# Negative regulation of IMD contributes to disease tolerance during systemic bacterial infection in *Drosophila*

Arun Prakash*, Katy M. Monteith, Pedro F. Vale*

Institute of Evolutionary Biology
School of Biological Sciences
University of Edinburgh
Edinburgh EH9 3FL

*Correspondence
arun.prakash@ed.ac.uk
pedro.vale@ed.ac.uk

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**Abstract**

Disease tolerance is an infection phenotype where hosts show relatively high health despite harbouring elevated pathogen loads. Compared to the mechanisms of immune clearance our knowledge of the mechanisms underlying increased tolerance remains incomplete. Variation in the ability to reduce immunopathology may explain why some hosts can tolerate higher pathogen burdens with reduced pathology. Negative immune regulation would therefore appear to be a clear candidate for a mechanism underlying disease tolerance but this has not been tested directly for bacterial infections. Here, we examined how the negative regulation of the immune deficiency (IMD) pathway affects disease tolerance in *Drosophila melanogaster* when infected with the gram-negative bacterial pathogen *Pseudomonas entomophila*. We find that $UAS^{RNAi}$-mediated reduced expression of the negative regulators of IMD (*pirk* and *caudal*) severely reduced the ability to tolerate infection in both males and females across a wide range of infectious doses. While flies unable to regulate the IMD response exhibited higher expression of antimicrobial peptides and lower bacterial loads as expected, this was not accompanied by a proportional reduction in mortality. Instead, tolerance (measured as fly survival relative to its microbe load) was drastically reduced, likely due to the combination of increased immunopathology and cytotoxicity of elevated AMP expression. Our results therefore highlight that in addition to regulating an efficient pathogen clearance response, negative regulators of IMD also contribute to disease tolerance.

39 **Introduction**

40 To survive infection hosts must not only identify and eliminate the pathogen but must also reduce
41 infection-induced tissue damage. Tissue damage may arise either directly from pathogen growth
42 and secreted virulence factors, or as a negative side-effect of the immune response in the form of
43 immunopathology (Graham, Allen, and Read 2005; Sears et al. 2011; Badinloo et al. 2018). For
44 example, the upregulation of antimicrobial peptides (AMPs) after pathogenic infection (Badinloo
45 et al. 2018; De Gregorio et al. 2002; Zerofsky et al. 2005), the accumulation of toxic/damage
46 molecules such as reactive oxygen species (*ROS*) (Han and Ulevitch 2005; Schneider 2007;
47 Lambeth 2007), and the phenoloxidase (*PO)* response (a fast-acting immune effector in insects)
48 are all known to cause tissue damage (Sadd and Siva-Jothy 2006; Cerenius, Lee, and Söderhäll
49 2008; Khan, Agashe, and Rolff 2017). Overall, the fine balance between clearing pathogens and
50 avoiding tissue damage requires tightly regulated induction and resolution of immune responses
51 (Sears et al. 2011; Soares, Gozzelino, and Weis 2014).

52

53 Understanding the distinct pathogen- or host-derived causes of tissue damage is important not
54 only from a therapeutic perspective (Vale, Fenton, and Brown 2014; Vale et al. 2016; Louie et al.
55 2016), it also offers important mechanistic explanations for the widely variable infection
56 phenotypes observed between individuals (Hill 1998; Råberg, Graham, and Read 2009; Kutzer and
57 Armitage 2016). The field of host-pathogen evolutionary ecology typically describes individuals
58 with low pathogen loads and relatively high health during infection as 'resistant' because they are
59 able to clear - or otherwise quantitatively reduce - the establishment and growth of pathogens
60 within the host (Hill 1998; Råberg, Graham, and Read 2009; Lazzaro, Sceurman, and Clark 2004;
61 Malo and Skamene 1994). Other infection phenotypes are more difficult to explain as the
62 consequence of immune clearance, such as the observation that some individuals exhibit low levels
63 of pathology (measured as either mortality or physiological performance), despite harbouring
64 relatively high pathogen loads (Råberg, Graham, and Read 2009; Kutzer and Armitage 2016;
65 Raberg, Sim, and Read 2007; Ayres and Schneider 2012). Instead, tolerating higher pathogen loads
66 is more likely to be under the control of mechanisms that prevent, limit or repair tissue damage
67 (Soares, Gozzelino, and Weis 2014; Vale, Fenton, and Brown 2014; Soares, Teixeira, and Moita
68 2017; R. Martins et al. 2019). However, compared to the detailed knowledge of immune clearance
69 mechanisms we associate with resistance, our knowledge of the specific mechanisms underlying
70 increased tolerance remains limited (Kutzer and Armitage 2016). Previous work in *Drosophila* has
71 employed GWAS and transcriptomics to uncover several candidate genes associated with disease

72 tolerance, including many that do not have an obvious immune related function such as *CrebA*
73 (Troha et al. 2018), *grainyhead and debris buster* (Howick and Lazzaro 2014), *dFOXO* (Dionne et al.
74 2006; Lissner and Schneider 2018). Curiously, genes related to canonical immune pathways are
75 conspicuously absent from these genome-wide screens of tolerance, even though we would expect
76 the regulation of inflammation to be a key mechanism underlying disease tolerance (Sears et al.
77 2011; Vale, Fenton, and Brown 2014; Ayres and Schneider 2012; Merkling et al. 2015).

78

79 In the present work, we take a more targeted approach to specifically investigate if the negative
80 regulation of immune responses has any measurable effect on the phenotype of disease tolerance.
81 We focused on the immune deficiency (IMD) pathway, one the best described immune signalling
82 pathways in *Drosophila* (Leulier et al. 2003; Bruno Lemaitre and Hoffmann 2007; Myllymäki,
83 Valanne, and Rämet 2014). Following infection, DAP-type peptidoglycans from gram-negative
84 bacteria are recognised by the pathogen-sensing receptors *PGRPs (LC* and *LE)* (Vodovar et al.
85 2005; Dieppois et al. 2015). These receptors then recruit the adaptor molecule IMD and activate
86 the intracellular signalling cascade that leads to the activation of a *NF-κB relish* (see **Fig**. 1) also
87 reviewed in (Myllymäki, Valanne, and Rämet 2014; Kuraishi, Hori, and Kurata 2013). Upon
88 activation, *relish* translocates to the nucleus and induces transcription of a set of IMD-responsive
89 AMPs. Minutes after pathogen recognition, a cocktail of AMPs such as *Diptericins, Attacins* and
90 *Cecropins* is secreted by the fat body (Bruno Lemaitre and Hoffmann 2007; B. Lemaitre, Reichhart,
91 and Hoffmann 1997). *Relish* also induces the transcription of negative regulators of IMD (*pirk*,
92 *caudal, PGRPs-LB, LC, LE*,) which ensures an appropriate level of immune response, while
93 avoiding immunopathology (Lee and Ferrandon 2011; Kleino and Silverman 2014). *Pirk*, for
94 example, interferes with the interaction of *PGRP-LC* and *-LE* with the molecule IMD, and limits
95 the activation of the IMD pathway (see **Fig**. 1) (Aggarwal et al. 2008; Kleino et al. 2008; Basbous
96 et al. 2011). Similarly, the homeobox protein *caudal* downregulates the expression of AMPs in flies
97 (Ryu et al. 2008). Taken together, the negative regulation of IMD-pathway ensures an appropriate
98 level of immune response and enables individuals to reduce immune activity in response to
99 reductions in bacterial numbers (Lee and Ferrandon 2011; Kleino et al. 2008; Paredes et al. 2011).

100

101 We measured disease tolerance during systemic infection with the gram-negative bacterium
102 *Pseudomonas entomophila* using *Drosophila* lines where individual components of the IMD-pathway
103 were knocked down using $UAS^{RNAi}$ including (i) the transcription factor- *relish*, its negative

regulators (ii) *pirk* and (iii) *caudal*, and (iv) a major IMD-responsive AMP *Diptericin-B*, because of substantial evidence that *Diptericins* are very effective during defense against Gram-negative bacteria (Bruno Lemaitre and Hoffmann 2007; Wicker et al. 1990). Given the widespread evidence of sex differences in immunity and response to infection, reviewed in (Klein and Flanagan 2016; Belmonte et al. 2020), we also specifically tested whether female and male flies differ in their ability to tolerate bacterial infection.

