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

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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* & *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.

Introduction

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

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

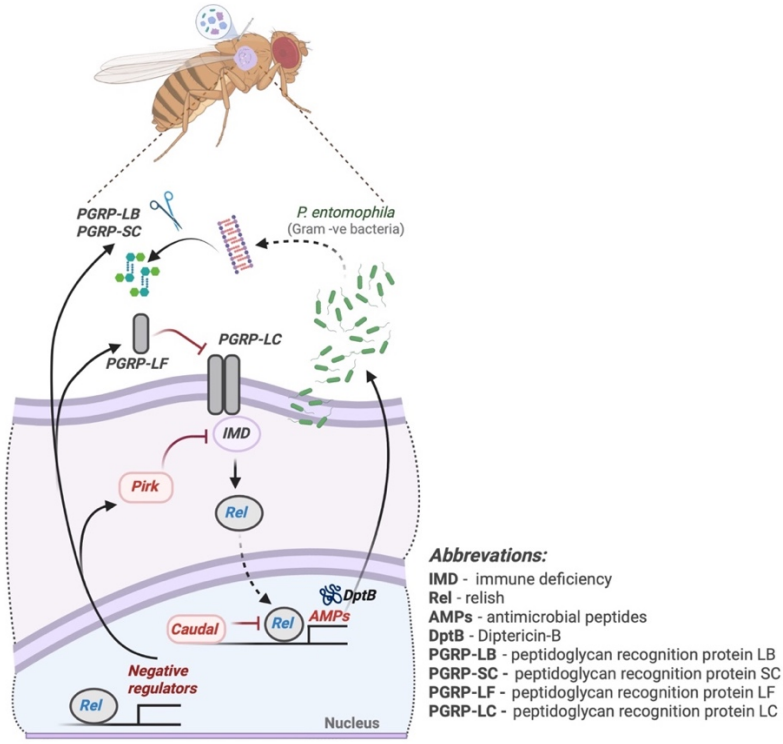
78

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 & LE)* (Vodovar et al. 2005;
85 Dieppois et al. 2015). These receptors then recruit the adaptor molecule IMD and activate the
86 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 &*
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 & -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

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



110

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

119

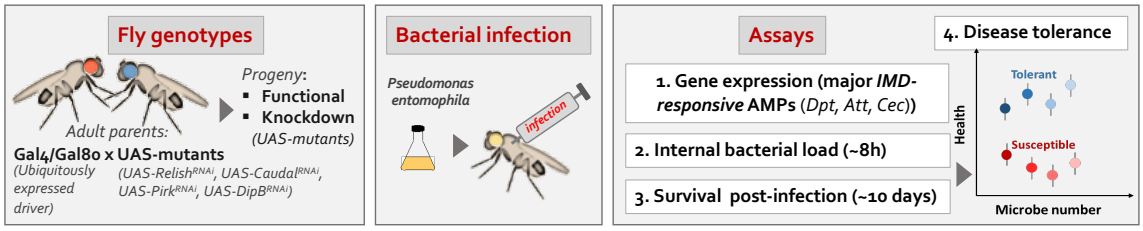
120 **Materials and methods**

121 Fly strains and husbandry: We used the following fly lines: UAS-*relish*^{RNAi} (*Bloomington stock#*
122 *33661*), UAS-*caudal*^{RNAi} (*Bloomington stock# 57546*), UAS-*pirk*^{RNAi} (*Bloomington stock# 67011*) & UAS-
123 *Diptericin*B^{RNAi} (*Bloomington stock# 28975*) also see Supplementary Information **Table** S1 for details
124 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±℃ 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 ~3 days after eclosion, offspring were transferred to agar vials
132 (~12 flies per vial) at the non-permissive temperature (29℃) for two days to facilitate silencing of
133 the candidate genes until experimental day.

134

135 Bacterial culture preparation: The gram-negative bacterium *Pseudomonas entomophila* has a broad
136 host range, infecting insects, nematodes, plants, and higher vertebrates (Dieppois et al. 2015). In
137 flies, *P. entomophila* infection results in pathology to the gut epithelium during systemic and oral
138 infections, eventually causing death (Vodovar et al. 2005; Dieppois et al. 2015; Troha and Buchon
139 2019). To obtain pure cultures, frozen bacterial stock cultures (-80℃) were streaked onto fresh LB
140 agar plates and single colonies were inoculated into 20 ml LB broth and incubated overnight at
141 37°C with shaking at 120 rpm (revolutions per minute). The overnight cultures were diluted 1: 100
142 into 500 ml of fresh LB broth and incubated again at 37°C with shaking at 120 rpm. At the mid-
143 log phase (optical density OD_{600} = ~0.75), we harvested the bacterial cells by centrifugation at
144 5000 rpm for 15 min at 4°C and re-suspended the bacterial pellet in 5% sucrose solution. The final
145 inoculum was adjusted to OD_{600} = 1.0, and this was the starting bacterial inoculum used for all
146 flies for systemic infection.

