The *Jak/Stat* pathway mediates disease tolerance during systemic bacterial infection in *Drosophila*

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Abstract

Disease tolerance describes a host's ability to maintain health independently of the ability to clear microbe loads. However, we currently know little about the mechanisms that underlie disease tolerance or how known mechanisms of tissue damage signalling and repair may contribute to variation in tolerance. The Jak/Stat pathway plays a pivotal role in *Drosophila* humoral innate immunity, signalling tissue damage and triggering cellular renewal, making it a potential mechanism underlying the disease tolerance phenotype. Here, we show that disrupting the Jak/Stat pathway in *Drosophila melanogaster* alters disease tolerance during *Pseudomonas entomophila* systemic infection. Overall, flies with disrupted Jak/Stat show variation in survival that is not explained by variation in pathogen loads. For instance, mutations disrupting the function of *ROS*-producing *dual oxidase (duox*) or the negative regulator of Jak/Stat*, Socs36E* render males less tolerant to systemic bacterial infection but not females. We also investigated whether the negative regulator of Jak/Stat*, G9a* - which has previously been associated with tolerance of viral infections – is also implicated in tolerance of bacterial infection. While female flies lacking *G9a* showed higher mortality and reduced bacterial clearance, disease tolerance did not differ between *G9a* mutants and the wildtype. This suggests that *G9a* does not affect tolerance during systemic bacterial infection as it appears to do with viral infection. Overall, our findings highlight that Jak/Stat signalling mediates disease tolerance during systemic bacterial infection and that this response differs between males and females. Our work therefore suggests that differences in Jak/Stat mediated disease tolerance may be a potential source of sexually dimorphic response to infection in *Drosophila*.

Introduction

When organisms acquire infection, they face two major challenges in order to return to a healthy state. The first challenge is to identify and clear the source of the infection. While crucial, pathogen clearance alone will not result in a healthy host, because after pathogen elimination, what is left is the tissue damage caused by pathogen growth and as a side-effect of immunopathology. The second challenge to return to healthy state is therefore to minimise damage even before it occurs, and to repair and regenerate tissue after it has been damaged during infection (Martins et al. 2019; McCarville and Ayres 2018; Oliveira, Bahia, and Vale 2020; Soares, Teixeira, and Moita 2017; Vale, Fenton, and Brown 2014).

Individuals capable of dealing with the first challenge are typically labelled 'resistant', and exhibit low microbe loads because their immune clearance mechanisms are very effective (Boon et al. 2009; Ganz and Ebert 2010; Lazzaro, Sackton, and Clark 2006; Wang, Lu, and Leger 2017). The mechanisms underlying host resistance have been well characterized empirically and often involve detecting pathogen-derived molecular patterns such as peptidoglycans and triggering signalling cascades that result in the expression of antimicrobial peptides (AMPs) which directly kill the replicating pathogen (Kleino and Silverman 2014; Henna Myllymäki, Valanne, and Rämet 2014; H. Myllymäki and Rämet 2014; Palmer, Varghese, and Van Rij 2018; Valanne, Wang, and Rämet 2011). These responses have been described in great detail in the fruit fly *Drosophila*, and include the immune deficiency (Imd), Toll & Jak/Stat pathways (Bang 2019; Ferrandon et al. 2007; Hoffmann 2003; Hultmark 2003; Lemaitre and Hoffmann 2007).

Individuals with very effective mechanisms of damage limitation and repair, however, may not necessarily have a strong capacity for pathogen clearance, but will be successful in preventing or repairing tissue damage (Martins et al. 2019; Medzhitov, Schneider, and Soares 2012; Schneider and Ayres 2008; Soares, Teixeira, and Moita 2017; Soares, Gozzelino, and Weis 2014). Effective mechanisms of damage signalling and repair may explain why some individuals are tolerant of infection, and are able to experience relatively high health even if their pathogen loads remain high or are not completely cleared (Martins et al. 2019; Soares, Gozzelino, and Weis 2014). Compared to well-described pathogen clearance mechanisms, we are only beginning to unravel the mechanistic basis of disease tolerance. Likely candidate mechanisms include those that regulate inflammation to reduce immunopathology (Adelman et al. 2013; Cornet et al. 2014; Sears et al.

2011); detoxification of host or pathogen derived metabolites (Ferreira et al. 2011; Soares, Teixeira, and Moita 2017; Vale, Fenton, and Brown 2014); or tissue protection and regeneration (Jamieson et al. 2013; Soares, Teixeira, and Moita 2017; Soares, Gozzelino, and Weis 2014). However, the few disease tolerance candidate genes arising from genome-wide association or transcriptomic studies - such as *ghd* (*grainyhead*), *dsb (debris buster), crebA (cyclic response element binding protein) and, dfoxo* (*forkhead box, sub-group O*) - do not appear to be associated with these functions, or with classical immune pathways (Dionne et al. 2006; Howick and Lazzaro 2014; Lissner and Schneider 2018; Troha et al. 2018). This may indicate that disease tolerance is a complex phenotype and that dissecting its mechanistic basis may be more successful through a more focused examination of the effect of specific candidate signalling pathways.

Here we take advantage of the detailed knowledge of *Drosophila* infection and immunity to investigate whether damage signalling plays a role in disease tolerance during systemic bacterial infection. We focus on the Janus kinase/signal transducer and activator of transcription (Jak/Stat) pathway which is known to have important roles in signalling damage and tissue renewal. The Jak/Stat pathway was originally shown to control multiple biological processes including development, homeostasis, metabolism, and immunity in insects and mammals (Brivanlou and Darnell 2002; Harrison 2012; H. Myllymäki and Rämet 2014; Stark and Darnell 2012; Zhou and Agaisse 2012). In response to different types of stresses such as mechanical injury, oxidative stress, and infection the Jak/Stat pathway is activated by cytokine-like ligands of the unpaired family namely *upd-1, upd-2* and *upd-3* (Agaisse et al. 2003; Chakrabarti et al. 2016; Dostert et al. 2005; Ekengren et al. 2001; Ekengren and Hultmark 2001; Gilbert et al. 2005; Harrison et al. 1998). *Upd-3* is produced during damage caused by reactive oxygen species (*ROS*), which in turn are produced by *dual oxidase* (*duox*) (Klebanoff 1974; Babior 1995; Lee and Kim 2014) (see ***Fig**. 1*). The extracellular binding of *upd-3* to *Domeless* (*dome*), leads to the phosphorylation of *Hopscotch* (*hop*). This then leads to the phosphorylation of *Stat92E*, and its translocation to the nucleus (H. Myllymäki and Rämet 2014). In the nucleus, in addition to the production of factors that are necessary for repairing cellar damage, *Stat92E* also induces the expression of *Socs36E*, a negative regulator of *Hopscotch* (Kiu and Nicholson 2012). Recent work has also highlighted the role of the histone H3 lysine 9 methyltransferase (also called *G9a*) in negatively regulating the expression of the Jak/Stat pathway during infection (Merkling et al. 2015).

