**Duox and Jak/Stat signalling influence disease tolerance in Drosophila during Pseudomonas entomophila infection**

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ABSTRACT

Disease tolerance describes an infected host’s ability to maintain health independently of the ability to clear microbe loads. The Jak/Stat pathway plays a pivotal role in humoral innate immunity by detecting tissue damage and triggering cellular renewal, making it a candidate tolerance mechanism. Here, we find that in Drosophila melanogaster infected with Pseudomonas entomophila disrupting ROS-producing dual oxidase (duox) or the negative regulator of Jak/Stat Socs36E render male flies less tolerant. Another negative regulator of Jak/Stat, G9a - which has previously been associated with variable tolerance of viral infections - did not affect the rate of mortality with increasing microbe loads compared to flies with functional G9a, suggesting it does not affect tolerance of bacterial infection as in viral infection. Our findings highlight that ROS production and Jak/Stat signalling influence the ability of flies to tolerate bacterial infection sex-specifically and may therefore contribute to sexually dimorphic infection outcomes in Drosophila.

1. Introduction

When organisms experience infection, they face two major challenges to return to a healthy state. The first challenge is to identify and clear the source of the infection. Individuals capable of dealing with the first challenge exhibit low microbe loads because their immune clearance mechanisms are very effective, and are typically labelled ‘resistant’ (Boon et al., 2009; Ganz and Ebert, 2015; Lazzaro et al., 2006; Wang et al., 2017). The mechanisms underlying host resistance have been well characterized empirically and often involve the detection of pathogen-derived molecular patterns such as peptidoglycans, and triggering signalling cascades including the immune deficiency (IMD) and Toll pathways, resulting in the downstream expression of antimicrobial peptides (AMPs) that directly kill pathogens (Kleino and Silverman, 2014; Myllymaki et al., 2014; Myllymaki and Rämet, 2014; Palmer et al., 2018; Valanne et al., 2011).

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 repair and regenerate damaged tissues. 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 et al., 2012; Prakash et al., 2022; Schneider and Ayres, 2008; Soares et al., 2014, 2017). 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 et al., 2014).

Compared to well-described pathogen clearance mechanisms, we are only beginning to unravel the mechanistic basis of disease tolerance (Martins et al., 2019; Medzhitov et al., 2012; Prakash et al., 2022; Soares et al., 2014, 2017). Likely candidate mechanisms underlying effective tolerance of infection include those that regulate inflammation to reduce immunopathology (Adelman et al., 2013; Cornet et al., 2014; Prakash et al., 2021; Sears et al., 2011); detoxification of host or pathogen derived metabolites (Ferreira et al., 2011; Soares et al., 2017; Vale et al., 2014); or tissue protection and regeneration (Jamieson et al., 2013; Prakash et al., 2022; Soares et al., 2014, 2017). 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, dfxox (forkhead box, sub-group O) - do not appear to be directly associated 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 damage signalling pathways.

Here we take advantage of the detailed knowledge of Drosophila immunity to investigate the role of damage signalling plays in disease tolerance during systemic bacterial infection. In response to 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) (Via et al., 2005; Lee and Kim, 2014). While the extra-cellular binding of upd-3 to Domeless (dom), leads to the phosphorylation of Hopscotch (hop). This then leads to the phosphorylation of Stat92E, and its translocation to the nucleus (Myllymäki and Rämet, 2014). In the nucleus, in addition to the production of factors that are necessary for repairing cell 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).

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

The Jak/Stat pathway is also important in humoral immunity to viral infection (Dostert et al., 2005), where a loss of regulation of Jak/Stat by the epigenetic negative regulators G9a results in reduced tolerance of Drosophila C virus infections due to increased immunopathology (Merkling et al., 2015). This specific result motivated us to question whether the effects of G9a-mediated Jak/Stat regulation on tolerance were specific to viral infection, or if the regulation of Jak/Stat also affects disease tolerance during bacterial infection.