147 

148 Figure 2: Design of experiments to assay **(1)** gene expression of the major IMD-regulated AMPs
149 **(2)** internal bacterial load and **(3)** survival following systemic bacterial infection with *Pseudomonas*
150 *entomophila* to test impact of the negative regulation of IMD pathway on phenotype of disease
151 tolerance **(4)**.

152

153 *Gene expression:* We analysed the antimicrobial peptides (AMPs) gene expression data to examine
154 the effects of knocking down negative regulators of IMD-pathway on the fly's immune response.
155 We quantified the expression of major IMD-responsive AMPs namely, *diptericin (Dpt), attacin-C*
156 *AttC), cecropin-A1 (CecA1*), by quantitative real-time polymerase chain reaction (qRT-PCR). We
157 randomly selected a subset of IMD-pathway mutants & functional flies both males & females at
158 8h post-infection for RNA extraction, we included 15 flies i.e., 5-7 replicates of 3 flies pooled
159 together for each treatment (Knockdown & functional) and sexes. We first homogenised whole
160 flies (3 in group) using sterile micro-pestles into 1.5µl microcentrifuge tubes containing 80µL of
161 TRIzol reagent (Invitrogen, Life Technologies). The tubes containing the fly homogenate were
162 kept frozen at -70°C until RNA extraction. We performed mRNA extractions using the standard
163 phenol-chloroform method and included a DNase treatment (Ambion, Life Technologies). We
164 confirmed the purity of eluted samples using a Nanodrop 1000 Spectrophotometer (version 3.8.1)
165 before proceeding with RT (reverse transcription). The cDNA was synthesized from 2µL of the
166 eluted RNA using M-MLV reverse transcriptase (Promega) and random hexamer primers, and
167 then diluted 1:1 in nuclease free water. We then performed quantitative RT-PCR (qRT-PCR) on
168 an Applied Biosystems StepOnePlus machine using Fast SYBR Green Master Mix (Invitrogen
169 Ltd.) using a 10 µL reaction containing 1.5 L of 1:1 diluted cDNA, 5 µL of Fast SYBR Green
170 Master Mix a 3.5 µL of a primer stock containing both forward and reverse primer at 1 µM
171 suspended in nuclease free water (final reaction concentration of each primer being 0.35 µM). For
172 each cDNA sample, we performed two technical replicates for each set of primers and the average
173 threshold cycle (Ct) was used for the analysis. We obtained the AMP primers from Sigma-Aldrich
174 Ltd (see supplementary **Table** S2 for information about primers). We optimised the annealing
175 temperature (T_a) and the efficiency (Eff) of the *Dpt* primer pair was calculated by 10-fold serial
176 dilution of a target template (each dilution was assayed in duplicate); *Dpt*: T_a= 59 °C, Eff= 102%;
177 *AttC*: T_a= 60 °C, Eff= 94%; *CecA1*: T_a= 59.5 °C, Eff= 109%.

178

179 *Fly survival following bacterial infection:* To test the impact of *Pseudomonas entomophila* infection on fly
180 survival, we challenged experimental flies to 4 different bacterial concentrations (infection doses)
181 ranging between (I) ~650 cells (II) ~1400 cells (III) ~2000 cells (IV) ~2900 cells/µl of *P.*
182 *entomophila* culture, obtained by serial dilution. Flies (n=10-18 fly vials /sex/infection dose/fly line)
183 were infected systemically by intra-thoracic pricking (Khalil et al. 2015) with a needle immersed in
184 bacterial suspension under minimal CO_2 anaesthesia. The effect of injury caused by pricking was

185 controlled by including mock infections performed using a needle dipped in sterile 5% sucrose
186 solution. We observed the mortality for 10 days post-infection every 3 hours (±0.5h) for the first
187 2 days (between 6 am and 11 pm) and every 12 hours for the following 7 days.

188

189 *Quantification of bacterial load following bacterial infection:* During the survival assay, flies were randomly
190 sampled (10-18 live fly vials/sex/infection dose/fly line) and the bacterial load was quantified at
191 ~8 hours following infection. Initially, flies (group of 3) were transferred to a 1.5 ml
192 microcentrifuge tubes and surface sterilized by adding 70% ethanol for ~30 secs to ensure we
193 counted only colony forming units (CFUs) inside the flies. Ethanol was then removed by briefly
194 washing flies twice with sterile distilled water. To ensure that this method was efficient in surface
195 cleaning flies, we plated 4 µl of the second wash on LB agar (from random flies) ensuring that the
196 flies are free from surface bacteria (i.e., no viable CFUs). We then placed in 1.5 ml Eppendorf
197 tubes and added 100 µl of LB broth to each tube and the flies were thoroughly homogenized using
198 a motorized pestle. We immediately plated this homogenate in serial dilutions ranging from 100 to
199 10^{-6} using 1xPBS (phosphate buffer) on LB agar plates and incubated at 30°C for ~18 hours after
200 which viable CFUs were counted manually (Siva-Jothy et al. 2018).