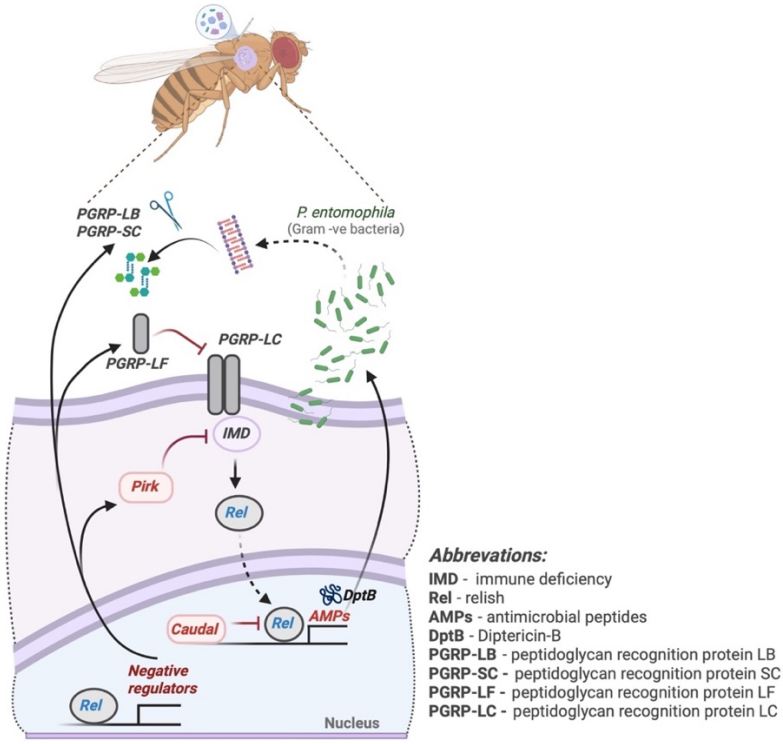



Figure 1: A simplified version of *Drosophila* IMD (immune deficiency) pathway activated by the DAP type pedptidoglycan following the gram-negative bacterial infection (*Pseudomonas entomophila*). These receptors then recruit the adaptor molecule Imd and activate the intracellular signalling cascade that leads to the activation of a NF-κB *relish*. Upon activation, relish translocates to the nucleus and induces transcription of a set of cocktail of IMD-responsive AMPs such as *Diptericins, Attacins* and *Cecropins*, minutes after pathogen recognition. The negative regulators of IMD such as *pirk, caudal, PGRPs-LB* ensure an appropriate level of immune response, while avoiding immunopathology

## Materials and methods

*Fly strains and husbandry*: We used the following fly lines: UAS-*relish*$^{RNAi}$ (*Bloomington stock# 33661*), UAS-*caudal*$^{RNAi}$ (*Bloomington stock# 57546*), UAS-*pirk*$^{RNAi}$ (*Bloomington stock# 67011*) and UAS-*DiptericinB*$^{RNAi}$ (*Bloomington stock# 28975*) also see Supplementary Information **Table** S1 for details about fly genotypes. All fly lines were obtained from the Bloomington *Drosophila* stock centre at

125 the Indiana University. Each of the transgenic fly line contains a homologous hairpin under the
126 control of the Upstream Activating Sequence ($UAS^{RNAi}$) to a Gal4 temperature-sensitive
127 transcriptional activator (*Bloomington stock# 67065*). This fly line drives the Gal4 expression in the
128 entire body of fly and contains a temperature sensitive Gal80, which inhibits Gal4 at the permissive
129 temperature (25℃). All fly stocks were reared in plastic vials (12 ml) on a standard Lewis medium
130 at 25±2℃ with a 12 hour light: dark on 14 days cycle (by transferring flies into new food vials). All
131 the crosses were made at 25℃ and after eclosion, offspring were transferred to agar vials (12 flies
132 per vial) at the non-permissive temperature (29℃) for two days to facilitate silencing of the
133 candidate genes until experimental day.

134

135 *Bacterial culture preparation:* The gram-negative bacterium *Pseudomonas entomophila* has a broad host
136 range, infecting insects, nematodes, plants, and higher vertebrates (Dieppois et al. 2015). In flies,
137 *P. entomophila* infection results in pathology to the gut epithelium during systemic infections,
138 eventually causing death (Vodovar et al. 2005; Dieppois et al. 2015; Troha and Buchon 2019). To
139 obtain pure cultures, frozen bacterial stock cultures (-70℃) were streaked onto fresh LB agar plates
140 and single colonies were inoculated into 15ml LB broth and incubated overnight at 37°C with
141 shaking at 120rpm (revolutions per minute). The overnight cultures were diluted 1: 100 into fresh
142 LB broth of 15ml and incubated again at 37°C with shaking at 120rpm. At the mid-log phase
143 (optical density $OD_{600}$ = 0.75), we harvested the bacterial cells by centrifugation at 5000 rpm for 5
144 min at 4°C and re-suspended the bacterial pellet in 1XPBS (phosphate buffer saline) solution. The
145 final inoculum was adjusted to $OD_{600}$ = 1.0, and this was the starting bacterial inoculum used for
146 all flies for systemic infection.

147

148 *Gene expression:* We quantified the expression of major IMD-responsive AMPs namely, *diptericin*
149 *(Dpt), attacin-C AttC), cecropin-A1 (CecA1*), by quantitative real-time polymerase chain reaction
150 (qRT-PCR). We randomly selected a subset of IMD-pathway mutants and functional flies both
151 males and females at 8 hours following infection for RNA extraction, we included 15 flies i.e., 5-7
152 replicates of 3 flies pooled together for each treatment (Knockdown and functional flies) and
153 sexes. We first homogenised whole flies (3 in group) using sterile micro-pestles into 1.5µl
154 microcentrifuge tubes containing 80µL of TRIzol reagent (Invitrogen, Life Technologies). The
155 tubes containing the fly homogenate were kept frozen at -70°C until RNA extraction. We

156 performed mRNA extractions using the standard phenol-chloroform method and included a
157 DNase treatment (Ambion, Life Technologies). We confirmed the purity of eluted samples using
158 a Nanodrop 1000 Spectrophotometer (version 3.8.1) before proceeding with RT (reverse
159 transcription). The cDNA was synthesized from 2µL of the eluted RNA using M-MLV reverse
160 transcriptase (Promega) and random hexamer primers, and then diluted 1:1 in nuclease free water.
161 We then performed quantitative RT-PCR (qRT-PCR) on an Applied Biosystems StepOnePlus
162 machine using Fast SYBR Green Master Mix (Invitrogen Ltd.) using a 10µL reaction containing
163 1.5L of 1:1 diluted cDNA, 5µL of Fast SYBR Green Master Mix a 3.5µL of a primer stock
164 containing both forward and reverse primer at 1µM suspended in nuclease free water (final
165 reaction concentration of each primer being 0.35µM). For each cDNA sample, we performed two
166 technical replicates for each set of primers and the average threshold cycle (Ct) was used for the
167 analysis. We obtained the AMP primers from Sigma-Aldrich Ltd (see supplementary **Table** S2 for
168 information about primers). We optimised the annealing temperature ($T_a$) and the efficiency (Eff)
169 of the *Dpt* primer pair was calculated by 10-fold serial dilution of a target template (each dilution
170 was assayed in duplicate); *Dpt*: $T_a$= 59 °C, Eff= 102%; *AttC*: $T_a$= 60 °C, Eff= 94%; *CecA1*: $T_a$=
171 59.5 °C, Eff= 109%.

172

173 *Fly survival following bacterial infection:* To test the impact of *Pseudomonas entomophila* infection on fly
174 survival, we challenged experimental flies to 4 different bacterial concentrations or infection doses
175 ranging between (I) ~650 cells (II) ~1400 cells (III) ~2000 cells (IV) ~2900 cells/µl of *P.*
176 *entomophila* culture, obtained by serial dilution. Flies (n=10-18 fly vials /sex/infection dose/fly line)
177 were infected systemically by intra-thoracic pricking (Khalil et al. 2015) with a needle immersed in
178 bacterial suspension under minimal $CO_2$ anaesthesia. The effect of injury caused by pricking was
179 controlled by including mock infections performed using a needle dipped in sterile 5% sucrose
180 solution. We observed the mortality for 10 days post-infection every 3 hours (±0.5h) for the first
181 2 days (between 6 am and 11 pm) and every 12 hours for the following 7 days.

182

183 *Quantification of bacterial load following bacterial infection:* During the survival assay, flies were randomly
184 sampled (10-18 live fly vials/sex/infection dose/fly line) and the bacterial load was quantified
185 around 8 hours following infection. Initially, flies (group of 3) were transferred to a 1.5ml
186 microcentrifuge tubes and surface sterilized by adding 70% ethanol for 30-60 seconds to ensure

187 we counted only colony forming units (CFUs) inside the flies. Ethanol was then removed by briefly
188 washing flies twice with sterile distilled water. To ensure that this method was efficient in surface
189 cleaning flies, we plated 4µl of the second wash on LB agar (from random flies) ensuring that the
190 flies are free from surface bacteria (i.e., no viable CFUs). We then placed in 1.5ml Eppendorf tubes
191 and added 100µl of LB broth to each tube and the flies were thoroughly homogenized using a
192 motorized pestle. We immediately plated this homogenate in serial dilutions ranging from 100 to
193 $10^{-6}$ using 1xPBS (phosphate buffer) on LB agar plates and incubated at 30°C for about 18 hours
194 after which viable colonies were counted manually (Siva-Jothy et al. 2018).

195

196 **Statistical analysis**

197 1. *Gene expression*: We analysed the gene expression data by first calculating the ΔCT value
198 (Livak and Schmittgen 2001) for the expression of a gene of interest relative to the
199 reference house-keeping gene rp49 (Gupta and Vale 2017). We then used analysis of
200 variance (ANOVA) model to analyse normally distributed data, i.e., to test for the effects
201 of the experimental treatment on the ΔCT values of whether the AMPs expression differed
202 significantly between knockdown and fully functional flies of each IMD-pathway fly line.
203 We used the R package 'ggplot2' for graphics and data visualization (Wickham 2016; Bunn
204 and Korpela, 2019)

205

206 2. *Bacterial load*: We then quantified differences in bacterial load following bacterial infection
207 by analysing the bacterial measurement ($\log_{10}$) around 8 hours following *P. entomophila*
208 infection. We found that residuals of bacterial load data were normally distributed when
209 tested with Shapiro-Wilks test and hence we used ANOVA and analysed the bacterial load
210 data by fitting 'knockdown' (functional flies and IMD-pathway knockdown flies) and 'sex'
211 as categorical fixed-effects, while infection dose (4 different infectious doses of *P.*
212 *entomophila*) and 'vials' (replicates) as a random-effects for each of the fly lines (functional
213 flies and IMD-pathway knockdown flies).