We investigated the tolerance response of Drosophila during septic infection with the bacterial pathogen Pseudomonas entomophila, using transgenic flies lacking various components of Jak/Stat signalling and regulation. We focused on P. entomophila because it is a well-established gram-negative bacterial infection model (Dieppois et al., 2015) and importantly, also a natural pathogen of D. melanogaster, having been originally isolated from a female fly of Guadeloupe (Vodovar et al., 2005). P. entomophila has been mainly characterised in the context of orally acquired gut infections, where it causes digestive obstruction and eventually death by severe damage and rupture of the gut through the production of insecticidal protease-based toxins (Dieppois et al., 2015; Vodovar et al., 2005, 2006). During gut infections, flies deficient in mechanism that either prevent, detect damage or that promote the regeneration of damaged tissues therefore experience reduced tolerance of infection (Prakash et al., 2022). A fascinating aspect of P. entomophila infection is that, while the expression of immune-related genes is rapidly induced in the gut following infection, P. entomophila is able to block the translation of these transcripts into functional antimicrobial peptides, thereby evading the host immune response (Chakrabarti et al., 2012). While enteric infections with P. entomophila are extremely well described, less is known about systemic infection with P. entomophila (Prakash et al., 2023), although it is known to cause death in otherwise healthy flies in a matter of hours (Dieppois et al., 2015). We therefore aimed to increase our understanding of P. entomophila systemic infection and the immune and infection phenotypes it causes in D. melanogaster. Further motivated by the widespread observation of sexually dimorphic immunity reviewed in (Belmonte et al., 2020; Klein and Flanagan, 2016) and particularly that the effects of G9a on tolerance of DCV infection are more pronounced in female flies (Gupta and Vale, 2017; Merkling et al., 2015), we also focused on quantifying sex differences in how Jak/Stat signalling affects tolerance of P. entomophila infection.

2. Materials and methods

2.1. Fly strains and maintenance

We used several D. melanogaster transgenic lines with TE mobilization using a P-element construct and subsequent loss-of-function for Duox - P(SUPor-P)DuoxKG07745 (Hurd et al., 2015), Domeless - P(SUPor-P)KG08434, Hopscotch - P(SUPor-P)hogKG01990 (Bellien et al., 2004), Socs36E - P(EPy2')Socs36E(Y2)EY06660 (Monahan and Starz-Gaiano, 2013). All lines were on the yg background (Eleftherianos et al., 2014) which served as a control genotype (detailed information is presented in Figs. S1 and S2 and Table S1). We also used G9a mutant flies (that is, G9aΔ/-), also known as G9a002 generated previously by mobilization of the P-element KG01242 located in the 5′ UTR of the gene (Kramer et al., 2011) and control G9a+/+ (Merkling et al., 2015). We maintained all the fly lines in a 12 ml plastic vials on a standard cornmeal diet see (Siva-Jothy et al., 2018), at 25 °C (±2 °C). We used 3-5-day-old adult flies for all our experiments (see below). First, we housed 2 males and 5 females for egg laying (48 h) in a vial containing fresh food. We then removed the adults and the vials containing the eggs were kept in 25 °C incubator for 14 days, or until pupation. We placed the newly eclosed individuals (males and females separately) in fresh food vials until the experimental day (3 days).

2.2. Bacterial culture preparation

We used P. entomophila cultured overnight in Luria broth (LB) at 37 °C under constant agitation that is, 120 revolutions per minute (rpm). P. entomophila is a gram-negative bacterium naturally found in soil and aquatic environments, known to be highly pathogenic for D. melanogaster (Dieppois et al., 2015; Vodovar et al., 2005). Upon reaching 0.75 OD600 we pelleted the culture by centrifuging during 5 min at 5000 rpm at 4 °C, and then removed the supernatant. We resuspended the bacteria in 1xPBS (phosphate buffer saline) and prepared the final infection inoculum of OD600 of 0.05 for all our infection assays.

2.3. Systemic infection assay

We used a split-vial experimental design (see Fig. S3), where, after infection, each vial containing 25 flies of each sex and fly line combination were divided into 2 vials for measuring (A) survival following infection (n = 15 vials of 15-17 flies/vial/infection treatment/sex/fly line) and (B) internal bacterial load (n = 15 vials of 8-10 flies/vial/infection treatment/sex/fly line). With this split-vial design we were able to use replicate-matched data for both survival and bacterial load to estimate disease tolerance for each fly line (that is, for each replicate group, mean fly survival with respect to mean internal bacterial load). We infected 3-5-day old male and female adult flies using a 0.14 mm
insect minute needles bent at 90° angle to avoid damaging the internal tissues by dipping in P. entomophila bacterial inoculum of OD$_{600}$ of 0.05, resulting in 50–70 bacterial cells/fly. For mock controls we substituted bacterial solution with sterile 1xPBS. After stabbed the flies in the sternopleural region of the thorax (Khalil et al., 2015). We then placed males and females separately onto fresh food vials and incubated at 25 °C. We scored the flies (both infected and control) every 2–3 h for the first 48-h following infection, then 2–3 times each day for the next 6 days (150 h).