201

202 **Statistical analysis**

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

210

211 2. *Bacterial load*: We then quantified differences in bacterial load following bacterial infection
212 by analysing the bacterial measurement ($\log_{10}$) at ~8h post-infection after *P. entomophila*
213 infections. We found that residuals of bacterial load data were normally distributed when
214 tested with Shapiro-Wilks test in 'R' and hence we used ANOVA and analysed the bacterial

load data by fitting 'knockdown' (functional flies & IMD-pathway knockdown flies) and 'sex' as categorical fixed-effects, while infection dose (4 concentrations of *P. entomophila*) and 'vials' (replicates) as a random-effects for each of the fly lines (functional flies & IMD-pathway knockdown flies).

3. *Survival post-infection*: We analysed the survival data after *P. entomophila* bacterial infection for 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 & knockdown)* x sex *(male & 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 (Package 'survival'). 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 & female flies (i.e., flies with functional & 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

247 same group of replicate individuals. Given the destructive sampling required to measure
248 microbe loads in flies we chose instead to use another commonly used metric of tolerance
249 using the average mortality of flies of a given line relative to the average bacterial load of
250 that line, see for example (Adelman et al. 2013; Knutie et al. 2017; Cornet et al. 2014). This
251 is sometimes referred to as 'point' tolerance and is intuitive to interpret: relative to a given
252 microbe load, does one line die more (less tolerant) or less (more tolerant) (Little et al.
253 2010).

254

255 **Results & discussion:**

256 1. <u>Loss of IMD negative regulation leads to increased AMP expression and decreased</u>
257 <u>bacterial load</u>

