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 egfr1 (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 (egfr1) 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; McCarrville 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
matter containing a large diversity of microorganisms, both commensal and pathogenic (Chandler et al. 2011; Corby-Harris et al. 2007). For example, recent genomic analyses of Drosophila samples collected across Europe revealed sequences belonging to multiple species of bacteria, fungi and viruses, many of which had not been previously described (Kapun et al. 2020; Wallace et al. 2021). 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 numbers of ingested pathogens.

Here, we aimed to specifically test how known mechanisms that either prevent, reduce, or repair tissue damage in the fly gut contribute to the phenotype of disease tolerance. The Drosophila gut is a compartmentalized tubular organ which is structurally and functionally similar to the vertebrate intestinal tract (Buchon, Broderick, and Lemaitre 2013; Buchon and Osman 2015; Kuraishi, Hori, and Kurata 2013; Lemaitre and Miguel-Aliaga 2013). We can consider several stages comprising gut defence in Drosophila. The first involves the physical barrier of the gut epithelia and the peritrophic matrix (PM), which is a layer of chitin and glycoproteins that lines the insect midgut lumen. The PM is functionally analogous to mammalian mucus membrane in the digestive tract and acts as the first line of defence against invading pathogens (Hegedus et al. 2009; Kuraishi, Hori, and Kurata 2013). A major component of the PM is drosocrystallin (dcy). Loss-of-function mutations in dcy increase the width of the peritrophic matrix, increasing its permeability to larger molecules and to leakage of microbial cells, including pathogens, into the haemolymph (Kuraishi, Hori, and Kurata 2013). Dcy deficient flies therefore exhibit increased susceptibility to oral bacterial infections.

As second mode of defence during gut infections is the production of reactive oxygen species (ROS) by the gut epithelia. For example, oral infection with P. entomophila in D. melanogaster causes massive destruction of the gut epithelium (Vodovar et al. 2005; Dieppois et al. 2015). In response to ingested P. entomophila, ROS production is induced by two NADPH enzymes—nox (NADPH oxidase) and duox (dual oxidase), while irc (immune-reactive catalase) negatively regulates ROS production once the infection threat is controlled, which otherwise, would lead to cytotoxic effects (Buchon et al. 2013; 2009; Kuraishi, Hori, and Kurata 2013). ROS production not only targets pathogens directly, but also plays additional roles in triggering signalling pathways that lead to the production of IMD or Toll responsive antimicrobial peptides (Buchon et al. 2009; Lemaitre and Hoffmann 2007; Myllymäki, Valanne, and Rämet 2014; Myllymäki and Rämet 2014; Valanne, 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* (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*) 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 lead 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 (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), ire (BL29191), egfr (BL2079), upd3 (BL19355). All the mutants were subsequently backcrossed into the wild type w1118 (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°C 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 (OD600=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 OD600=10 (low dose), OD600=25 (medium dose) and OD600=45 (high dose).

**Oral infection assay**: Before infecting flies we initially prepared infection vials by pipetting around 350µl of standard agar (1L triple distilled H2O, 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
the experimental flies on 12ml agar vials for 4-5 hours. Once the agar in the bijou lids dried, we placed a filter disc (Whatmann-10) in the lid and pipetted 80µl of bacterial culture directly onto the filter disc. For control (mock) infections, we replaced bacterial culture with 5% sucrose solution. We then orally exposed flies inside the bijou vials for about 18-hours and then transferred the flies onto fresh vials containing standard cornmeal (Siva-Jothy et al. 2018).

**Experimental design:** To test how mechanisms of damage repair contribute to disease tolerance we employed a split vial design (see Fig. 2). Here, after oral bacterial exposure each vial containing about 25 flies of each infection treatment, sex and fly line combination were divided into 2 vials for measuring (1) survival following infection (15 flies/combination) and (2) internal bacterial load (10 flies/combination) see Fig. 2. With this split-vial design we were able to use replicate-matched data for both survival and bacterial load in order to estimate (3) disease tolerance for each fly line that is, the linear relationship between fly survival and internal bacterial load.