214

215 3. *Survival post-infection*: We analysed the survival data after *P. entomophila* bacterial infection for
216 all the IMD-pathway mutants by a mixed effects Cox model using the R package 'coxme'

(Therneau 2015) for both males and females respectively. In each case, we specified the model as: survival ~ knockdown *(functional and knockdown)* x sex *(male and female)* x dose *(4 concentrations of P. entomophila)* (1|vial *(n replicates)*), with 'knockdown' and 'sex' as fixed effects, and vials as a random effect for all the fly lines (IMD-pathway mutants). We fit separate models for each fly lines during *P. entomophila* infections, since they were assayed on different days. Next, to understand how different knockdown flies (i.e., having fully functional vs. knockdown of IMD-pathway) differ in survival to bacterial infections in detail, we also analysed survival data for *P. entomophila* infections for each of the IMD-pathway mutant/fly lines separately using Cox Proportional Hazard survival analysis. We estimated the impact of harbouring IMD-pathway knockdowns as the hazard ratio of IMD-knockdowns vs. fully functional fly lines. A hazard ratio significantly greater than one indicates higher risk of mortality in the IMD-mutants relative to fully functional individuals; hence, a significant impact on fly's survival.

4. *Measuring disease tolerance*: Finally, to understand how the negative immune regulation contribute to disease tolerance during bacterial infections we plotted tolerance by calculating (median lifespan/mean bacterial load) for male and female flies (i.e., flies with functional and knockdown IMD pathway regulators) after bacterial exposure with 4 different infection doses. Tolerance is usually measured as a reaction-norm or slope which could be either linear or non-linear, where the pathogen burden is plotted on the x-axis and health or fitness is plotted on the y-axis (Raberg, Sim, and Read 2007; Ayres and Schneider 2012; Medzhitov, Schneider, and Soares 2012; Louie et al. 2016; Gupta and Vale 2017; Oliveira, Bahia, and Vale 2020). This standard reaction norm approach that measures tolerance as the rate of health decrease for increasing microbe loads is only statistically sound when mortality and microbe loads measurements are taken from the same individual or from the same group of replicate individuals. Given the destructive sampling required to measure microbe loads in flies we chose instead to use another commonly used metric of tolerance using the average mortality of flies of a given line relative to the average bacterial load of that line, see for example (Adelman et al. 2013; Knutie et al. 2017; Cornet et al. 2014). This is sometimes referred to as 'point' tolerance and is intuitive to interpret: relative to a given microbe load, does one line die more (less tolerant) or less (more tolerant) (Little et al. 2010).

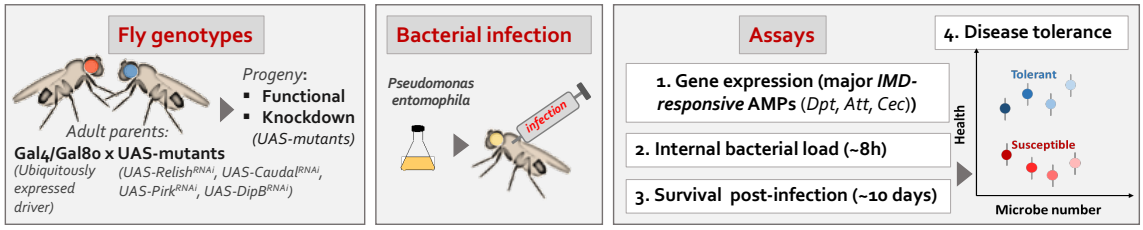



Figure 2: Design of experiments to assay **(1)** gene expression of the major IMD-regulated AMPs (n=15 flies; 5-7 replicates of 3 flies pooled together for each treatment, sex and fly line combination) **(2)** internal bacterial load (n=10-18 fly vials /sex/infection dose/fly line) and **(3)** survival following systemic bacterial infection with *Pseudomonas entomophila* n=10-18 fly vials /sex/infection dose/fly line) to test impact of the negative regulation of IMD pathway on phenotype of disease tolerance **(4)**.

## Results and discussion:

1. Loss of IMD negative regulation leads to increased AMP expression and decreased bacterial load

We first began by quantifying major IMD-regulated AMP expression (*AttC, CecA1* and *Dpt*) following infection with *P. entomophila* in both male and female flies (**Fig. 3A, Table-S3)**. As expected, *relish* knockdown flies had lower AMP expression while loss of negative regulators *caudal* and *pirk* resulted in higher AMP expression (**Fig. 3A, Table-S3)**. Next, we wanted to test whether AMP expression correlated with the bacterial replication in flies. Consistent with their AMP expression, both male and female flies with reduced negative regulation (*caudal* and *pirk*) exhibited a reduction in bacterial numbers while flies lacking *relish* exhibited higher levels of bacterial numbers (compare **Fig. 3A** gene expression and **Fig. 3B** bacterial load data), clearly indicating the expected importance of AMPs in defence against this bacterial pathogen (**Fig. 3B, Table-S4)**.

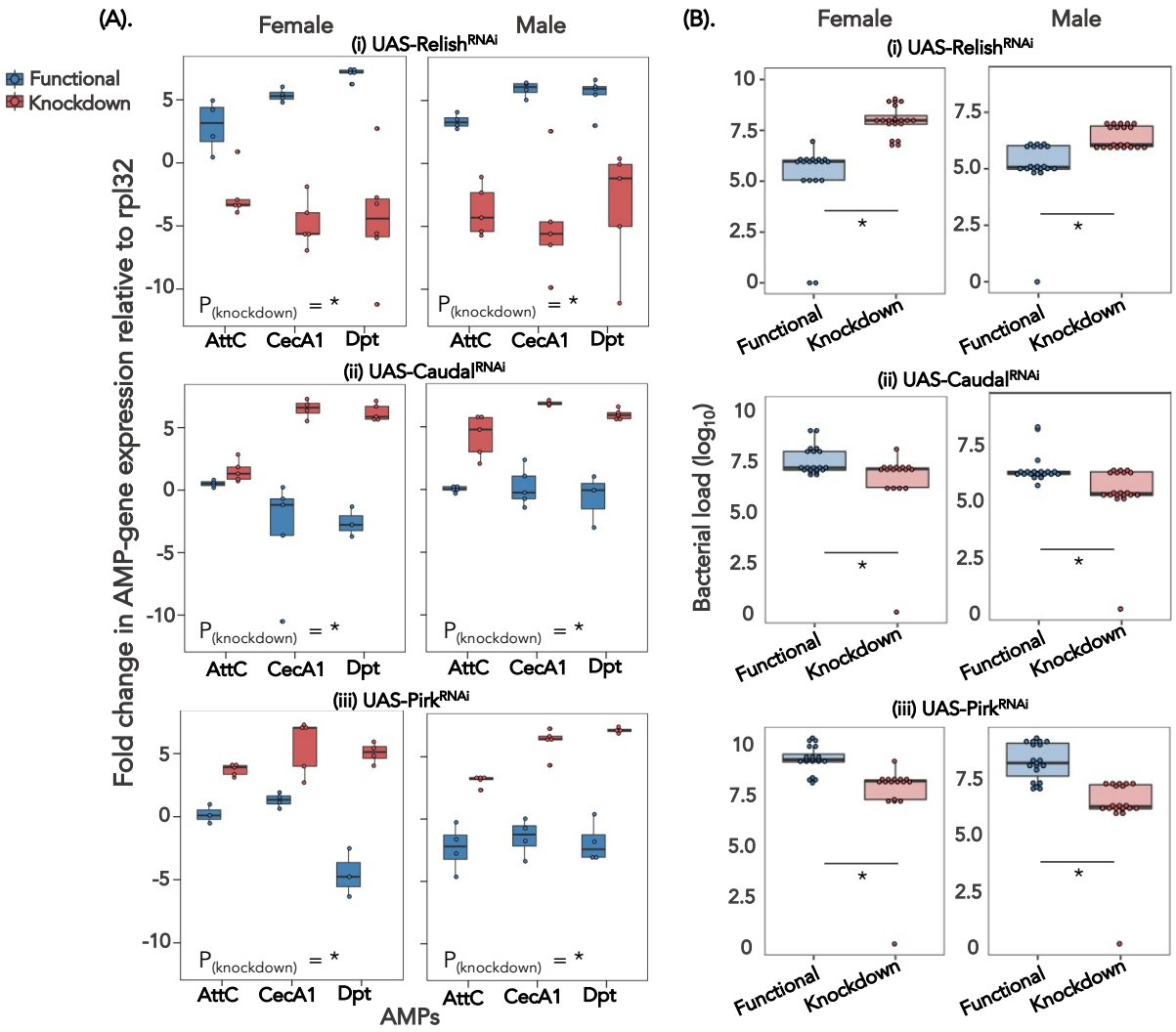



Figure 3: **(A)** Quantification of major IMD-responsive AMPs by qRT-PCR (n=15 flies/sex/treatment) for IMD-pathway mutants and functional flies (males and females) around 8 hours following *P. entomophila* systemic infection (0.05 OD or ~2000 bacterial cells/µl). Each data point represents 5-7 replicates of 3 flies pooled together for each infection treatment, fly line and sex **(B)** internal bacterial load after 8 hours following infection (n=5 replicates, 3-pooled flies/sex/treatment). Asterisks inside the panels indicate significant differences in AMP expression and bacterial load between knockdown flies and flies with fully intact IMD functioning ($p<0.05$).