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

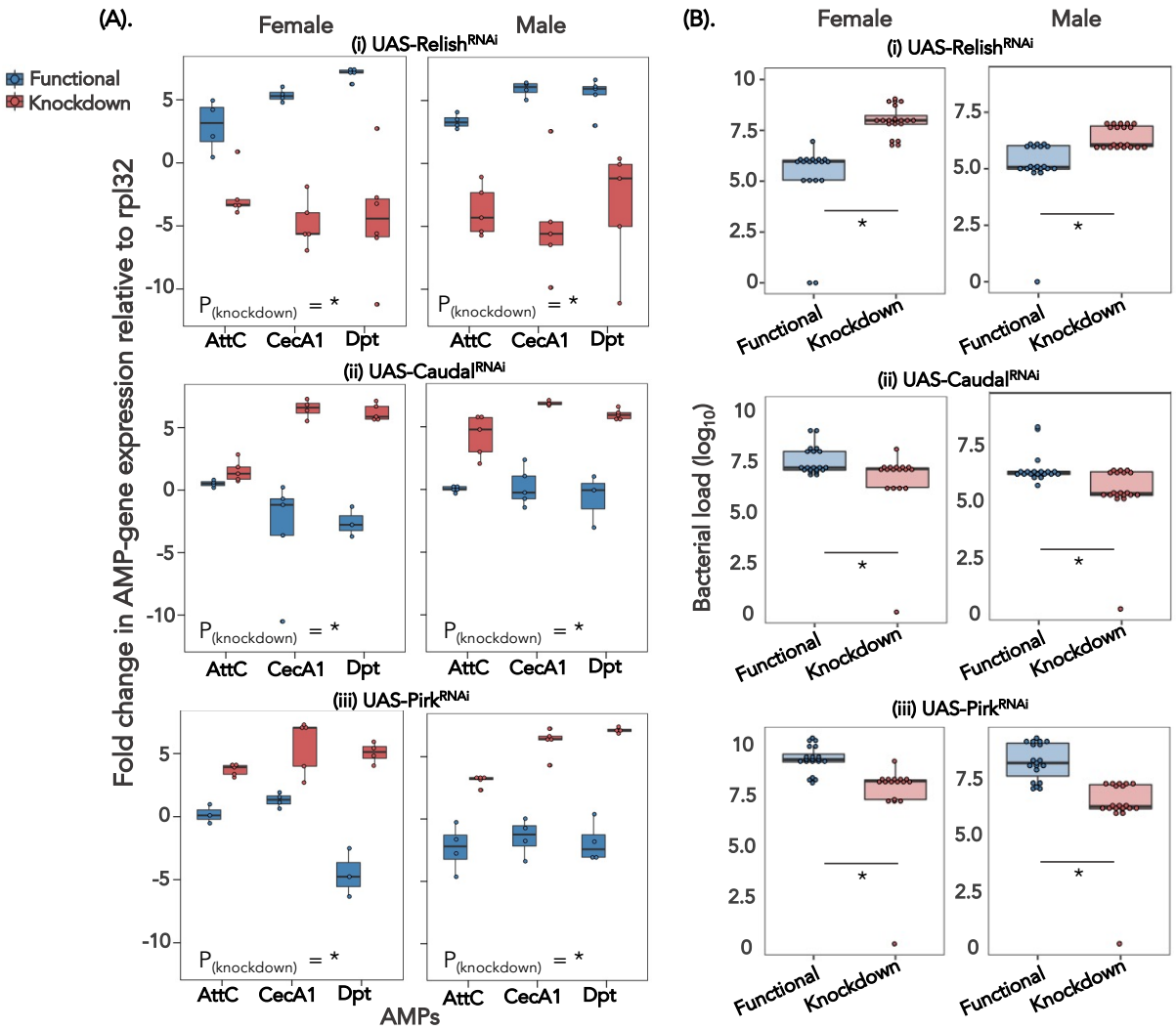


Figure 3: **(A)** Quantification of major IMD-responsive AMPs by qRT-PCR (n=~15 flies/sex/treatment) for IMD-pathway mutants & functional flies (males and females) after ~8 hours following *P. entomophila* systemic infection (0.05 OD or ~2000 bacterial cells/µl). Each data point represents 5-7 replicates 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.

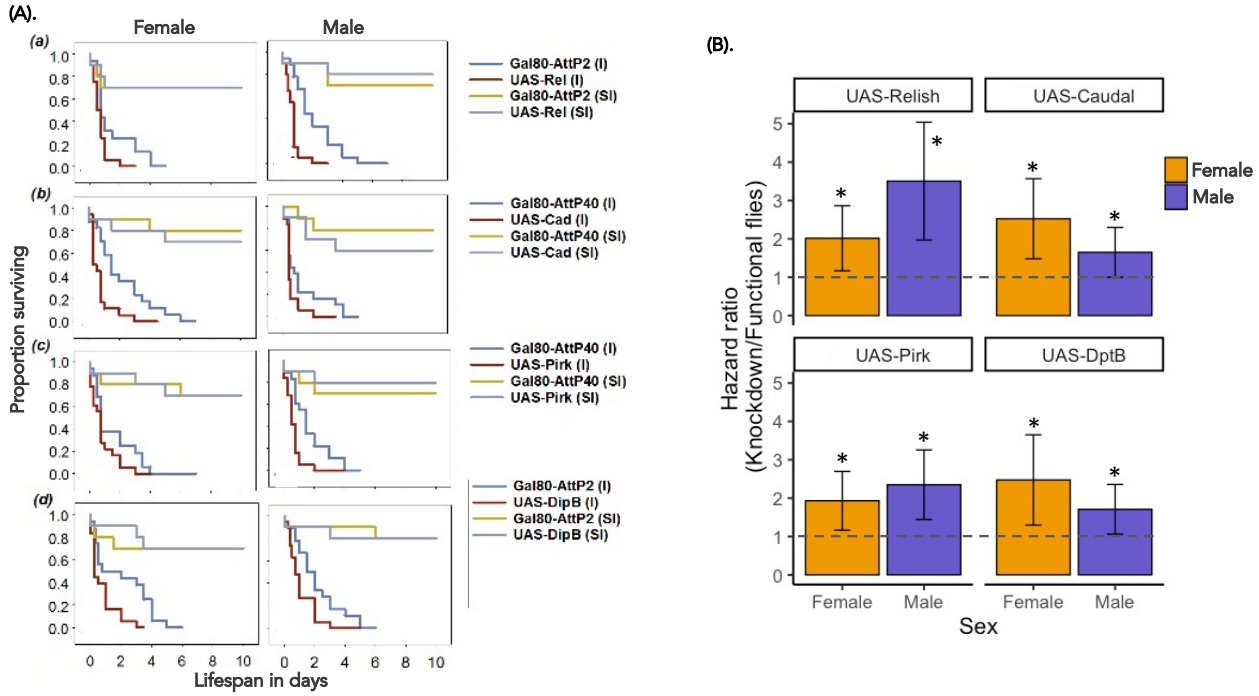


Figure 4: **(A)** Survival curves for male and female UAS^{RNAi} flies (n=16-24 fly vial/treatment/sex/fly line) (a) *UAS-Relish*^{RNAi} (b) *UAS-Caudal*^{RNAi} (c) *UAS-Pirk*^{RNAi} (d) *UAS-DptB*^{RNAi} after systemic infection of *P. entomophila* (dose ~2000 cells/µl). **(B)** hazard ratio calculated 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