**Bacterial load measurement:** To test whether variation in mortality of experimental flies after *P. entomophila* infection is explained by the ability to clear infection, we measured bacterial load at three timepoints (immediately after oral exposure 0-15 minutes, 24-hours, and 96-hours) by exposing 3–5-day old flies with OD$_{600}$=25 *P. entomophila* cells. To confirm oral bacterial infection, we thoroughly surface-sterilised flies (group of 3) with 70% ethanol for 30-60 seconds and then rinsed twice with sterile distilled water. We plated the second wash on LB agar plates and incubated overnight at 30°C to confirm that surface bacteria were successfully removed after alcohol sterilization. We transferred flies onto 1.5ml micro centrifuge tubes and homogenized using a motorized pestle for approximately 30-60 seconds in 100µl LB broth (n=30 homogenates/sex/infection treatment/fly line). We performed serial dilution of each homogenate up to 10$^{-6}$-fold and added 4µl aliquot on a LB agar plate. After this, we incubated the plate overnight for about 18-hours at 30°C and counted the resultant bacterial colonies manually. We note that mock-infected control fly homogenates did not produce any colonies on LB agar plates (Siva-Jothy et al. 2018). We also measured bacterial load at 24-hour following oral *P. entomophila* infection for low dose (OD$_{600}$=10) and high (OD$_{600}$=45) infection dose (See Supplementary Information Figs. S1 and S2).

**Statistics**

1. **Survival following oral infection:** We analysed survival data using a mixed effects Cox model using the R package ‘coxme’ (Therneau 2015). We specified the model as: survival ~ fly...
line * treatment * sex * (1 | vials/block), with ‘fly line’, ‘treatment’ and ‘sex’ and their
interactions as fixed effects, and ‘vials’ nested in ‘block’ as a random effect for wild type
\( w^{1118} \) and mutant flies.

2. **Internal bacterial load**: We found that residuals of bacterial load data were non-normally
distributed when tested using Shapiro–Wilks’s test. Hence, we first log-transformed the
data and then confirmed that the log-transformed residuals were still non-normally
distributed. Subsequently, we used a non-parametric one-way ANOVA (Kruskal-Wallis
test) to test the effects of each fly line, that is, wild-type \( w^{1118} \) and mutant flies on males and
females separately following oral \( P. \ entomophila \) infection.

3. **Measuring disease tolerance**: Finally, to understand how tissue damage signalling and repair
mechanisms affect disease tolerance in males and females during oral \( P. \ entomophila \)
infection, we analysed the linear relationship between fly survival against bacterial load by
fitting linear models (Raberg, Sim, and Read 2007; Schneider and Ayres 2008; Medzhitov,
Schneider, and Soares 2012; Louie et al. 2016; Gupta and Vale 2017; Oliveira, Bahia, and
Vale 2020). We assessed differences in disease tolerance (fly survival with increasing
bacterial load) using ANCOVA by fitting ‘fly line’ and ‘sex’ as categorical fixed effects,
‘average bacterial load’ as a continuous covariate and their interactions as fixed effects.
Significant interaction effects between fly line and bacterial load would indicate that the
slope of the relationship between fly survival and load varies between fly lines, that is, the
tolerance response differs between lines. Because our interest was to find out to what
extent loss-of damage prevention and tissue repair mechanisms differed from wildtype
\( w^{1118} \) in tolerating enteric bacterial infections we compared the estimates of slope for wild
type \( w^{1118} \) line with each of the mutant line for both males and females separately using a
pairwise comparison (f-test).
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' and controlling irs, were all significantly more susceptible to oral *P. entomophila* infections than the wild type *w1118* 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 w1118 and mutant flies exposed to oral P. entomophila of OD600=25 (medium dose, n=30 vials of ~30 flies/treatment/sex/fly line). (C) Estimated hazard ratios for males and female flies (wild type w1118 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 w1118 flies for males and females respectively. See Figs. S1 and S2 for similar results at lower dose (OD600=10) and higher dose (OD600=45).

