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Immune regulation of disease tolerance and immune priming in Drosophila

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Thesis abstract

To regain health following infection, hosts must not only identify and eliminate the source of infection, but also be able to reduce the resulting tissue damage in order to tolerate immunopathology. Compared to the mechanisms of pathogen clearance, we currently know less about how the mechanisms of damage prevention and repair contribute to disease tolerance phenotype. The aim of this thesis is to improve our understanding of mechanisms that enhance disease tolerance during bacterial infections. Here, I employed genetically manipulated fruit flies (*Drosophila melanogaster*) with disrupted mechanisms of immune regulation, damage prevention and repair to test how these mechanisms contribute to disease tolerance.

We find that reduced expression of the negative regulators of IMD (immune deficiency pathway) or absence of regulation of Jak/Stat (Janus kinase/signal transducer and activator of transcription pathway), severely reduced the ability of flies to tolerate systemic infection with *Pseudomonas entomophila*. Therefore, in addition to regulating an efficient pathogen clearance response, negative immune regulators also contribute to disease tolerance. We also found that loss-of-function mutants lacking damage preventing *dcy* (drosocrystallin - a major component of the peritrophic matrix), damage signalling *upd3* (unpaired protein, a cytokine-like molecule), damage repairing *egfr*^{1} (epidermal growth factor receptor) and damage controlling *irc* (immune-regulated catalase, a negative regulator of reactive oxygen species), affect the ability of flies to tolerate enteric infection, and that these effects are sexually dimorphic.

Finally, we also investigated an additional defence mechanism of immune priming in *Drosophila*. Using UAS^{-RNAi} knockdown, loss-of-function or immune deletion mutants and CRISPR knockout transgenic flies we found that immune priming is a long-lasting response, occurring in several backgrounds and is particularly stronger in male flies. Priming requires the regulation of the IMD-responsive antimicrobial peptide *Diptercin* in the fat body against the gram-negative bacteria *Providencia rettgeri*. We further found that priming has the potential to reduce disease spread and transmission by affecting pathogen shedding. The thesis concludes with an outlook on future research in the field of disease tolerance and damage limitation mechanisms to bacterial infections in *Drosophila*.

Lay Summary

When faced with infection threat, individuals must not only identify and eliminate the infection source, but also be able to endure the damage caused to cells and tissues. Activation of immune response is costly, while excessive immune system activity causes self-damage to cells and tissues, an insufficient immune response can result in pathology. To regain health and minimize the damage following infection, hosts must balance the costs of immunity by closely regulating immune responses. Compared to previously identified processes that clears infection, we know less about how hosts regulate, control, prevent and repair the cellular damage during infection.

In this thesis, I use several genetically manipulated fruit flies and their bacterial pathogens to test how the immune regulation, tissue damage prevention and repair processes contribute to host's ability to tolerate infections. Largely, reducing the negative immune regulation of a major immunological control pathway in fruit flies known as IMD (immune deficiency pathway), absence of damage sensing, control, prevention, and repair processes reduces the fly's ability to tolerate bacterial infection. Some of these effects differ between males and females highlighting how sex differences in some of these mechanisms could generate sexual dimorphism in immunity and infection.

In addition, regulation of another defense mechanism know as immune priming in fruit flies was addressed. Priming in fruit flies is a long-standing response, that is usually stronger in males, and requires the regulation of antimicrobial peptide (known as *Diptericin*) through immune signalling pathway (IMD) in the fat body. Further, priming has the potential to reduce disease spread.

Declarations

I declare that the work within this thesis is my own, except when stated. I have written this thesis under the guidance of my supervisor. I conducted all experimental work with laboratory assistance as mentioned below. All the thesis ***chapters*** are in the process of submission to journals, and therefore I use "We" throughout my thesis.

Chapter 2 and 3: All experiments were designed with the help of my supervisor, Dr. Pedro Vale. Katy Monteith (*lab manager*) assisted me during gene expression assay. I collected all the data presented in this chapter, performed all the analyses, and wrote the subsequent chapter in collaboration with Pedro.

Chapter 4: All experiments were designed with Pedro. Mickael Bonnet (*master's student*) assisted me during survival assays and Katy helped me during gene-expression experiments. I collected most of the data presented in this chapter with Mickael's help, performed all the analyses, and wrote the subsequent chapter in collaboration with Pedro.

Chapter 5: All experiments were designed with Pedro. I collected survival and bacterial load the data with Florence Fenner's (*undergrad student*) help, while Biswajit Shit (*project assistant*), Tiina Salminen (*post-doc*) and Katy assisted me during other experiments. I performed all the analyses with help from Florence and wrote the subsequent chapter in collaboration with Pedro.

Chapter 6: All experiments were designed with Pedro. I collected most of the data with Katy. I performed all the analyses and wrote the subsequent chapter in collaboration with Pedro.

This thesis is submitted in accordance with the requirements for a Doctor of Philosophy by the School of Biological Sciences at the University of Edinburgh. The work included in this thesis has not been submitted for any other degree or professional qualification.

Arun Prakash

List of preprints and published articles

The following chapters are under review or preparation for journal submission.

- **Prakash A**, Monteith KM, Vale PF. 2021. (*In revision*). Negative regulation of IMD contributes to disease tolerance during systemic bacterial infection in *Drosophila. bioRxiv,* 2021.09.23.461574. September 2021.

- **Prakash A**, Monteith KM, Vale PF. 2021. (*In revision*). Mechanisms of damage prevention, signalling and repair impact the ability of *Drosophila* to tolerate enteric bacterial infection. *bioRxiv,* 2021.10.03.462916. October 2021.

- **Prakash A**, Bonnet M, Monteith KM, Vale PF. 2021. (*In revision*). The *Jak/Stat* pathway mediates disease tolerance during systemic bacterial infection in *Drosophila. bioRxiv,* 2021.09.23.461578. September 2021.

- **Prakash A**, Fenner F, Shit B, Salminen TS, Monteith KM, Khan I, Vale PF. (*In prep*) Regulation of the *IMD*-responsive antimicrobial peptide *Diptericin* is required for immune priming in *Drosophila.*

- **Prakash A**, Monteith KM, Vale PF. (*In prep*). Immune priming reduces pathogen transmission in *Drosophila.*

I contributed to following papers during my PhD, but these do not form part of my thesis:

- Siva-Jothy JA, **Prakash A,** Vasanthakrishnan RB, Monteith KM, Vale PF[#]. (2018). Oral Bacterial Infection and Shedding in *Drosophila melanogaster*. *Journal of Visualized Experiments,* (135), e57676, doi:10.3791/57676

- Khan I[#], **Prakash A**, Agashe D[#]. (2019). Pathogen susceptibility and fitness costs may explain inter-population variation in immune priming. *Journal of Animal Ecology,* 88 (9), 1332–1342, doi: 10.1111/1365-2656.13030

- **Prakash A**, Agashe D[#], Khan I[#]. (2022). The costs and benefits of basal infection resistance vs immune priming responses in an insect. *Development and Comparative Immunology,* 104261 doi: https://doi.org/10.1016/j.dci.2021.104261

- **Prakash A**[#] and Khan I[#]. (2022). Why do insects evolve immune priming? A search for crossroads. ([#]*correspondence*) *Development and Comparative Immunology,* 104246 doi: https://doi.org/10.1016/j.dci.2021.104246

See Appendix section for manuscripts in their published format.

Glossary

AMPs – antimicrobial peptides
ANCOVA – analysis of co-variance
ANOVA – analysis of variance
AttC – attacin C
AttD – attacin D
cDNA – complementary DNA (deoxyribonucleic acid)
CecA1 – cecropin A1
CFUs – colony forming units
CO_2 – carbon dioxide
CrebA – Cyclic response element binding protein A
CRISPR – Clustered regularly interspaced short palindromic repeats
Ct – cycle threshold value
DAM – *Drosophila* activity monitor
DAMPs – damage associated molecular patterns
DAP – type (Diaminopimelic acid) peptidoglycans
dbs – debris buster
DCV – *Drosophila* C virus
Dcy – drosocrystallin protein
dFOXO – forkhead transcription factor encoded by daf-16
DGRP – *Drosophila* genetic resource panel
Dif – dorsal related immunity factor
DNA – deoxyribonucleic acid
Dom – domeless
Dpt – diptericin
Dro – drosocin
Duox – dual oxidase
E. coli – Escherichia coli
E. faecalis – Enterococcus faecalis
Ecc-15 – Erwinia carotovora spp.
ECs – enterocytes
EFGR – epidermal growth factor receptor
gh – grainyhead
GLM – general linear models
GNBPs – gram-negative binding proteins
GWAS – genome-wide association study
H_2O_2 – hydrogen peroxidase
Hops – hopscotch
Imd – immune deficiency pathway
Irc – immune regulated catalase
ISCs – intestinal stem cells
Jak/Stat – Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway
K-W test – Kruskal-Wallis statistical test
LB – Luria broth
M-MLV – Moloney Murine Leukaemia Virus Reverse Transcriptase
mtDNA – mitochondrial DNA
mtROS – mitochondrial ROS
NF-kB – nuclear factor kappa B
NK-cells – natural killer cells

Nox – NADPH oxidase
OD – optical density
OXPHOS – oxidative phosphorylation
P. entomophila – Pseudomonas entomophila
P. rettgeri – Providencia rettgeri
PAMPs – pathogen associated molecular patterns
PM – peritrophic matrix
PO – phenoloxidase
PRRs/PGRPs – pattern recognition receptors/ peptidoglycan recognition proteins
qRT-PCR – Real-Time Quantitative Reverse Transcription PCR
Rel – relish
RNA – ribonucleic acid
RNAi – RNA interference pathway
RNAseq – RNA sequencing
ROS – reactive oxygen species
rp49 – ribosomal protein 49
rpm – revolutions per minute
S. aureus – Staphylococcus aureus
S. pneumoniae – Streptococcus pneumoniae
Socs36E – suppressor of cytokine signalling 36E
spz – spaetzle
Stat92E – Signal-transducer and activator of transcription protein at 92E
T_a – annealing temperature
TALEN – Transcription activator-like effector nucleases
TCA – tricarboxylic acid cycle (Krebs cycle)
TNF – tumour necrosis factor
Tukey's HSD – Tukey's honest significant difference test
UAS-GAL4/80 – upstream activation system - encoding the yeast transcription activator protein Gal4/80
Upds – unpaired proteins
VDRC – The Vienna *Drosophila* Resource Centre
w^{1118} – *Drosophila* white fly strain, originates from the wild-collected Oregon-R
yw – *Drosophila* yellow white fly strain

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To family,

always present not only in the good times

Chapter 1

General introduction and Outline of the thesis

1.1 *Preface*

In the past two decades, two major host immune defense strategies, called resistance and tolerance, have been conceptualized in immunology and infection biology. Resistance to infection involves all the mechanisms that either eliminate or clear the invading pathogens by immune activation. On the other hand, hosts might tolerate infections, by minimizing the negative impacts of the pathogen without reducing the pathogen burden. Although the majority of studies on pathogen defence primarily focus on the mechanisms that hosts employ to resist infections, recent experiments provide ample evidence for disease tolerance. For instance, many hosts are able to coexist with pathogens and withstand their negative fitness impacts by reducing pathogen or immune-induced damage (Raberg et al., 2007; Ayres and Schneider, 2012; Medzhitov et al., 2012; Louie et al., 2016; McCarville and Ayres, 2018; Oliveira et al., 2020). However, in contrast to the mechanisms that eliminate pathogens, we currently know less about mechanisms that promote disease tolerance. The overall aim of this thesis is to improve our understanding of mechanisms that enhance or boost disease tolerance during bacterial infections. The following introductory chapter first provides a brief introduction to insect immunity, mainly focusing on the fruit fly *Drosophila*. This is followed by a detailed overview of *Drosophila* innate immune signalling pathways and immune regulation. In the second part, we investigate an additional defence mechanism of immune-like priming in *Drosophila*. We further address important implications of immune priming for disease ecology and epidemiology, and particularly how it may affect pathogen transmission.

1.2 *Insect immunity*

Insects are among the most diverse organisms, populating almost every habitat on earth (Chapman, 2009). Insects also pose a major threat to humans and other organisms as agricultural pests and vectors for numerous pathogens including bacteria, protozoa, nematodes and viruses (Schmid-Hempel, 2021). The majority of work on insect immunity mainly focus on arboviruses (viral arthropod borne diseases), however, arthropod-borne bacterial diseases are often ignored (Weaver and Reisen, 2010; Ehounoud et al., 2017). For instance, Lyme disease, different spotted fevers, tick-borne and louse-borne relapsing fevers are a few among many life-threating bacterial arthropod-borne diseases (Ehounoud et al., 2017). Due to climate change, global travel, urbanization, and adaptation to new habitats, outbreaks of arthropod-borne diseases are often difficult to predict and control. Hence, insect vector control tools are immediately needed to control bacterial and viral arthropod-borne diseases.

1.1.1 *Drosophila immunity in a nutshell:*

The fruit fly, *Drosophila melanogaster* is host to a wide range of natural microbial pathogens including bacteria, virus, fungi and parasites, reviewed in (Keebaugh and Schlenke, 2014; Troha and Buchon, 2019; Kapun et al., 2020). *D. melanogaster* is also a powerful and genetically tractable model to study innate immunity, making it an ideal system for studying host-pathogen interactions. Their highly conserved innate immune systems and cellular processes between flies and vertebrates are commonly hijacked by pathogens (Mirzoyan et al., 2019). The availability of versatile tools for genetic manipulation for instance, the potential to express a candidate (gene or protein) either one at a time or in conjunction with each other in order to dissect the molecular functions, especially in a cell-type-specific manner. Together, all these features, with a brief generation time, low maintenance costs, combine to make *Drosophila* a powerful system for dissecting out host-pathogen interactions ***Fig****. 1*.

Drosophila immunity, in general, relies on humoral and cell-mediated innate components for its defense against pathogens, such as bacteria, viruses, fungi etc. Immune system mechanisms in fly against these invading pathogens usually share a basic and similar immune architecture and briefly include: (i) first, recognition of these pathogens via the pattern recognition receptors (such as *PGRPs* – pathogen recognition receptors proteins). Followed by (ii) the activation of appropriate signalling pathways (such as IMD-immune deficiency, Toll, and Jak/Stat – The Janus kinase/signal transducer and activator of transcription), which involves (iii) production of effector molecules - antimicrobial peptides (*AMPs*), which aids in (iv) elimination of infecting pathogens. After which (v) the wound and tissue damage repair /renewal mechanisms in the gut-epithelia (Enterocytes; *ECs* – Jak/Stat activation and *EGFR* epidermal growth factor receptor signalling), and finally, (vi) the circulating hemocytes that is, lamellocytes, crystal cells and plasmacytes, macrophages (cellular arm of immunity) involved in the melanisation process, phagocytosis or encapsulation of the foreign particles.

These processes are similar to first line defense mechanisms that can be found in other vertebrates including mammals and plants. **(1).** Humoral innate immunity: The humoral innate immune response involves the production of inducible antimicrobial peptides (*AMPs*) in response to systemic infections such as bacterial and fungal infections (Lehrer and Ganz, 1999; Hultmark, 2003; Hoffmann, 2003; Lemaitre and Hoffmann, 2007). *AMP* production is mediated by the immune deficiency (IMD) and Toll signalling pathways (Myllymäki et al., 2014; Valanne et al., 2011). Apart from IMD and Toll signalling pathway, in response to viral infections, insects also rely on the Jak/Stat pathway (Janus kinase/signal transducers and

activators of transcription)to signal tissue damage, and the *RNAi* pathway during viral infection (Merkling and van Rij, 2013; Palmer et al., 2018; Bang, 2019). **(2)** Cellular innate immunity: Cell-mediated innate immunity mainly consists of haemocytes (such as plasmatocytes, lamellocytes, and crystal cells,) which are similar to human macrophages in function as they destroy pathogens by phagocytosis and by encapsulating foreign material (Stofanko et al., 2010; Vlisidou and Wood, 2015). Haemocytes are particularly important during wasps and other parasite infection. **(3)** The gut-epithelial response following oral or enteric infection is key for tissue damage prevention, control and repair processes in the *Drosophila*. Gut-epithelial response is important during oral/enteric infections. Finally, other physiological processes such as (4) energetics and metabolism may also influence fly's response against pathogens (Salminen and Vale, 2020) (see ***Fig.*** *1*). Below I will describe the functioning of these pathways in greater detail.

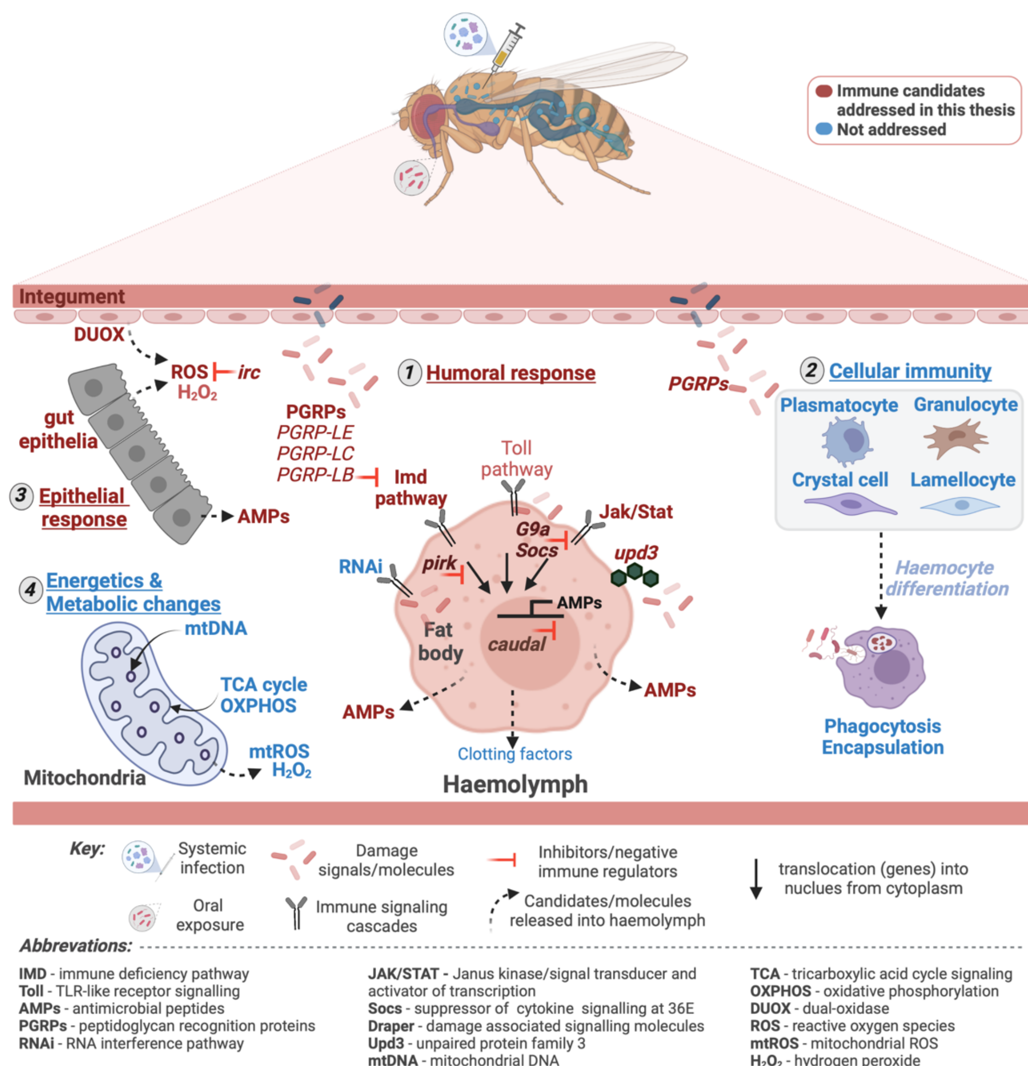

Figure 1: Graphical summary of *Drosophila* innate immunity in a nutshell

1.2.2 *Inducible immune pathways*:

The Toll and IMD (Immune deficiency) pathways mediate systemic immunity against bacteria (gram-positive and gram-negative) and fungi in *Drosophila* (De Gregorio et al., 2002; Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007), and have more recently been implicated in antiviral defense responses (Merkling and van Rij, 2013; Palmer et al., 2018).