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

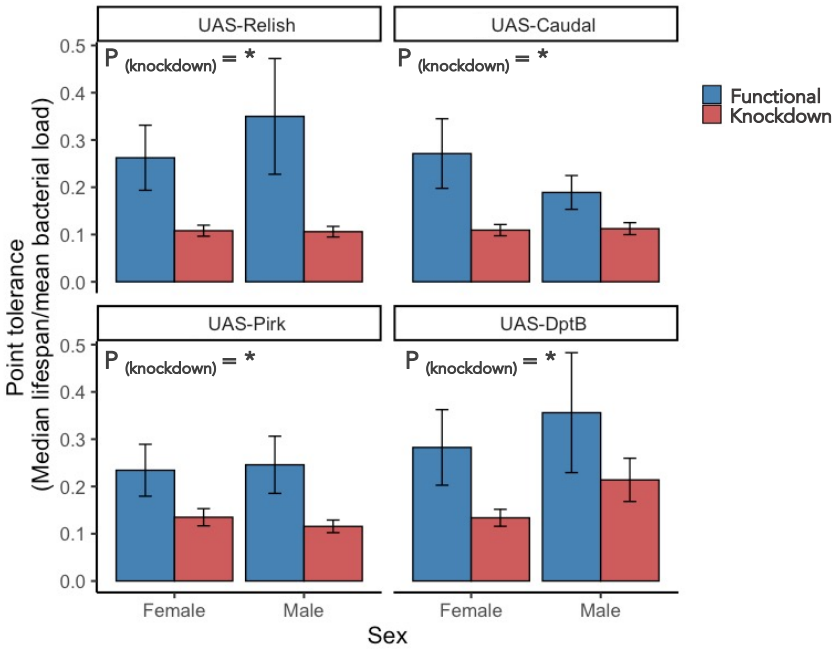
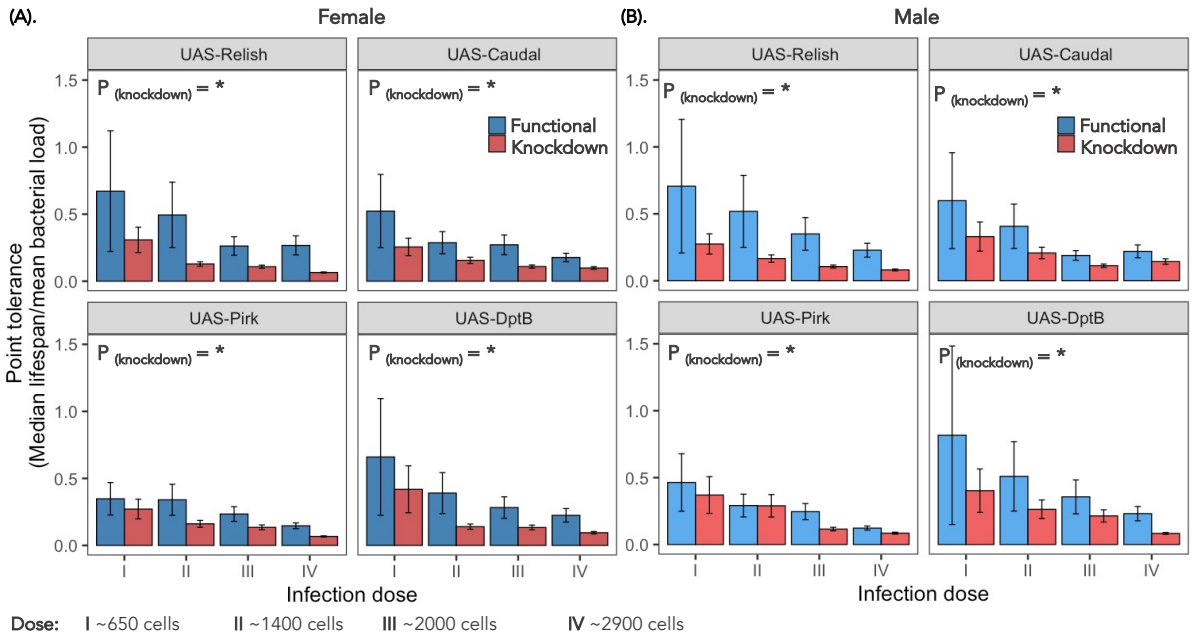


Figure 5: Disease tolerance in IMD-pathway mutants and fully functional flies. The relationship between host (fly) survival and bacterial load (single infection dose) analysing using point tolerance at ~8 hours post *P. entomophila* infection for male and female flies. Each data represents 'point tolerance',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