2.4. Measuring bacterial load

To quantify internal bacterial load after 24-h following systemic P. entomophila infection first, we thoroughly washed each fly with 70% ethanol for 30 s to surface sterilize and then rinsed twice with autoclaved distilled water. We plated the second wash on LB agar plates and confirmed that the surface bacteria were successfully removed after sterilization. We then transferred individual fly onto 1.5 ml micro centrifuge tubes and homogenized using a motorized pestle for approximately 30–60 s in 100 μl LB broth (n = 30 fly homogenates/sex/infection treatment/fly line). We performed serial dilution of each fly homogenate up to 10$^{-6}$ fold and added 4 μL aliquot on a LB agar plate. We incubated the plate overnight for 18h at 30 °C and counted the resultant bacterial colonies manually (Siva-Jothy et al., 2018). We note that mock-infected control fly homogenates did not produce any colonies on LB agar plates.

2.5. Statistical analyses

2.5.1. Survival

We analysed the survival data with a Cox mixed effects model using the R package ‘coxme’ (Therneau, 2023) for different treatment groups Fig. 1. (A) Survival curves for control 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 = 15 vials with 15–17 flies each vial/fly line/treatment/sex/infection dose). ‘SI’ indicates ‘sham-infection control; ‘I’ indicates infection; ‘*’ indicates that the Jak/Stat transgenic lines are significantly different from yw flies]. (B) Estimated hazard ratios calculated from the survival curves for males and female flies (yw and with flies lacking components of Jak/Stat signalling and duox). A greater hazard ratio (>1) indicates higher susceptibility of Jak/Stat mutants than control while (<1) indicates transgenic lines have better survival than control flies to systemic bacterial infection (p<0.05). (C) Bacterial load (mean log$_{10}$) measured 24 h following infection (n = 15 vials with 8–10 flies each vial/fly line/treatment/sex/infection dose). [significantly different fly lines are connected by different letters using Tukey’s HSD as a post hoc analysis of pairwise comparisons].
(P. entomophila systemic infection and mock controls) across males and females. We specified the model as: survival ~ fly line * treatment * sex * (1|vials/block), with ‘fly line’, ‘treatment’ and ‘sex’ and their interactions as fixed effects, and ‘vials’ nested within a ‘block’ as a random effect.

2.5.2. Bacterial load

We found that the bacterial load data were not normally distributed (tested with Shapiro–Wilks’s test for normality). We therefore used a non-parametric one-way ANOVA Kruskal-Wallis test to test the effects of each fly line and sex on internal bacterial load.

2.5.3. Measuring disease tolerance

We analysed disease tolerance as the linear relationship between fly survival against bacterial load (Ayers and Schneider, 2012; Louie et al., 2016; Oliveira et al., 2020; Raberg et al., 2007). To this end, we employed ANCOVA by fitting ‘fly line’ and ‘sex’ as categorical fixed effects and ‘bacterial load’ as a continuous covariate, and their interactions as fixed effects. Since we were interested in identifying how each transgenic line differed from the control line, we compared the estimates of the model slope using pairwise comparison (t-test; yw vs. different transgenic lines) to test the extent to which each transgenic line significantly differed from the control in tolerating bacterial infections.

3. Results

3.1. Following systemic bacterial infection, disruption of Duox or different components of Jak/Stat pathway result in variable survival outcomes

Overall, we found that disruption of Duox or the Jak/Stat pathway (either by disrupting the positive regulators upd3 and domeless, or overactivation by disrupting the negative regulator socs36E) affected fly survival during bacterial P. entomophila infections (Fig. 1A and B, Table 1 and SI-2). Both male and female flies lacking duox (ROS producing dual oxidase) were more susceptible to P. entomophila infections compared to the control line (yw) (Fig. 1A and B, Table 1 and SI-2). However, other transgenic lines showed slightly improved survival relative to the functional control line. These included male and female flies lacking the transmembrane receptor domeless, and males lacking the negative regulator Soc36E (see hazard ratio in Fig. 1B, Table 1 and SI-2).