1.2.2.1 *The IMD (immune deficiency) pathway*:

During gram-negative bacterial infection, the immune response in *Drosophila* is mainly regulated by the immune deficiency (IMD) signalling pathway, which activates NF-κB transcription factor (*relish*) that controls the expression of the majority of the *AMPs* such as *Diptericins, Attacins and Cecropins* that are distinct from those induced by *Toll* immune pathway (*Drosomycin and Defensin*). Gram-negative bacteria are recognised by the pathogen-sensing receptors *PGRPs* (*LC and LE*) via peptidoglycans (see ***Fig**. 2*). These receptors then recruit the adaptor molecule IMD and activate the intracellular signalling cascade that leads to the activation of a NF-κB *relish* [reviewed in (De Gregorio et al., 2002; Kleino and Silverman, 2014; Myllymäki et al., 2014)]. Upon activation, *relish* translocates to the nucleus and induces transcription of a set of IMD-responsive *AMPs* and also negative regulators of IMD pathway.

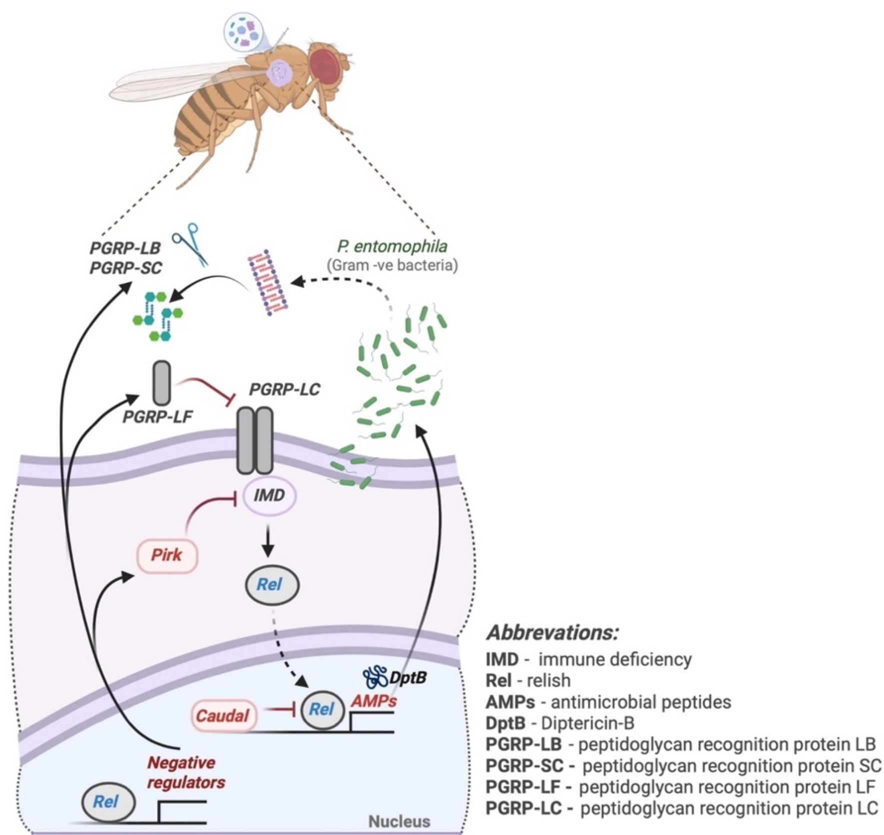

Figure 2: Graphical summary of IMD-pathway. *This figure also appears in the data chapter-2.*

Minutes after pathogen recognition, a cocktail of *AMPs* is secreted by the fat body (Lemaitre et al., 1997). The negative regulators such as *PGRP-LB, pirk and caudal* ensure an appropriate level of immune response (Kleino and Silverman, 2014; Lee and Ferrandon, 2011). *Pirk*, a negative regulator of the IMD pathway, interferes with the interaction of *PGRP-LC and -LE* with the molecule IMD, and limits the activation of the IMD pathway (Aggarwal et al., 2008; Kleino et al., 2008; Basbous et al., 2011). Similarly, the homeobox protein caudal downregulates the expression of *AMPs* in flies (Ryu et al., 2008). Taken together, the negative regulators of IMD enables individuals to reduce immune activity in response to reductions in bacterial numbers.

1.2.2.2 *The Toll pathway*:

Upon infection by Gram-positive bacteria or fungal pathogens, the *Toll* pathway is activated by two pathogen-sensing systems or pattern-recognition receptors (Gobert et al., 2003) (i) *PAMPs* by beta-glucans of entomopathogenic fungi and (ii) Gram- negative binding protein (*GNBP*)-1 and -3, and peptidoglycan recognition proteins *PGRP-SA* and *-SD*, respectively by Lysine-type peptidoglycan of gram-positive bacteria. The proteolytic activity in the haemolymph due to pathogenic invasion, also considered as danger signal, is sensed by the protease *Persephone* (Gottar et al., 2006). This initiates an extracellular signaling cascade that leads to activation of cytokine *Spätzle*, which then binds the transmembrane receptor *Toll*. Subsequently, an intracellular signaling pathway leads to the translocation of the NF-κB like transcription factors *Dif* (Dorsal-related immunity factor, in adults) and *Dorsal* into the nucleus. Thereby inducing the expression of many genes, including antimicrobial peptides (*AMPs*), such as *Drosomycin* and *Defensin* (Lemaitre and Hoffmann, 2007; N. Buchon et al., 2009; Valanne et al., 2011).

1.2.2.3 *The Jak-Stat pathway:*

In response to different types of stresses such as mechanical injury, oxidative stress, and during infections the Jak/Stat pathway is activated (Chakrabarti et al., 2016; Dostert et al., 2005; Ekengren et al., 2001; Ekengren and Hultmark, 2001; Lemaitre et al., 1996), by ligands of unpaired family namely *upd-1, upd-2* and *upd-3*) (Harrison et al., 1998; Agaisse et al., 2003; Gilbert et al., 2005). Due to their resemblance to mammalian cytokine Interleukin-6, the upd ligands are also known as cytokines (Brown et al., 2001). The cytokine ligand upd-3 then interacts with a transmembrane receptor *Domeless* (Brown et al., 2001). The *upd-3*'s are produced during damage (cellular/infectious) and are induced by reactive oxygen species

(*ROS*) and damage-associated molecular patterns (*DAMPs*), which in turn are produced by the *duox* (dual oxidase) (Lee and Kim, 2014; Srinivasan et al., 2016; Wang et al., 2017) (see ***Fig.*** *3*).

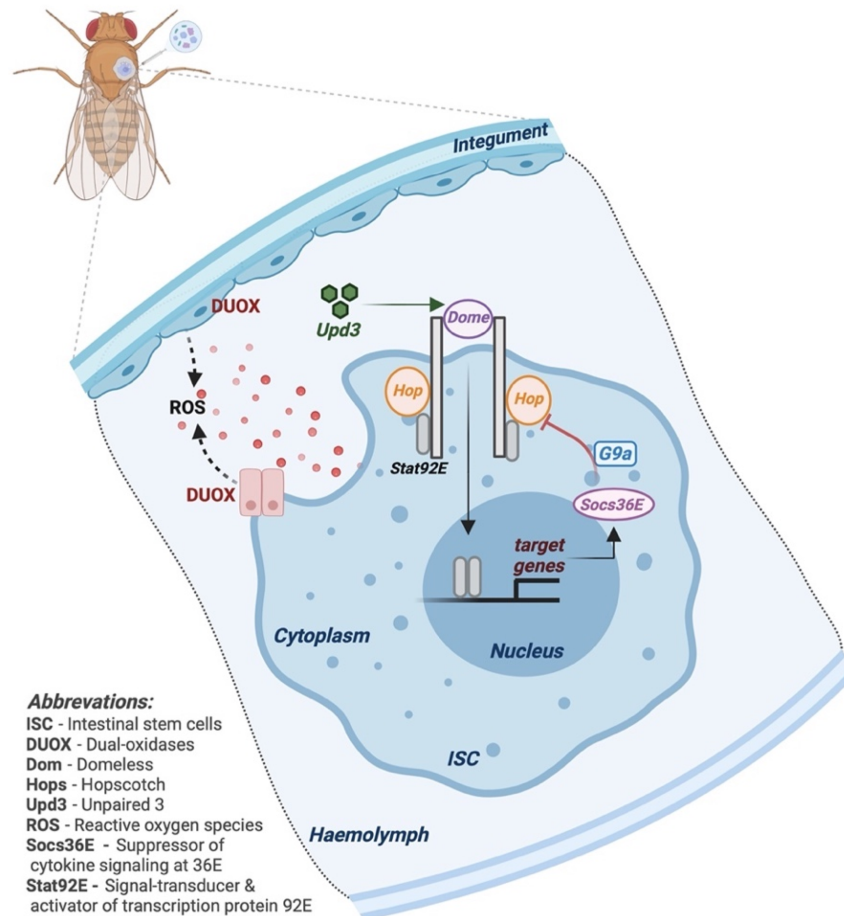

Figure 3: Graphical summary of Jak/Stat-pathway. *This figure also appears in data chapter-4.*

The extracellular binding of *upd-3* on *Domeless* (*dome*), leads to the phosphorylation of *Hopscotch* (*hop*), known as the "JAK" (Janus kinase) part of the pathway. This then leads to the phosphorylation of *Stat92E*, once activated the Stat92E, enters the nucleus and binds to the DNA under dimers or more complex oligomers (Hou et al., 1996). This binding leads to the production of repair factors that are necessary for repairing the cellar damage. Among these factors, the binding of *Stat92E* on the DNA induces the production of Socs36E, a negative regulator of *Hopscotch* (Kiu and Nicholson, 2012) However, the production of *ROS* also triggers another signaling pathway, the epidermal growth factor (*EGFR*) which is also implicated in cell repair (Weng et al., 2018) and damage control (Buchon et al., 2010; Jiang et al., 2011) ***Fig.*** *3*. Meanwhile, the epigenetic regulator histone H3 lysine 9 methyltransferase (also known as *G9a*) is known to regulate the expression of the Jak/Stat proteins and mediate tolerance to RNA virus infection (Merkling et al., 2015).

1.3 ***The Drosophila gut-epithelial immunity:***

The *Drosophila* gut-epithelium mainly consists of a barrier, a peritrophic matrix (PM) that physically separates from lumen. The PM is analogous to mammalian mucus membrane in the digestive tract and acts as first line of defense against invading pathogens (Hegedus et al., 2009; Kuraishi et al., 2013). Previous evidence suggests that *dcy* (drosocrystallin protein) in the adult *Drosophila* midgut plays a protective role of the peritrophic matrix against microbial pathogens . The loss-of-function mutation in *dcy* increases the peritrophic matrix width which in turn increases its permeability to larger molecules such as pathogens (Kuraishi et al., 2011). The *dcy* deficient flies exhibit-increased susceptibility to oral bacterial infections (Vodovar et al., 2005; Kuraishi et al., 2013).

Oral infection with *P. entomophila in D. melanogaster* causes destruction of the gut epithelium due the production of pore-forming toxin produced by *P. entomophila* (Vodovar et al., 2005; Dieppois et al., 2015; Kuraishi et al., 2013). In response to the ingested *P. entomophila*, flies induce two key responses (i) the production of *ROS* and the (ii) expression of *AMPs* (antimicrobial peptides). Although, *ROS* productions induced by two *NADPH* enzymes- *nox* (NADPH oxidase) and *duox* (dual oxidase), *irc* (immune-reactive catalase), negatively regulates *ROS* production during infections (Nicolas Buchon et al., 2009; Kuraishi et al., 2013), by suppressing the cytotoxic reactive oxygen species once the infection threat is lessened, which otherwise, would lead to immunopathological consequences ***Fig****. 4*.

To repair the infection-induced damage during oral bacterial infections, the *Drosophila* gut possess *ISCs* (intestinal stem cells). These *ISCs* are activated under stressful conditions such as infection threat, leading to proliferation and differentiation into *ECs* (enterocytes), thereby maintaining host homeostasis (Kuraishi et al., 2013). Meanwhile, the damage sensing cytokine molecules *upd3* released from the haemocytes stimulates the *ISCs* and initiate the damage repair process (Chakrabarti et al., 2016; Weng et al., 2018). Finally, the damage repairing *egfr*[1] (epidermal growth factor receptor) along with Jak/Stat-pathways in *ISCs* are indispensable for repairing damage and maintaining the homeostasis. Flies lacking *egfr* are highly susceptible to bacterial infections (Jiang et al., 2009; Buchon et al., 2010; Jiang et al., 2011; Osman et al., 2012; Marianes and Spradling, 2013).

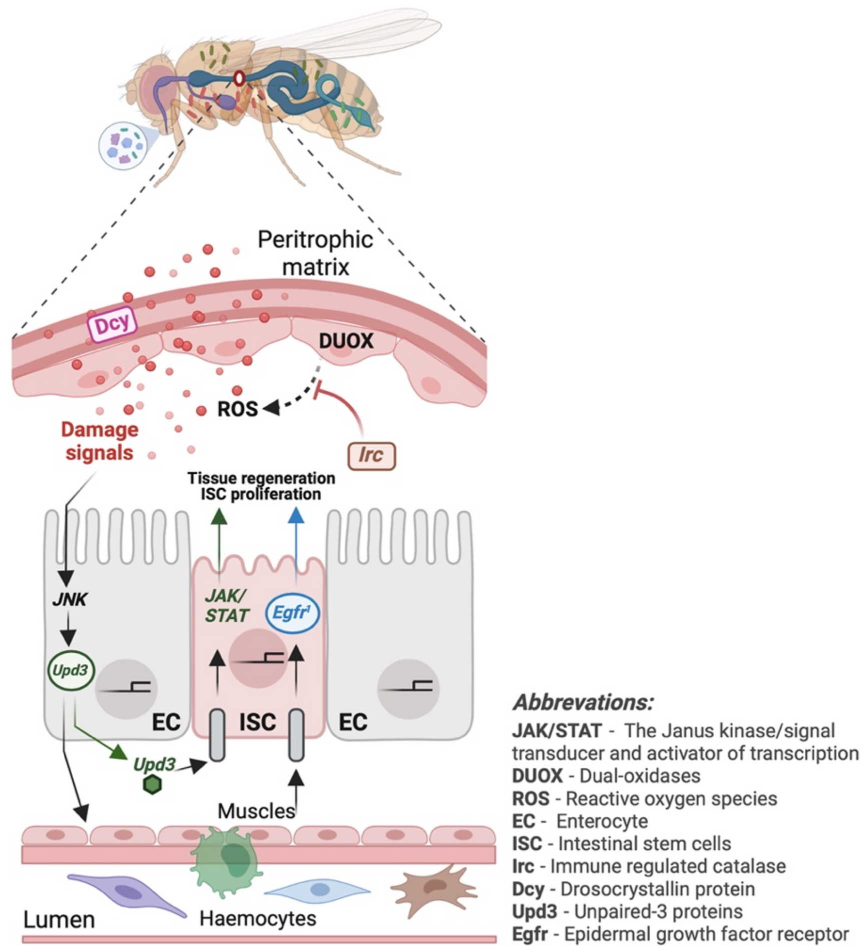

Figure 4: Graphical summary of gut-epithelial immunity. *This figure also appears in data chapter-3.*

In recent decades, insects have gained considerable attention as a model system for immunology due to their wide similarity with human innate immune pathways (Schmid-Hempel, 2021) The importance of *Drosophila* as a model for innate immunity mainly relies on the evolutionary conservation of innate immune signaling pathways between insects and mammals. Although the core signaling pathways have remained highly conserved, however, the upstream receptors and downstream effectors have significantly diverged during evolution between *Drosophila* and other insects such as mosquitoes, beetles (Bartholomay et al., 2010). The evolutionary conservation of immune pathways even extends to vertebrates as well. The *Drosophila* genome is 60% homologous to that of humans, less redundant, and about 75% of the genes responsible for human diseases have homologs in flies (Ugur et al., 2016).

Insect IMD and Toll pathways have striking similarities to the mammalian Tumor Necrosis Factor (TNF) and Toll-like receptor (TLR) pathways, respectively. In mammals, the TNF signaling pathway regulates basic cellular processes, such as inflammation, and is essential

for both innate and adaptive immune functions (Aggarwal, 2003), while mammalian TLRs contribute to innate antiviral immunity and production of cytokines, such as interferons (Kawai and Akira, 2010). Among these, Interleukin-6 resembles the *upd* (unpaired protein family) ligand in *Drosophila*, hence the *upd* ligands are often known as cytokines (Brown et al., 2001) see ***Fig**. 4*. The mammalian pathway is comprised of four JAKs and seven STATs, whereas the invertebrate pathway has fewer family members for each component. However, the overall mechanisms for signal transduction remain identical (Rawlings et al., 2004).

1.4 *Immune regulation of defence strategies: tolerance vs. resistance vs. priming*

Pathogenic infection threat must be immediately met with immune defence before the infection is completely established. Following infection, hosts must not only identify and eliminate infection sources but also reduce the infection-induced damage. Excessive immune response activity can lead to immunopathology while an optimal immune system which is rapidly activated during infection and is immediately deactivated once infection threat is lessened. This raises another important question about the importance of optimal immune regulation in response to pathogens. A heightened immune response can be detrimental to fly's survival, for instance, the elevated expression of *AMPs,* increased *PO* activity*,* and *ROS* production can lead to increased mortality due to the cytotoxic effects (Zerofsky et al., 2005; Han and Ulevitch, 2005; Sadd and Siva-Jothy, 2006; Khan et al., 2017; Badinloo et al., 2018). While an underwhelming immune response provides ample time to pathogens to become established. Yet, it remains challenging – how hosts optimize the immune activity in order to sufficiently control infections while avoiding and/or minimizing immunopathology (tissue or cellular damage). Thus, highlighting the importance of understanding innate immune regulation of major immune defense strategies.

While addressing innate immune regulation, it is important to understand the host-derived as well as pathogen-derived causes of tissue damage to identify the mechanisms of variable disease outcomes. One such infection outcome includes individuals that exhibit low levels of pathology despite harbouring high pathogen burden (Raberg et al., 2007; Råberg et al., 2009; Ayres and Schneider, 2012; Medzhitov et al., 2012). Individuals with higher tolerance ability are more likely to avoid immunopathology, inflammation, reduce the tissue or cellular damage and enhance fitness (Soares et al., 2014, 2017; Vale et al., 2014, 2016; Martins et al., 2019). Theoretical models suggest that disease tolerance is beneficial and can be fixed in a population under directional selection [see (Roy and Kirchner, 2000; Miller et al., 2005)]. While resistance on the other hand, the pathogen clearance mechanisms, often involves costly

immune responses potentially involving other life-history trade-offs (Prakash and Khan, 2022). Previous studies suggest a link between immune priming and tolerance exists, for instance, in fruit flies, priming with the *Drosophila C virus* increases tolerance against the subsequent exposure to the same viral pathogen (Mondotte et al., 2018, 2020). In contrast, priming in other insects for instance flour beetles *Tribolium castaneum*, showed enhanced survival by suppressing/reducing pathogen burden, suggesting a form of resistance (Khan et al., 2019). Despite the contrasting results, the immune regulation of diverse immune strategies remains largely unclear. Certainly, more studies are needed because understanding the regulation of disease tolerance, resistance and immune priming might provide valuable insights in identifying active immune molecules (pathway networks) that can impart a deeper holistic understanding of immune defense networks.

Damage limitation mechanisms such as tissue damage signalling, prevention, and repair mechanisms are important from therapeutic perspective. These mechanisms aid in identifying drug targets for treating diseases since they have the potential to boost/enhance host health (Soares et al., 2014; Vale et al., 2016; Salas et al., 2020). Then, how do hosts regulate the damage limiting mechanisms such as disease tolerance and immune priming which promote hosts health during infections? In this thesis, we aim to address this important question of understanding immune regulation of diseases tolerance and priming during bacterial infection using the fruit fly *Drosophila* and their bacterial pathogens *Pseudomonas entomophila* and *Providencia rettgeri*. Also, see (Prakash and Khan, 2022) in Appendix section for more details about the role of competing immune strategies such as disease tolerance vs. infection resistance vs. immune priming.

1.4.1 ***Measuring tolerance***: Tolerance can be estimated as either 'point tolerance' or 'range tolerance' (Oliveira et al., 2020) and is usually measured as a reaction-norm or slope which could be either linear or non-linear, wherein pathogen burden is plotted on the x-axis and health or fitness is plotted on the y-axis (Louie et al., 2016; Gupta and Vale, 2017). Throughout the thesis we assessed disease tolerance phenotype using a linear model, we confirmed AICc (Akaike information criterion) scores as a measure of the goodness of fit to the data (*data not shown*) see (Louie et al., 2016; Gupta and Vale, 2017). Given that disease tolerance refers to the rate at which hosts lose health with increasing bacterial loads (in our case), the analysis of interest is how the slope of the fly lines (wild type vs. mutant) differs, that is, if survival is significantly affected by the 'fly line × load' interaction (*also see methods section of each results chapter for more details*). We analysed the Log_{10} bacterial load data measured after infection (hours post-

infection) using ANCOVA and fitted 'fly line' and 'sex' as categorical fixed effects, 'bacterial load' as a continuous covariate and their interactions as fixed effects for wildtypes and different immune mutants.