## 2. Flies with reduced expression of *pirk* or *caudal* show increased mortality despite having lower bacterial loads

In flies, systemic infection with *P. entomophila* results in severe pathology, eventually causing death (N. E. Martins et al. 2013). Induction of AMPs can occur as early as 2 hours after infection (B. Lemaitre, Reichhart, and Hoffmann 1997). *Relish* knockdown flies are known to be short-lived and also exhibit increased bacterial loads after infection. For instance, even a single *E. cloacae* cell

infection is sufficient to kill the fly (Hedengren et al. 1999). Given our observation that knocking down negative regulators of IMD resulted in an increase in AMP expression, we would expect mortality in these flies to be lower than controls if variation in mortality was mainly explained by pathogen clearance. However, while mortality was higher in *relish* and *DiptericinB* knockdowns as expected, we also observed higher mortality when knocking down the expression of both negative regulators, *pirk* and *caudal* (**Fig. 4, Table-S5)**, despite the increased expression of AMPs and their relatively lower bacterial load **(Fig. 3)**. This increased mortality of flies lacking negative regulators of IMD such as *pirk* and *caudal* has been observed previously (Kleino et al. 2008; Ryu et al. 2008; Morris et al. 2016) and suggests that mechanisms other than pathogen clearance actively contribute to the variation in mortality during systemic bacterial infection.

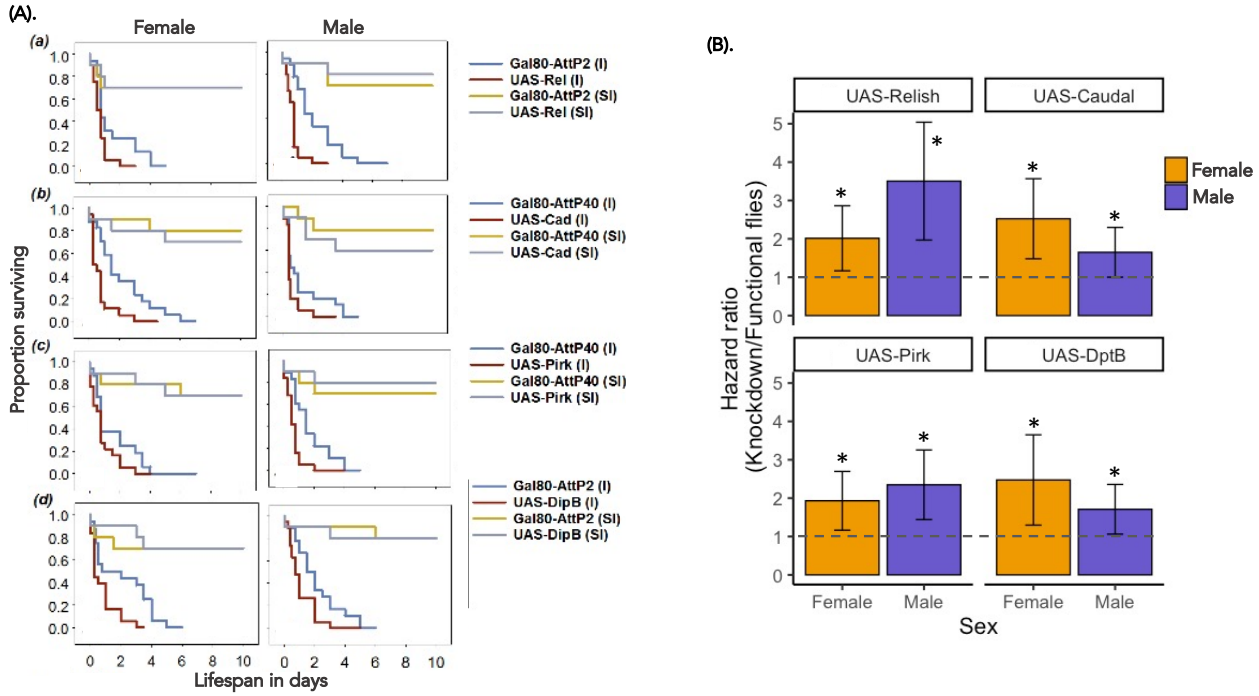
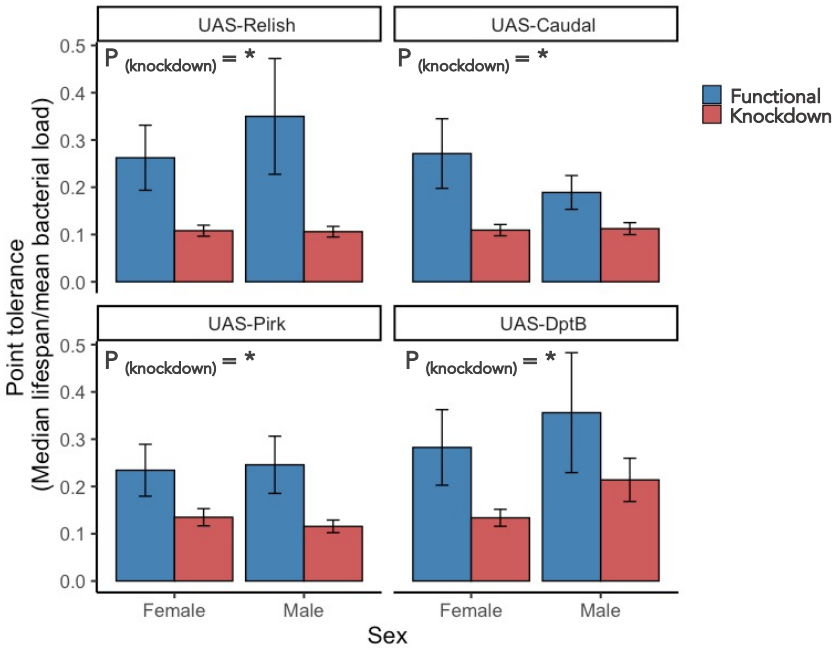



Figure 4: **(A)** Survival curves for male and female *UAS*$^{RNAi}$ flies (n=16-24 fly vial/treatment/sex/fly line) (a) *UAS-Relish*$^{RNAi}$ (b) *UAS-Caudal*$^{RNAi}$ (c) *UAS-Pirk*$^{RNAi}$ (d) *UAS-DptB*$^{RNAi}$ after systemic infection of *P. entomophila* (dose ~2000 cells/µl). **(B)** hazard ratio calculated from the survival curves. Asterisks in panel B indicate increased susceptibility of knockdown flies compared to functional flies ($p<0.05$).

3. Knocking down of *caudal* or *pirk* expression in flies results in reduced disease tolerance

By affecting the mortality of flies independently of their microbe loads, negative regulators of IMD would fit the functional definition of a tolerance mechanisms (Kutzer and Armitage 2016; Ayres and Schneider 2012; Lissner and Schneider 2018). To quantify the tolerance response of each fly

305 line more precisely, we measured the tolerance phenotype (measured as fly survival relative to its
306 bacterial load) of male and female flies differing in functional components of IMD-pathway (*relish,*
307 *caudal, pirk* and *DptB*). Overall, we found that flies without the negative IMD regulators *caudal* and
308 *pirk* showed reduced tolerance to systemic *P. entomophila* infection compared to flies with fully intact
309 immune system (**Fig. 5, Table-S6**). Flies without the transcription factor *relish* or the AMP *DptB*
310 also showed reduced tolerance, which may reflect feedbacks between increased pathogen growth
311 and decreased ability to limit damage when microbe loads get too high (that is, the per-pathogen
312 damage becomes higher in flies unable to clear infection in the first place) (Råberg 2014).




313

314 Figure 5: Disease tolerance in IMD-pathway mutants and fully functional flies. The relationship
315 between host (fly) survival and bacterial load (single infection dose) analysing using point tolerance
316 around 8 hours post *P. entomophila* infection for male and female flies. Each data represents 'point
317 tolerance', the ratio estimates calculated from median lifespan and average bacterial load for IMD-
318 pathway knockdown and flies with fully intact IMD functioning. Asterisks inside the panel
319 indicates significant difference in disease tolerance between knockdown flies and flies with fully
320 intact IMD functioning ($p<0.05$).

321

322 To further characterise the tolerance response of each IMD fly line across a wide range of pathogen
323 exposure, we infected the IMD-pathway mutants with different doses of *P. entomophila* (ranging
324 from ~650 bacterial cells infection dose to leading up to ~2900 cells). When IMD mutant flies
325 were exposed to very high bacterial infection, all fly lines showed reduced tolerance (**Fig. 6, Table-**
326 **S6**). We found similar effect for both females and males suggesting that overall tolerance effects

327 both sexes similarly when exposed to systemic *P. entomophila* bacteria. Altogether, our data show
328 that the negative regulators of IMD pathway play a major role in antibacterial protection and
329 mutant flies without negative regulators of IMD are less tolerant to infection.