| Fly line | Sex | Estimate | p   | lower 95% | upper 95% |
|----------|-----|----------|-----|-----------|-----------|
| dy       | Female | 1.520   | <0.01 | 1.32      | 1.74      |
|          | Male   | 1.516   | <0.01 | 1.32      | 1.73      |
| Egrf1    | Female | 1.370   | <0.01 | 1.20      | 1.55      |
|          | Male   | 1.374   | <0.01 | 1.21      | 1.55      |
| ir-c     | 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 w1118 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 suppress 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*<sub>600</sub>=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<sup>1118</sup>* 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*<sub>600</sub>=10) and higher dose (*OD*<sub>600</sub>=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 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 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 ISC3 (intestinal stem cells, a major part of egfr-mediated 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 OD600=10 and high dose OD600=45 survival data).
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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|

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

Discussion

In the present work we tested how mechanisms of tissue damage prevention (dcy), signalling (upd3) control (irc) and renewal (egfr^1) contribute to disease tolerance during enteric infection. We present evidence that all of these mechanisms contribute to disease tolerance, and that some of these effects are sexually dimorphic. Previous studies have identified several candidate genes associated with disease tolerance, including - CrebA, grainyhead and debris buster, dFOXO (Troha et al. 2018; Howick and Lazzaro 2017; Dionne et al. 2006; Lissner and Schneider 2018). However, these genes arising from transcriptomic, genome-wide association (GWAS) or global profiling of gene expression (microarrays) studies have been screened from flies that are systemically infected to bacterial pathogens and do not seem to be associated with the well described damage prevention and tissue repair processes in the Drosophila gut, or with any canonical immune response. Here, we took a more targeted approach to specifically investigate how some of the mechanisms that prevent, sense, control and repair tissue damage affect disease tolerance during enteric bacterial infections.

Though repairing infection-damage is crucial to fly survival, we found that mutant flies with disrupted tissue damage-preventing (dcy) are particularly susceptible to oral infections compared to those lacking components that minimise, signal or repair tissue damage. In other words, preventing tissue damage would appear to be better than repairing from the perspective of fly survival. This result is consistent with previously described dcy knockout phenotypes. The loss-of-function in dcy increases the peritrophic matrix width by half and increases its permeability to larger molecules, including pathogens (Kuraishi, Hori, and Kurata 2013), making the gut leaky and compromising gut barrier function during oral bacterial infections (Opota et al. 2011; Kuraishi et al. 2011; Chakrabarti et al. 2012; Blemont et al. 2013). We also found increased bacterial loads measured after 24-hours following infection in both male and female dcy mutants, This is likely because of
the combination of leaky gut and pore-forming toxin produced by *P. entomophila* (Kuraishi, Hori, and Kurata 2013) resulted in higher bacterial growth in the fly haemolymph.

In the case of *upd3*-deficient flies, we found reduced survival and higher bacterial loads compared to wild type *w*1118 flies. Previous work has shown that in response to *P. entomophila* infections, excessive ROS (reactive oxygen species) produced by host cells destroy the gut epithelia and block the gut repair process (Han and Ulevitch 2005; Lambeth 2007; Chakrabarti et al. 2012). The JNK and Hippo pathways are activated in damaged ECs (enterocytes), which produce *upd3*, in turn activating the Jak/Stat pathway in ISCs (intestinal stem cells). Meanwhile, *upd3* also triggers the activation of EGFR signalling in ISCs to promote their proliferation and differentiation to renew the damaged cells/tissues (Chakrabarti et al. 2012; Kuraishi, Hori, and Kurata 2013; Buchon, Broderick, and Lemaître 2013). We also found that loss-of-function in *irc* (immune-regulated catalyse – negative regulator of ROS), results in lower bacterial loads, and this is likely because ROS is higher leading to greater clearance.