A second approach which is commonly used to quantify disease tolerance include a non-linear model - that is, analysing tolerance data using a non-linear (4) parametric logistic model. A 4-parameter logistic model can be used to different components of host disease tolerance including (i) host vigour (general good-health condition), (ii) infection sensitivity (iii) slope and (iv) severity of the infection (Gupta and Vale, 2017). In our case we found that linear model outperformed other non-linear models (*data not shown*), based on AICc scores.

1.5 *Sexual dimorphism in disease tolerance and immune priming*

Majority of insects, including *D. melanogaster*, show strong dimorphism in immune responses, where both males and females respond differently to infections, these include differences in baseline levels, pathogenic infections, systemic and oral infection routes (Simon, 2005; Zuk, 2009; Klein and Flanagan, 2016; Belmonte et al., 2020). The difference in sexes have repeatedly been found in various immune pathway components (IMD and Toll) including function and expression. For instance, following a *P. rettgeri* systemic infection, males of *D. melanogaster* induced several IMD- and Toll-regulated effectors at a higher level than females, followed by greater survivorship in males (Duneau et al., 2017). Therefore, it will be interesting to see if this sexual dimorphism influenced response to disease tolerance and immune priming. Since most immunology studies do not distinguish between sexes (Beery and Zucker, 2011).

A recent *D. melanogaster* study found that the Toll signalling pathway was crucial in producing a sexually dimorphic response to a gram-negative *P. rettgeri* bacterial infection and that the IMD pathway AMP- *Diptericins* was not involved in the dimorphism (Duneau et al., 2017). In contrast, our results indicate that the IMD pathway is key to the priming response, while the Toll pathway is not involved in *P. rettgeri* priming (unpublished results from *Chapter 5*). More recently (Hanson et al., 2019), showed a high degree of non-redundancy and pathogen specificity of IMD and Toll pathway regulated AMPs. In some cases, even a single IMD-regulated AMP could confer complete defence against specific pathogens. But we know little about whether and to what extent males and females differ in the role of these innate immune pathway regulation during disease tolerance and immune priming? We aim to address this as a subsidiary question in throughout our thesis. Further investigating the basis (immunological drivers) of dimorphism in disease tolerance and priming would allow us to gain a better

understanding of the disease transmission through males and females in insect populations. *Also see discussion section of each data chapter for more details about sexual dimorphism in disease tolerance and immune priming.*

1.6 ***Candidate gene approach vs. association studies***

Association studies like genome wide association studies (GWAS), transcriptomics etc., investigate the genetic variants/variations spanning across the entire genome while, candidate gene studies analysis to a relatively fewer number of genes (of interest) (Tabor et al., 2002; Modena et al., 2019). As a result, candidate gene studies have increased statistical power to detect differences compared to association studies like GWAS and transcriptome approach. Moreover, the candidate gene approach tends to be unavoidably biased toward genes and candidates of interest especially biological pathways (i.e., candidate genes are hand-picked) (Modena et al., 2019).

Further, criticisms such as non-replication (sample size), lack of thoroughness (sensitivity and resolution) makes it difficult to investigate some of the canonical immune pathway candidates that are involved in the disease tolerance and immune priming. Of course, previous *Drosophila* work has employed GWAS and transcriptomic approach to uncover candidate genes associated with disease tolerance and priming. However, most of the genome wide screens did not pick up canonical immune pathway regulators and genes, possibly due to lack of variation or genetic architecture of disease tolerance [*Please refer to introduction section in each results chapter for examples and comparison of candidate vs. association studies in disease tolerance*].

1.7 ***Thesis Outline***

Research on damage limitation mechanisms such as disease tolerance and immune priming, not only provides fundamental insights into highly conserved innate immune processes, but it might also contribute to the fight against infectious diseases. The aim of this thesis is to improve our understanding of mechanisms that enhance disease tolerance in *Drosophila melanogaster* during bacterial infection. First, ***in chapter (2),*** we examined how the negative immune regulators of IMD (immune deficiency) pathway affect disease tolerance during systemic bacterial infection. The major insights to derive from this chapter is that the *UAS*$^{-RNAi}$ mediated reduced expression of the negative regulators of the IMD*-pathway*, *caudal* and *pirk*, reduces the fly's ability to tolerate infection (measured as survival relative to its pathogen load) in males and females across a wide range of infectious doses. Our results

highlight that in addition to efficient pathogen clearance, the negative regulators of tolerance are also important for disease tolerance.

In chapter (3), we focus on the natural infection route, that is, oral or enteric infection and address how the damage - sensing, control, prevention and repair/renewal mechanisms in the gut mediate disease tolerance during oral bacterial infections. We find that disrupting the major component of peritrophic matrix (*dcy*) resulted in the highest loss of tolerance, while loss of function of either the negative regulator the *irc* (immune-regulated catalase) or *upd3* (a cytokine-like, unpaired protein) also reduced tolerance in both sexes. The absence of tissue damage repair signalling *egfr*[1] (epidermal growth factor receptor) resulted in a severe loss in tolerance in male flies but had no substantial effect on the ability of female flies to tolerate *P. entomophila* infection, despite carrying greater microbe loads than males. Together, our findings provide empirical evidence for the role of damage limitation mechanisms in disease tolerance.

We address the role of Jak/Stat signalling pathway in disease tolerance ***in chapter (4),*** during systemic bacterial infections. 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. Together, our findings highlight that Jak/Stat signalling mediates disease tolerance during systemic bacterial infection and that this response differs between males and females.

In chapter (5), we investigated the occurrence, generality, and mechanistic basis of immune priming in *Drosophila melanogaster* when infected with the gram-negative bacterial pathogen *Providencia rettgeri*. we find that priming in *Drosophila* is a long-lasting response, occurring in several genetic backgrounds and is particularly stronger in male flies. Mechanistically, we show that flies lacking major components of the IMD immune signalling pathway are no longer able to improve survival following initial heat-killed exposure with *P. rettgeri*. We show that the enhanced survival of individuals primed with an initial non-lethal bacterial inoculum coincides with a transient decrease in bacterial loads, and that this is likely driven by the IMD-responsive antimicrobial-peptide *Diptericin-B* in the fat body through the regulation by peptidoglycan recognition proteins *PGRP-LB, PGRP-LC* and *PGRP-LE*. In ***chapter (6)***, we further show that priming has the potential to reduce disease transmission by affecting pathogen shedding.

Finally, ***chapter (7)*** provides a general discussion on the results of this thesis, and an outlook on future research in the field of disease tolerance and damage limitation mechanisms to bacterial infections in *Drosophila*.

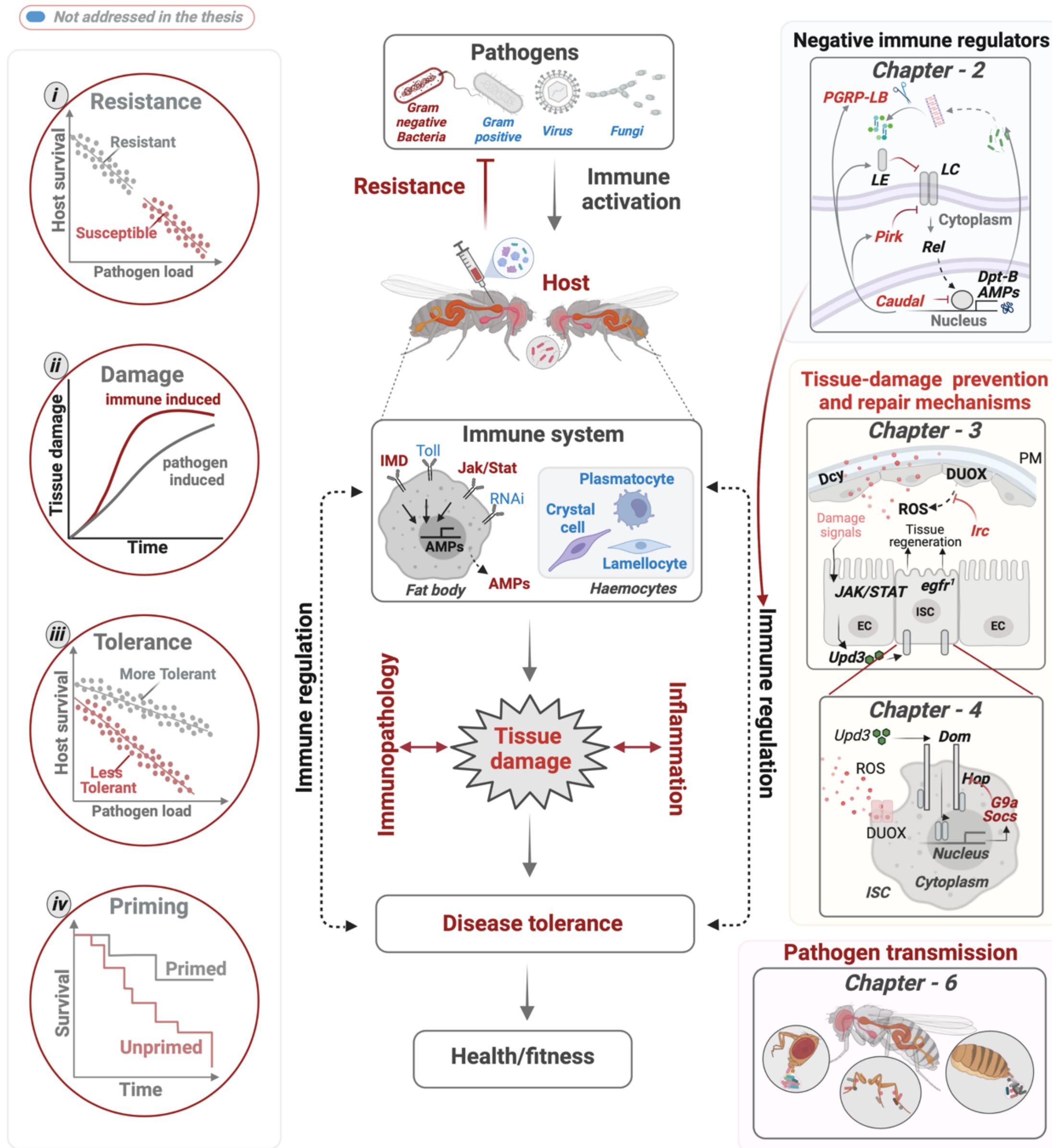

Figure 5: Thesis Summary. The host-pathogen interactions are usually summarized by the activation of immune system by pathogens (bacteria, virus, and fungi), triggering immune response. Hosts must resist or eliminate pathogens to survive infection. Apart from identifying and eliminating infection source, hosts must also avoid immunopathology and inflammation to reduce the tissue or cellular damage. The immune regulatory mechanisms such as negative regulator of major immune signalling pathways, for instance, IMD-immune deficiency pathway (*addressed in* ***Chapter-2***) limit the potentially damaging effects of immunity that is, immunopathology. The counteracting response to these processes, are the tissue damage control, prevention, and repair mechanisms (***Chapter-3*** *and* ***Chapter-4***). These mechanisms underlie the establishment of disease tolerance to infection while promoting host's health and fitness. In addition, immune memory-like priming defence mechanism enhances survival following sub-lethal pathogenic exposure (***Chapter-5***). Further, immune priming has the potential to reduce pathogen transmission by affecting pathogen shedding (***Chapter-6***). Panel

on the left, diagrammatic representation linking hosts ability to **(i)** resist pathogen growth by immune activation; **(ii)** control tissue or cellular damage by induced by pathogen and immune response; **(iii)** tolerate the pathogen burden by reducing the fitness costs of infection or immune activation without directly reducing the pathogen numbers; **(iv)** improve survival following initial sub-lethal pathogenic exposure known as immune priming.

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Chapter 2

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

Keywords: Cytotoxicity, Immune deficiency (IMD) pathway, immune effectors, disease tolerance, immunopathology, negative immune regulators

2.1 *Abstract*

Disease tolerance is an infection phenotype where hosts show relatively high health despite harbouring elevated pathogen loads. Compared to the mechanisms of immune clearance our knowledge of the mechanisms underlying increased tolerance remains incomplete. Variation in the ability to reduce immunopathology may explain why some hosts can tolerate higher pathogen burdens with reduced pathology. Negative immune regulation would therefore appear to be a clear candidate for a mechanism underlying disease tolerance, but this has not been tested directly for bacterial infections.

Here, we examined how the negative regulation of the immune deficiency (IMD) pathway affects disease tolerance in *Drosophila melanogaster* when infected with the gram-negative bacterial pathogen *Pseudomonas entomophila*. We find that UAS^{RNAi}-mediated reduced expression of the negative regulators of IMD (*pirk* and *caudal*) severely reduced the ability to tolerate infection in both males and females across a wide range of infectious doses. While flies unable to regulate the IMD response exhibited higher expression of antimicrobial peptides and lower bacterial loads as expected, this was not accompanied by a proportional reduction in mortality. Instead, tolerance (measured as fly survival relative to its microbe load) was drastically reduced, likely due to the combination of increased immunopathology and cytotoxicity of elevated AMP expression.

Our results therefore highlight that in addition to regulating an efficient pathogen clearance response, negative regulators of IMD also contribute to disease tolerance.

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

In the present work, we take a more targeted approach to specifically investigate if the negative regulation of immune responses has any measurable effect on the phenotype of disease tolerance. We focused on the immune deficiency (IMD) pathway, one the best described immune signalling pathways in *Drosophila* (Leulier et al. 2003; Bruno Lemaitre and Hoffmann 2007; Myllymäki, Valanne, and Rämet 2014). Following infection, DAP-type (Diaminopimelic acid) peptidoglycans from gram-negative bacteria are recognised by the pathogen-sensing receptors *PGRPs (LC* and *LE)* (Vodovar et al. 2005; Dieppois et al. 2015). These receptors then recruit the adaptor molecule IMD and activate the intracellular signalling cascade that leads to the activation of a *NF-κB relish* (see ***Fig****. 1*) also reviewed in (Myllymäki, Valanne, and Rämet 2014; Kuraishi, Hori, and Kurata 2013).

Upon activation, *relish* translocates to the nucleus and induces transcription of a set of IMD-responsive AMPs. Minutes after pathogen recognition, a cocktail of AMPs such as *Diptericins, Attacins* and *Cecropins* is secreted by the fat body (Bruno Lemaitre and Hoffmann 2007; B. Lemaitre, Reichhart, and Hoffmann 1997). *Relish* also induces the transcription of negative regulators of IMD (*pirk*, *caudal, PGRPs-LB, LC, LE*,) which ensures an appropriate level of immune response, while avoiding immunopathology (Lee and Ferrandon 2011; Kleino and Silverman 2014). *Pirk*, for example, interferes with the interaction of *PGRP-LC* and *-LE* with the molecule IMD, and limits the activation of the IMD pathway (see ***Fig****. 1*) (Aggarwal et al. 2008; Kleino et al. 2008; Basbous et al. 2011). Similarly, the homeobox protein *caudal* downregulates the expression of AMPs in flies (Ryu et al. 2008). Taken together, the negative regulation of IMD-pathway ensures an appropriate level of immune response and enables individuals to reduce immune activity in response to reductions in bacterial numbers (Lee and Ferrandon 2011; Kleino et al. 2008; Paredes et al. 2011).

We measured disease tolerance during systemic infection with the gram-negative bacterium *Pseudomonas entomophila* using *Drosophila* lines where individual components of the IMD-pathway were knocked down using UAS^{RNAi} including (i) the transcription factor- *relish*, its negative regulators (ii) *pirk* and (iii) *caudal*, and (iv) a major IMD-responsive AMP *Diptericin-B*, because of substantial evidence that *Diptericins* are very effective during defense against Gram-negative bacteria (Bruno Lemaitre and Hoffmann 2007; Wicker et al. 1990). Given the widespread evidence of sex differences in immunity and response to infection, reviewed in (Klein and Flanagan 2016; Belmonte et al. 2020), we also specifically tested whether female and male flies differ in their ability to tolerate bacterial infection.

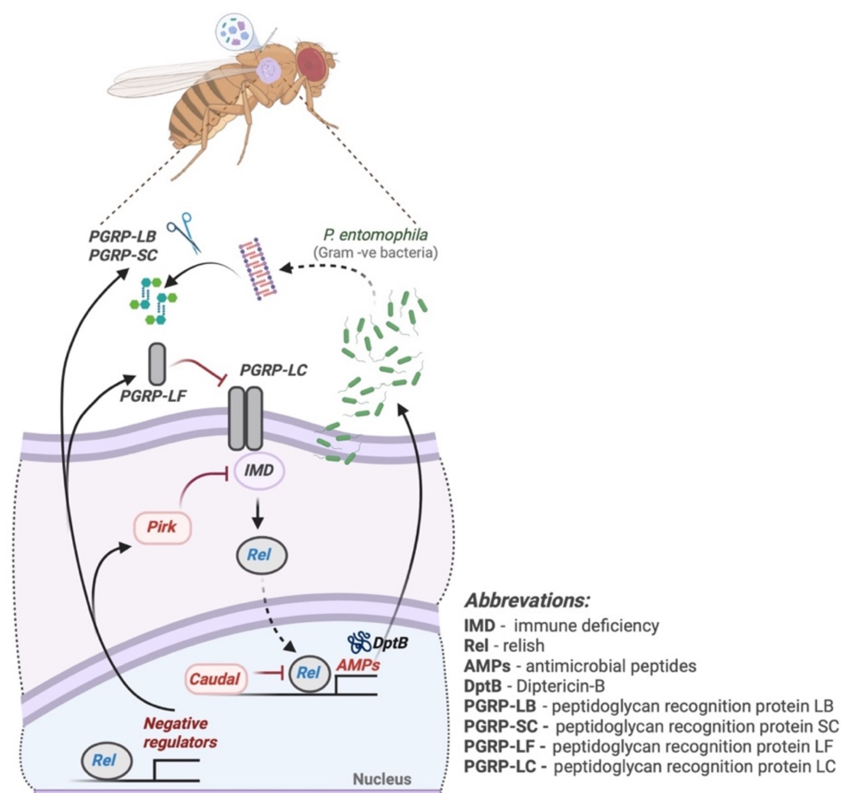

Figure 1*:* A simplified version of *Drosophila* IMD (immune deficiency) pathway activated by the DAP (diaminopimelic acid) type pedptidoglycan following the gram-negative bacterial infection (*Pseudomonas entomophila*). These receptors then recruit ↘ the adaptor molecule IMD and activate the intracellular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

2.3 *Materials and methods*

1. *Fly strains and husbandry:* We used the following fly lines: UAS-*relish*^{RNAi} (*Bloomington stock# 33661*), UAS-*caudal*^{RNAi} (*57546*), UAS-*pirk*^{RNAi} (*67011*) and UAS-*DiptericinB*^{RNAi} (*28975*) also see ***Supplementary Information Table** SI-1* for details about fly genotypes. All fly lines were obtained from the Bloomington *Drosophila* stock centre at the Indiana University. Each of the transgenic fly line contains a homologous hairpin under the control of the Upstream Activating Sequence (UAS^{RNAi}) to a Gal4 temperature-sensitive transcriptional activator (*Bloomington stock# 67065*). This fly line drives the Gal4 expression in the entire body of fly and contains a temperature sensitive Gal80, which inhibits Gal4 at the permissive temperature (25℃) where all the relevant genes are expressed normally. We used the driver fly line as a functional fly line (wild type). All fly stocks were reared in plastic vials (12 ml) on a standard Lewis medium (fly food) at 25±2˚C with a 12 hour light: dark on 14 days cycle (by transferring flies into new food vials). All the crosses were made at 25℃ and after eclosion, offspring were transferred to agar vials (12 flies per vial) at the non-permissive temperature (29℃) for two days to facilitate silencing of the candidate genes until experimental day.

2. *Bacterial culture preparation:* The gram-negative bacterium *Pseudomonas entomophila* has a broad host range, infecting insects, nematodes, plants, and higher vertebrates (Dieppois et al. 2015). In flies, *P. entomophila* infection results in pathology to the gut epithelium during systemic infections, eventually causing death (Vodovar et al. 2005; Dieppois et al. 2015; Troha and Buchon 2019). To obtain pure cultures, frozen bacterial stock cultures (-70℃) were streaked onto fresh LB agar plates and single colonies were inoculated into 15ml LB broth and incubated overnight at 37°C with shaking at 120rpm (revolutions per minute). The overnight cultures were diluted 1: 100 into fresh LB broth of 15ml and incubated again at 37°C with shaking at 120rpm. At the mid-log phase (optical density OD_{600} = 0.75), we harvested the bacterial cells by centrifugation at 5000 rpm for 5 min at 4°C and re-suspended the bacterial pellet in 1XPBS (phosphate buffer saline) solution. The final inoculum was adjusted to OD_{600} = 1.0, and this was the starting bacterial inoculum used to prepare 4 different dilutions of infection doses (OD_{600} = 0.1, 0.05, 0.01 and 0.005) for all flies for systemic infection.