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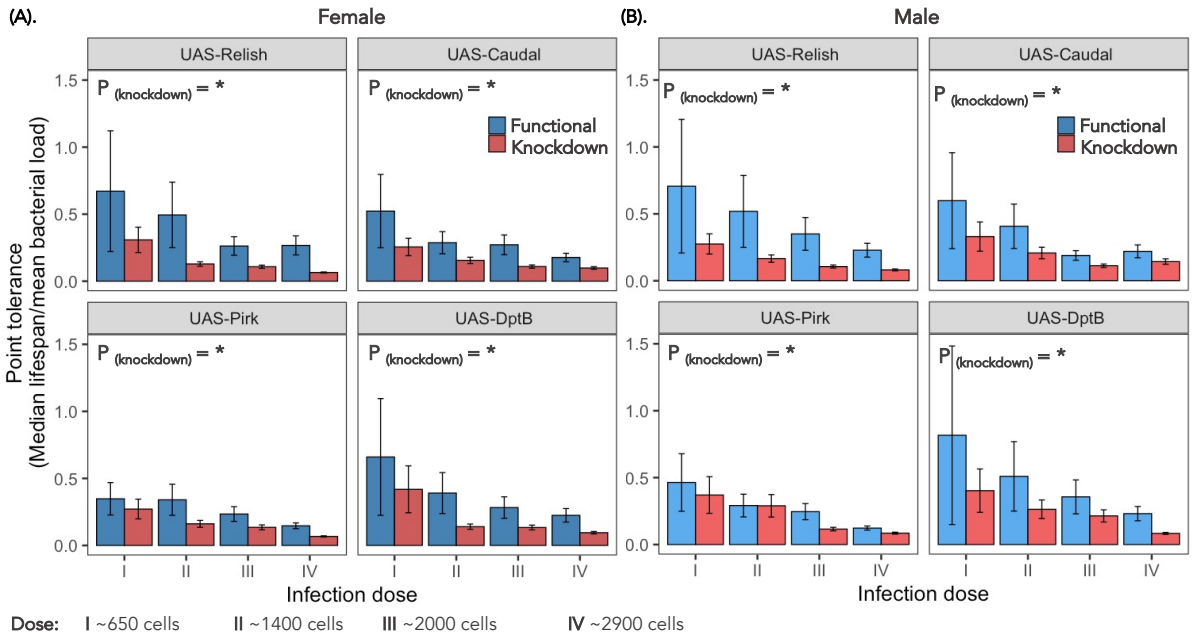


331

332 Figure 6: Disease tolerance in IMD-pathway mutants and fully functional flies. The relationship
333 between host (fly) survival and bacterial load of 4 infection doses [i.e., (I) ~650 cells (II) ~1400
334 cells (III) ~2000 cells (IV) ~2900 cells] of *P. entomophila*, analysing using point tolerance around 8
335 hours following *P. entomophila* infection for **(A)** male and **(B)** female flies. Each data represents
336 'point tolerance', the ratio estimates calculated from median lifespan and average bacterial load for
337 IMD-pathway knockdown and flies with fully functional IMD. Asterisks inside the panel indicates
338 significant difference in disease tolerance between knockdown flies and with fully intact IMD
339 functioning ($p<0.05$).

340

341 One likely cause of flies lacking negative IMD regulation still succumb to death despite reducing
342 pathogen burden is immunopathology due to continued immune activation. There is a precedent
343 for the effects of negative regulators on disease tolerance. For example, *G9a*, a negative regulator
344 of Jak/Stat, was identified as being important in tolerating *Drosophila C Virus* infections by reducing
345 immunopathology during the antiviral immunity (Merkling et al. 2015). During bacterial infections,
346 *PGRP-LB* functions as a negative regulator of the *Imd* pathway by regulating the IMD-responsive
347 AMP *Diptericin* (Zaidman-Rémy et al. 2006). More recently it has been shown that *PGRP-LB*
348 mutant flies experience sex-differences in survival despite undetectable differences in their
349 bacterial loads (Vincent and Dionne 2021). Though this study did not directly measure disease
350 tolerance (i.e., survival relative to microbe load), its findings are consistent with the phenotype of

351 disease tolerance. Here, we have explicitly analysed the tolerance response in the IMD mutants
352 and found a clear effect of its negative regulators on disease tolerance, though this was comparable
353 in both males and females. How these negative regulators contribute to disease tolerance is likely
354 linked to their role in reducing immunopathology. In the case of *caudal* and *pirk*, there is a possible
355 source of tissue damage through the cytotoxic effects of AMPs, because we observed higher AMP
356 expression in these mutants. AMPs are known to be harmful to both prokaryotic and eukaryotic
357 cells and tissues (Song et al. 2005; Paredes-Gamero et al. 2012) and overexpression of AMPs in
358 flies leads to cytotoxicity, causing neurodegeneration and age-related mortality (Badinloo et al.
359 2018; Cao et al. 2013). This would support the idea that elevated levels of several AMPs are
360 sufficient to cause cytotoxicity to cells/tissues, contributing to immunopathological consequences.

361 **Concluding remarks**

362 In the present work, we used a highly virulent fly pathogen (*P. entomophila*) and flies lacking various
363 components of IMD-signalling. Our data show that flies with reduced expression of the negative
364 regulators of IMD *pirk* and *caudal* experienced reduced tolerance despite having higher AMP
365 expression and reducing the bacterial numbers. We hypothesise that this is likely due to
366 immunopathology arising through the accumulation of cytotoxicity (AMPs and ROS). Our results
367 highlight that the negative regulators *caudal* and *pirk* are important not only in regulating pathogen
368 clearance by the IMD pathway, but also play a role in disease tolerance (**Fig. 7**). Uncovering the
369 role of these regulators in disease tolerance phenotype opens up the possibility of using them as
370 therapeutic targets where the aim is to enhance host disease tolerance (R. Martins et al. 2019; Vale
371 et al. 2016; Vale, Fenton, and Brown 2014; Schneider and Ayres 2008). Future work may also
372 investigate if natural genetic variation in disease tolerance can be explained by variation in negative
373 immune regulators.

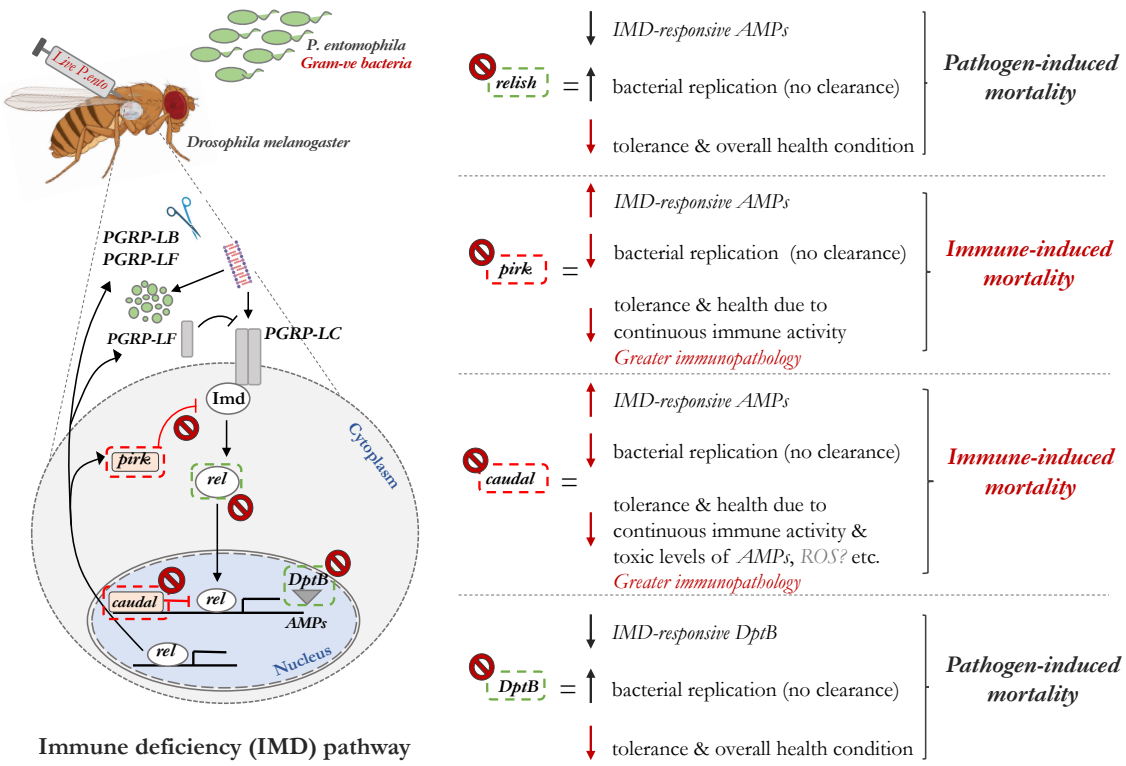


Figure 7: Summary highlighting that in addition to regulating an efficient pathogen clearance response, negative regulators of IMD also contribute to the phenotype of disease tolerance. Flies with reduced expression of the negative regulators *pirk* and *caudal* show reduced tolerance (overall health state) despite having higher AMP expression and reducing the bacterial numbers. Flies with reduced *relish* and *DptB* expression also exhibited reduced tolerance and survival mostly due to pathogen-induced mortality because of lower AMPs to target the bacterial replication.