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

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

329



330

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

339

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

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

360 **Concluding remarks**

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

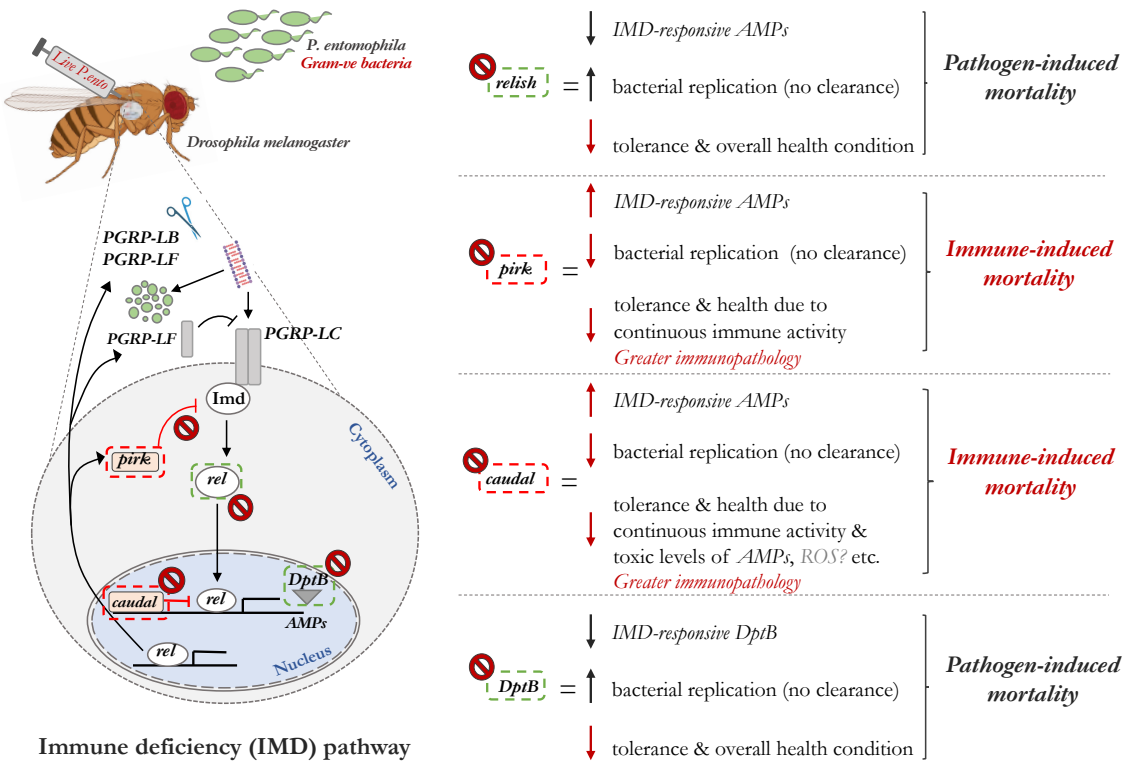


Figure 7: Summary highlighting that in addition to regulating an efficient pathogen clearance response, negative regulators of IMD also contribute to the phenotype of disease tolerance. Flies with reduced expression of the negative regulators *pirk* & *caudal* show reduced tolerance (overall health state) despite having higher AMP expression and reducing the bacterial numbers. Flies with reduced *relish* & *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 & P.F.V designed the experiments; A.P carried out experiments; A.P & K.M.M performed qPCR experiments; A.P & P.F.V analysed the data and wrote the manuscript.

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Competing interests:

None.

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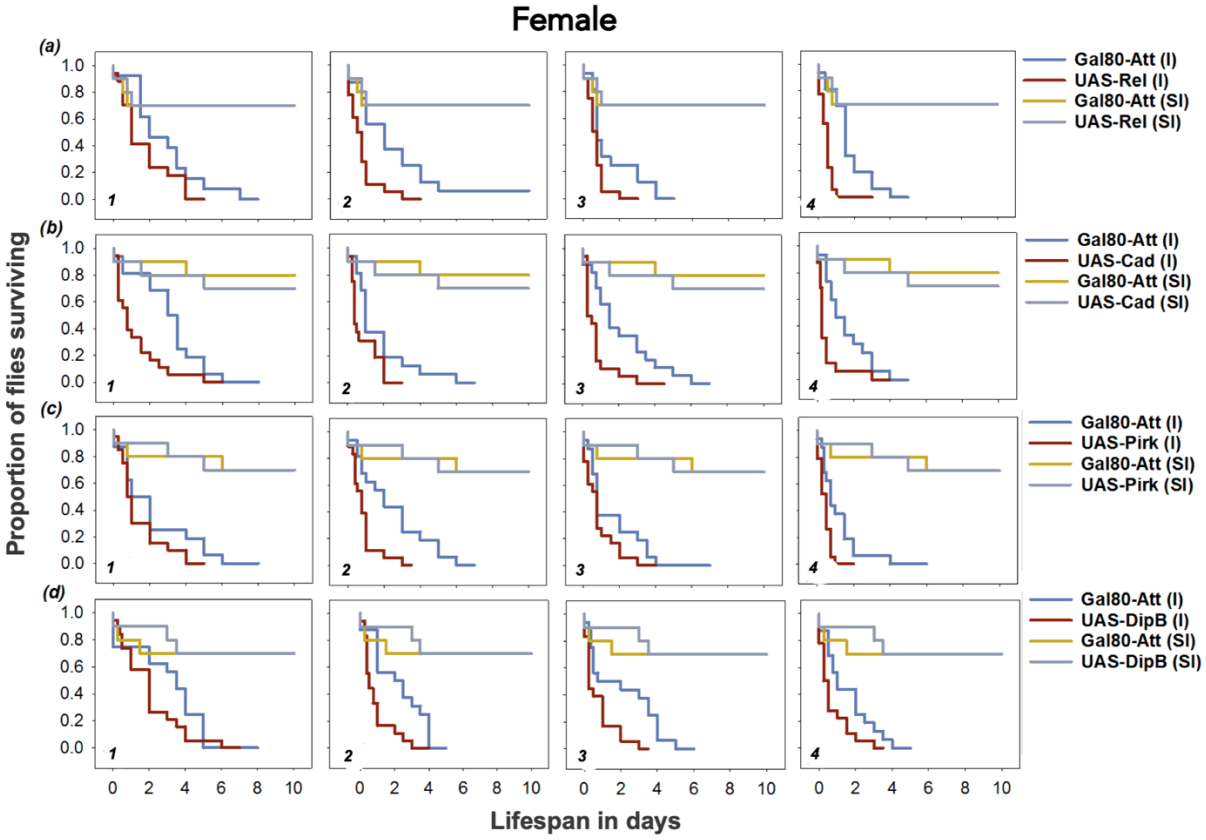
647 **Supplementary information**