3. *Gene expression:* We quantified the expression of major IMD-responsive AMPs namely, *diptericin (Dpt), attacin-C AttC), cecropin-A1 (CecA1*), by quantitative real-time polymerase chain reaction (qRT-PCR). We randomly selected a subset of IMD-pathway mutants and functional flies both males and females at 8 hours following infection for RNA extraction, we included 15-21 flies that is, 3 flies pooled together for each treatment (Knockdown and functional flies) and sexes. We first homogenised whole flies (3 in group) using sterile micro-pestles into 1.5µl microcentrifuge tubes containing 80µL of TRIzol reagent (Invitrogen, Life Technologies). The tubes containing the fly homogenate were kept frozen at -70°C until RNA extraction.

   We performed mRNA extractions using the standard phenol-chloroform method and included a DNase treatment (Ambion, Life Technologies). We confirmed the purity of eluted samples using a Nanodrop 1000 Spectrophotometer (version 3.8.1) before proceeding with RT (reverse transcription). The cDNA was synthesized from 2µL of the eluted RNA using M-MLV reverse transcriptase (Promega) and random hexamer primers, and then diluted 1:1 in nuclease free water. We then performed quantitative RT-PCR (qRT-PCR) on an Applied Biosystems StepOnePlus machine using Fast SYBR Green Master Mix (Invitrogen Ltd.) using a 10µL reaction containing 1.5L of 1:1 diluted cDNA, 5µL of Fast SYBR Green Master Mix a 3.5µL of a primer stock containing both forward and reverse primer at 1µM suspended in nuclease free water (final reaction concentration of each primer being 0.35µM).

   For each cDNA sample, we performed two technical replicates for each set of primers and the average threshold cycle (Ct) was used for the analysis. We obtained the AMP primers from Sigma-Aldrich Ltd (see supplementary ***Table*** *SI-2* for information about primers). We optimised the annealing temperature (T_a) and the efficiency (Eff) of the *Dpt* primer pair was calculated by 10-fold serial dilution of a target template (each dilution was assayed in duplicate); *Dpt*: T_a= 59 °C, Eff= 102%; *AttC*: T_a= 60 °C, Eff= 94%; *CecA1*: T_a= 59.5 °C, Eff= 109%.

4. *Fly survival following bacterial infection:* To test the impact of *Pseudomonas entomophila* infection on fly survival, we challenged experimental flies to 4 different bacterial concentrations or infection doses ranging between (I) ~650 cells (II) ~1400 cells (III) ~2000 cells (IV) ~2900 cells/µl of *P. entomophila* culture, obtained by serial dilution.

Flies (n=16-24 flies /sex/infection dose/fly line) were infected systemically by intra-thoracic pricking (Khalil et al. 2015) with a needle immersed in bacterial suspension under minimal CO_2 anaesthesia. The effect of injury caused by pricking was controlled by including mock infections performed using a needle dipped in 1xPBS (phosphate buffer saline). We observed the mortality for 10 days post-infection every 3 hours (±0.5 hours) for the first 2 days (between 6 am and 11 pm) and every 12 hours for the following 7 days.

5. *Quantification of bacterial load following bacterial infection:* During the survival assay, flies were randomly sampled (16-24 flies/sex/infection dose/fly line) and the bacterial load was quantified around 8 hours following infection. Initially, flies (group of 3) were transferred to a 1.5ml microcentrifuge tubes and surface sterilized by adding 70% ethanol for 30-60 seconds to ensure we counted only colony forming units (CFUs) inside the flies. Ethanol was then removed by briefly washing flies twice with sterile distilled water. To ensure that this method was efficient in surface cleaning flies, we plated 4μl of the second wash on LB agar (from random flies) ensuring that the flies are free from surface bacteria (that is, no viable CFUs). We then placed in 1.5ml Eppendorf tubes and added 100μl of LB broth to each tube and the flies were thoroughly homogenized using a motorized pestle. We immediately plated this homogenate in serial dilutions ranging from 100 to 10^{-6} using 1xPBS (phosphate buffer) on LB agar plates and incubated at 30°C for about 18 hours after which viable colonies were counted manually (Siva-Jothy et al. 2018).

Statistical analysis

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

2. *Bacterial load*: We then quantified differences in bacterial load following bacterial infection by analysing the bacterial measurement (log_{10}) around 8 hours following *P. entomophila* infection. We found that residuals of bacterial load data were normally distributed when tested with Shapiro-Wilks test and hence we used ANOVA and analysed the bacterial load data by fitting 'knockdown' (functional flies and IMD-pathway knockdown flies), 'sex' and 'infection dose' (4 different infectious doses of *P. entomophila*) as categorical fixed-effects, while 'vials' (replicates) as a random-effects for each of the fly lines (functional flies and 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 and knockdown)* x sex *(male and female)* x dose *(4 concentrations of P. entomophila)* (1|vial *(n replicates)*), with 'knockdown' and 'sex' as fixed effects, and vials as a random effect for all the fly lines (IMD-pathway mutants). We fit separate models for each fly lines during *P. entomophila* infections, since they were assayed on different days that is, each knockdown fly line and functional (wild type) fly line. Next, to understand how different knockdown flies (that is, having fully functional vs. knockdown of IMD-pathway) differ in survival to bacterial infections in detail, we also analysed survival data for *P. entomophila* infections for each of the IMD-pathway mutant/fly lines separately using Cox Proportional Hazard survival analysis. We estimated the impact of harbouring IMD-pathway knockdowns as the hazard ratio of IMD-knockdowns vs. fully functional fly lines. A hazard ratio significantly greater than one indicates higher risk of mortality in the IMD-mutants relative to fully functional individuals; hence, a significant impact on fly's survival.

4. *Measuring disease tolerance*: Finally, to understand how the negative immune regulation contribute to disease tolerance during bacterial infections we plotted tolerance by calculating (median lifespan/mean bacterial load) for male and female flies (that is, flies with functional and knockdown IMD pathway regulators) after bacterial exposure with 4 different infection doses. Tolerance is usually measured as a reaction-norm or slope which could be either linear or non-linear, where the pathogen burden is plotted on the x-axis and health or fitness is plotted on the y-axis (Raberg, Sim, and Read 2007; Ayres and Schneider 2012; Medzhitov, Schneider, and Soares 2012; Louie et al. 2016; Gupta and Vale 2017; Oliveira, Bahia, and Vale 2020).

This standard reaction norm approach that measures tolerance as the rate of health decrease for increasing microbe loads is only statistically sound when mortality and microbe loads measurements are taken from the same individual or from the same group of replicate individuals. Given the destructive sampling required to measure microbe loads in flies we chose instead to use another commonly used metric of tolerance using the average mortality of flies of a given line relative to the average bacterial load of that line, see for example (Adelman et al. 2013; Knutie et al. 2017; Cornet et al. 2014). This is sometimes referred to as 'point' tolerance and is intuitive to interpret: relative to a given microbe load, does one line die more (less tolerant) or less (more tolerant) (Little et al. 2010).

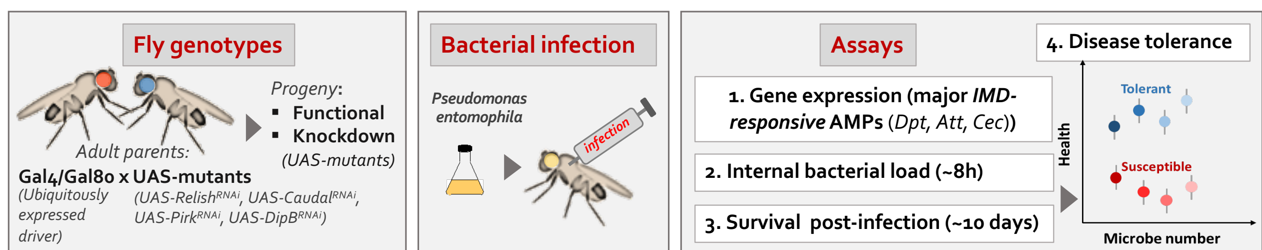

Figure 2: Design of experiments to assay **(1)** gene expression of the major IMD-regulated AMPs (n=15-21 flies; 3 flies pooled together for each treatment, sex and fly line combination) **(2)** internal bacterial load (n=16-24 flies /sex/infection dose/fly line) and **(3)** survival following systemic bacterial infection with *Pseudomonas entomophila* n=16-24 fly vials /sex/infection dose/fly line) to test impact of the negative regulation of IMD pathway on phenotype of disease tolerance **(4)**. The data represented on disease tolerance panel-4 is a sketch, not actual data.

2.4 ***Results and discussion:***

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

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

bacterial pathogen (***Fig.*** *3B,* ***Table****-SI-4*). See ***Fig.*** *SI-1 and SI-2* for bacterial load across different infection doses.

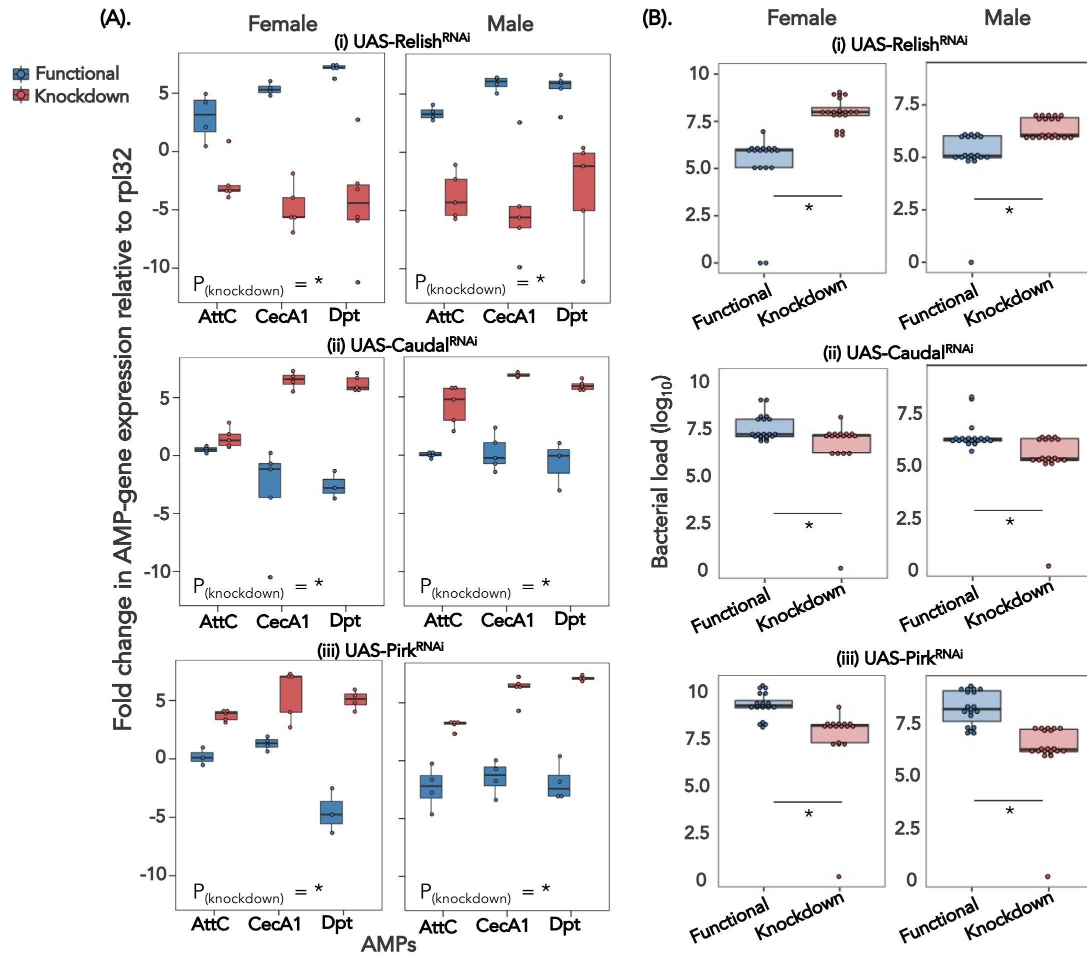

Figure 3: **(A)** Quantification of major IMD-responsive AMPs by qRT-PCR (n=15-21 flies/sex/treatment) for IMD-pathway mutants and functional flies (males and females) around 8 hours following *P. entomophila* systemic infection (0.05 OD or ~2000 bacterial cells/µl). Each data point represents pooled data of 3 flies for each infection treatment, fly line and sex **(B)** internal bacterial load after 8 hours following infection (n=16-24 flies/sex/treatment). Asterisks inside the panels indicate significant differences in AMP expression (that is, impact of knocking down on overall AMPs measured) and bacterial load between knockdown flies and flies with fully intact IMD functioning ($p<0.05$). The error bars represent standard error.

2. <u>Flies with reduced expression of *pirk* or *caudal* show increased mortality despite having lower bacterial loads</u>

In flies, systemic infection with *P. entomophila* results in severe pathology, eventually causing death (N. E. Martins et al. 2013). In fact studies suggest that induction of higher levels 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****-SI-5; Fig. SI-3 and SI-4 for survival curves and Fig. SI-5 for hazard ratio across different infection doses*), 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.

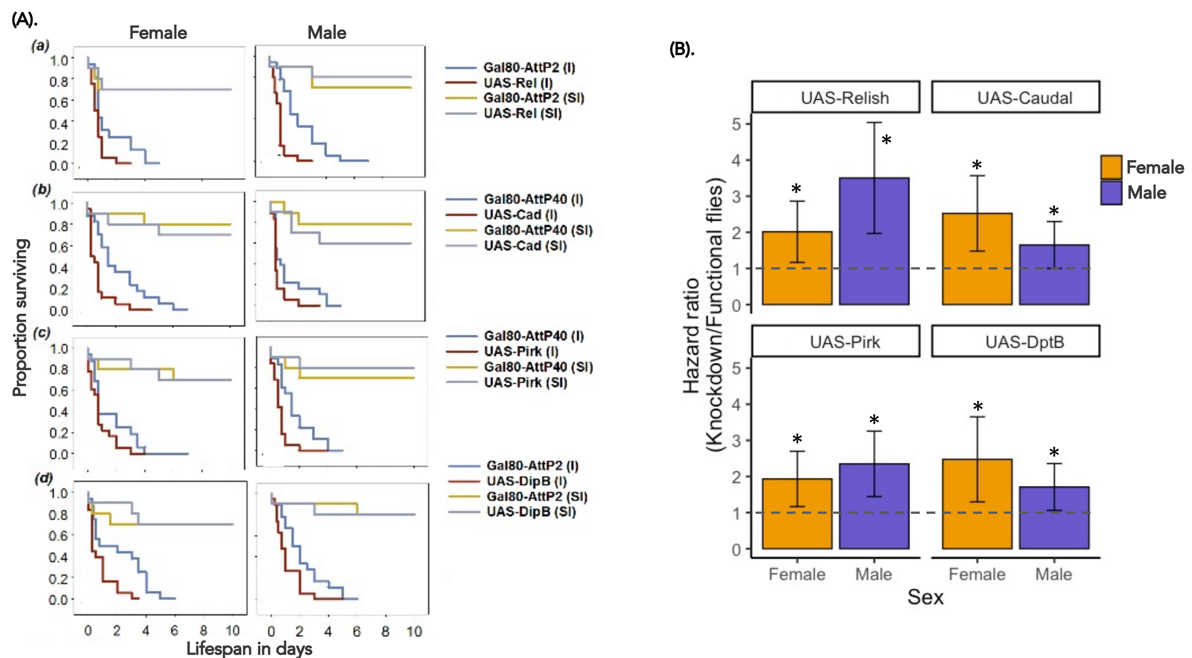

Figure 4: **(A)** Survival curves for male and female *UAS*^{RNAi} flies (n=16-24 vial/treatment/sex/fly line) (a) *UAS-Relish*^{RNAi} (b) *UAS-Caudal*^{RNAi} (c) *UAS-Pirk*^{RNAi} (d) *UAS-DptB*^{RNAi} after systemic infection of *P. entomophila* (dose ~2000 cells/μl). 'I' – indicates infection treatment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

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

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 and female flies differing in functional components of IMD-pathway (*relish, caudal, pirk* and *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**-SI-6*). 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).

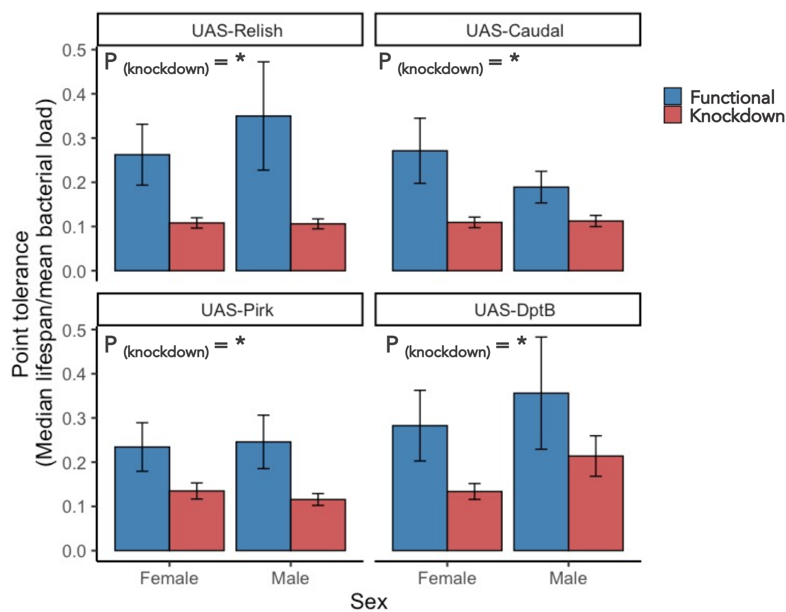

Figure 5: Disease tolerance in IMD-pathway mutants and fully functional flies. The relationship between host (fly) survival and bacterial load (single infection dose) analysing using point tolerance around 8 hours post *P. entomophila* infection for male and female flies. Each data represents 'point 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 ($p<0.05$) across males and females. The error bars represent standard error.

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 a general trend of reduced tolerance both functional (wild type) and knockdown flies (***Fig.** 6,* ***Table**-SI-6*). We found similar effect for both females and males suggesting that overall

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

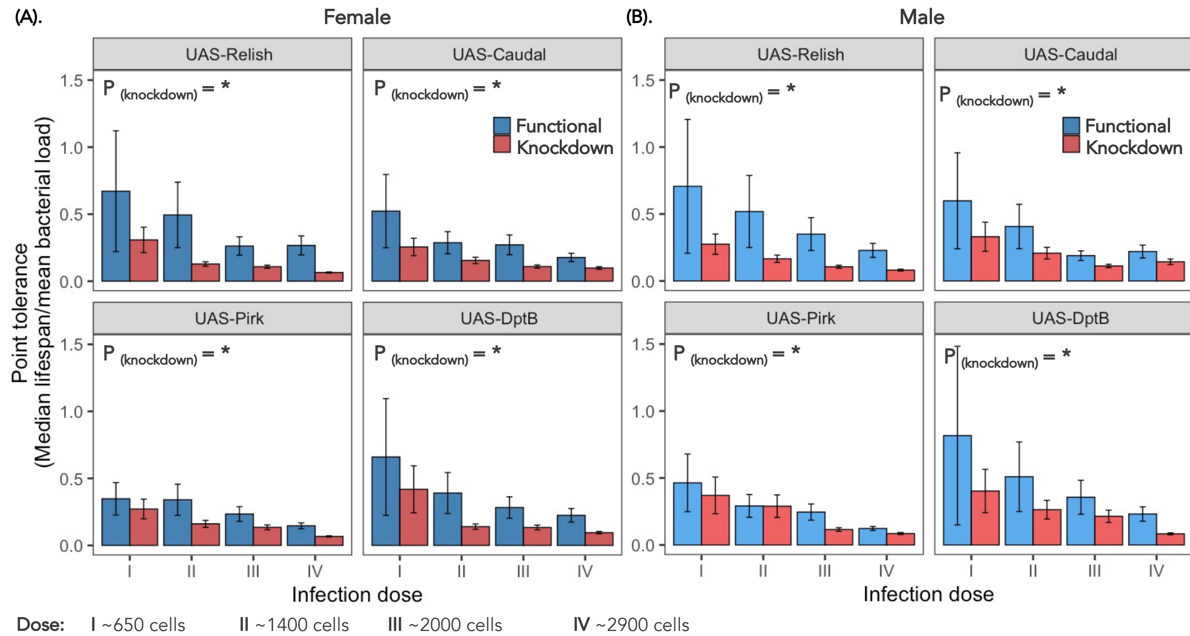

Figure 6: Disease tolerance in IMD-pathway mutants and fully functional flies. The relationship between host (fly) survival and bacterial load of 4 infection doses [that is, (I) ~650 cells (II) ~1400 cells (III) ~2000 cells (IV) ~2900 cells] of *P. entomophila*, analysing using point tolerance around 8 hours following *P. entomophila* infection for **(A)** male and **(B)** 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 functional IMD. Asterisks inside the panel indicates significant difference in disease tolerance between knockdown flies and with fully intact IMD functioning ($p<0.05$) across different infection doses. The error bars represent standard error.