3.2. Control yw and Duox/Jak/Stat transgenic deletion lines exhibit similar bacterial loads

We investigated whether the variation we observed between transgenic lines in mortality could be explained by differences in their bacterial load. Given that most mortality occurred just after 24 h for most of our fly genotypes (Fig. 1A) we quantified bacterial load at 24 h following infection. Both control and transgenic lines exhibited similar levels of bacterial load 24 h following infection with P. entomophila (Fig. 1C, Table SI-3). Therefore, despite no substantial difference in microbe loads at 24-h post infection, transgenic lines showed variable survival. This would fit the functional definition of disease tolerance as for the same bacterial load some lines appear to be more tolerant (survive longer, such as domeless) while others are less tolerant (e.g., duox).

3.3. Disrupted expression of Duox or Jak-Stat signalling leads to differences in disease tolerance phenotypes

While the results above are indicative of variable tolerance depending on the Jak/Stat disruption, 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 and Fig. S3 for description of split-vial design). Here, the differences in tolerance between Jak/Stat deletion and the control fly line 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 between fly lines. Overall, we found that the transgenic lines showed differences in disease tolerance phenotypes compared to control in both males and females, and this effect was driven mainly the Duox-deficient lines, which showed a much steeper decline in survival with increasing P. entomophila bacterial loads (Fig. 2A and B, Tables 2 and 3).

3.4. Disruption of G9a does not affect tolerance of P. entomophila

The negative regulator of Jak/Stat, G9a, was previously identified as being important for tolerating Drosophila C Virus (DCV) infections (Merkling et al., 2015), so we inquired if loss of function of G9a also affected disease tolerance in response to bacterial infections. Overall, we found that loss of G9a makes both males and females more susceptible to P. entomophila infections, (Fig. 3A for survival and Fig. 3B for hazard ratio, Table 4 and Table SI-4). To test if this increased mortality in G9a+/- flies was associated with higher bacterial replication we measured bacterial load following 24 h P. entomophila systemic infection. We found that G9a+/- females exhibited higher bacterial load than G9a+/-/+ (control) flies, while males showed similar bacterial load as G9a+/-/+ flies (Fig. 3C, Table SI-5). However, the overall ability to tolerate P. entomophila bacterial infections (that is, measured as G9a fly’s survival relative to its bacterial load) remained similar across both males and females G9a flies that is, both G9a+/- and G9a+/-/+ controls (Fig. 3D, Table 5, and Table 6 for comparison between estimates of tolerance slope).

4. Discussion

Our data show that loss-of-function in Jak/Stat pathway components reduces overall survival following P. entomophila septic infection and that this is not caused by impaired pathogen clearance but due to lower disease tolerance. We have previously found similar effects of Jak/Stat signalling on tolerance of P. entomophila during oral infection, where fly lines with the loss-of-function Jak/Stat ligand upd3 showed sharp declines in tolerance in both males and females (Prakash et al., 2022). While we did not test the role of upd3 directly in the present study, our results are consistent with a general role for Jak/Stat signalling in mediating infection tolerance, in both systemic and oral infections.

An important point regarding the action of duox, is that its role has been mainly established during enteric infections by producing ROS in response to epithelial damage (Ha et al., 2005; Lee and Kim, 2014; Vodovar et al., 2005). This makes the effects of duox on tolerance of septic infection that we observe in the current study less intuitive. However, previous work has shown that injury or septic infection with either gram positive or gram-negative bacteria can trigger the expression of upd3, leading to the remote activation of the Jak/Stat pathway in the gut as well as intestinal stem cell (ISC)-mediated renewal of gut

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**Table 1**

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

| 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     | 0.968     |
|            | Male    | 0.694    | <0.001| 0.585     | 0.824     |
| Soc36E     | Female  | 0.990    | 0.91  | 0.843     | 1.167     |
|            | Male    | 0.795    | 0.006 | 0.676     | 0.937     |
epithelial cells (Chakrabarti et al., 2016). Further, by inhibiting this intestinal renewal, flies became more susceptible following septic infection. While that study did not investigate the role of duox in this remote activation of gut renewal, given the role of duox in ROS production and in triggering intestinal epithelial renewal it is plausible that duox may also be required for increased protection following septic infection, which may explain why we observed increased mortality without and substantial effect on microbe loads (decreased tolerance – Figs. 1 and 2) in duox loss-of-function fly lines.