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



654

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

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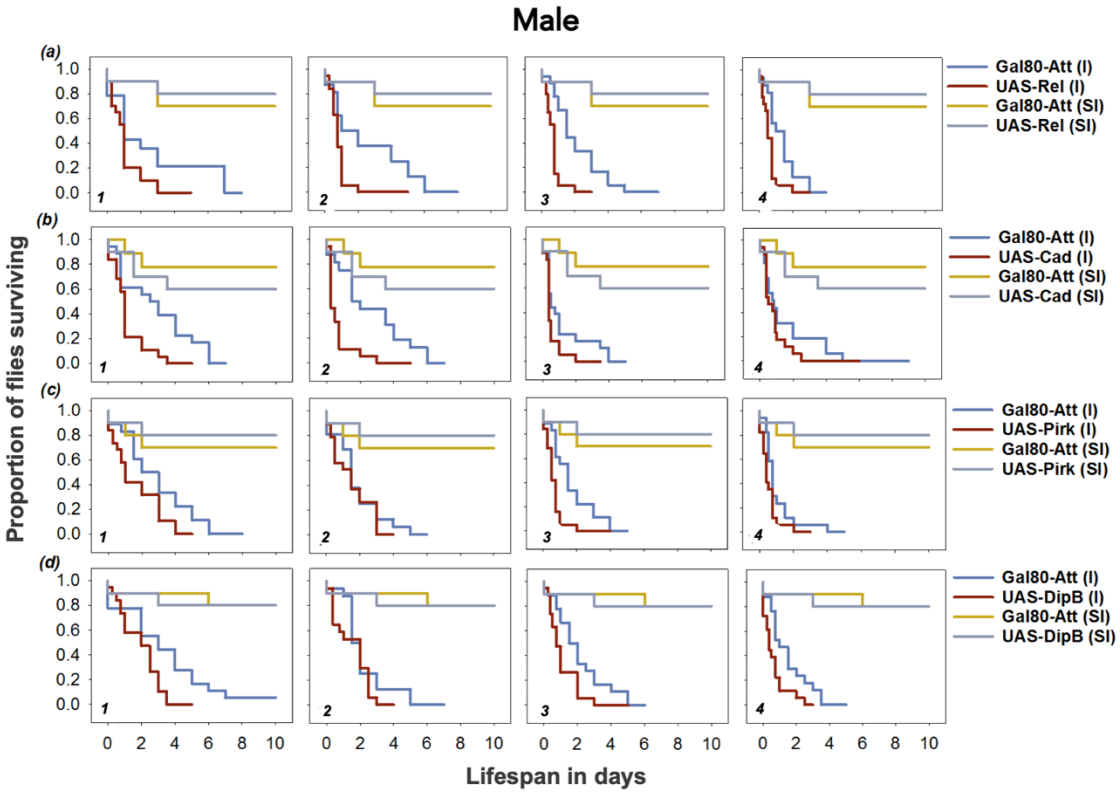