One likely cause of flies lacking negative IMD regulation still succumb to death despite reducing pathogen burden is immunopathology due to continued immune activation. There is a precedent for the effects of negative regulators on disease tolerance. For example*, G9a*, a negative regulator of Jak/Stat, was identified as being important in tolerating *Drosophila C Virus* infections by reducing immunopathology during the antiviral immunity (Merkling et al. 2015). During bacterial infections, *PGRP-LB* functions as a negative regulator of the IMD pathway by regulating the IMD-responsive AMP *Diptericin* (Zaidman-Rémy et al. 2006). More recently it has been shown that *PGRP-LB* mutant flies experience sex-differences in survival despite undetectable differences in their bacterial loads (Vincent and Dionne 2021). Though this study

did not directly measure disease tolerance (that is, survival relative to microbe load), its findings are consistent with the phenotype of disease tolerance. Here, we have explicitly analysed the tolerance response in the IMD mutants and found a clear effect of its negative regulators on disease tolerance, though this was comparable in both males and females.

How these negative regulators contribute to disease tolerance is likely linked to their role in reducing immunopathology. In the case of *caudal* and *pirk*, there is a possible source of tissue damage through the cytotoxic effects of AMPs, because we observed higher AMP expression in these mutants. AMPs are known to be harmful to both prokaryotic and eukaryotic cells and tissues (Song et al. 2005; Paredes-Gamero et al. 2012) and overexpression of AMPs in flies leads to cytotoxicity, causing neurodegeneration and age-related mortality (Badinloo et al. 2018; Cao et al. 2013). This would support the idea that elevated levels of several AMPs are sufficient to cause cytotoxicity to cells/tissues, contributing to immunopathological consequences.

2.5 *Concluding remarks*

In the present work, we used a highly virulent fly pathogen (*P. entomophila*) and flies lacking various components of IMD-signalling. Our data show that flies with reduced expression of the negative regulators of IMD *pirk* and *caudal* experienced reduced tolerance (overall health with increased pathogen burden). The knockdown flies with reduced expression of negative regulators despite having higher AMP expression (at their disposal) and reducing the bacterial numbers still experienced increased mortality following wide range of infection doses. We hypothesise that this is likely due to immunopathology arising through the accumulation of cytotoxicity (AMPs and ROS).

Our results highlight that the negative regulators *caudal* and *pirk* are important not only in regulating pathogen clearance by the IMD pathway, but also play a role in disease tolerance (***Fig.*** *7*). Uncovering the role of these regulators in disease tolerance phenotype opens up the possibility of using them as therapeutic targets where the aim is to enhance host disease tolerance (R. Martins et al. 2019; Vale et al. 2016; Vale, Fenton, and Brown 2014; Schneider and Ayres 2008). Future work may also investigate if natural genetic variation in disease tolerance can be explained by variation in negative immune regulators.

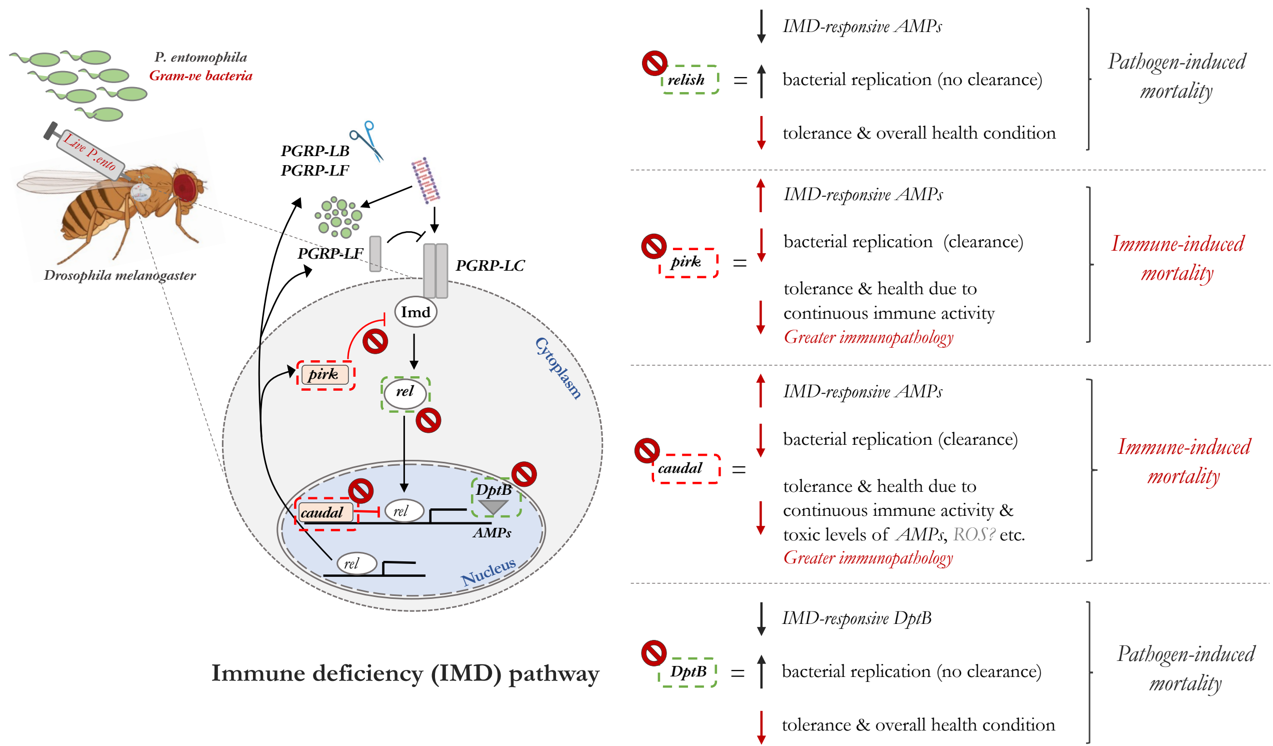

Figure 7: Summary highlighting that in addition to the already known role of regulating an efficient pathogen clearance response, negative regulators of IMD also contribute to the phenotype of disease tolerance. Flies with reduced expression of the negative regulators *pirk* and *caudal* show reduced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

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2.7 *Supplementary information*

Figure SI-1. Bacterial loads around 24 hours after different concentrations of systemic *P. entomophila* infection for female flies for IMD-pathway mutants **(i)** *UAS-Relish*^{RNAi} **(ii)** *UAS-Caudal*^{RNAi} **(iii)** *UAS-Pirk*^{RNAi} **(iv)** *UAS-DptB*^{RNAi}.

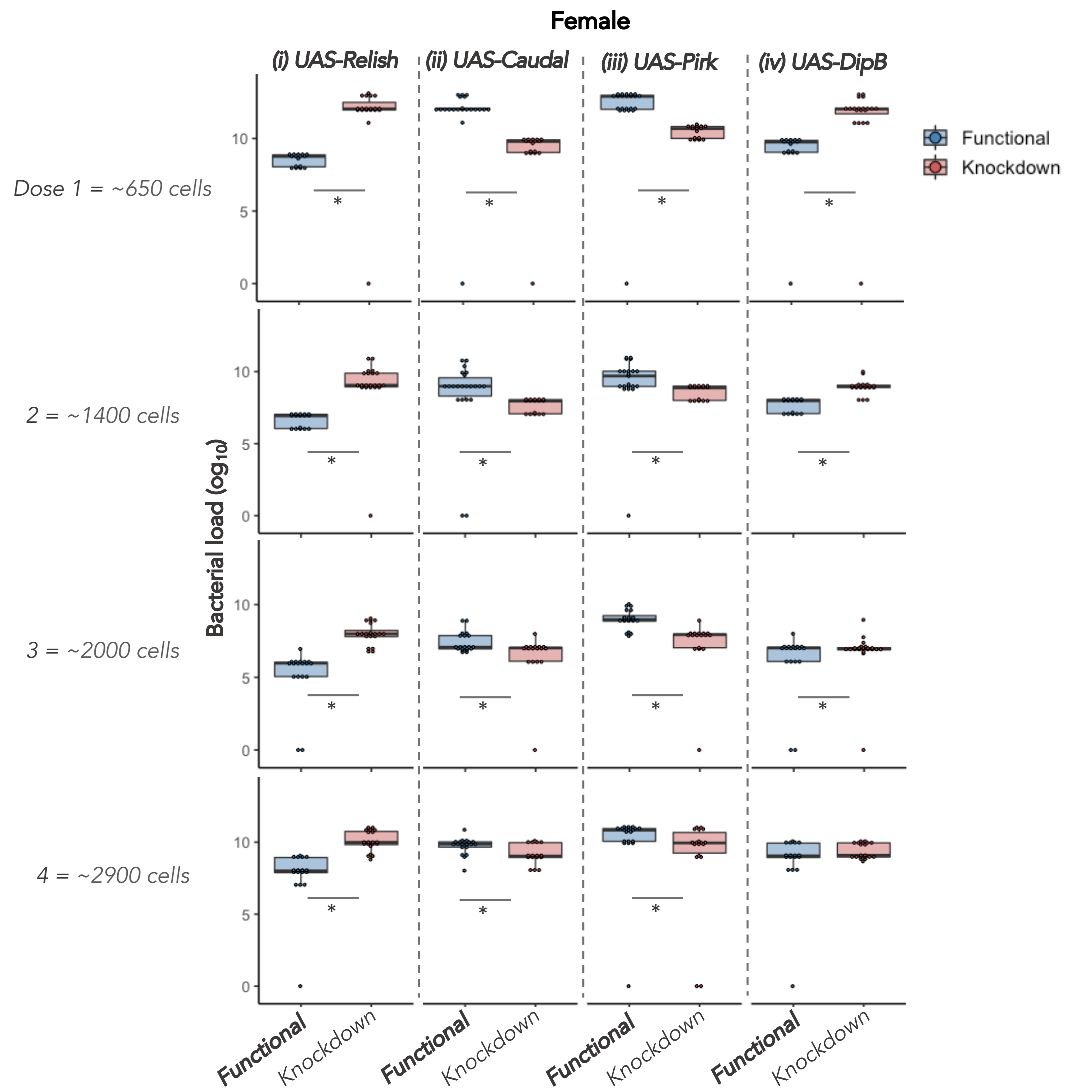

Figure SI-2. Bacterial loads around 24 hours after different concentrations of systemic *P. entomophila* infection for male flies for IMD-pathway mutants **(i)** *UAS-Relish*^{RNAi} **(ii)** *UAS-Caudal*^{RNAi} **(iii)** *UAS-Pirk*^{RNAi} **(iv)** *UAS-DptB*^{RNAi}.

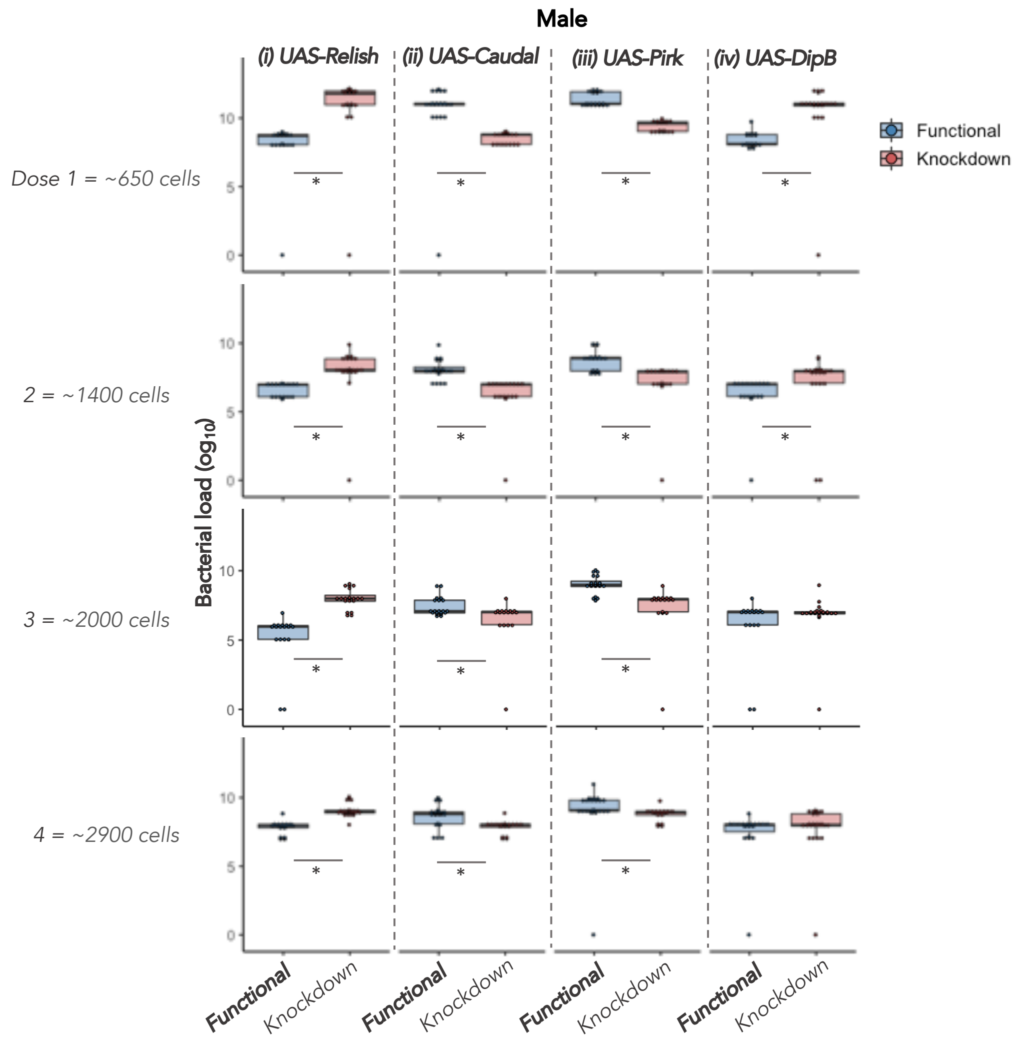

Figure SI-3. Survival curves of female flies of different IMD-pathway mutants (n=16-24 flies/treatment/sex/fly line) **(a)** *UAS-Relish*^{RNAi} **(b)** *UAS-Caudal*^{RNAi} **(c)** *UAS-Pirk*^{RNAi} **(d)** *UAS-DptB*^{RNAi} during 4 concentrations of systemic *Pseudomonas entomophila* infection ranging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

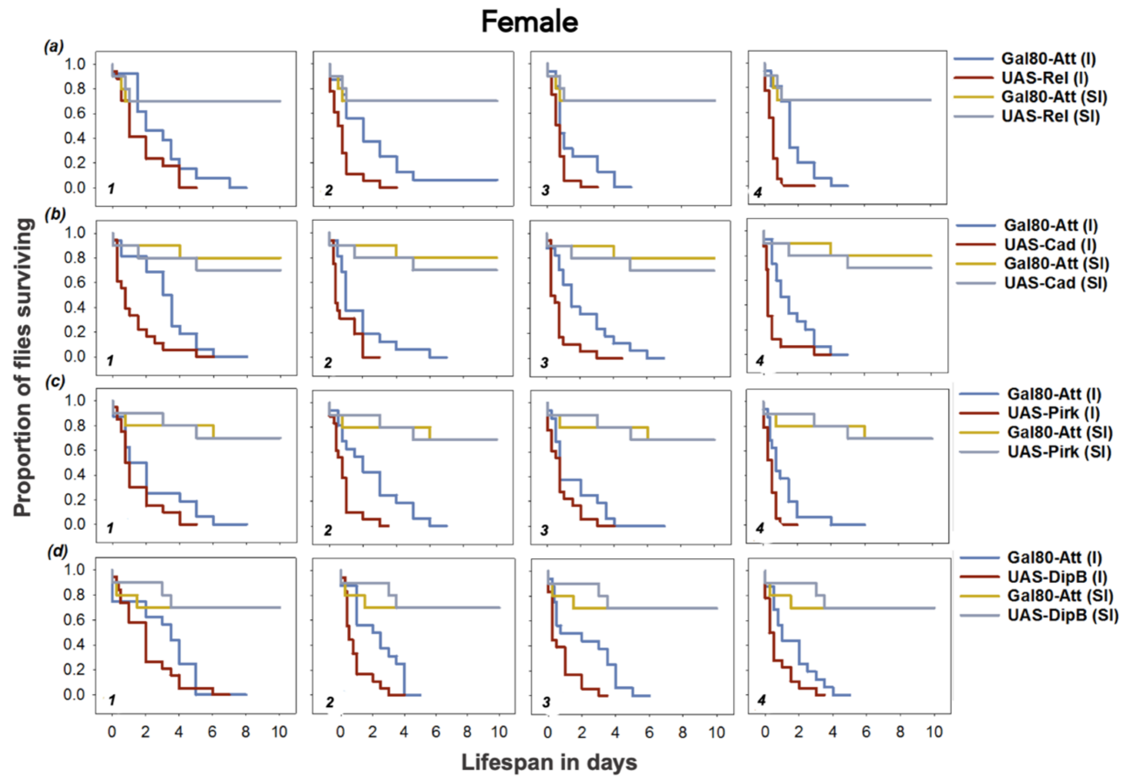

Figure SI-4. Survival curves of male flies of different IMD-pathway mutants (n=16-24 flies/treatment/sex/fly line) **(a)** *UAS-Relish*^{RNAi} **(b)** *UAS-Caudal*^{RNAi} **(c)** *UAS-Pirk*^{RNAi} **(d)** *UAS-DptB*^{RNAi} during 4 concentrations of systemic *Pseudomonas entomophila* infection ranging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

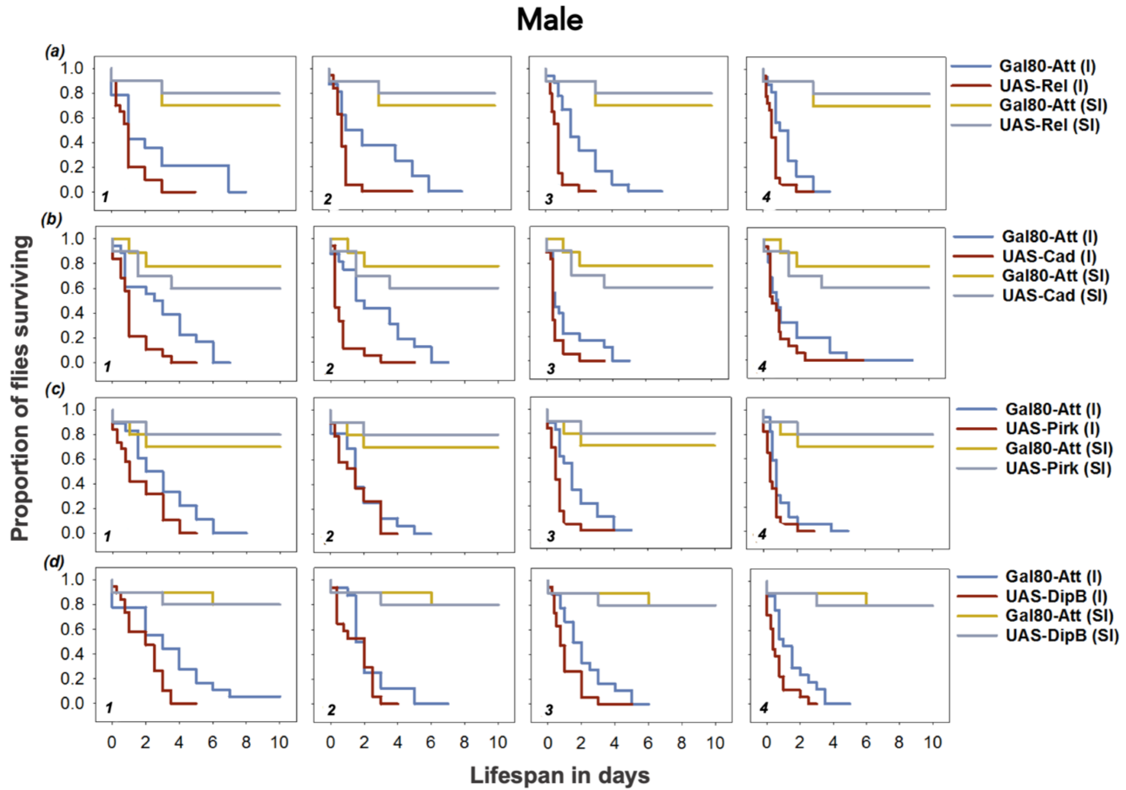

Figure SI-5. The estimated hazard ratios calculated from survival curves (***Fig.*** *S1 and S2*) for flies with fully functional immune system and IMD-pathway mutants for both Sexes. The different IMD-pathway mutants include **(a)** *UAS-Relish*^{RNAi} **(b)** *UAS-Caudal*^{RNAi} **(c)** *UAS-Pirk*^{RNAi} **(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.