Another potential explanation for duox-mediated disease tolerance during septic infection is that haemocytes also produce ROS in response to injury – even in the absence of infection – triggering a Jak/Stat-mediated tissue repair response at the site of tissue injury (Chakrabarti and Visweswariah, 2020). Given that P. entomophila produce a cocktail of protease-based toxins which cause severe tissue degradation during infection, it is plausible that tolerating such pathology would require an intracellular duox-mediated oxidative burst for the activation of cellular responses during wounding and injury (Chakrabarti and Visweswariah, 2020). This response is also likely to be sexually dimorphic; wild type (w^{Dahomey}) males showed higher levels of duox expression and ROS following Ecc (Erwinia caterovora) infection (Regan et al., 2016), which may suggest that loss of function of duox might impact males more than females, as observed in this experiment (Fig. 2B).

Sex differences in disease tolerance were also apparent in flies lacking Soc36E, and one potential explanation may be related to sex differences in the 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 than males during viral infections (Gupta and Vale, 2017). Similar examples also

Table 2
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, 24 h following infection. We analysed ANCOVA and fitted ‘sex’ as categorical fixed effects, ‘mean bacterial load (log_{10})’ as a continuous covariate and their interactions as fixed effects for the transgenic lines.

| sex line | Source | DF | Sum of Sq. | F ratio | P |
|----------|--------|----|------------|---------|---|
| Female   | Fly line | 4  | 24817.1    | 15.27   | <0.001 |
|          | Bac. load | 1  | 4482.9     | 11.03   | 0.0012 |
|          | Fly line X Bac. load | 4  | 7647.2     | 4.7     | 0.0015 |
| Male     | Fly line | 4  | 16964.8    | 9.22    | <0.001 |
|          | Bac. load | 1  | 6122.6     | 13.32   | 0.0004 |
|          | Fly line X Bac. load | 4  | 5737.5     | 3.12    | 0.017  |

Table 3
Summary of F-test pairwise comparisons of estimates of linear slopes (from the linear model) transgenic lines compared to the yw control.

| sex | line | SSE | df | slope | std | slope | diff | std | F | ratio | p |
|-----|------|-----|----|-------|-----|-------|------|-----|---|-------|---|
| Female | Duox | 10018.68 | 30  | 13.15 | 3.09 | 0.53  | 0.37 | 0.058 |
|       | Domeless | 16075.94 | 45  | 1.83  | 2.25 | 8.38  | 2.30 | 0.047 |
|       | Hopscotch | 11897.99 | 34  | 5.99  | 2.64 | 1.39  | 0.24 |
|       | Soc36E | 27135.53 | 47  | 3.77  | 2.28 | 1.28  | 0.22 |
| Male  | Duox | 17512.77 | 34  | 11.25 | 4.47 | 5.25  | 0.028 |
|       | Domeless | 21066.63 | 49  | 1.46  | 2.67 | 0.09  | 0.65 |
|       | Hopscotch | 15019.55 | 36  | 3.2   | 2.8  | 0.01  | 0.91 |
|       | Soc36E | 22825.12 | 47  | 1.46  | 2.75 | 0.54  | 0.46 |
exist from other innate immune pathways where, disrupting the negative regulator of IMD, PGRP-LB (peptidoglycan receptor-LB), affected survival to a greater extent in females following E. coli infection, suggesting the sex-specific role of some of these regulators (Vincent and Dionne, 2021). The combination of these observations might explain why flies lacking Socs36E showed sex differences in disease tolerance, particularly in light of the crosstalk between Jak/Stat and IMD pathways (Bang, 2019; Dostert et al., 2005; Kemp et al., 2013).

An unexpected observation was that flies lacking domeless showed slightly increased survival relative to the yw control (Fig. 1) (and a trend for increased tolerance, though not statistically significant, Fig. 2). Given the role of domeless as an activator of Jak-Stat signalling, this might suggest that Jak/Stat activation may be costly to flies. While

Table 4
Summary of estimated hazard ratio from the cox proportional model. A greater hazard ratio (>1) indicates that G9a⁻⁻ flies are more susceptible to P. entomophila infection than control (G9a⁺⁺) flies.

| sex  | Fly line | estimate | p      | Std err |
|------|----------|----------|--------|---------|
| Female | G9a⁻⁻    | 2.2      | <0.001 | 0.75    |
| Male  | G9a⁻⁻    | 1.41     | <0.001 | 0.45    |

Table 5
Summary of ANCOVA. To assess differences in infection tolerance (fly survival with increasing bacterial burden) following systemic P. entomophila infection with OD₆₀₀ = 0.05 infection dose, 24 h following infection. We analysed ANCOVA and fitted ‘sex’ as categorical fixed effects, ‘mean bacterial load (log₁₀)’ as a continuous covariate and their interactions as fixed effects for each of the fly lines (G9a).