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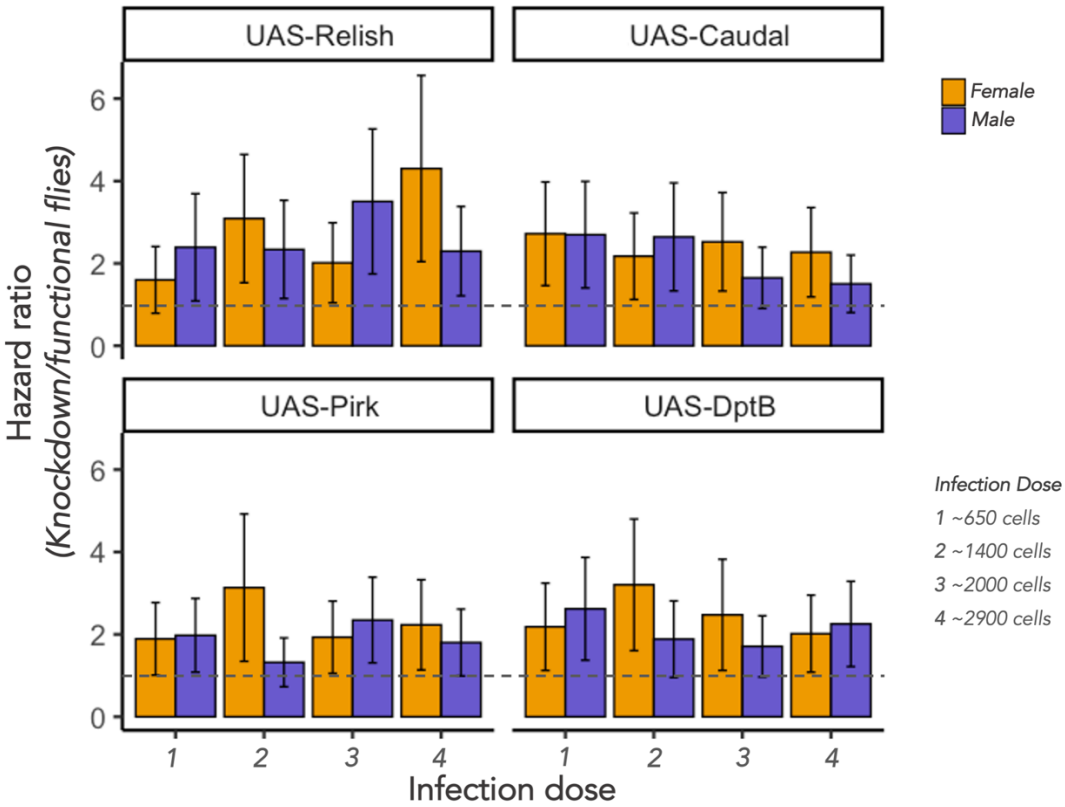
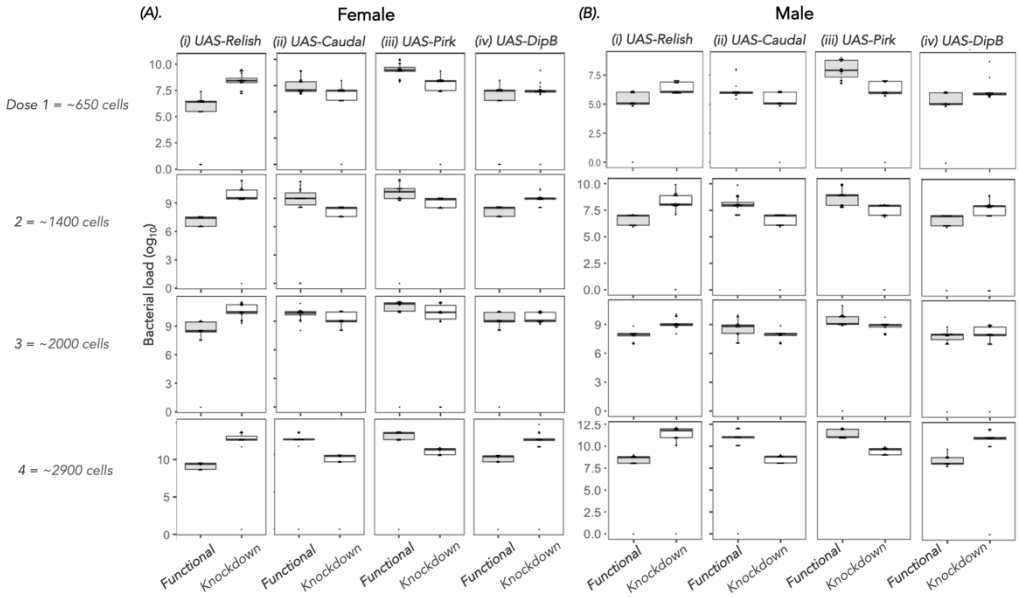
Figure S3. The estimated hazard ratios calculated from survival curves (**Fig. S1 & S2**) for flies with fully functional immune system & IMD-pathway mutants for both Sexes. The different IMD-pathway mutants include **(a)** *UAS-Relish*^{RNAi} **(b)** *UAS-Caudal*^{RNAi} **(c)** *UAS-Pirk*^{RNAi} **(d)**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



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



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

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}

679
680
681 **Table S2**: Primers used in this study
682

	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'

683

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

Genotype	*Source*	*df*	*Sum Sq*	*F value*	*p*
UAS-Relish^{RNAi}	Knockdown *(knockdown & 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

691
692 **Table S4:** Summary of $\log_{10}$ transformed bacterial load data after exposure to 4 different
693 concentrations (doses) of *P. entomophila* systemic infection, analysed using ANOVA by fitting
694 'Knockdown', 'Sex' and 'dose' as categorical fixed-effects and replicate 'vials' as a random-effects
695 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-day adult males and females infected with 4 concentration (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*

704
705 **Table S6:** 'Point tolerance' for all doses - Summary of ANCOVA. To assess differences bacterial
706 loads following infection with 4 concentrations (dose) of *P. entomophila*, we analysed the average
707 bacterial load, measured at ~8 hours following infection using ANCOVA and fitted 'Knockdown'
708 and 'Sex' as categorical fixed effects and 'bacterial load' as continuous co-variate for each fly line.
709
710

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

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