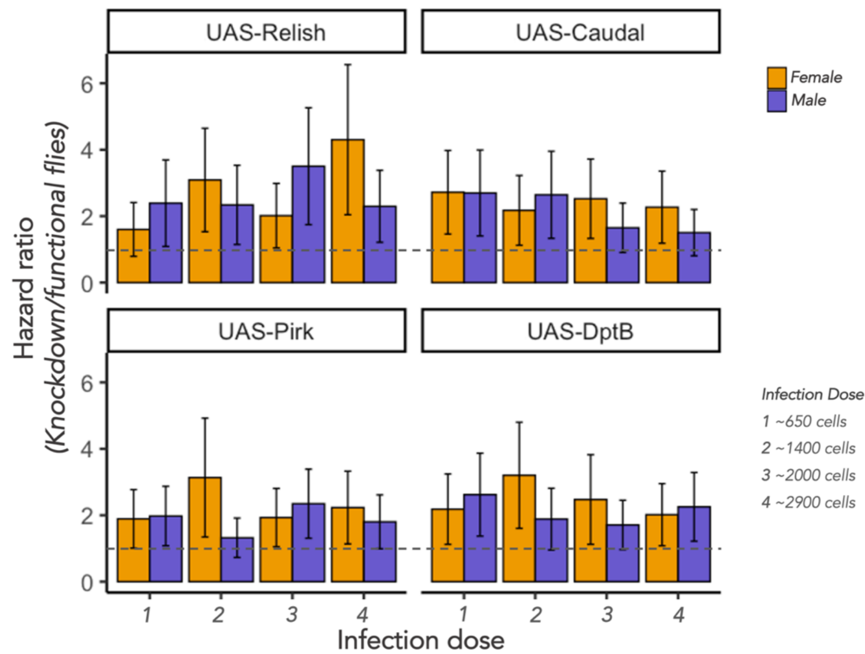

Table SI-1: Fly strains used in this study

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}*

Table SI-2*:* Primers used in this study

	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’

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

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

Table SI-4: Summary of log_{10} transformed bacterial load data after exposure to 4 different concentrations (doses) of *P. entomophila* systemic infection, analysed using ANOVA by fitting 'Knockdown', 'Sex' and 'dose' as categorical fixed-effects and replicate 'vials' as a random-effects 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 SI-5: Summary of mixed effects Cox model, fitting the model to estimate response to *P. entomophila* infection in flies with fully intact immune system and flies with deficiencies in IMD-pathway. We used data from the individuals of 3-5-day adult males and females infected with 4 concentrations (doses) of *P. entomophila* for each fly lines (IMD-pathway mutants) and specified the model as: survival ~ Knockdown * Sex * dose (1|vial), with 'Knockdown' and 'Sex' as fixed effects, and 'vials' as a random effect. The table shows model output (ANOVA) for survival post-infection for flies with fully function immune system and defects in IMD functioning.

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

	Knockdown x Sex	1331.0	0.1187	1	0.73
	Knockdown x dose	1330.8	0.3249	4	0.98
	Sex x dose	1329.1	3.5419	4	0.47
	Knockdown x Sex x dose	1328.0	2.1198	4	0.71
	Random effect	*Std dev*			
	Vials	*0.15*			

Table SI-6: 'Point tolerance' for all doses - Summary of ANCOVA. To assess differences bacterial loads following infection with 4 concentrations (dose) of *P. entomophila*, we analysed the average bacterial load, measured around 8 hours following infection using ANCOVA and fitted 'Knockdown' and 'Sex' as categorical fixed effects and 'bacterial load' as continuous co-variate for each fly line (functional wild type and IMD knockdown).

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

Chapter 3

Mechanisms of damage prevention, signalling, and repair impact the ability to tolerate enteric bacterial infection

Keywords: Disease tolerance, Gut-epithelial immunity, Tissue damage prevention and renewal, Oral bacterial infection

3.1 *Abstract*

Many insects thrive on decomposing and decaying organic matter containing a large diversity of both commensal and pathogenic microorganisms. The insect gut is therefore frequently exposed to pathogenic threats and must be able not only to detect and clear these potential infections, but also be able to repair the resulting damage to gut tissues in order to tolerate relatively high microbe loads. In contrast to the mechanisms that eliminate pathogens, we currently know less about the mechanisms of disease tolerance, and most of this knowledge stems from systemic infections.

Here we investigated how well-described mechanisms that either prevent, signal, control, or repair tissue damage during infection contribute to the phenotype of disease tolerance during gut infection. We orally infected adult *Drosophila melanogaster* flies with the bacterial pathogen *Pseudomonas entomophila* in several loss-of-function mutants lacking epithelial responses including damage preventing *dcy* (drosocrystallin - a major component of the peritrophic matrix), damage signalling *upd3* (unpaired protein, a cytokine-like molecule), damage controlling *irc* (immune-regulated catalase, a negative regulator of reactive oxygen species) and tissue damage repairing $egfr^{1}$ (epidermal growth factor receptor).

Overall, we detect effects of all these mechanisms on disease tolerance. The deterioration of the peritrophic matrix in *dcy* mutants resulted in the highest loss of tolerance, while loss of function of either *irc* or *upd3* also reduced tolerance in both sexes. The absence of tissue damage repair signalling ($egfr^{1}$) resulted in a severe loss in tolerance in male flies but had no substantial effect on the ability of female flies to tolerate *P. entomophila* infection, despite carrying greater microbe loads than males. Together, our findings provide empirical evidence for the role of damage limitation mechanisms in disease tolerance and highlight how sex differences in these mechanisms could generate sexual dimorphism in immunity.

3.2 *Introduction*

Animals have evolved diverse responses to infection, including behavioural avoidance of infection, physical barriers to pathogen entry and a variety of humoral and cellular immune responses (Buchon, Silverman, and Cherry 2014; Kuraishi, Hori, and Kurata 2013; Vale et al. 2018). These responses have been particularly well described in the fruit fly *Drosophila*, where signalling pathways such as *IMD* and *Toll* are recognised as major contributors to pathogen clearance during infection (Apidianakis and Rahme 2011; Buchon, Silverman, and Cherry 2014; Hoffmann 2003; Hultmark 2003; Kuraishi, Hori, and Kurata 2013; Lemaitre and Hoffmann 2007).

In addition to mechanisms that clear or otherwise quantitatively reduce pathogen burdens, there is an increasing appreciation that mechanisms that promote disease tolerance are at least equally as important during infection and in recovery to a healthy state (Raberg, Sim, and Read 2007; Ayres and Schneider 2012; Medzhitov, Schneider, and Soares 2012; Soares, Teixeira, and Moita 2017; McCarville and Ayres 2018). Disease tolerance is defined as the ability of hosts to maintain health despite harbouring relatively high pathogen loads, a phenotype that has been observed in several species, including insects (Howick and Lazzaro 2014; Lissner and Schneider 2018; Oliveira, Bahia, and Vale 2020) rodents (Palaferri Schieber et al. 2015; Raberg, Sim, and Read 2007; Cumnock et al. 2018); birds (Adelman et al. 2013; Bonneaud et al. 2019); and humans (Regoes et al. 2014; Nahrendorf, Ivens, and Spence 2021; Soares, Teixeira, and Moita 2017).

The mechanisms of pathogen clearance are well-described in many animal species (Cooper 2018), but we currently know less about the mechanisms underlying disease tolerance. Given that tolerance reflects a distinct ability to maintain health independently of pathogen clearance, we might expect these mechanisms to be related to processes such as detoxification, reduction of inflammation, or tissue damage control and cellular renewal (Ayres and Schneider 2012; Soares, Teixeira, and Moita 2017; Soares, Gozzelino, and Weis 2014). Genome-wide association or transcriptomic studies in *Drosophila* have highlighted potential candidate genes underlying variation in disease tolerance, reviewed in (Lissner and Schneider 2018), but it remains unclear how these genes interact with known mechanisms of immunity and recovery.

Furthermore, almost all candidate genes for disease tolerance in *Drosophila* arise from systemic infections(Howick and Lazzaro 2017; Troha et al. 2018; Lissner and Schneider 2018), leaving a gap in our knowledge about disease tolerance during oral infections. Orally acquired

infections are especially relevant given the pervasive nature of oral-faecal transmission routes, and in the context of the ecology of most insects, like *Drosophila*, that thrive on decomposing and decaying organic matter containing a large diversity of microorganisms, both commensal and pathogenic (Chandler et al. 2011; Corby-Harris et al. 2007). For example, recent genomic analyses of *Drosophila* samples collected across Europe revealed sequences belonging to multiple species of bacteria, fungi and viruses, many of which had not been previously described (Kapun et al. 2020; Wallace et al. 2021). The insect gut is therefore frequently exposed to pathogenic threats and must be able not only to detect and clear these potential infections, but also be able to repair the resulting damage to gut tissues in order to tolerate relatively high numbers of ingested pathogens.

Here, we aimed to specifically test how known mechanisms that either prevent, reduce, or repair tissue damage in the fly gut contribute to the phenotype of disease tolerance. The *Drosophila* gut is a compartmentalized tubular organ which is structurally and functionally similar to the vertebrate intestinal tract (Buchon, Broderick, and Lemaitre 2013; Buchon and Osman 2015; Kuraishi, Hori, and Kurata 2013; Lemaitre and Miguel-Aliaga 2013). We can consider several stages comprising gut defence in *Drosophila.* The first involves the physical barrier of the gut epithelia and the peritrophic matrix (*PM*), which is a layer of chitin and glycoproteins that lines the insect midgut lumen.

The *PM* is functionally analogous to mammalian mucus membrane in the digestive tract and acts as the first line of defence against invading pathogens (Hegedus et al. 2009; Kuraishi, Hori, and Kurata 2013). A major component of the *PM* is *drosocrystallin* (*dcy*). Loss-of-function mutations in *dcy* increase the width of the peritrophic matrix, increasing its permeability to larger molecules and to leakage of microbial cells, including pathogens, in to the haemolymph (Kuraishi, Hori, and Kurata 2013). *Dcy* deficient flies therefore exhibit increased susceptibility to oral bacterial infections.

As second mode of defence during gut infections is the production of reactive oxygen species (*ROS*) by the gut epithelia. For example, oral infection with *P. entomophila* in *D. melanogaster* causes massive destruction of the gut epithelium (Vodovar et al. 2005; Dieppois et al. 2015). In response to ingested *P. entomophila*, *ROS* production is induced by two NADPH enzymes-*nox* (NADPH oxidase) and *duox* (dual oxidase), while *irc* (immune-reactive catalase) negatively regulates *ROS* production once the infection threat is controlled, which otherwise, would lead to cytotoxic effects (Buchon et al. 2013; 2009; Kuraishi, Hori, and Kurata 2013).

ROS production not only targets pathogens directly, but also plays additional roles in triggering signalling pathways that lead to the production of IMD or Toll responsive antimicrobial peptides (Buchon et al. 2009; Lemaitre and Hoffmann 2007; Myllymäki, Valanne, and Rämet 2014; Myllymäki and Rämet 2014; Valanne, Wang, and Rämet 2011).

The final stage in gut defence is to repair the tissue damage caused during the infection. Damage-signalling cytokine-like molecules *upd3* are released from damaged cells which trigger the Jak/Stat-pathway, stimulating the proliferation of intestinal stem cells (*ISCs*) and their differentiation into enterocytes (*ECs*) via $egfr^{1}$ (epidermal growth factor receptor) signalling (Buchon et al. 2010; Chakrabarti et al. 2016; Jiang et al. 2011; Weng et al. 2018). Flies lacking Jak/Stat or *Egfr* are therefore highly susceptible to bacterial infections due to their inability to repair and renew damaged tissue (Buchon et al. 2010; Jiang et al. 2011; 2009; Marianes and Spradling 2013; Myllymäki and Rämet 2014).

To investigate how these mechanisms of damage prevention (*dcy*), signalling (*upd3*) control (*irc*) and renewal ($egfr^{1}$) contribute to disease tolerance during gut infections we employed oral infections in *Drosophila* lines carrying loss-of-function mutations in each of these genes on a common genetic background (w^{1118}). We orally challenged these flies with a range of infection doses of *Pseudomonas entomophila* and then quantified their effects on survival, pathogen loads and disease tolerance responses during period of peak infection burden.

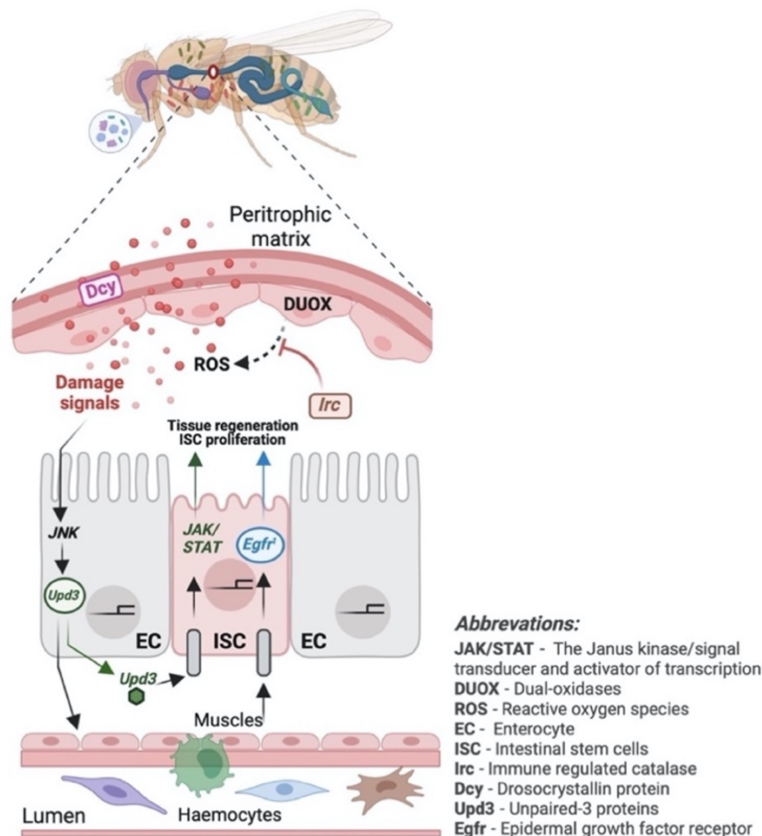

Figure 1: Schematic representation of *Drosophila* epithelial immune response following enteric infection. Several stages of tissue damage prevention and repair epithelial response in the *Drosophila* can be found. ***Stage-I***: involves the physical barrier the peritrophic matrix (*PM* - layer of chitin and glycoproteins) that lines the *Drosophila* midgut lumen. A major component of the *PM* is ***dcy*** (*drosocrystallin*) and loss-of-function mutations in *dcy* increase the width of the *PM*, increasing permeability to pathogens. ***II***: production of reactive oxygen species (*ROS*) by the gut epithelia - enteric infections cause massive destruction of the gut epithelium and in response to ingested pathogens, *ROS* production is induced by *nox* (NADPH oxidase) and *duox* (dual oxidase), while ***irc*** (immune-reactive catalase) negatively ⊣ regulates *ROS* production by suppressing the cytotoxic effects of *ROS* once the infection threat is lessened, avoiding immunopathology. In addition, *ROS* production is known to trigger signalling pathways the IMD or Toll that leads to the production of antimicrobial peptides (*AMPs*) critical for pathogen clearance in the gut. ***III***: tissue renewal process after infection-induced damage - The final stage in gut defence is to repair the damage caused during enteric infections. Damage-signalling cytokine-like molecules ***upd3***, released from damaged cells trigger the Jak/Stat-pathway, stimulating the proliferation of intestinal stem cells (*ISCs*) and their differentiation into enterocytes (*ECs*) via ***egfr¹*** (epidermal growth factor receptor) signalling which are indispensable for maintaining homeostasis and renewing the damaged cells/tissues. Figure created using Biorender.

3.3 *Materials and methods*

1. *Fly strains:* The following fly stocks were obtained from VDRC (Vienna *Drosophila* Resource Centre) and Bloomington Stock Centre, Indiana: *dcy* (*BL26106*), *irc* (*BL29191*), *egfr¹* (*BL2079*), *upd3* (*BL19355*). All the mutants were subsequently backcrossed into the wild type w^{1118} (*VDRC stock# 60000*) for at least 10 generations.

All fly lines were maintained in plastic vials (12 ml) on a standard cornmeal diet (Lewis's medium) at a constant temperature of 25°C (±2°C).

2. *Bacterial culture preparation:* To test the impact of bacterial infection on fly survival we used the gram-negative bacteria *Pseudomonas entomophila*, that commonly infects a broad range of insects and other invertebrates. In flies, *P. entomophila* infection mainly occurs in the intestinal epithelium and eventually causes death (Buchon et al. 2009; Vodovar et al. 2005). To obtain bacterial cultures for oral exposure, we inoculated frozen bacterial stock cultures stored at -80℃ onto fresh 15ml LB broth (media composition) and incubated overnight at 37°C with shaking at 120rpm (revolutions per minute). The overnight cultures were diluted 1: 100 into 500ml of fresh LB broth and incubated again at 30°C with shaking at 120rpm. At the mid-log phase (OD_{600}=0.75), we harvested the bacterial cells by centrifugation at 5000rpm for 15 min and re-suspended the bacterial pellet in 5% sucrose (Siva-Jothy et al. 2018). The final inoculum was adjusted to three different bacterial concentrations or infection dose $OD_{600}=10$ (low dose), $OD_{600}=25$ (medium dose) and $OD_{600}=45$ (high dose).

3. *Oral infection assay:* Before infecting flies we initially prepared infection vials by pipetting around 350µl of standard agar (1L triple distilled H_2O, 20g agar, 84g brown sugar, 7ml Tegosept anti-fungal agent) onto lid of a 7ml tubes (bijou vials) and allowed it to dry. Simultaneously, we starved the experimental flies on 12ml agar vials for 4-5 hours. Once the agar in the bijou lids dried, we placed a filter disc (Whattmann-10) in the lid and pipetted 80µl of bacterial culture directly onto the filter disc. For control (mock) infections, we replaced bacterial culture with 5% sucrose solution. We then orally exposed flies inside the bijou vials for about 18-hours and then transferred the flies onto fresh vials containing standard cornmeal (Siva-Jothy et al. 2018).

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

survival and bacterial load in order to estimate (3) disease tolerance for each fly line that is, the linear relationship between fly survival and internal bacterial load.

5. _Bacterial load measurement:_ To test whether variation in mortality of experimental flies after *P. entomophila* infection is explained by the ability to clear infection, we measured bacterial load at three timepoints (immediately after oral exposure 0-15 minutes, 24-hours, and 96-hours) by exposing 3–5-day old flies with $OD_{600}=25$ *P. entomophila* cells. To confirm oral bacterial infection, we thoroughly surface-sterilised flies (group of 3) with 70% ethanol for 30-60 seconds and then rinsed twice with sterile distilled water. We plated the second wash on LB agar plates and incubated overnight at 30°C to confirm that surface bacteria were successfully removed after alcohol sterilization. We transferred flies onto 1.5ml micro centrifuge tubes and homogenized using a motorized pestle for approximately 30-60 seconds in 100µl LB broth (n=30 homogenates/sex/infection treatment/fly line). We performed serial dilution of each homogenate up to 10^{-6}-fold and added 4µl aliquot on a LB agar plate. After this, we incubated the plate overnight for about 18-hours at 30°C and counted the resultant bacterial colonies manually. We note that mock-infected control fly homogenates did not produce any colonies on LB agar plates (Siva-Jothy et al. 2018). We also measured bacterial load at 24-hour following oral *P. entomophila* infection for low dose ($OD_{600}=10$) and high ($OD_{600}=45$) infection dose (See Supplementary Information ***Figs***. *SI-1* and *SI-2*).