108 Focusing on its role in immunity, there is substantial evidence that Jak/Stat signalling plays a key
109 role in wound healing, gut immunity, and downstream AMP production (Chakrabarti et al. 2016;
110 Kemp et al. 2013; Lamiable and Imler 2014; Tafesh-Edwards and Eleftherianos 2020). For
111 instance, during enteric bacterial infection in *Drosophila*, the Jak/Stat pathway contributes to
112 intestinal immunity by regulating intestinal stem cell (ISC) proliferation and epithelial cell renewal
113 via epidermal growth factor (*EGFR) signalling* (Buchon et al. 2010; Chakrabarti et al. 2016; Ohlstein
114 and Spradling 2006). The absence of epithelial renewal leads to a loss of structural integrity and
115 increased susceptibility to bacterial infections (Buchon et al. 2009). In cellular immunity, Jak/Stat
116 signalling is central to the production, differentiation and maintenance of blood cells in insects
117 (Banerjee et al. 2019; Meister and Lagueux 2003).

118

119 Jak/Stat is also important in humoral immunity to viral infection (Dostert et al. 2005), where a loss
120 of regulation of Jak/Stat by the epigenetic negative regulators *G9a* results in reduced tolerance of
121 *Drosophila C virus* infections due to increased immunopathology (Merkling et al. 2015). This specific
122 result led us to question whether the effects of *G9a*-mediated Jak/Stat regulation on tolerance
123 were specific to viral infection, or if the regulation of Jak/Stat also affects disease tolerance during
124 bacterial infection. Here we investigated the tolerance response of *Drosophila* during septic
125 infection with the bacterial pathogen *P. entomophila*, using deletion mutant flies lacking various
126 components of Jak/Stat signalling and regulation. Further motivated by the widespread
127 observation of sexually dimorphic immunity *reviewed in* (Belmonte et al. 2020; Klein and Flanagan
128 2016) and particularly that the effects of *G9a* on tolerance of *DCV* infection are more pronounced
129 in female flies (Gupta and Vale 2017; Merkling et al. 2015), we also focused on assessing how
130 Jak/Stat affects males and females separately.

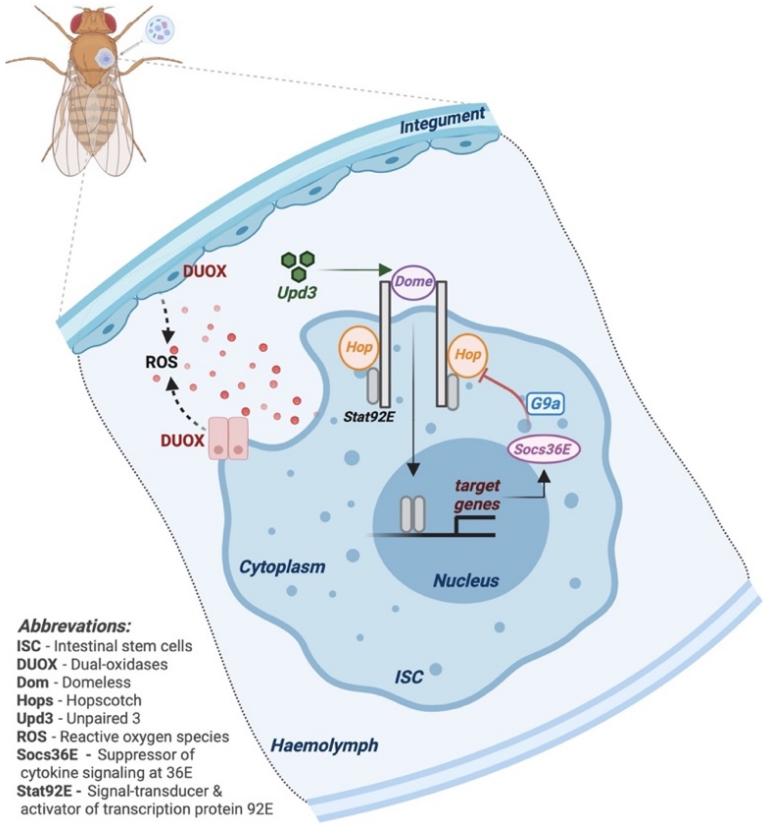

Figure 1. A simplified version of *Drosophila* Jak/Stat pathway known to have important roles in signalling damage and tissue renewal. In response to infection, the Jak/Stat pathway is activated by cytokine-like ligands of the unpaired family namely *upd-3*, produced ↘ during damage caused by reactive oxygen species (*ROS*), which in turn are produced by *dual oxidase* (*duox*). The extracellular binding of *upd-3* to *Domeless* (*dome*), leads to the phosphorylation of *Hopscotch* (*hop*). This then leads to the phosphorylation of *Stat92E*, and its translocation ↘ to the nucleus. In the nucleus, *Stat92E* induces the expression of *Socs36E*, a negative regulator ⊣ of *Hopscotch*. Recent work has also highlighted the role of the *histone H3 lysine 9 methyltransferase* (also called *G9a*) in negatively regulating ⊣ the expression of the Jak/Stat pathway during infection.

Materials and methods

<u>*Fly strains and maintenance:*</u> we used several *D. melanogaster* strains with loss-of-function mutations for the *Jak/Stat* pathway. All the fly strains were on the same genetic background as that of *yw (189)* including *Domeless* (*Bloomington stock# 14726*), *Hopscotch* (*15097*), *Socs36E* (*16744*), *Duox* (*16468)*. We also used *G9a* mutant flies (i.e., $G9a^{-/-}$) and control ($G9a^{+/+}$), also known as $G9a^{DD2}$ see (Merkling et al. 2015) for details about *G9a* mutants. Previous work has shown that *yw* flies have lower basal levels of nitric oxide and are more susceptible to infection compared to other lab wild types such as w^{1118}, *Oregon R, Canton-S* (Eleftherianos et al. 2003). However, we found that males and females of both *yw* and another wildtype of *outcrossed* flies showed comparable survival following *P. entomophila* systemic infection and exhibited similar levels of internal bacterial loads when measured ~24 hours following infection (see supplementary ***Fig****. S1A & B,* ***Table*** *S1* for

153 survival; ***Fig**. S1C,* for bacterial load). We maintained all the fly lines in a 12ml plastic vials on a
154 standard cornmeal diet or Lewis's medium *see* (Siva-Jothy et al. 2018), at 25°C (±2°C). We used
155 ~3-day old adult flies for all our experiments (see below). First, we housed 2 males and 5 females
156 for egg laying (48 hours) onto a vial containing fresh food. We then removed the adults and the
157 vials containing the eggs were kept in 25°C incubator for ~14 days, or until pupation. We placed
158 the newly eclosed individuals (males and females separately) in fresh food vials until the
159 experimental day (~3 days).