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## Author contribution

A.P and P.F.V designed the experiments; A.P carried out experiments; A.P and K.M.M performed qPCR experiments; A.P and P.F.V analysed the data and wrote the manuscript.

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**Competing interests**

None.

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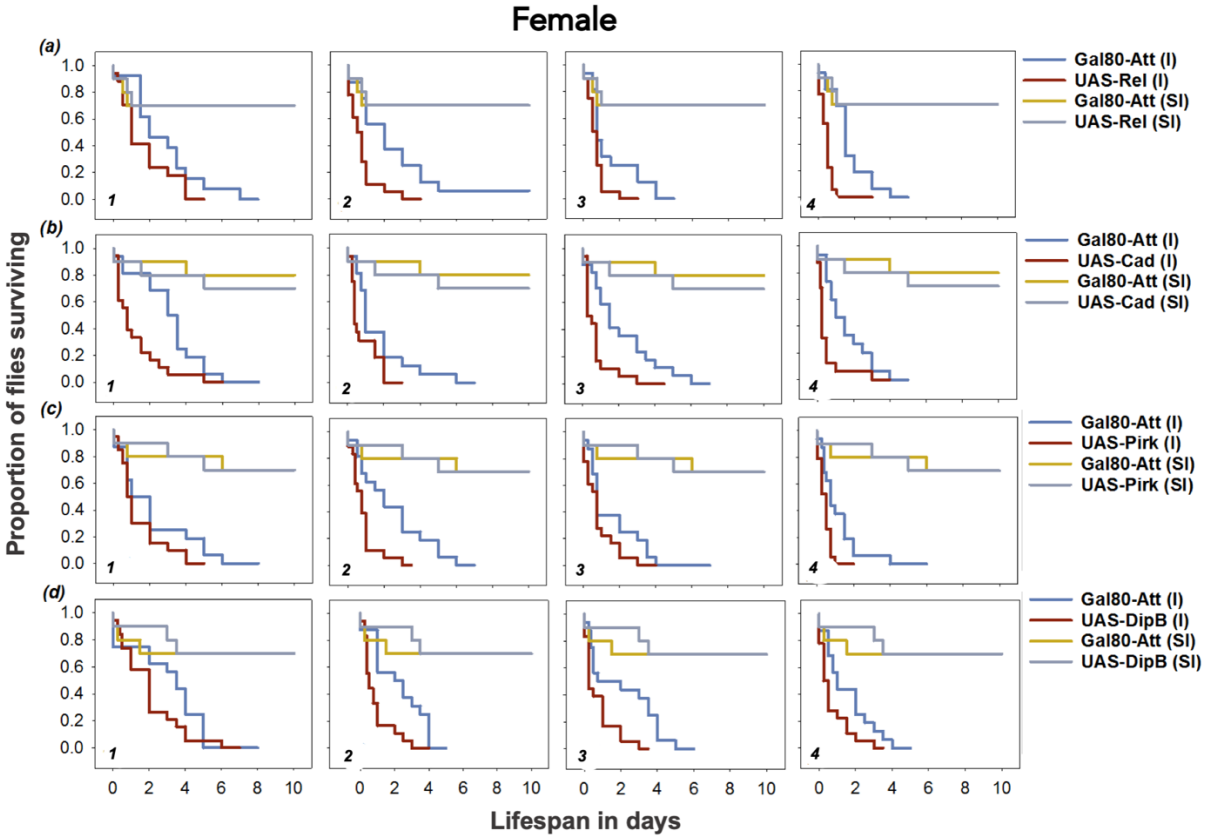
648 **Supplementary information**

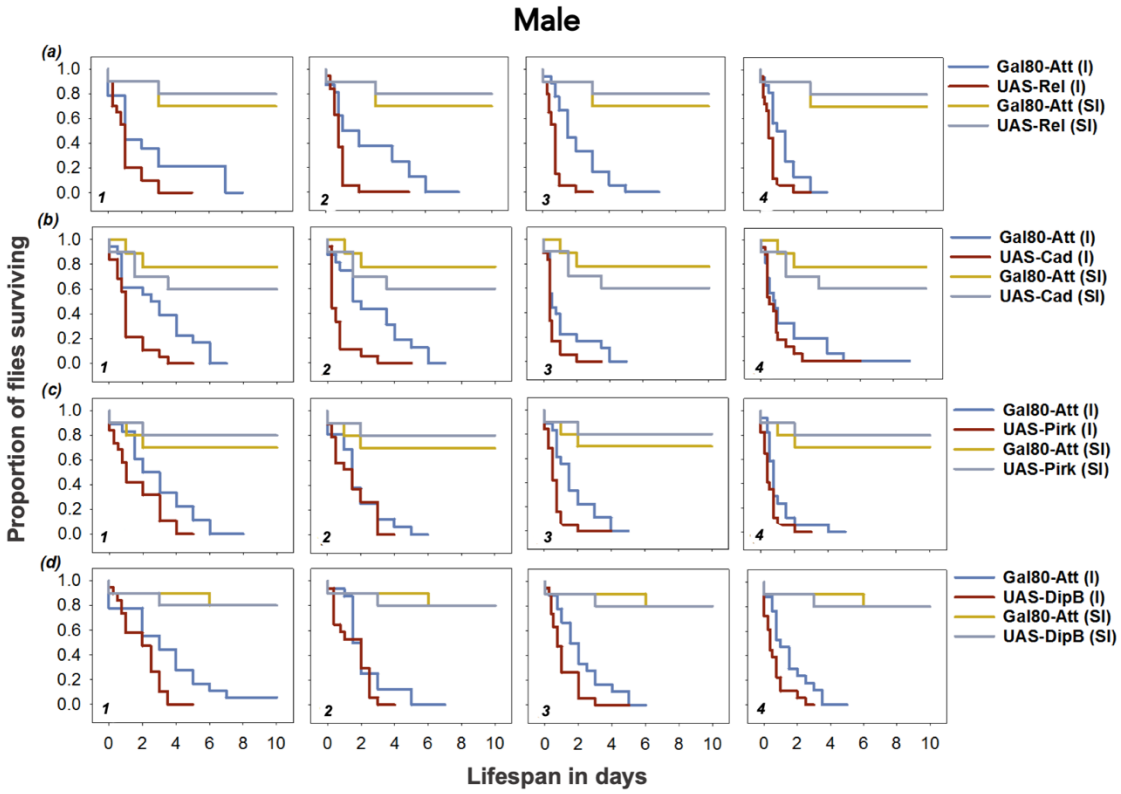
649 **Figure S1.** Survival curves of female flies of different IMD-pathway mutants (n=16-24
650 flies/treatment/sex/fly line) **(a)** *UAS-Relish*$^{RNAi}$ **(b)** *UAS-Caudal*$^{RNAi}$ **(c)** *UAS-Pirk*$^{RNAi}$ **(d)** *UAS-*
651 *DptB*$^{RNAi}$ during 4 concentrations of systemic *Pseudomonas entomophila* infection ranging between (1)
652 ~650 cells (2) ~1400 cells (3) ~2000 cells (4) ~2900 cells/µl of *P. entomophila* culture. Since all 4
653 infection concentrations were performed simultaneously for each of the fly line, we used a single
654 mock-controls (SI) across all 4 different infection doses.




655

656 **Figure S2.** Survival curves of male flies of different IMD-pathway mutants (n=16-24
657 flies/treatment/sex/fly line) **(a)** *UAS-Relish*$^{RNAi}$ **(b)** *UAS-Caudal*$^{RNAi}$ **(c)** *UAS-Pirk*$^{RNAi}$ **(d)** *UAS-*
658 *DptB*$^{RNAi}$ during 4 concentrations of systemic *Pseudomonas entomophila* infection ranging between (1)
659 ~650 cells (2) ~1400 cells (3) ~2000 cells (4) ~2900 cells/µl of *P. entomophila* culture. Since all 4
660 infection concentrations were performed simultaneously for each of the fly line, we used a single
661 mock-controls (SI) across all 4 different infection doses.