Statistics

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

2. _Internal bacterial load:_ We found that residuals of bacterial load data were non-normally distributed when tested using Shapiro–Wilks's test. Hence, we first log-transformed the data and then confirmed that the log-transformed residuals were still non-normally distributed. Subsequently, we used a non-parametric one-way ANOVA (Kruskal-

Wallis test) to test the effects of each fly line, that is, wild-type w^{1118} and mutant flies on males and females separately following oral *P. entomophila* infection.

3. *Measuring disease tolerance:* Finally, to understand how tissue damage signalling and repair mechanisms affect disease tolerance in males and females during oral *P. entomophila* infection, we analysed the linear relationship between fly survival against bacterial load by fitting linear models (Raberg, Sim, and Read 2007; Schneider and Ayres 2008; Medzhitov, Schneider, and Soares 2012; Louie et al. 2016; Gupta and Vale 2017; Oliveira, Bahia, and Vale 2020).

We assessed differences in disease tolerance (fly survival with increasing bacterial load) using ANCOVA by fitting 'fly line' and 'sex' as categorical fixed effects, 'Mean bacterial load (log_{10})' for wild type and mutant flies as a continuous covariate and their interactions as fixed effects. Significant interaction effects between fly line and bacterial load would indicate that the slope of the relationship between fly survival and load varies between fly lines, that is, the tolerance response differs between lines. Because our interest was to find out to what extent loss-of damage prevention and tissue repair mechanisms differed from wildtype w^{1118} in tolerating enteric bacterial infections we compared the estimates of slope for wild type w^{1118} line with each of the mutant line for both males and females separately using a pairwise comparison (f-test).

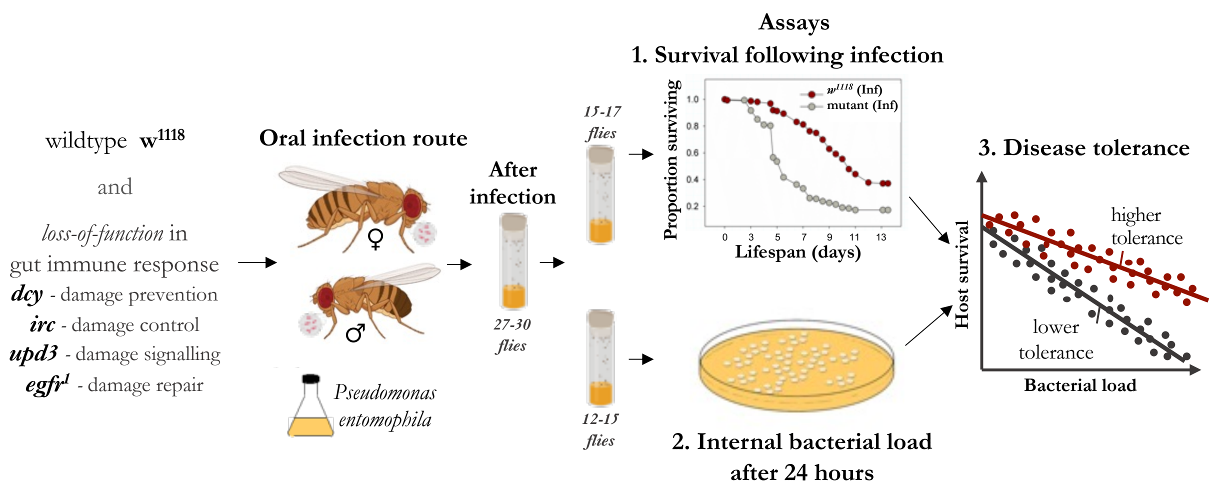

Figure 2: Split-vial experimental design to assay **(1)** survival (15-17 flies/combination in a vial) and **(2)** internal bacterial load (12-15 flies/combination in a vial) following oral bacterial infection with *Pseudomonas entomophila* to test how epithelial immune response including damage prevention and tissue repair mechanisms in *Drosophila* gut contribute to **(3).** disease tolerance. For the split-vial design, after oral exposure with each vial containing about 25 flies (of each infection treatment, sex, fly line combination) were divided into 2 vials, each for survival (around 15-17 flies) and bacterial load (around 12-15 flies). Each point in disease tolerance

panel (3) represents replicate-matched data [n=30 vials/infection treatment/sex/fly line – with each vial containing around 27-30 flies] from survival (1) and bacterial load (2). The data points shown in panel 1 and 3 are diagrammatic representation (*not actual data*).

3.4 *Results*

1. <u>Flies lacking damage-preventing *dcy* are more susceptible to oral *P. entomophila* infections than those lacking components that minimise, signal or repair tissue damage</u>

Following oral infection with three different doses of *Pseudomonas entomophila*, we found that flies lacking major components of gut immunity such as tissue damage - preventing *dcy*, signalling *upd3*, repairing $egfr^1$ and controlling *irc*, were all significantly more susceptible to oral *P. entomophila* infections than the wild type w^{1118} flies (***Fig****. 3B* and *1A,* ***Table*** *1*; infection dose $OD_{600}=25$*;* see Supplementary ***Figs****. SI-1 and SI-2,* ***Table*** *SI-1* for low $OD_{600}=10$ and high $OD_{600}=45$ infection doses). Overall, flies lacking major components of gut immunity showed increased susceptibility than wild type w^{1118} flies during oral *P. entomophila* infections (***Fig****. 3*, ***Table*** *1*). Among these mutants, *dcy* mutants showed increased susceptibility to infection compared to other mutants (***Fig****. 3B* and *1A* for survival*,* ***Table*** *1* for hazard ratio; infection dose $OD_{600}=25$). The effect of each mutation on the survival of flies following infection was similar in males and females (fly line x sex x treatment interaction = non-significant, ***Table*** *SI-1*), and was consistent at both lower ($OD_{600}=10$) and higher infection ($OD_{600}=45$) doses (see Supplementary ***Figs****. SI-1 and SI-2,* ***Table*** *SI-1* for low $OD_{600}=10$ and high $OD_{600}=45$ infection dose).

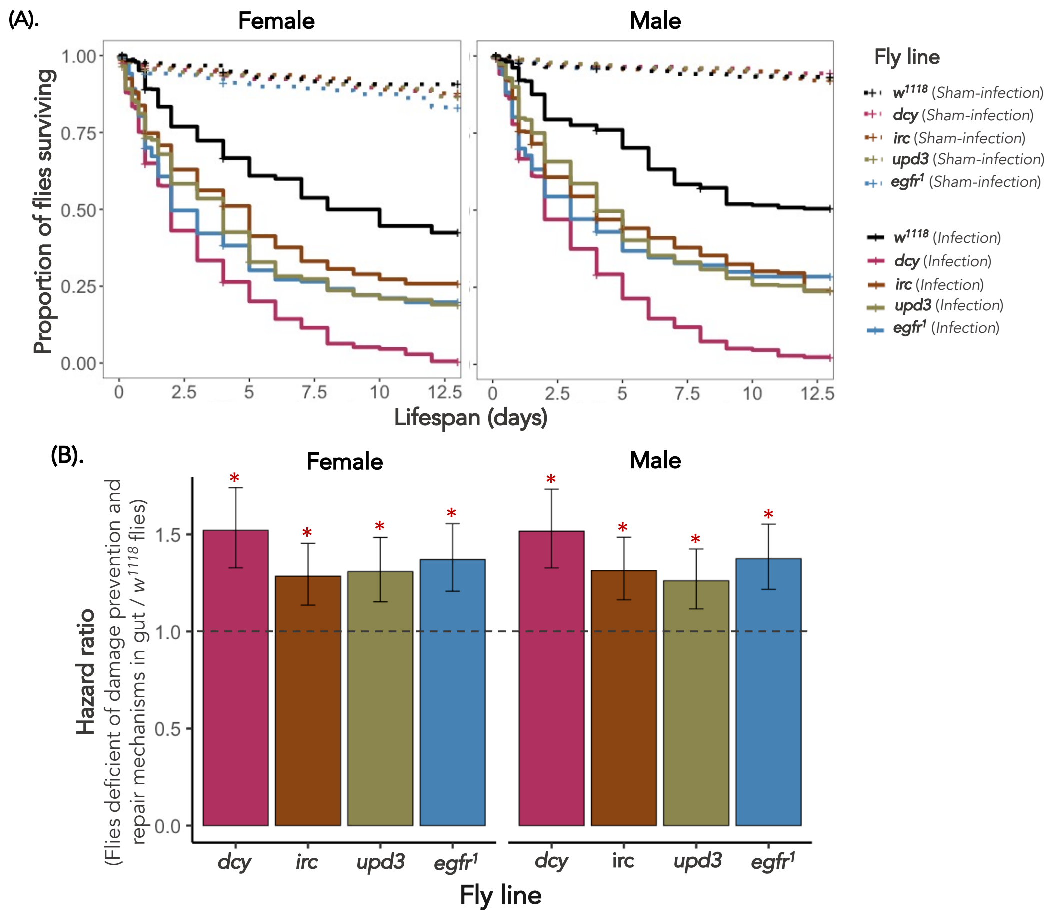

Figure 3. Survival curves for **(*A*)** females and males of wildtype w^{1118} and mutant flies exposed to oral *P. entomophila* of $OD_{600}=25$ (medium dose, n=30 vials of which 15-17 flies/treatment/sex/fly line were used for survival). **(*B*)** Estimated hazard ratios for males and female flies (wild type w^{1118} and mutant flies) calculated from the survival curves. A greater hazard ratio (>1) indicates higher susceptibility to infection. ['*' indicates that the mutant flies are significantly different from wild type w^{1118} flies for males and females respectively]. See ***Figs****. S1* and *S2* for similar results at lower dose ($OD_{600}=10$) and higher dose ($OD_{600}=45$).

Table 1: Summary of estimated hazard ratio from the cox proportional model. A greater hazard ratio (>1) indicates that mutant flies are more susceptible to oral *P. entomophila* infection than w^{1118} wildtype flies.

Fly line	***Sex***	***Estimate***	***p***	***lower 95%***	***upper 95%***
dcy	Female	1.520	<0.01	1.32	1.74
	Male	1.516	<0.01	1.32	1.73
Egfr¹	Female	1.370	<0.01	1.20	1.55
	Male	1.374	<0.01	1.21	1.55
irc	Female	1.284	<0.01	1.13	1.45
	Male	1.314	<0.01	1.16	1.48
upd3	Female	1.307	<0.01	1.15	1.48
	Male	1.261	<0.01	1.11	1.42

2. Both wild-type and flies with disrupted tissue damage prevention and repair mechanisms show sex differences in bacterial load during oral infections

The higher susceptibility of mutants to oral bacterial exposure could either be caused by their inability to supress the bacterial growth or due to their inability to tolerate the damage inflicted during oral infection. To distinguish between these mechanisms, we first quantified internal bacterial loads across several time points, that is, 0-15 minutes, 24-hours and 96-hours following oral exposure to *P. entomophila*. All fly lines showed sex-differences at 24-hours peak load, though by 96-hours this sex difference was no longer present in flies lacking damage sensing (*upd3*) and tissue renewal mechanisms (*egfr*) (***Fig****. 4,* ***Table*** *SI-2*). Flies lacking the negative regulator of ROS *irc* always exhibited lower levels of bacterial load compared to wild type flies (***Fig****. 4B,* ***Table*** *SI-2*).

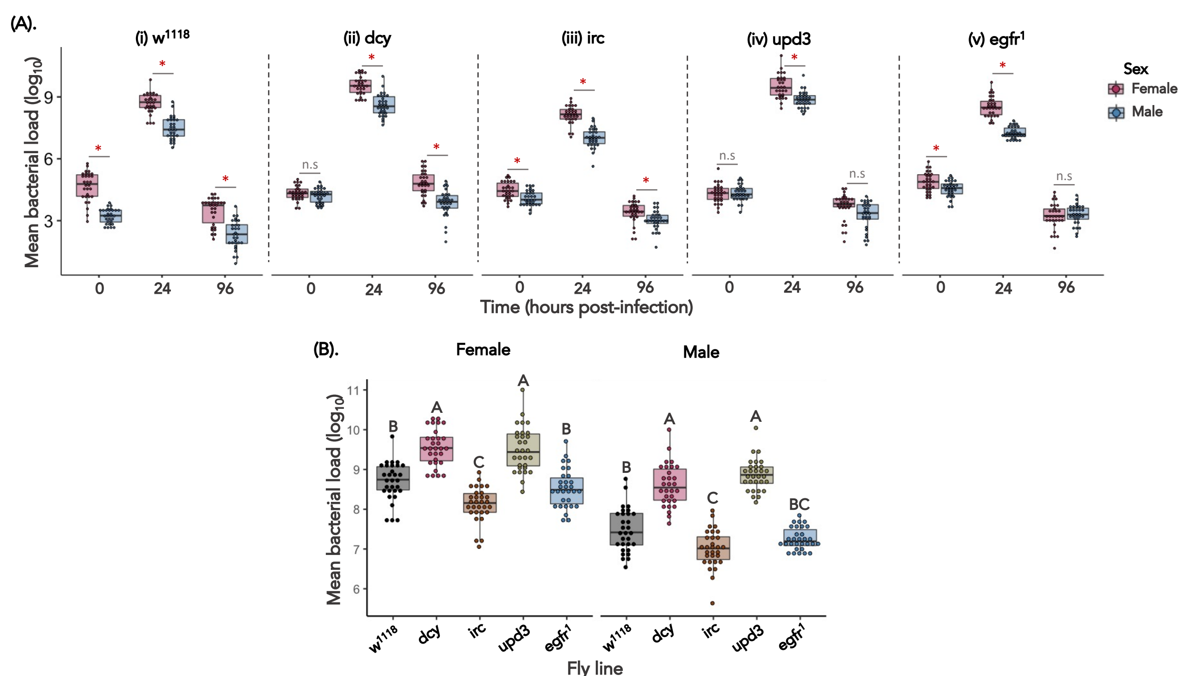

Figure 4. **(A)** Bacterial load measured as CFUs- colony forming units using infection dose (medium $OD_{600}=25$) after different time points 0-15 minutes (immediately after the oral bacterial exposure), 24-hours and 96-hours following infection with *P. entomophila* for wildtype *w*^{1118} flies and mutants [n=30 vials of which 12-15 flies/treatment/sex/fly line for bacterial load measurement)]. **(B)** bacterial load at 24-hours following infection [significantly different fly lines are connected with different alphabets using Tukey's HSD as a post hoc analysis of pairwise comparisons between wild type and mutants for males and females respectively]. See ***Figs****. SI-1* and *SI-2* for similar results at lower dose ($OD_{600}=10$) and higher dose ($OD_{600}=45$).

3. <u>Tissue damage repair mechanisms in gut-epithelia mediate sex differences in disease tolerance to oral bacterial *P. entomophila* infections</u>

While some of the variation in survival between mutants (***Fig****. 3*) may be explained by variation in resistance [that is, their ability to clear infection (***Fig****. 4*)], some of that variation may also arise due to differences in tolerance. We were therefore interested in measuring

disease tolerance in these lines using the reaction norm of survival relative to bacterial loads, where the slope of the linear relationship reflects the degree of tolerance: steep negative slopes indicate a rapid mortality with increases in pathogen loads (low tolerance), while less steep or flat slopes reflect relatively more tolerant host (Raberg, Sim, and Read 2007; Schneider and Ayres 2008; Louie et al. 2016; Kutzer and Armitage 2016). In statistical terms, the differences in tolerance between lines are indicated by a significant interaction between the bacterial load and the fly line for survival, which reflects the overall rate at which fly health (survival) changes with bacterial load (tolerance) between fly lines (Howick and Lazzaro 2014; Kutzer and Armitage 2016; Louie et al. 2016).

Overall, we found that most mutant lines lacking major components of tissue damage limitation showed reduced tolerance to oral bacterial *P. entomophila* infections compared to wild type w^{1118} flies (***Fig**. 5, **Table** 3*). Here, the differences in tolerance between wild type w^{1118} and mutant flies are indicated by a significant interaction between the fly line bacterial load for survival, which reflects the overall rate at which fly health (survival) changes with bacterial load (tolerance) between fly lines in both males and females (***Fig.** 5. **Table** 2*). Males lacking $egfr^1$ show lower tolerance to bacterial infection than females (***Fig**. 5, **Table** 2*), suggesting sexual dimorphism in gut cell renewal. However, both males and females lacking major component of the peritrophic matrix that is, *dcy* showed significantly reduced tolerance (***Fig**. 5*), indicating the general importance of tissue damage preventing epithelial barrier in defence against oral infections (Kuraishi, Hori, and Kurata 2013).

One notable finding was that $egfr^1$ flies, lacking tissue repair and renewal signalling, showed sex-differences in tolerance during oral *P. entomophila* bacterial infections (***Fig**. 5, **Table** 2 and 3*). Previous studies suggest that *D. melanogaster* males are more susceptible to intestinal infections possibly due to males having fewer *ISCs* (intestinal stem cells, a major part of *egfr*-mediated tissue repair process) (Regan et al. 2016; Rera, Clark, and Walker 2012). Males also have lower basal rate of division, overall, making them more susceptible to intestinal oral infection, compared to females (Regan et al. 2016; Rera, Clark, and Walker 2012). Instead, we found that loss of function of tissue renewal mechanisms makes females more susceptible to *P. entomophila* oral infections across wide range of infection doses (see *Supplementary **Figs**. SI-1 and SI-2, **Table** SI-1* for low $OD_{600}=10$ and high dose $OD_{600}=45$ survival data).

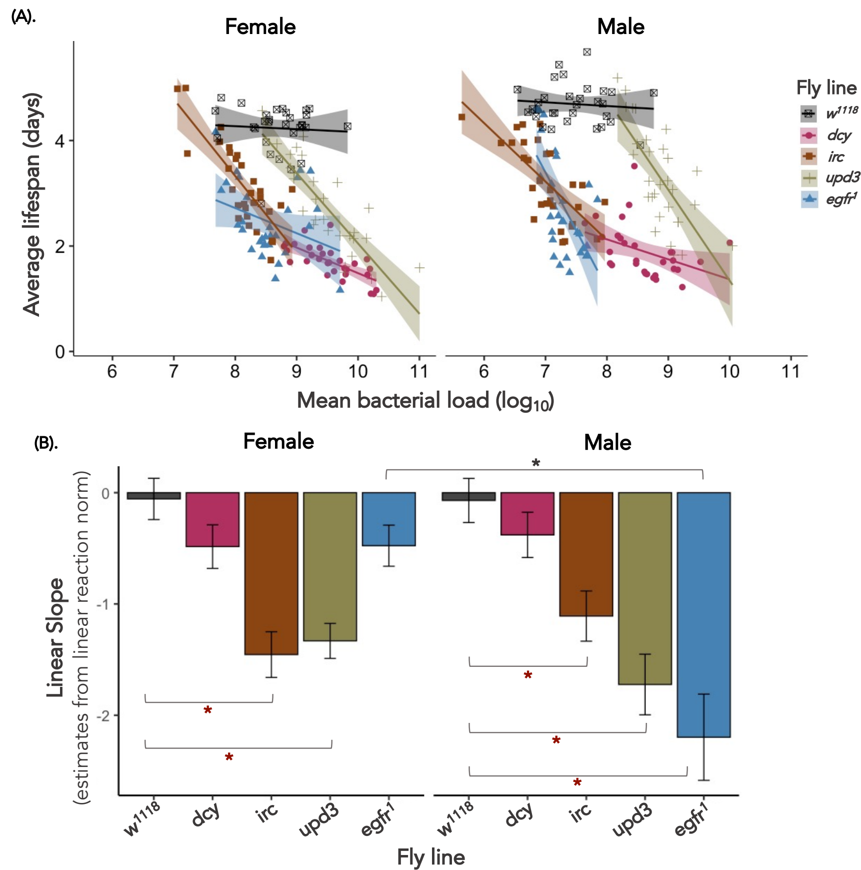

Figure 5. **(A).** The relationship between fly survival (measured as average lifespan) and bacterial load (as mean CFUs – colony forming units), analysed using linear models for female and male flies (w^{1118} and mutant flies). We confirmed AICc (Akaike information criterion) scores as a measure of the goodness of fit to the data (*not shown*). Each point shows data for average lifespan and mean bacterial load (CFUs) of all the flies in one vial repeated over 30 vials (with each vial containing about 25 individual flies/fly line/sex combination) after 24-hours post oral bacterial exposure. The data shown here are for the infection doses (medium $OD_{600}=25$). **(B).** shows the slope of the linear reaction norm extracted from the linear models. [grey colour '*' indicates significant difference in tolerance between males and females in tolerance to bacterial infections (interaction between the bacterial load and the sex for each fly line measured using ANCOVA, Table 2), maroon colour '*' indicates that mutant fly lines are significantly different from wild type w^{1118}, analysed using pairwise f-test from linear norm estimates see Table 3].