160

161 *Bacterial culture preparation:* We used *P. entomophila* cultured overnight in Luria broth (LB) at 37°C
162 under constant agitation i.e., 120 revolutions per minute (rpm). *P. entomophila* is a gram-negative
163 bacterium naturally found in soil and aquatic environments, known to be highly pathogenetic for
164 *D. melanogaster* (Dieppois et al. 2015; Vodovar et al. 2005)). Upon reaching ~0.75 OD_{600} we pelleted
165 the culture using a 4°C cold centrifuge during 5 minutes at 5000rpm and then removed the
166 supernatant. We resuspended the bacteria in 1X phosphate buffer saline (PBS) and prepared the
167 final infection dose of OD_{600} of 0.05 for all our infection assays.

168

169 *Systemic infection assay:* We used a split-vial experimental design (see ***Fig**. 2*), wherein after infection
170 each vial containing about 25 flies (of each treatment, sex and fly line combination) were divided
171 into 2 vials for measuring **(A)**. survival following infection (~15 flies/combination) and **(B)**.
172 internal bacterial load (see ***Fig**. 2II*). With this split-vial design we were able to use replicate-
173 matched data for both survival and bacterial load in order to estimate disease tolerance for each
174 fly line (i.e., fly survival with respect to internal bacterial load). We infected ~3-day old male and
175 female adult flies using a 0.15mm insect minutein needles bent at ~90° angle to avoid damaging
176 the internal tissues by dipping in *P. entomophila* bacterial solution. For mock controls we substituted
177 bacterial solution with sterile 1X PBS. After stabbed the flies in the sternopleural region of the
178 thorax (Khalil et al. 2015). We then placed males and females separately onto fresh food vials and
179 incubated at 25°C. We scored the flies (both infected and control) every 2-3 hours for the first 48
180 hours following infection, then 2-3 times each day for the next 6 days.

181

182 *Measuring bacterial load:* To quantify internal bacterial load after *P. entomophila* infection first, we
183 thoroughly washed each fly with 70% ethanol for ~30 sec to surface sterilize and then rinsed twice
184 with autoclaved distilled water. We plated the second wash on LB agar plates to confirm whether
185 the surface bacteria were successfully removed after sterilization. We then transferred individual

186 fly onto 1.5ml micro centrifuge tubes and homogenized using a motorized pestle for approximately
187 30-60 sec in 100µl LB broth (n=30 homogenates/sex/infection treatment/ fly line). We
188 performed serial dilution of each fly homogenate up to 10^{-6} fold and added 4µL aliquot on a LB
189 agar plate. We incubated the plate overnight for ~18h at 30°C and counted the resultant bacterial
190 colonies manually (Siva-Jothy et al. 2018). We note that mock-infected control fly homogenates
191 did not produce any colonies on LB agar plates.

192

193 ***Statistics***.

194 ***1.*** *Survival post oral bacterial infection*: We analysed the survival data with a Cox mixed effects
195 model using the R package 'coxme' (*Therneau 2015*) for different treatment groups (*P.*
196 *entomophila* systemic infection and mock controls) across males and females. We specified
197 the model as: survival ~ fly line * treatment * sex * (1|vials/block), with 'fly line',
198 'treatment' and 'sex' and their interactions as fixed effects, and 'vials' nested within a 'block'
199 as a random effect for wild type *yw* and *Jak/Stat* mutant fly lines.

200 ***2.*** *Bacterial load*: We found that the bacterial load data were not normally distributed (tested
201 with Shapiro–Wilks's test for normality). Hence, we first log transformed the data and then
202 confirmed that the transformed residuals were still not normally distributed. Hence, we
203 used a non-parametric one-way ANOVA Kruskal-Wallis test to test the effects of each fly
204 lines (wild type *yw* and *Jak/Stat* mutants) on males and females following bacterial
205 infection.

206 ***3.*** *Measuring infection tolerance*: Finally, to understand how the Jak/Stat signalling contributes to
207 the phenotype disease we analysed tolerance to bacterial infection by linear reaction norm
208 (i.e., measuring the linear relationship between fly survival against bacterial load) (Raberg,
209 Sim, and Read 2007; Ayres and Schneider 2012; Louie et al. 2016; Oliveira, Bahia, and Vale
210 2020). We analysed using ANCOVA for each of the fly lines (wild type *yw* and *Jak/Stat*
211 mutants) by fitting 'fly line' and 'sex' as categorical fixed effects and 'bacterial load' as a
212 continuous covariate and their interactions as fixed effects. Because we were interested in
213 identifying how the Jak/Stat mutants differ from wildtype flies during disease tolerance -
214 we compared the estimates of the slope using pairwise comparison (f-test; w^{1118} vs. different
215 *Jak/Stat* mutants) to find out what extent these Jak/Stat mutants significantly differed
216 from *yw* wild type in tolerating bacterial infections.

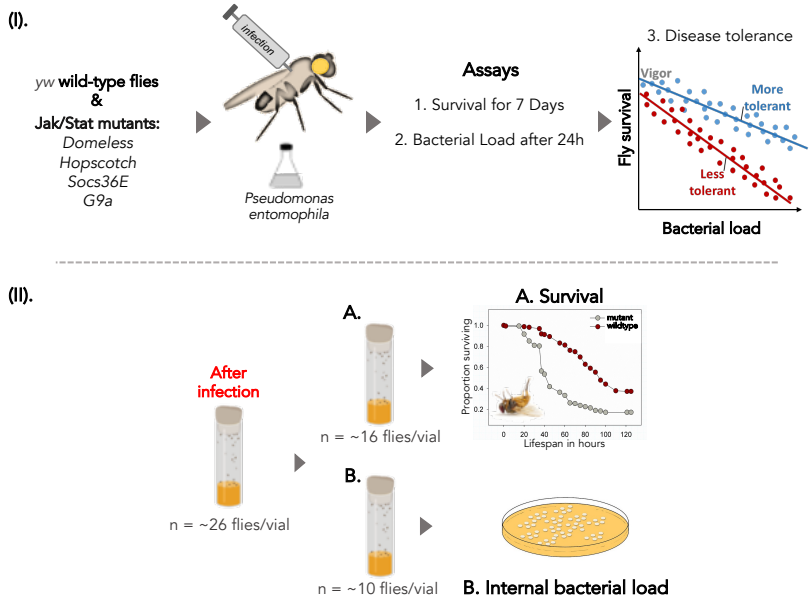

Figure 2: **(I)** Design of experiments to assay **(1)** survival and **(2)** internal bacterial load following systemic bacterial infection with *Pseudomonas entomophila* to test the role of the Jak/Stat pathway on **(3)** disease tolerance. **(II)** A split-vial experimental design, wherein after infection each vial containing about 25 flies of each treatment, sex and fly line combination were divided into 2 vials for measuring **(A)**. survival following infection (~15 flies/combination) and **(B)**. internal bacterial load (~10 flies/combination).