662




663

664 **Figure S3.** The estimated hazard ratios calculated from survival curves (**Fig. S1** and **S2**) for flies
665 with fully functional immune system and IMD-pathway mutants for both Sexes. The different
666 IMD-pathway mutants include **(a)** *UAS-Relish*$^{RNAi}$ **(b)** *UAS-Caudal*$^{RNAi}$ **(c)** *UAS-Pirk*$^{RNAi}$ **(d)** *UAS-*
667 *DptB*$^{RNAi}$. We used 4 different concentrations of systemic *P. entomophila* infection. A greater hazard
668 ratio >1 shown as horizontal dashed grey lines indicates higher susceptibility of IMD-mutant flies
669 than the functional flies to *P. entomophila* infection.
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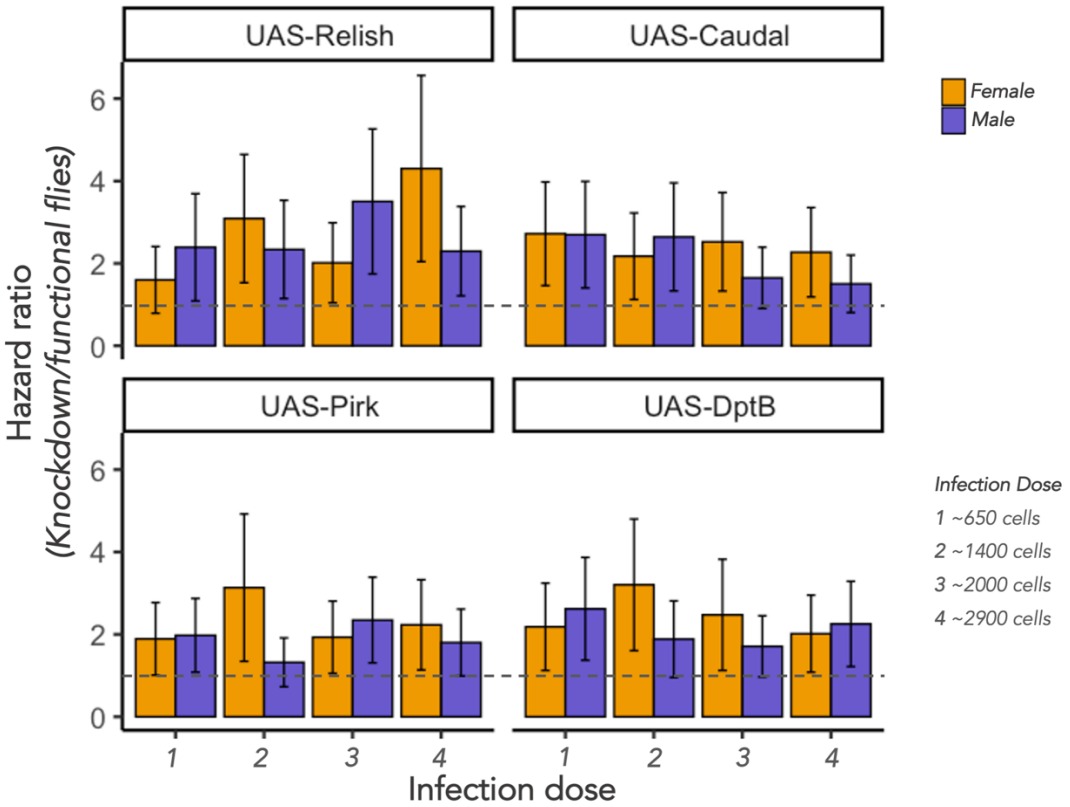
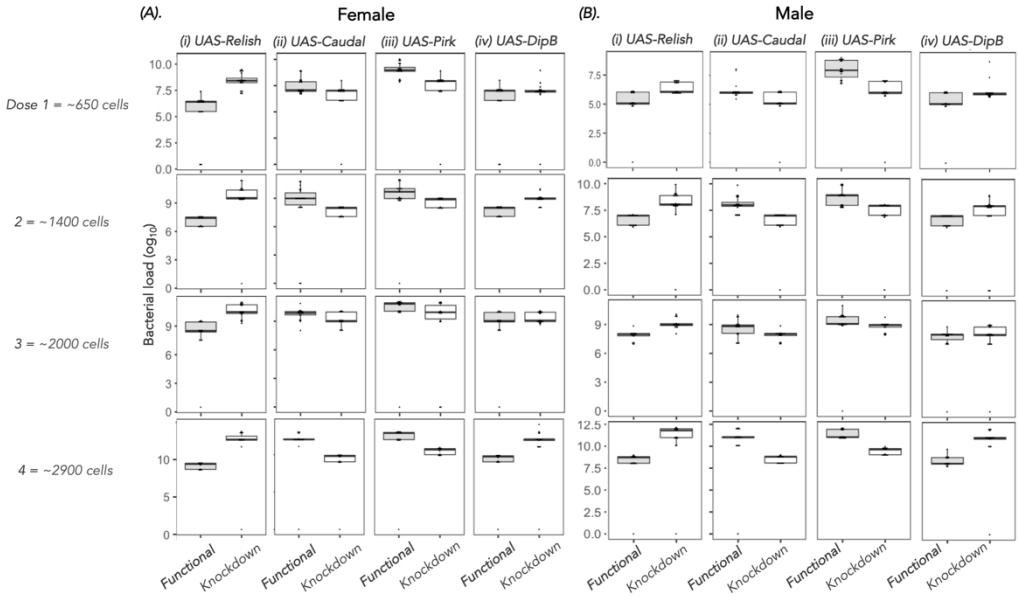



671
672

673 **Figure S4.** Bacterial loads around 24 hours after different concentrations of systemic *P.*
674 *entomophila* infection for **(A)** female and **(B)** male flies for IMD-pathway mutants **(i)** *UAS-*
675 *Relish*$^{RNAi}$ **(ii)** *UAS-Caudal*$^{RNAi}$ **(iii)** *UAS-Pirk*$^{RNAi}$ **(iv)** *UAS-DptB*$^{RNAi}$.




676
677

678 **Table S1:** Fly strains used in this study
679

| *Fly line* | *Bloomington stock #* | *Genotype* |
|---|---|---|
| UAS-*relish*$^{RNAi}$ | *33661* | *y[1]sc[*]v[1]; P{y[+t7.7]v[+t1.8]=TRiP.HMS00070}attP2* |
| UAS-*caudal*$^{RNAi}$ | *57546* | *y[1]sc[*]v[1];P{y[+t7.7] v[+t1.8]=TRiP.HMC04863}attP40*), |
| UAS-*pirk*$^{RNAi}$ | *67011* | *y[1]sc[*]v[1];P{y[+t7.7] v[+t1.8]=TRiP.HMS05477}attP40* |
| UAS-*Diptericin*B$^{RNAi}$ | *28975* | *y[1]v[1];P{y[+t7.7] v[+t1.8]=TRiP.HM05186}attP2* |
| (*UAS*$^{RNAi}$) ubiquitous driver | *67065* | *w*;P{UAS-3xFLAG.dCas9.VPR}attP40, P{tubP-GAL80*$^{ts}$*}10; P{tubP-GAL4}LL7/TM6B, Tb*$^{1}$ |

680
681
682 **Table S2**: Primers used in this study
683

| | *Obtained from* | *AMPs* | *Sequence* |
|---|---|---|---|
| *1.* | (Troha et al. 2018) | *Dpt*_Forward: | 5' GACGCCACGAGATTGGACTG 3' |
| | | *Dpt*_Rverese | 5' CCCACTTTCCAGCTCGGTTC 3' |
| | | *AttC*_Forward: | 5' TGCCCGATTGGACCTAAGC 3' |
| | | *AttC*_Reverse: | 5' GCGTATGGGTTTTGGTCAGTTC 3' |
| *2.* | (Loch et al. 2017) | *CecA1*_Forward | 5' TCTTCGTTTTCGTCGCTCTCA 3', |
| | | *CecA1*_Reverse | 5' ATTCCCAGTCCCTGGATT GTG 3'. |
| *3.* | (Gupta and Vale 2017) | *Rp49*_Forward: | 5' ATGCTAAGCTGTCGCACAAATG 3' |
| | | *Rp49*_Reverse: | 5' GTTCGATCCGTAACCGATGT 3' |

684

685 **Table S3:** Gene expression of major IMD-pathway regulated AMPs. Gene expression quantified
686 relative to the internal control gene rp49 was quantified in 5-6 replicate individuals (3 pooled
687 flies) of males and females exposed to *P. entomophila* systemic bacterial infection (dose 0.05 OD
688 or ~2000 bac. cells). Analysed using ANOVA and specified the model as gene expression ~
689 knockdown *(functional and knockdown)* x sex *(male and female)* x AMPs *(major IMD-regulated)* for each of the fly line (IMD-
690 mutants).
691

| *Genotype* | *Source* | *df* | *Sum Sq* | *F value* | *p* |
|---|---|---|---|---|---|
| *UAS-Relish*$^{RNAi}$ | Knockdown *(knockdown and functional flies)* | 1 | 1160.3 | 148.38 | <0.001 |
| | AMPs *(major IMD-responsive AMPs)* | 2 | 11.2 | 0.717 | 0.49 |
| | Sex | 1 | 0.4 | 0.051 | 0.82 |
| | Knockdown x AMPs | 2 | 49.1 | 3.139 | 0.05 |
| | Knockdown x Sex | 1 | 0.3 | 0.033 | 0.85 |
| | AMPs x Sex | 2 | 1.4 | 0.087 | 0.91 |
| | Knockdown x AMPs x Sex | 2 | 11.7 | 0.748 | 0.47 |
| *UAS-Caudal*$^{RNAi}$ | Knockdown | 1 | 460.4 | 159.2 | <0.001 |
| | AMPs | 2 | 10.6 | 1.835 | 0.17 |
| | Sex | 1 | 23.5 | 8.137 | <0.001 |
| | Knockdown x AMPs | 2 | 82.3 | 14.24 | <0.001 |
| | Knockdown x Sex | 1 | 1.1 | 0.394 | 0.53 |
| | AMPs x Sex | 2 | 4.4 | 0.770 | 0.46 |
| | Knockdown x AMPs x Sex | 2 | 25.2 | 4.365 | <0.001 |
| *UAS-Pirk*$^{RNAi}$ | Knockdown | 1 | 538.3 | 338.9 | <0.001 |
| | AMPs | 2 | 34.2 | 10.77 | <0.001 |
| | Sex | 1 | 0.0 | 0.000 | 0.99 |
| | Knockdown x AMPs | 2 | 47.1 | 14.82 | <0.001 |
| | Knockdown x Sex | 1 | 8.5 | 5.360 | <0.001 |
| | AMPs x Sex | 2 | 35.6 | 11.22 | <0.001 |
| | Knockdown x AMPs x Sex | 2 | 7.2 | 2.255 | 0.11 |