Regarding the effects of these damage limitation mechanisms on disease tolerance, overall, we found that both male and female wild type *w*1118 flies were quite tolerant of enteric bacterial infections (reflected in their relatively flat tolerance slopes, Fig. 5, Table 3), while disrupting any damage prevention and tissue repair mechanism lowered disease tolerance (steep decline in slopes for all the mutant lines). While we found reduced tolerance in all mutants, disrupting some components of damage limitation had particularly severe effects on disease tolerance. Significant reductions in disease tolerance were observed in flies with loss-of-function in *irc* and *upd3*, and in these mutants the effect was comparable in both sexes. *Irc*-deficient flies are unable to regulate ROS levels which would lead to increased cytotoxic effects (Buchon et al. 2009; Kuraishi, Hori, and Kurata 2013) while *upd3* mutants are unable to trigger the activation of the Jak/Stat pathway (Jiang et al. 2009; Buchon et al. 2010). Our results suggest that the absence of these mechanisms affects fly health independently of pathogen load, thereby resulting in changes in disease tolerance.

We observed, the fastest decline in tolerance in male flies lacking *egfr*, but the disease tolerance of female *egfr* mutants appeared unaffected. This sex difference in tolerance may arise as the result of sex differences in gut physiology and repair. Recent work has demonstrated that during oral *E. coli* infection, males showed significantly lower gut ISCs in response to infection, while female had higher ISCs and were resistant to infection and other stress (Regan et al. 2016). The differentiation and proliferation of ISCs (intestinal stem cells) via Jak/Stat signalling into ECs (enterocytes) via
egfr is indispensable for tissue renewal. Loss of egfr signalling might therefore be felt more severely in males than in females, explaining why male but not female egfr mutants showed severe a decrease in disease tolerance. To date, only a small proportion of studies have compared sex-differences in intestinal immunity, with the majority of work focusing on one particular sex, usually females (Ayyaz, Li, and Jasper 2015; Chakrabarti et al. 2016; Regan et al. 2016; Belmonte et al. 2020). Another possibility for the observed sex-difference in tissue renewal process, could relate to sexual dimorphism in gut-plasticity and remodelling. For instance, females of mammals such as mice extensively remodel their guts, increasing both digestive and absorptive capacity depending on the nutritional demands of lactation (Speakman 2008). The remodelling of the gut might be one of the possible driving factors for dimorphism in gut immunity, since males and females differ in their nutritional needs (Belmonte et al. 2020). Studies using Drosophila have shown that males and females can make different diet or nutritional choices in accordance with their reproductive role and demand (Camus, Piper, and Reuter 2019) and that the Drosophila midgut plastically resizes in response to changes in dietary sugar and yeast (Bonfini et al. 2021). Whether gut remodelling and nutritional choice-demand causes sex-differences in tissue damage repair process during disease tolerance remains a question for future research.

Although host mechanisms of immune-mediated clearance are key for pathogen defence and elimination, there is an increasing appreciation that additional defence mechanisms which prevent, signal, repair or renew the extent of tissue damage are also key to infection outcomes by promoting disease tolerance (Soares, Gozzelino, and Weis 2014; Vale, Fenton, and Brown 2014). Tissue repair mechanisms that promote disease tolerance are interesting from a therapeutic perspective (Ayres and Schneider 2012; Medzhitov, Schneider, and Soares 2012; Vale et al. 2016). For instance, in mice, mechanisms that prevent damage or repair tissues have been shown to confer disease tolerance during malarial Plasmodium infection and also during co-infections by pneumonia causing bacteria (Legionella pneumophila) and influenza virus (Ferreira et al. 2011; Jamieson et al. 2013). Understanding how tissue damage prevention and repair mechanisms contribute to disease tolerance may also help explain how other arthropods are able to vector bacterial and viral infections without substantial health loss (Taracena et al. 2018; Oliveira, Bahia, and Vale 2020; Lambrechts and Saleh 2019). In summary, our results show that the absence of tissue repair processes resulted in severe loss of disease tolerance, and highlight how sex differences in some 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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Supplementary information:

**Figure S1.** (A) Survival curves for male and female flies of wild-type w^1118 and mutants with disrupted tissue damage prevention and repair process in adult *Drosophila*, exposed to OD_{600}=10 (low dose) of oral *Pseudomonas entomophila* infection. (B) Bacterial load measured at 24-hours after OD_{600}=10 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.