225 ***Results & discussion***

226 1. **Following systemic bacterial infection, loss of different components of Jak/Stat**
227 **pathway result in variable survival outcomes**

228 Overall, we found that disruption of Jak/Stat signalling affected fly survival during bacterial *P.*
229 *entomophila* infections (***Fig.*** *3A and B,* ***Table*** *1 & S2*). Both male and female flies lacking *duox*
230 (*ROS* producing *dual oxidase*) were more susceptible to *P. entomophila* infections compared to
231 the wildtype (*yw*) (***Fig.*** *3A and B,* ***Table*** *1 & S2*). However, other mutants showed improved
232 survival relative to wild types. These included male and female flies lacking the transmembrane
233 receptor *domeless*, males lacking the negative regulator *Soc36E* and both sexes lacking *hopscotch*
234 (see hazard ratio in ***Fig.*** *3B,* ***Table*** *1 & S2*).

235

236 2. **Both wildtype (*yw*) and Jak/Stat mutant flies exhibit similar bacterial loads**

237 Next, we tested whether the variation we observed between mutants in mortality could be
238 explained by differences in their bacterial load. Given that most mortality occurred just after
239 24 hours for most of our fly genotypes (***Fig.*** *3A*) we quantified bacterial load at 24 hours
240 following infection. Both wild type and Jak/Stat mutants exhibited similar levels of bacterial
241 load around 24 hours following infection with *P. entomophila* (***Fig.*** *3C,* ***Table*** *S3*). Therefore,
242 despite no substantial difference in microbe loads at 24 hours post infection, Jak/Stat mutants
243 showed variable survival. This would fit the functional definition of disease tolerance as for
244 the same bacterial load some lines appear to be more tolerant (survive longer, such as *domeless*)
245 while others are less tolerant (e.g., *duox*).

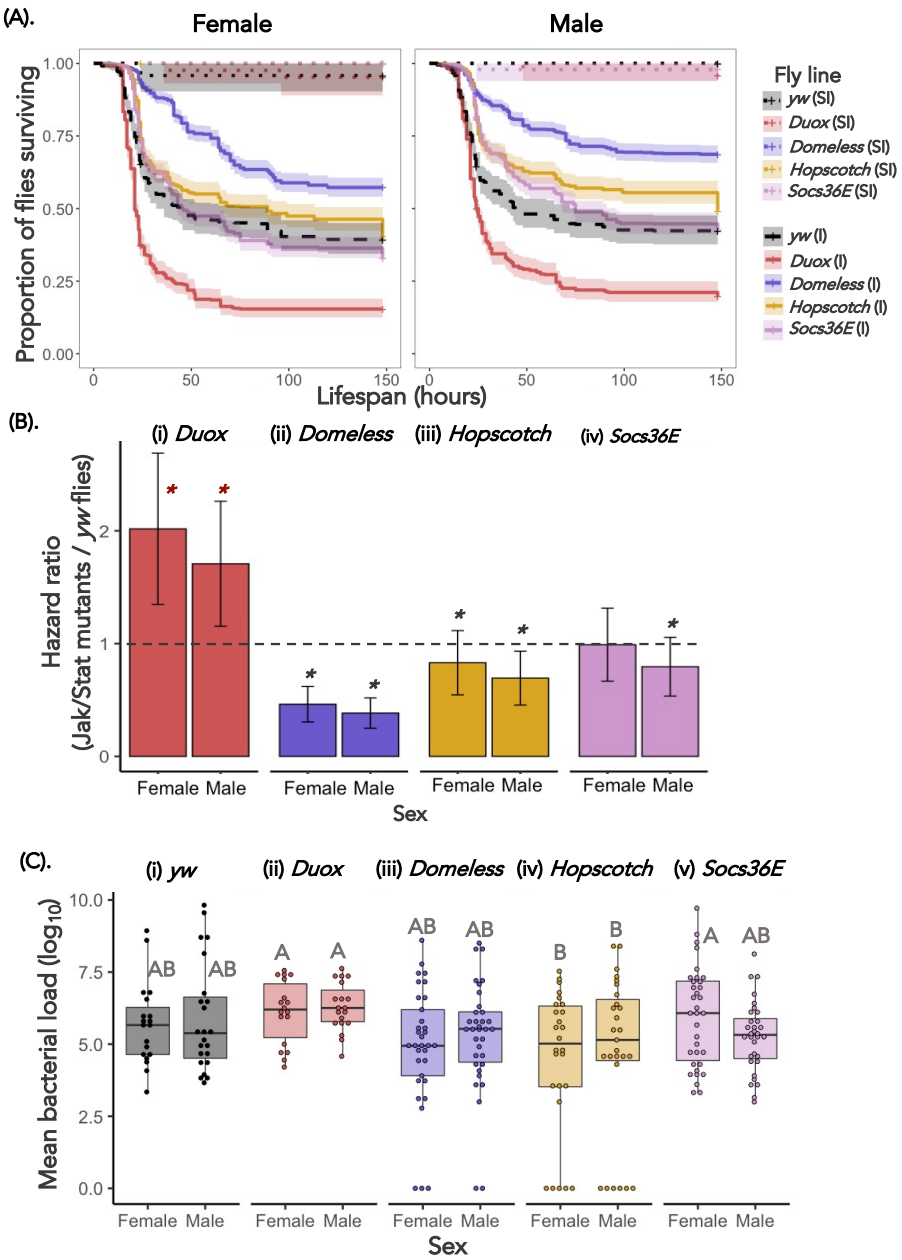

Figure 3. **(A)** Survival curves for wildtype *yw* flies and flies lacking Jak/Stat pathway components for females and males exposed to systemic *P. entomophila* of infection dose OD_{600}=0.05 (n=20-30 flies/vial (~15 vials)/fly line/treatment/sex/infection dose). ['*' indicates that the *Jak/Stat* mutants are significantly different from *yw* flies]. **(B)** Estimated hazard ratios calculated from the survival curves for males and female flies (*yw* & with flies lacking components of Jak/Stat). A greater hazard ratio (>1) indicates higher susceptibility of Jak/Stat mutants than wild type while (<1) indicates Jak/Stat mutants have better survival than wild type *yw* flies to systemic bacterial infection (p=<0.05). **(C)** Bacterial load (mean ln10) measured around 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].

