exposure to $OD_{600}=25$ of oral *P.*
755 *entomophila* infection for male and female wildtype w^{1118} and mutant flies, analysed using non-parametric
756 one-way ANOVA (Kruskal–Wallis test) at 3 different timepoints (0-15 minutes, 24-hours and 96-hours
757 following oral infection)
758

	Sex	***Fly line***		***Mean diff***	***Z***	***p***
0-15 min	*Female*	*egfr1*	w^{1118}	3.50	0.77	0.43
		irc	w^{1118}	-7.83	-1.73	0.08
		upd-3	w^{1118}	-11.03	-2.44	0.01
		dcy	w^{1118}	-11.16	-2.47	0.01
	Male	*egfr1*	w^{1118}	29.56	6.55	< 0.001
		dcy	w^{1118}	28.96	6.423	< 0.001
		upd-3	w^{1118}	28.96	6.42	< 0.001
		irc	w^{1118}	28.033	6.21	< 0.001

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760

	Sex	***Fly line***		***Mean diff***	***Z***	***p***
24-hours	*Female*	*dcy*	w^{1118}	24.30	5.388	<0.001
		upd-3	w^{1118}	22.23	4.930	<0.001
		egfr1	w^{1118}	-7.233	-1.604	0.108
		irc	w^{1118}	-18.96	-4.206	<0.001
	Male	*upd-3*	w^{1118}	28.70	6.364	<0.001
		dcy	w^{1118}	25.90	5.743	<0.001
		egfr1	w^{1118}	-7.233	-1.604	0.10
		irc	w^{1118}	-14.16	-3.141	0.001

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	Sex	***Fly line***		***Mean diff***	***Z***	***p***
96-hours	*Female*	*dcy*	w^{1118}	26.90	5.965	< 0.001
		upd-3	w^{1118}	6.100	1.352	0.17
		irc	w^{1118}	-5.166	-1.145	0.25
		egfr1	w^{1118}	-8.566	-1.899	0.057
	Male	*dcy*	w^{1118}	26.50	5.876	< 0.001
		egfr1	w^{1118}	22.73	5.041	< 0.001
		upd-3	w^{1118}	20.36	4.516	< 0.001
		irc	w^{1118}	17.56	3.895	< 0.001

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