| Fly line | Source | DF | Sum of Sq. | F ratio | p         |
|----------|--------|----|------------|---------|-----------|
| G9a⁻⁻    | Female | 1  | 6802.2     | 20.21   | <0.001    |
|          | Bac. load | 1  | 53.19      | 0.158   | 0.69      |
|          | Fly line X Bac. load | 1  | 1.610      | 0.004   | 0.94      |
| G9a⁺⁺    | Female | 1  | 3042.5     | 8.685   | 0.005     |
|          | Bac. load | 1  | 533.13     | 1.521   | 0.22      |
|          | Fly line X Bac. load | 1  | 166.21     | 0.474   | 0.49      |

Table 6
Summary of F-test pairwise comparisons of estimates of the linear slopes (linear reaction norm) for G9a⁻⁻ relative to G9a⁺⁺ control fly lines.

| sex  | Fly line | Fly line | F Ratio | p       |
|------|----------|----------|---------|---------|
| Female | G9a⁻⁻    | G9a⁺⁺    | 0.005   | 0.94    |
| Male  | G9a⁻⁻    | G9a⁺⁺    | 0.474   | 0.49    |
immune deployment and regulation is highly energy demanding across most species (McKean et al., 2008; Nystrand and Dowling, 2020; Schwenke et al., 2016; Vale et al., 2015), the physiological costs of specific individual immune components and pathways remains understudied and an open question for future research.

The negative regulator of Jak/Stat, G9a, was previously identified as being important for tolerating Drosophila C Virus (DCV) infections (Merkling et al., 2015). Subsequent work exploring sex differences in this response found that G9a+/− (control) females had higher tolerance than G9a−/− females, when measured across a range of viral DCV doses (Gupta and Vale, 2017). We therefore aimed to test whether the loss of function of G9a also affects fly survival and disease tolerance in response to bacterial infections. While G9a+/− flies showed higher susceptibility to infection, likely driven by elevated microbe loads compared to control flies with functional G9a, we did not observe an effect on disease tolerance. It is unclear why G9a loss-of-function would result in a reduced ability to control microbe growth. Like Socs36E, G9a is described as a negative regulator of Jak/Stat. However, while Socs36E acts by interacting physically with hopscotch (thereby interfering with its phosphorylation and downstream signalling), G9a is a histone H3 lysine 9 methyltransferase and negatively regulates Jak/Stat signalling by epigenetic methylation of Socs36E and Drosophila C Virus (DCV) infections (Merkling et al., 2015). While previous work investigating the role of G9a on viral tolerance focused specifically on genes involved in Jak/Stat signalling, it is possible that G9a also acts epigenetically on other immune responsive genes that would directly affect bacterial clearance, as we observe here (Riahi et al., 2021). However, despite the previously identified role of this negative regulator of Jak/Stat in tolerating viral infections by reducing immunopathology (Gupta and Vale, 2017; Merkling et al., 2015), G9a does not appear to affect bacterial disease tolerance in a comparable way.

Linking tissue damage signalling and repair mechanisms such as Jak/Stat to disease tolerance is important from a therapeutic perspective because they have the potential to boost host tolerance by minimising disease severity (Soares et al., 2014; Vale et al., 2016). For instance, dysregulation of cytokines and interferons (JAK signalling - Tyrosine kinase2) result in drugs that inhibit Jak have been shown to be effective in treating several autoimmune diseases, while STAT inhibitors have been promising candidates in the context of cancer (Mikløssy et al., 2013; Pérez-Jeldres et al., 2019; Salas et al., 2020). It may therefore be possible to repurpose these existing drugs to improve host tolerance of infection.

In summary, our work highlights that Jak/Stat directly impacts the ability to tolerate septic bacterial infection and that this response differs between males and females. Jak/Stat mediated disease tolerance may be a potential source of sexually dimorphic responses to infection in Drosophila.

Author contributions
AP and PFV conceived and designed the experiments. AP, MB, KMM, conceived and designed the experiments. AP, MB, KMM, Dieppois, G., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Filloux, A. (Eds.), Pseudomonas Entomophila: A Versatile Bacterium with Applications in Neurobiology and Parasitology (2001), J. Animal Ecology 83, 256–265. https://doi.org/10.1017/S0021879001005518.

Data availability
All data and code for analysis is available at doi:10.5281/zenodo.7867071

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2023.104756.

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