Fly line	*sex*	*estimate*	*P*	*lower 95%*	*upper 95%*
Domeless	Female	0.462	<0.001	0.391	0.548
	Male	0.383	<0.001	0.322	0.457

Duox	Female	2.017	<0.001	1.712	2.384
	Male	1.707	<0.001	1.455	2.009
Hopscotch	Female	0.830	0.03	0.701	0.986
	Male	0.694	<0.001	0.585	0.824
Socs36e	Female	0.990	0.91	0.843	1.167
	Male	0.795	0.006	0.676	0.937

Table 1: Summary of estimated hazard ratio from the cox proportional model. A greater hazard ratio (>1) indicates that Jak/Stat mutant flies are more susceptible to *P. entomophila* infection than *yw* wildtype flies while lower ratio (<1) indicates that Jak/Stat mutants have better survival than wildtype.

3. During systemic bacterial infection, loss of Jak-Stat signalling leads to sex differences in disease tolerance phenotypes

While the results above are indicative of variable tolerance depending on the Jak/Stat mutation, we carried out a formal analysis of disease tolerance using the slope of the linear reaction norm between fly survival and microbe load, where each data point is the matched survival / CFU data for one replicate vial (see methods for description of split-vial design and ***Fig***. *2*). Here, the differences in tolerance between Jak/Stat mutant and *yw* fly 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. Overall, we found that the Jak/Stat mutants showed differences in disease tolerance phenotypes compared to wild type *yw* in both males and females (***Fig.*** *4,* ***Table*** *2*). Females lacking *duox* or *Socs36E* showed higher tolerance than males to *P. entomophila* (***Fig.*** *4B and 4A,* ***Table*** *3*). Given the role of *duox* in producing *ROS*, one possible explanation for increased tolerance in *duox* mutants is that *ROS*-derived damage is lower. However, flies also require intracellular *ROS* (oxidative burst) such as H_2O_2 (hydrogen peroxide) for the activation of macrophages during wounding and injury, in addition to Toll and Jak/Stat activation (Chakrabarti and Visweswariah 2020). It is possible that these effects are more pronounced in males which would explain our observation that female *duox* mutants have comparatively higher tolerance. In other work, wild type ($w^{Dahomey}$) males showed higher levels of *duox* expression and *ROS* following *Ecc* (*Erwinia catovora*) infection (Regan et al. 2016), which may suggest that loss of function of *duox* might impact males more than females.

Regarding the sex difference in disease tolerance in flies lacking *Socs36E,* it could be due to sex differences in regulation of Jak/Stat, and the resulting differences in immunopathology. For example, female flies lacking the negative epigenetic regulator *G9a*, were found to be more tolerant

287 than males during viral infections (Gupta and Vale 2017). Similar examples also exist from other
288 innate immune pathways where, disrupting the negative regulator of IMD, *PGRP-LB* (*peptidoglycan*
289 *receptor-LB*), affected survival to a greater extent in females following *E. coli* infection, suggesting
290 the sex-specific role of some of these regulators (Vincent and Dionne 2021). The combination of
291 these observations might explain why flies lacking *Socs36E* showed sex differences in disease
292 tolerance, particularly in light of the crosstalk between Jak/Stat and IMD pathways (Kemp et al.
293 2013; Bang 2019; Dostert et al. 2005). An unexpected result is that we found that female flies
294 lacking *domeless* showed increased survival (and tolerance) relative to the *yw* wildtype (***Fig.*** *3* -
295 survival *and* ***Fig.*** *4* - tolerance), suggesting that Jak/Stat activation may in fact be costly to flies.
296 Immune deployment and regulation is highly energy demanding (McKean et al. 2008; Schwenke,
297 Lazzaro, and Wolfner 2016; Nystrand and Dowling 2020). The physiological cost of specific
298 individual immune components and pathways remains an open question for future research.

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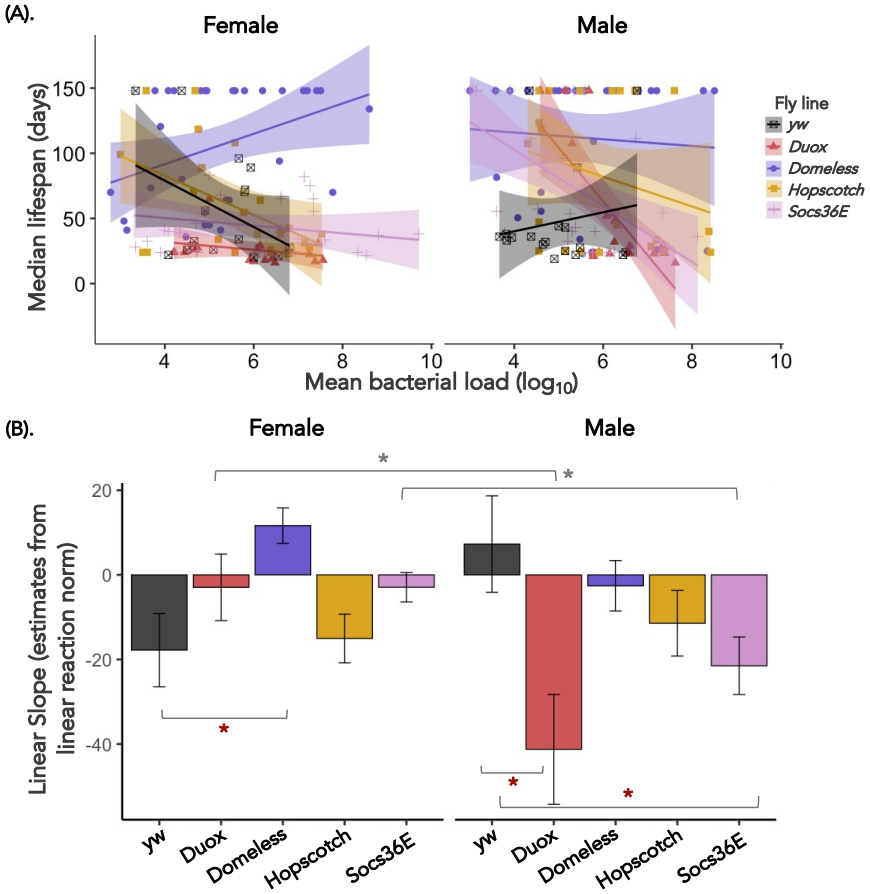


300

301 ***Figure 4.*** **(A).** The relationship between fly survival (measured as median lifespan) and bacterial
302 load (as mean CFUs - Colony Forming Units) analysed using linear models for female and male
303 flies (*yw* & Jak/Stat mutants). Each point shows data for median lifespan and mean CFUs of 15
304 vials (with each vial containing ~25 flies/sex/fly line combination after 24 hours post systemic
305 bacterial exposure. The data shown here are for the infection doses (OD_{600}=0.05). **(B).** Represents

306 estimates of negative slope of the linear reaction norm extracted from the linear models. [Asterix
307 '*' on the lower side of the panel B indicates that Jak/Stat mutants are significantly different from
308 wild type *yw*, analysed using the F-test pairwise comparisons of estimates of the linear reaction
309 norm for both males and females separately (see Table-3)]. Asterix '*' on the upper side of the
310 panel B indicates sex differences within the fly line i.e., males and females significantly differ in
311 tolerance to systemic bacterial *P. entomophila* infection.