692
693 **Table S4:** Summary of $\log_{10}$ transformed bacterial load data after exposure to 4 different
694 concentrations (doses) of *P. entomophila* systemic infection, analysed using ANOVA by fitting
695 'Knockdown', 'Sex' and 'dose' as categorical fixed-effects and replicate 'vials' as a random-effects
696 for each of the genotype.

| *Genotype* | *Source* | *df* | *Sum Sq* | *F value* | *p* |
|---|---|---|---|---|---|
| *UAS-Relish*$^{RNAi}$ | Knockdown | 3 | 351.07 | 45.4374 | <0.001 |
| | Sex | 1 | 39.32 | 15.2654 | <0.001 |
| | Dose | 3 | 518.53 | 67.1097 | <0.001 |
| | Knockdown x Sex | 1 | 15.53 | 6.0297 | <0.001 |
| | Knockdown x dose | 3 | 14.43 | 1.8681 | 0.13 |
| | Sex x dose | 3 | 1.64 | 0.2120 | 0.88 |
| | Knockdown x Sex x dose | 1 | 3.72 | 1.4450 | 0.23 |
| | *Random effect* | *Std err* | | | |
| | *Vials* | *1.605* | | | |
| *UAS-Caudal*$^{RNAi}$ | Knockdown | 3 | 157.03 | 20.6574 | <0.001 |
| | Sex | 1 | 115.72 | 45.6679 | <0.001 |
| | Dose | 3 | 528.70 | 69.5504 | <0.001 |
| | Knockdown x Sex | 1 | 0.38 | 0.1497 | 0.69 |
| | Knockdown x dose | 3 | 27.01 | 3.5529 | 0.014 |
| | Sex x dose | 3 | 3.55 | 0.4665 | 0.70 |
| | Knockdown x Sex x dose | 1 | 1.10 | 0.4360 | 0.50 |
| | *Random effect* | *Std err* | | | |
| | *vials* | *1.592* | | | |
| *UAS-Pirk*$^{RNAi}$ | Knockdown | 3 | 164.02 | 18.3271 | <0.001 |
| | Sex | 1 | 98.90 | 33.1544 | <0.001 |
| | Dose | 3 | 407.46 | 45.5296 | <0.001 |
| | Knockdown x Sex | 1 | 2.57 | 0.8601 | 0.35 |

| | | | | | |
|---|---|---|---|---|---|
| | Knockdown x dose | 3 | 3.17 | 0.3541 | 0.78 |
| | Sex x dose | 3 | 2.17 | 0.2428 | 0.86 |
| | Knockdown x Sex x dose | 1 | 0.06 | 0.0215 | 0.88 |
| | *Random effect* | *Std err* | | | |
| | *vials* | *1.727* | | | |
| *UAS-DptB*$^{RNAi}$ | Knockdown | 3 | 128.94 | 13.2207 | <0.001 |
| | Sex | 1 | 87.43 | 26.8915 | <0.001 |
| | Dose | 3 | 615.60 | 63.1179 | <0.001 |
| | Knockdown x Sex | 1 | 0.44 | 0.1354 | 0.71 |
| | Knockdown x dose | 3 | 27.20 | 2.7885 | 0.04 |
| | Sex x dose | 3 | 7.48 | 0.7674 | 0.51 |
| | Knockdown x Sex x dose | 1 | 1.43 | 0.4411 | 0.50 |
| | *Random effect* | *Std err* | | | |
| | *vials* | *1.803* | | | |

**Table S5:** Summary of mixed effects Cox model, fitting the model to estimate response to *P. entomophila* infection in flies with fully intact immune system and flies with deficiencies in IMD-pathway. We used data from the individuals of 3-5-day adult males and females infected with 4 concentrations (doses) of *P. entomophila* for each fly lines (IMD-pathway mutants) and specified the model as: survival ~ Knockdown * Sex * dose (1|vial), with 'Knockdown' and 'Sex' as fixed effects, and 'vials' as a random effect. The table shows model output (ANOVA) for survival post-infection for flies with fully function immune system and defects in IMD functioning.

| ***Genotype*** | ***Source*** | ***Loglik*** | ***Chisq*** | ***df*** | ***p*** |
|---|---|---|---|---|---|
| *UAS-Relish*$^{RNAi}$ | Knockdown | 1344.5 | 33.429 | 1 | <0.001 |
| | Sex | 1344.5 | 0.0003 | 1 | 0.98 |
| | Dose | 1273.7 | 141.55 | 4 | <0.001 |
| | Knockdown x Sex | 1273.5 | 0.4192 | 1 | 0.51 |
| | Knockdown x dose | 1270.0 | 7.0930 | 4 | 0.13 |
| | Sex x dose | 1269.5 | 0.9557 | 4 | 0.91 |
| | Knockdown x Sex x dose | 1266.8 | 5.4275 | 4 | 0.24 |
| | *Random effect* | *Std dev* | | | |
| | *Vials* | *0.244* | | | |
| *UAS-Caudal*$^{RNAi}$ | Knockdown | 1337.6 | 28.624 | 1 | <0.001 |
| | Sex | 1337.5 | 0.0156 | 1 | 0.9 |
| | Dose | 1285.3 | 104.43 | 4 | <0.001 |
| | Knockdown x Sex | 1285.3 | 0.0005 | 1 | 0.98 |
| | Knockdown x dose | 1283.6 | 3.4040 | 4 | 0.49 |
| | Sex x dose | 1280.6 | 6.0031 | 4 | 0.19 |
| | Knockdown x Sex x dose | 1279.2 | 2.8850 | 4 | 0.57 |
| | *Random effect* | *Std dev* | | | |
| | *vials* | *0.02* | | | |
| *UAS-Pirk*$^{RNAi}$ | Knockdown | 1412.1 | 21.126 | 1 | <0.001 |
| | Sex | 1412.1 | 0.0001 | 1 | 0.99 |
| | Dose | 1357.4 | 109.30 | 4 | <0.001 |
| | Knockdown x Sex | 1356.6 | 1.6275 | 1 | 0.20 |
| | Knockdown x dose | 1355.3 | 2.5330 | 4 | 0.63 |
| | Sex x dose | 1353.9 | 2.9391 | 4 | 0.56 |
| | Knockdown x Sex x dose | 1352.3 | 3.2265 | 4 | 0.52 |
| | *Random effect* | *Std dev* | | | |
| | *Vials* | *0.14* | | | |
| *UAS-DptB*$^{RNAi}$ | Knockdown | 1386.1 | 29.146 | 1 | <0.001 |
| | Sex | 1386.0 | 0.2848 | 1 | 0.59 |
| | Dose | 1331.1 | 109.81 | 4 | <0.001 |
| | Knockdown x Sex | 1331.0 | 0.1187 | 1 | 0.73 |
| | Knockdown x dose | 1330.8 | 0.3249 | 4 | 0.98 |
| | Sex x dose | 1329.1 | 3.5419 | 4 | 0.47 |
| | Knockdown x Sex x dose | 1328.0 | 2.1198 | 4 | 0.71 |
| | *Random effect* | *Std dev* | | | |

| | | |
|---|---|---|
| | *Vials* | *0.15* |

705
706 **Table S6:** 'Point tolerance' for all doses - Summary of ANCOVA. To assess differences bacterial
707 loads following infection with 4 concentrations (dose) of *P. entomophila*, we analysed the average
708 bacterial load, measured around 8 hours following infection using ANCOVA and fitted
709 'Knockdown' and 'Sex' as categorical fixed effects and 'bacterial load' as continuous co-variate for
710 each fly line.
711
712

| ***Genotype*** | ***Source*** | ***df*** | ***F ratio*** | ***p*** |
|---|---|---|---|---|
| *UAS-Relish*$^{RNAi}$ | Knockdown | 1 | 17.584 | 0.003 |
| | Sex | 1 | 2.2509 | 0.17 |
| | Dose | 3 | 5.2387 | 0.02 |
| | Bacterial load | 1 | 0.0728 | 0.79 |
| | Knockdown * Bac. load | 1 | 13.549 | 0.006 |
| *UAS-Caudal*$^{RNAi}$ | Knockdown | 1 | 9.1734 | 0.01 |
| | Sex | 1 | 0.5894 | 0.46 |
| | Dose | 3 | 3.7512 | 0.059 |
| | Bacterial load | 1 | 0.0386 | 0.84 |
| | Knockdown * Bac. load | 1 | 5.9732 | 0.04 |
| *UAS-Pirk*$^{RNAi}$ | Knockdown | 1 | 25.071 | 0.001 |
| | Sex | 1 | 0.9570 | 0.35 |
| | Dose | 3 | 2.8131 | 0.10 |
| | Bacterial load | 1 | 8.6071 | 0.01 |
| | Knockdown * Bac. load | 1 | 0.1030 | 0.75 |
| *UAS-DptB*$^{RNAi}$ | Knockdown | 1 | 25.486 | 0.001 |
| | Sex | 1 | 0.0266 | 0.87 |
| | Dose | 3 | 10.069 | 0.004 |
| | Bacterial load | 1 | 4.2575 | 0.07 |
| | Knockdown * Bac. load | 1 | 17.282 | 0.003 |

713