(A) **Female**

![Survival curves for male and female flies](image)

(A) **Male**

![Survival curves for male and female flies](image)

(B) **Fly line**

- (i) w^1118
- (ii) dcy
- (iii) irc
- (iv) upd3
- (v) egfr

![Bacterial load measured at 24-hours after OD_{600}=10 of oral *Pseudomonas entomophila* infection](image)
**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<sub>600</sub>=10 | Treatment | -12880 | 968.61 | 1 | < 0.001 |
| OD<sub>600</sub>=10 | Sex | -12872 | 17.367 | 1 | < 0.001 |
| OD<sub>600</sub>=10 | Fly line | -12669 | 404.70 | 4 | < 0.001 |
| OD<sub>600</sub>=10 | Treatment x sex | -12669 | 0.9114 | 1 | 0.33 |
| OD<sub>600</sub>=10 | Treatment x fly line | -12652 | 33.944 | 4 | < 0.001 |
| OD<sub>600</sub>=10 | Sex x fly line | -12650 | 3.9894 | 4 | 0.40 |
| OD<sub>600</sub>=10 | Treatment x sex x fly line | -12647 | 6.4149 | 4 | 0.17 |
| Random effect | Std dev | vial/block | 8.88E-04 | |
| OD<sub>600</sub>=25 | Treatment | -37499 | 3058.87 | 1 | < 0.001 |
| OD<sub>600</sub>=25 | Sex | -37487 | 25.046 | 1 | < 0.001 |
| OD<sub>600</sub>=25 | Fly line | -37257 | 459.75 | 4 | < 0.001 |
| OD<sub>600</sub>=25 | Treatment x sex | -37250 | 14.685 | 1 | < 0.001 |
| OD<sub>600</sub>=25 | Treatment x fly line | -37240 | 18.773 | 4 | < 0.001 |
| OD<sub>600</sub>=25 | Sex x fly line | -37236 | 7.8494 | 4 | 0.09 |
| OD<sub>600</sub>=25 | Treatment x sex x fly line | -37235 | 2.9252 | 4 | 0.57 |
| Random effect | Std dev | vial/block | 1.92E-03 | |
| OD<sub>600</sub>=45 | Treatment | 21144 | 3256.2 | 1 | < 0.001 |
| OD<sub>600</sub>=45 | Sex | 21141 | 5.5753 | 1 | < 0.001 |
| OD<sub>600</sub>=45 | Fly line | 21101 | 80.198 | 4 | < 0.001 |
| OD<sub>600</sub>=45 | Treatment x sex | 21101 | 1.0625 | 1 | 0.30 |
| OD<sub>600</sub>=45 | Treatment x fly line | 21098 | 5.7909 | 4 | 0.21 |
| OD<sub>600</sub>=45 | Sex x fly line | 21094 | 6.6203 | 4 | 0.15 |
| OD<sub>600</sub>=45 | Treatment x sex x fly line | 21094 | 1.8525 | 4 | 0.76 |
| Random effect | Std dev | vial/block | 0.019 | |
**Table S2.** Summary of log<sub>10</sub> transformed bacterial load data after exposure to \( OD\textsubscript{600}=25 \) of oral *P. entomophila* infection for male and female wildtype \( w^{1118} \) and mutant flies, analysed using non-parametric one-way ANOVA (Kruskal–Wallis test) at 3 different timepoints (0-15 minutes, 24-hours and 96-hours following oral infection)

| 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 |
| **24-hours** |        |           |     |        |
| Female  | dcy \( w^{1118} \)  | 24.30      | 5.388| <0.001 |
|         | upd-3 \( w^{1118} \) | 22.23      | 4.93| <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 |
| **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 |