312

	Fly line	***Source***	***DF***	***Sum of Sq.***	***F ratio***	***P***
Jak/Stat	***Female***	Fly line	1	100928.9	21.6	<0.001
		Bac. load	1	4273.8	3.66	0.06
		Fly line X bac. load	1	22262.4	4.77	0.001
	Male	Fly line	1	48485.2	5.72	0.0003
		Bac. load	1	23206.2	10.9	0.001
		Fly line X bac. load	1	26181.0	3.09	0.01

313 ***Table 2.*** Summary of ANCOVA. To assess differences in infection tolerance (fly survival with
314 increasing bacterial burden) following systemic *P. entomophila* infection with OD_{600}=0.05 infection
315 dose, around 24 hours following infection. We analysed ANCOVA and fitted 'sex' as categorical
316 fixed effects, 'average bacterial load (ln_{10})' as a continuous covariate and their interactions as fixed
317 effects for the Jak/Stat mutants.

318

Sex	***Fly line***	***Difference (slope)***	***Std err.***	***F Ratio***	***p***
Female	*duox vs. yw*	-18.11	7.32	2.13	0.15
	domeless vs. yw	28.04	6.28	6.32	0.01
	hopscotch vs. yw	7.87	6.76	0.05	0.81
	socs36E vs. yw	0.87	6.21	2.92	0.09
Male	*duox vs. yw*	7.20	8.64	12.51	0.001
	domeless vs. yw	34.74	6.80	0.52	0.47
	hopscotch vs. yw	23.30	7.52	1.65	0.20
	socs36E vs. yw	11.67	6.94	6.23	0.01

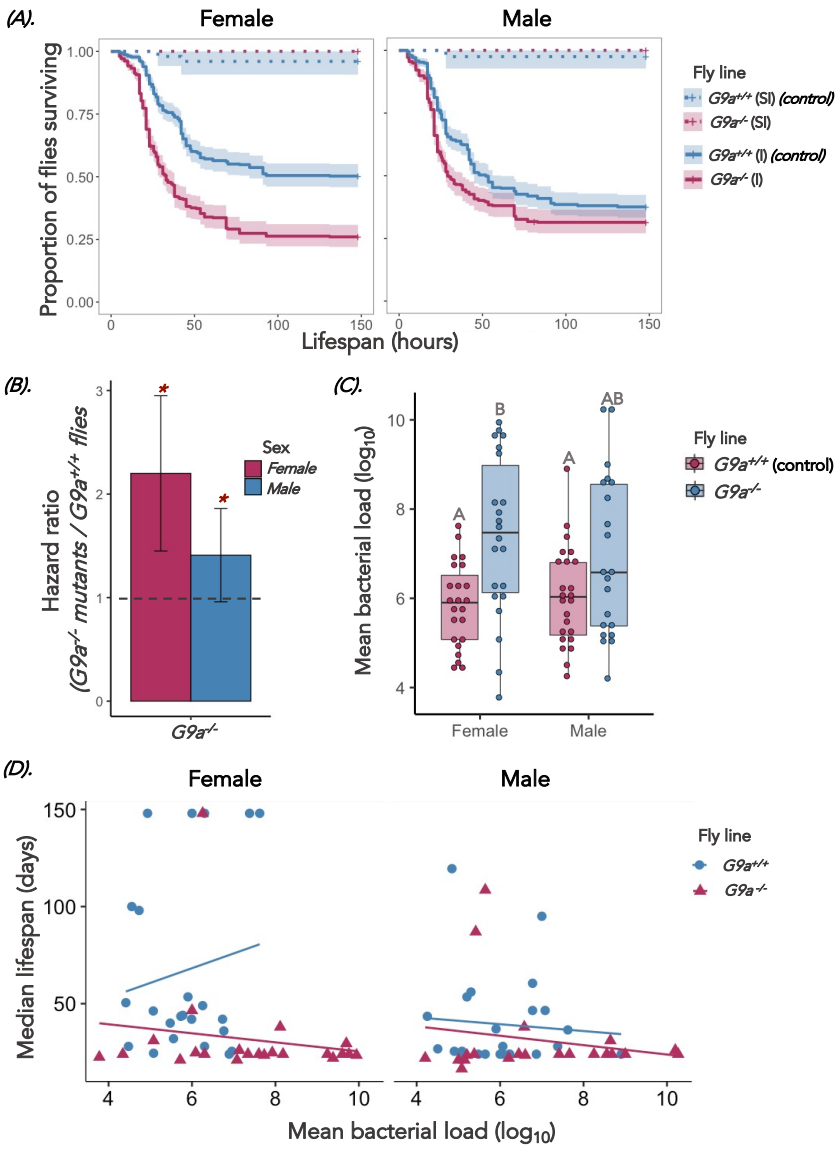
319 ***Table 3:*** Summary of F-test pairwise comparisons of estimates of linear slopes (from the linear
320 model) Jak/Stat-mutants compared to the *yw* wildtype.

321

322 4. **Loss of *G9a* does not affect bacterial disease tolerance**

323 The epigenetic negative regulator of Jak/Stat *G9a* was previously identified as being important for
324 tolerating *Drosophila C Virus* (DCV) infections (Merkling et al. 2015). Subsequent work exploring
325 sex differences in this response found that $G9a^{+/+}$ (control) females had higher tolerance than
326 $G9a^{-/-}$ females, when measured across a range of viral DCV doses (Gupta and Vale 2017). We
327 wanted to test whether the loss of function of *G9a* also affects fly survival and disease tolerance
328 in response to bacterial infections. Overall, we found that loss of *G9a* makes both males and
329 females more susceptible to *P. entomophila* infections, (***Fig***. *5A* for survival and ***Fig***. *5B* for hazard

330 ratio, ***Table*** *4* and ***Table*** *S4*). To test if this increased mortality in *G9a*$^{-/-}$ flies was associated with
331 higher bacterial replication we measured bacterial load following ~24 hours *P. entomophila* systemic
332 infection. We found that *G9a*$^{-/-}$ females exhibited higher bacterial load than *G9a*$^{+/+}$ (control) flies,
333 while males showed similar bacterial load as *G9a*$^{+/+}$ flies (***Fig.*** *5C,* ***Table*** *S5*). However, the overall
334 ability to tolerate *P. entomophila* bacterial infections (i.e., measured as *G9a* fly's survival relative to
335 its bacterial load) remained similar across both males and females *G9a* flies i.e., both *G9a*$^{-/-}$ and
336 *G9a*$^{+/+}$ controls (see ***Fig.*** *5D,* ***Table*** *5,* and ***Table*** *6* for comparison between estimates of tolerance
337 slope). Thus, despite the previously identified role of this negative regulator of Jak/Stat in
338 tolerating viral infections by reducing immunopathology (Gupta and Vale 2017; Merkling et al.
339 2015), *G9a* does not appear to affect bacterial disease tolerance in either sex.



340

341 ***Figure 5.*** **(A)** Survival curves for wildtype *yw* flies and flies lacking *G9a* the epigenetic regulator of
342 Jak/Stat for female and male flies exposed to systemic *P. entomophila* of infection dose OD_{600}=0.05

(n=20-30 flies/vial (~15 vials)/fly line/treatment/sex/infection dose). **(B)** Estimated hazard ratios calculated from the survival curves for males and female flies (*yw* and flies without *G9a*). A greater hazard ratio (>1) indicates higher susceptibility of *G9a*$^{-/-}$ mutants than control flies to bacterial infection. ['*' indicates that the *G9a*$^{-/-}$ flies are significantly different from *G9a*$^{+/+}$ flies]. **(C)** Bacterial load (mean $\ln_{10}$) measured around 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]. **(D)** Linear tolerance to *P. entomophila* infection – the relationship between *G9a* fly survival (measured as median lifespan) and bacterial load (as mean CFUs - Colony Forming Units) analysed using linear models for female and male flies (both *G9a*$^{-/-}$ and *G9a*$^{+/+}$).

sex	***flyline***	***estimate***	***p***	***Std err***
Female	G9a-/-	2.2	<0.001	0.75
Male	G9a-/-	1.41	<0.001	0.45

***Table 4*:** Summary of estimated hazard ratio from the cox proportional model. A greater hazard ratio (>1) indicates that *G9a*$^{-/-}$ mutant flies are more susceptible to *P. entomophila* infection than control (*G9a*$^{+/+}$) flies.

	Fly line	***Source***	***DF***	***Sum of Sq.***	***F ratio***	***P***
G9a	***Female***	Fly line	1	12373.5	8.028	0.007
		Bac. load	1	414.4	0.268	0.60
		Fly line X bac. load	1	1469.8	0.953	0.33
	Male	Fly line	1	394.8	0.692	0.41
		Bac. load	1	343.4	0.602	0.44
		Fly line X bac. load	1	8.480	0.014	0.90

***Table 5*.** Summary of ANCOVA. To assess differences in infection tolerance (fly survival with increasing bacterial burden) following systemic *P. entomophila* infection with OD_{600}=0.05 infection dose, around ~24 hours following infection. We analysed ANCOVA and fitted 'sex' as categorical fixed effects, 'average bacterial load ($\ln_{10}$)' as a continuous covariate and their interactions as fixed effects for each of the fly lines (*G9a*).

Sex	***Fly line***	***Fly line***	***F Ratio***	***p***
Female	*G9a* $^{-/-}$	*G9a* $^{+/+}$	0.95	0.33
Male	*G9a* $^{-/-}$	*G9a* $^{+/+}$	0.01	0.90

Table 6: Summary of F-test pairwise comparisons of estimates of the linear slopes (linear reaction norm) for *G9a* $^{-/-}$ *relative to G9a* $^{+/+}$ wildtype fly lines.

5. Concluding remarks

Tissue damage signalling and repair mechanisms such as Jak/Stat are important from a therapeutic perspective because they have the potential to boost host tolerance by minimising disease severity (Soares, Gozzelino, and Weis 2014; Vale et al. 2016). Our data show that loss of Jak/Stat pathway components following bacterial infection disrupts disease tolerance and overall survival. These observations have parallels in human infection. For instance, dysregulation of cytokines and

interferons (JAK signalling - Tyrosinekinase2) result in immunodeficiency while defective STAT increases the risk of autoimmunity (O'Shea, Holland, and Staudt 2013; O'Shea et al. 2014). Drugs that inhibit JAK have been shown to be effective in treating several autoimmune diseases by targeting cytokine-dependent pathways, while STAT inhibitors have been promising candidates in the context of cancer (Salas et al. 2020; Pérez-Jeldres et al. 2019; Miklossy, Hilliard, and Turkson 2013). It may therefore be possible to repurpose these existing drugs to improve host tolerance of infection. In summary, our work highlights that Jak/Stat signalling mediates disease tolerance during systemic bacterial infection and that this response differs between males and females. Therefore, we suggest that differences in Jak/Stat mediated disease tolerance may be a potential source of sexually dimorphic response to infection in *Drosophila*.

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Authors contribution:

A.P and P.F.V conceived the study and designed the experiments; A.P carried out the experimental work with assistance from M.B. and K.M.M; A.P and M.B. acquired the data; A.P analysed the data and drafted the manuscript; A.P & P.F.V wrote the manuscript.

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***Competing interests*:**

None.

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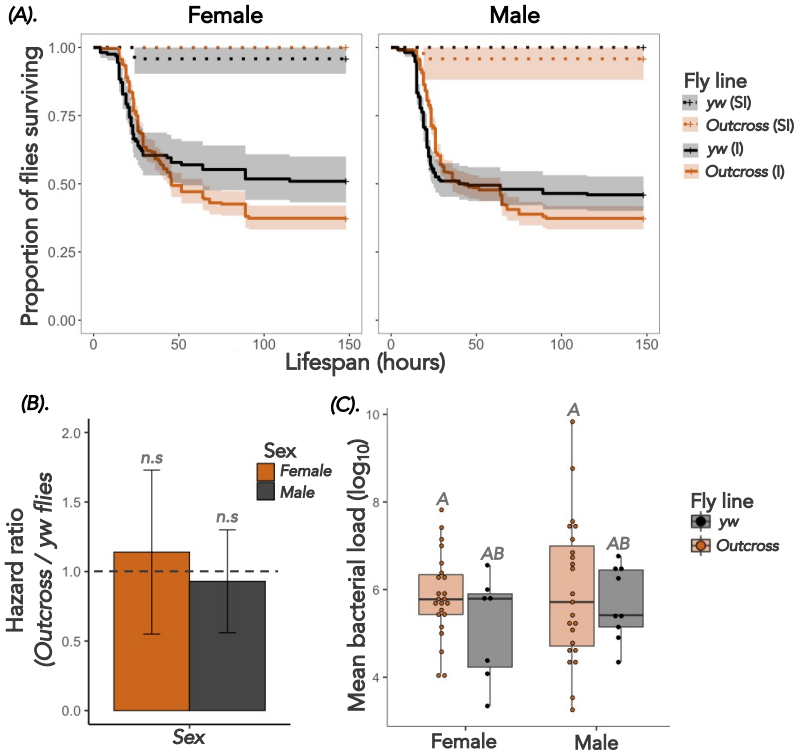
Supplementary Information

Figure S1. **(A)** Survival curves for males and females of wildtype *yw* and *Outcross* population exposed to systemic *P. entomophila* of infection dose OD_{600}=0.05 (n=20-30 flies/vial (~15 vials)/fly line/treatment/sex/infection dose). **(B)** Estimated hazard ratios calculated from the survival curves for males and female flies (*Outcross* and *yw* flies). A greater hazard ratio (>1) indicates higher susceptibility to bacterial infection. ['n.s' in the panel indicates that *Outcross* flies are not significantly different from *yw* flies]. **(C)** Internal bacterial load (as mean CFUs- colony forming units) measured around 24 hours post systemic *P. entomophila* infection. [significantly different fly lines are connected with different alphabets using Tukey's HSD as a post hoc analysis of pairwise comparisons].



Both wildtype (*yw*) & *Outcrossed* flies show similar response to systemic *P. entomophila* infection: All our *Jak/Stat*-pathway mutants were in *yw* genetic background. Previous work has shown that *yw* flies have lower basal levels of nitric oxide and are more susceptible to infection compared to other lab wild types such as w^{1118}, *Oregon R, Canton-S* (*Eleftherianos et al., 2014*). Before addressing whether the *Jak/Stat* pathway is involved in disease tolerance of bacterial infection, we wanted to better understand how wild type (*yw*) flies survive *P. entomophila* infections relative to other wild-type strains. We used an *Outcrossed* fly population as another control for comparison, which was originally created using 100 pairwise crosses of 113 *DRGP* lines and maintained as an outcrossed population for over 19 generations (see *Savola et al., 2021* for generation of *DGRP Outcrossed* population). We found that males and females of both wild type *yw* and the *Outcross*ed flies showed comparable survival following *P. entomophila* systemic infection and exhibited similar levels of internal bacterial loads when measured ~24h post-infection (***Fig***. *S1A & S1B,* ***Table*** *S1* for survival; ***Fig***. *S1C,* for bacterial load). Since both the wildtype (*yw*) and *Outcross* fly lines showed similar infection responses to *P. entomophila*, all remaining experiments only used *yw* as the wildtype control line.

695 ***Table S1.*** Summary of mixed effects Cox model, for wildtype *yw* & flies without *Jak/Stat* pathway
696 components during systemic *P. entomophila* infection in. We used data from the individuals of 3-
697 day adult males and females infected with OD_{600} = 0.05 infection dose of *P. entomophila* for each
698 fly lines (*Jak/Stat* -pathway mutants) and specified the model as: survival ~ treatment x sex x fly
699 line (1|vial), with 'treatment', 'sex' & 'fly line' as fixed effects, and 'vials' as a random effect. The
700 table shows model output (ANOVA) for survival post-infection for flies with fully function
701 immune system and lacking *Jak/Stat* - pathway.

	Source	***Log lik.***	***Chi sq.***	***df***	***p***
Wildtype vs. outcross	Treatment (SI vs I)	-5956.3	500.18	1	<0.001
	Sex	-5953.8	5.0844	1	0.38
	Fly line	-5945.8	15.929	1	0.54
	Treatment x sex	-5945.7	0.3030	1	0.58
	Treatment x fly line	-5945.7	0.0077	1	0.93
	Sex x fly line	-5945.3	0.7848	1	0.37
	Treatment x sex x fly line	-5942.8	4.9490	1	0.02
	Random effect	*Std dev*			
	vial	*0.61*			

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705 ***Table S2.*** Summary of mixed effects Cox model, for each fly line, wildtype *yw* & flies without
706 *Jak/Stat* pathway mutants. We used data from the individuals of 3-day adult males and females
707 infected with OD_{600} = 0.05 infection dose of *P. entomophila* for each fly lines (*Jak/Stat* -pathway
708 mutants) and specified the model as: survival ~ treatment x sex x fly line (1|vial), with 'treatment',
709 'sex' & 'fly line' as fixed effects, and 'vials' as a random effect. The table shows model output
710 (ANOVA) for survival post-infection for flies with fully function immune system (*yw*) and flies
711 lacking *Jak/Stat* – pathway components.

	Source	***Log lik.***	***Chi sq.***	***df***	***p***
***Jak/Stat* mutants**	Treatment	-30378	1042.2	1	<0.001
	Sex	-30357	42.39	1	<0.001
	Fly line	-29746	1222.9	4	<0.001
	Treatment x sex	-29746	0.050	1	0.82
	Treatment x fly line	-29744	2.732	4	0.60
	Sex x flyline	-29736	15.57	4	0.003
	Treatment x fly line x sex	-29734	4.506	4	0.34
	Random effect	*Std dev*			
	vial/block	*0.49*			

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716 ***Table S3.*** Summary of $\ln_{10}$ transformed bacterial load data around 24 hours following
717 OD_{600}=0.05 *P. entomophila* systemic infection for wildtype (*yw*) flies & *Jak/Stat* pathway mutants,
718 analysed using non-parametric one-way ANOVA (K-W test) by fitting 'fly line' (*yw* and Jak/Stat
719 mutants) as categorical fixed-effects for males and females separately.
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K-W	***Sex***	***Source***	***DF***	***Chi Sq.***	***P***
Jak/Stat mutants	Female	Fly line	4	9.1704	0.057
	Male	Fly line	4	9.4565	0.0506

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723 ***Table S4.*** Summary of mixed effects Cox model, for G9a *Jak/Stat* pathway components during
724 systemic *P. entomophila* infection in. We used data from the individuals of 3-day adult males and
725 females infected with OD_{600} = 0.05 infection dose of *P. entomophila* for each fly lines (*G9a*) and
726 specified the model as: survival ~ treatment x sex x fly line (1|vial), with 'treatment', 'sex' & 'fly
727 line' as fixed effects, and 'vials' as a random effect. The table shows model output (ANOVA) for
728 survival post-infection for flies with fully function immune system and lacking *G9a*.

	Source	***Log lik.***	***Chi sq.***	***df***	***p***
G9a	Treatment	-8485.2	310.1	1	<0.001
	Sex	-8481.2	8.07	1	0.004
	Fly line	-8436.6	89.1	1	<0.001
	Treatment x sex	-8436.5	0.22	1	0.63
	Treatment x fly line	-8433.9	5.13	1	0.002
	Sex x fly line	-8426.3	15.2	1	<0.001
	Treatment x sex x fly line	-8426.3	0.00	1	1.0
	Random effect	*Std dev*			
	vial/block	*0.25*			

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733 ***Table S5.*** Summary of $\log_{10}$ transformed bacterial load data around 24 hours following OD_{600}=0.05
734 *P. entomophila* systemic infection for *G9a* mutants, analysed using non-parametric one-way
735 ANOVA (K-W test) by fitting 'Fly line' as categorical fixed-effects for males and females
736 separately.

Fly line	***Sex***	***S***	***Z***	***p***
G9a	Female	641.5	3.065	0.002
	Male	548.5	1.479	0.139

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