Table 2. Summary of ANCOVA. To assess differences in infection tolerance (fly survival with increasing bacterial burden) following oral *P. entomophila* infection with $OD_{600}=25$ infection dose, after 24-hours. We analysed ANCOVA and fitted 'sex' as categorical fixed effects, 'average bacterial load' as a continuous covariate and their interactions as fixed effects for each of the fly lines (w^{1118} and mutants).

Sex	*Source*	*df*	*Sum of Sq.*	*F ratio*	*p*
Female	Fly line	4	66.33	71.6	<0.01
	Bact. load	1	19.27	83.3	<0.01
	Fly line x Bact. load	4	10.02	10.8	<0.01
Male	Fly line	4	84.60	62.8	<0.01
	Bact. load	1	28.39	84.3	<0.01
	Fly line x Bact. load	4	14.88	11.0	<0.01

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

Sex	*Fly line*	SSE	*F ratio*	*p*
Female	*dcy* vs. w^{1118}	7.32	4.85	0.03
	egfr^{1} vs. w^{1118}	18.32	1.88	0.17
	irc vs. w^{1118}	17.75	28.00	<0.01
	upd3 vs. w^{1118}	18.33	29.79	<0.01
Male	*dcy* vs. w^{1118}	10.66	2.17	0.14
	egfr^{1} vs. w^{1118}	29.07	21.41	<0.01
	irc vs. w^{1118}	16.06	18.72	<0.01
	upd3 vs. w^{1118}	24.87	27.01	<0.01

3.5 *Discussion*

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

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

toxin produced by *P. entomophila* (Kuraishi, Hori, and Kurata 2013) resulted in higher bacterial growth in the fly haemolymph.

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

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

We observed, the fastest decline in tolerance in male flies lacking $egfr^{1}$, but the disease tolerance of female $egfr^{1}$ mutants appeared unaffected. This sex difference in tolerance may arise as the result of sex differences in gut physiology and repair. Recent work has demonstrated that during oral *Ecc15* infection, males showed significantly lower gut *ISCs* in response to infection, while female had higher *ISCs* and were resistant to infection and other stress (Regan et al. 2016). The differentiation and proliferation of *ISCs* (intestinal stem cells)

via Jak/Stat signalling into *ECs* (enterocytes) via *egfr* is indispensable for tissue renewal. Loss of *egfr*^{1} signalling might therefore be felt more severely in males than in females, explaining why male but not female *egfr*^{1} mutants showed severe a decrease in disease tolerance. To date, only a small proportion of studies have compared sex-differences in intestinal immunity, with the majority of work focusing on one particular sex, usually females (Ayyaz, Li, and Jasper 2015; Chakrabarti et al. 2016; Regan et al. 2016; Belmonte et al. 2020).

Another possibility for the observed sex-difference in tissue renewal process, could relate to sexual dimorphism in gut-plasticity and remodelling. For instance, females of mammals such as mice extensively remodel their guts, increasing both digestive and absorptive capacity depending on the nutritional demands of lactation (Speakman 2008). The remodelling of the gut might be one of the possible driving factors for dimorphism in gut immunity, since males and females differ in their nutritional needs (Belmonte et al. 2020). Studies using *Drosophila* have shown that males and females can make different diet or nutritional choices in accordance with their reproductive role and demand (Camus, Piper, and Reuter 2019) and that the *Drosophila* midgut plastically resizes in response to changes in dietary sugar and yeast (Bonfini et al. 2021). Whether gut remodelling and nutritional choice-demand causes sex-differences in tissue damage repair process during disease tolerance remains a question for future research.

Although host mechanisms of immune-mediated clearance are key for pathogen defence and elimination, there is an increasing appreciation that additional defence mechanisms which prevent, signal, repair or renew the extent of tissue damage are also key to infection outcomes by promoting disease tolerance (Soares, Gozzelino, and Weis 2014; Vale, Fenton, and Brown 2014). Tissue repair mechanisms that promote disease tolerance are interesting from a therapeutic perspective (Ayres and Schneider 2012; Medzhitov, Schneider, and Soares 2012; Vale et al. 2016). For instance, in mice, mechanisms that prevent damage or repair tissues have been shown to confer disease tolerance during malarial *Plasmodium* infection and also during co-infections by pneumonia causing bacteria (*Legionella pneumophila*) and influenza virus (Ferreira et al. 2011; Jamieson et al. 2013).

Understanding how tissue damage prevention and repair mechanisms contribute to disease tolerance may also help explain how other arthropods are able to vector bacterial and viral infections without substantial health loss (Taracena et al. 2018; Oliveira, Bahia, and Vale 2020; Lambrechts and Saleh 2019). In summary, our results show that the absence of tissue

repair processes resulted in severe loss of disease tolerance, and highlight how sex differences in some tissue damage repair mechanisms could generate sexual dimorphism in gut immunity.

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3.7 *Supplementary information*

Figure SI-1. **(A)** Survival curves for male and female flies of wild-type w^{1118} and mutants with disrupted tissue damage prevention and repair process in adult *Drosophila*, exposed to OD_{600}=10 (low dose) of oral *Pseudomonas entomophila* infection **(B)** Bacterial load measured at 24-hours after OD_{600}=10 of oral *P. entomophila* infection for male and female flies of wild-type w^{1118} and mutant flies. Significantly different fly lines are connected with different alphabets using Tukey's HSD as a post hoc analysis of pairwise comparisons in panel B. SI=sham/mock-infection, I=infection.

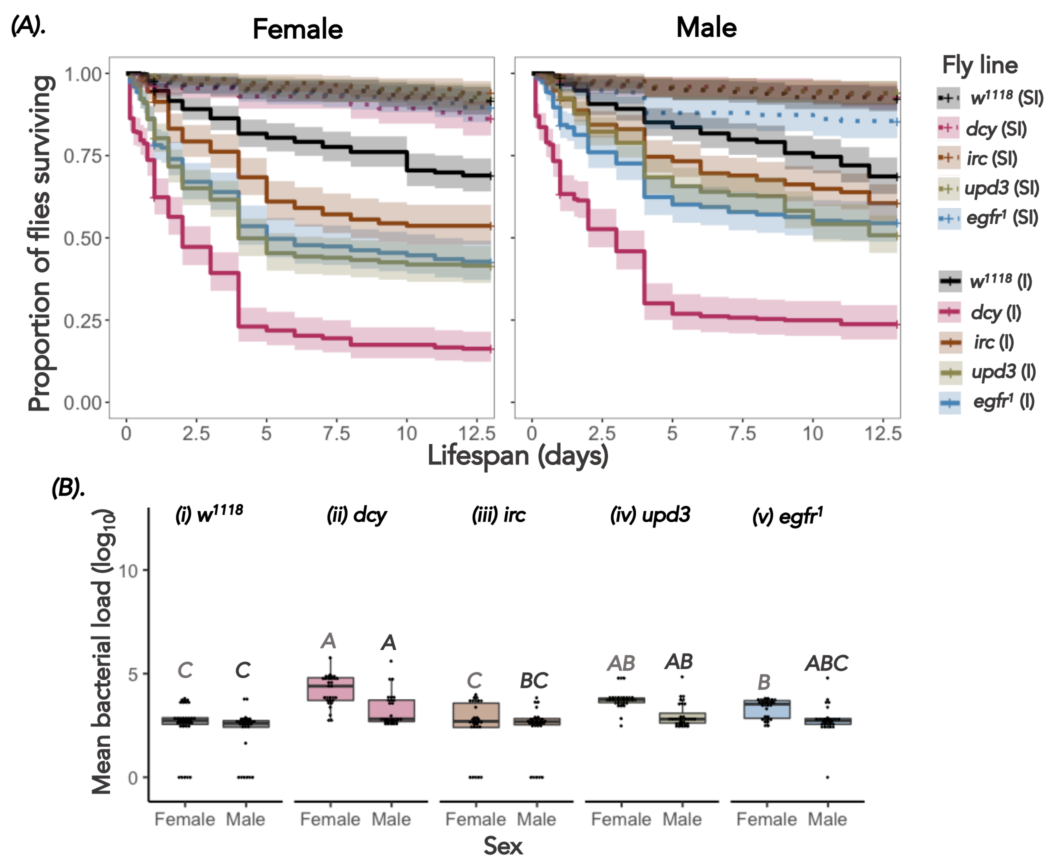

Figure SI-2. **(A)** Survival curves of male and female flies of wild-type w^{1118} and mutant flies exposed to OD_{600}=45 (high dose) of oral *Pseudomonas entomophila* infection **(B)** Bacterial load measured at 24-hours after OD_{600}=45 of oral *P. entomophila* infection for male and female flies of wild-type w^{1118} and mutant flies. Significantly different fly lines are connected with different alphabets using Tukey's HSD as a post hoc analysis of pairwise comparisons in panel B. SI=sham/mock-infection, I=infection.

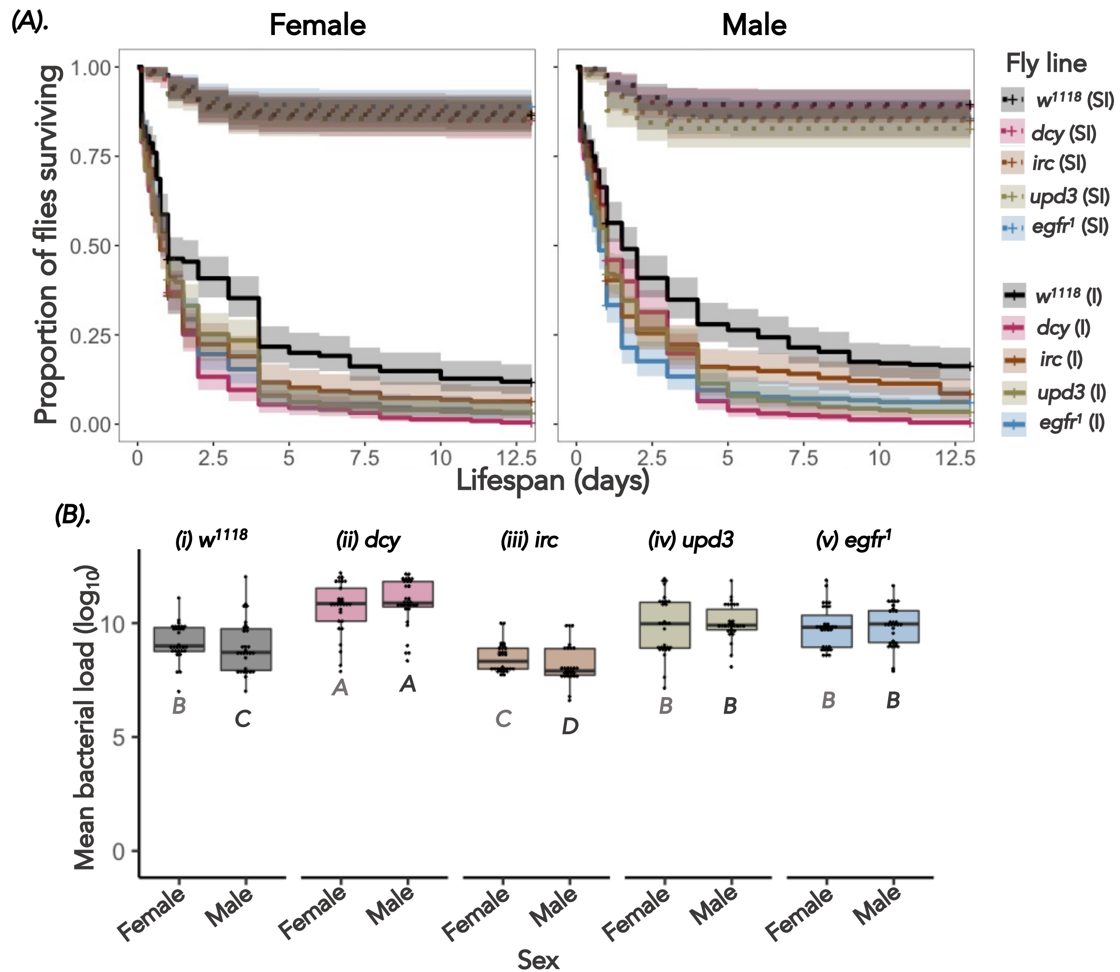

Table SI-1. Summary of mixed effects Cox model, for male and female wildtype w^{1118} and mutant flies orally infected with 3 different doses of *P. entomophila*. We used 3-5-day old adult males and females of each fly lines (wildtype and mutants) and specified the model as: Survival ~ Treatment x Sex x Fly line (1|vial/block), with 'Treatment', 'Sex' and 'Fly line' as fixed effects, and 'vials' nested in 'block' as a random effect. The table shows model output (ANOVA) for survival post oral infection for wildtype w^{1118} flies and mutant flies.

Infection dose	***Source***	***Loglik***	***Chisq***	***df***	***p***
OD_{600}=10	Treatment	-12880	968.61	1	< 0.001
	Sex	-12872	17.367	1	< 0.001
	Fly line	-12669	404.70	4	< 0.001
	Treatment x sex	-12669	0.9114	1	0.33
	Treatment x fly line	-12652	33.944	4	< 0.001
	Sex x fly line	-12650	3.9894	4	0.40
	Treatment x sex x fly line	-12647	6.4149	4	0.17
	Random effect	*Std dev*			
	vial/block	*8.88E-04*			
OD_{600}=25	Treatment	-37499	3058.87	1	< 0.001
	Sex	-37487	25.046	1	< 0.001
	Fly line	-37257	459.75	4	< 0.001
	Treatment x sex	-37250	14.685	1	< 0.001
	Treatment x fly line	-37240	18.773	4	< 0.001
	Sex x fly line	-37236	7.8494	4	0.09
	Treatment x sex x fly line	-37235	2.9252	4	0.57
	Random effect	*Std dev*			
	vial/block	*1.92E-03*			
OD_{600}=45	Treatment	21144	3256.2	1	< 0.001
	Sex	21141	5.5753	1	< 0.001
	Fly line	21101	80.198	4	< 0.001
	Treatment x sex	21101	1.0625	1	0.30
	Treatment x fly line	21098	5.7909	4	0.21
	Sex x fly line	21094	6.6203	4	0.15
	Treatment x sex x fly line	21094	1.8525	4	0.76
	Random effect	*Std dev*			
	vial/block	*0.019*			

Table SI-2. Summary of log_{10} transformed bacterial load data after exposure to *OD_{600}=25* of oral *P. entomophila* infection for male and female wildtype w^{1118} and mutant flies, analysed using non-parametric one-way ANOVA (Kruskal–Wallis test) at 3 different timepoints (0-15 minutes, 24-hours and 96-hours following oral infection)

	Sex	***Fly line***		***Mean diff***	***Z***	***p***
0-15 min	*Female*	*egfr1*	w^{1118}	3.50	0.77	0.43
		dcy	w^{1118}	-11.16	-2.47	0.01
		upd-3	w^{1118}	-11.03	-2.44	0.01
		irc	w^{1118}	-7.83	-1.73	0.08
	Male	*egfr1*	w^{1118}	29.56	6.55	< 0.001
		dcy	w^{1118}	28.96	6.423	< 0.001
		upd-3	w^{1118}	28.96	6.42	< 0.001
		irc	w^{1118}	28.033	6.21	< 0.001

	Sex	Fly line		Mean diff	Z	p
24-hours	*Female*	*dcy*	w^{1118}	24.30	5.388	<0.001
		upd-3	w^{1118}	22.23	4.930	<0.001
		egfr1	w^{1118}	-7.233	-1.604	0.108
		irc	w^{1118}	-18.96	-4.206	<0.001
	Male	*upd-3*	w^{1118}	28.70	6.364	<0.001
		dcy	w^{1118}	25.90	5.743	<0.001
		egfr1	w^{1118}	-7.233	-1.604	0.10
		irc	w^{1118}	-14.16	-3.141	0.001

	Sex	Fly line		Mean diff	Z	p
96-hours	*Female*	*dcy*	w^{1118}	26.90	5.965	< 0.001
		upd-3	w^{1118}	6.100	1.352	0.17
		irc	w^{1118}	-5.166	-1.145	0.25
		egfr1	w^{1118}	-8.566	-1.899	0.057
	Male	*dcy*	w^{1118}	26.50	5.876	< 0.001
		egfr1	w^{1118}	22.73	5.041	< 0.001
		upd-3	w^{1118}	20.36	4.516	< 0.001
		irc	w^{1118}	17.56	3.895	< 0.001

Chapter 4

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

Keywords: Antibacterial immunity, Damage signalling mechanisms, Disease tolerance, Negative immune regulators

4.1 *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*.

4.2 *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 and 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).

Focusing on its role in immunity, there is substantial evidence that Jak/Stat signalling plays a key role in wound healing, gut immunity, and downstream AMP production

(Chakrabarti et al. 2016; Kemp et al. 2013; Lamiable and Imler 2014; Tafesh-Edwards and Eleftherianos 2020). For instance, during 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 (Banerjee et al. 2019; Meister and Lagueux 2003).

Jak/Stat 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 led 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. Here we investigated the tolerance response of *Drosophila* during septic infection with the bacterial pathogen *P. entomophila*, using deletion mutant flies lacking various components of Jak/Stat signalling and regulation.

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 assessing how Jak/Stat affects males and females separately.

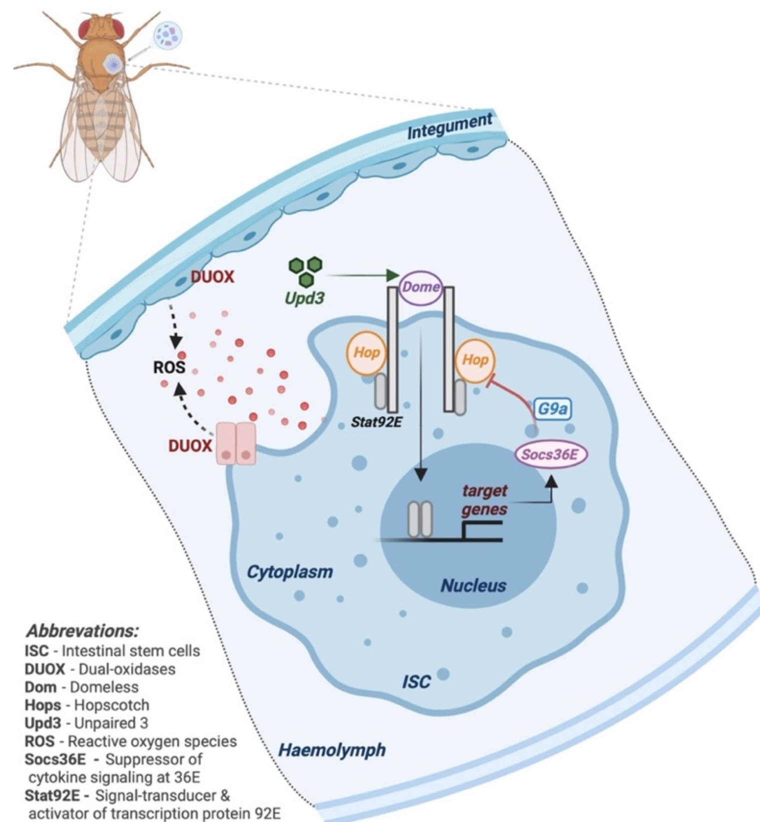

Figure 1. A simplified version of *Drosophila* Jak/Stat pathway known to have important roles in signalling damage and tissue renewal. In response to infection, the Jak/Stat pathway is activated by cytokine-like ligands of the unpaired family namely *upd-3*, produced ↘ during damage caused by reactive oxygen species (*ROS*), which in turn are produced by *dual oxidase* (*duox*). The extracellular binding of *upd-3* to *Domeless* (*dome*), leads to ↘ 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.

4.3 *Materials and methods*

1. <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 wild type *yw (189)* including *Domeless* (*Bloomington stock# 14726*), *Hopscotch* (*15097*), *Socs36E* (*16744*), *Duox* (*16468).* We also used *G9a* mutant flies (that is, $G9a^{-/-}$) and control ($G9a^{+/+}$), also known as $G9a^{DD2}$ see (Merkling et al. 2015) for details about *G9a* mutants. Previous work has shown that wild type *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 wild type *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 around 24 hours following infection (see supplementary ***Fig****. SI-1A and B,* ***Table*** *SI-1* for survival; ***Fig****. SI-1C,* for bacterial load).

We maintained all the fly lines in a 12ml plastic vials on a standard cornmeal diet or Lewis's medium *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 hours) onto 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 (around 3 days).

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 pathogenetic for *D. melanogaster* (Dieppois et al. 2015; Vodovar et al. 2005)). Upon reaching ~0.75 OD_{600} we pelleted the culture using a 4°C cold centrifuge during 5 minutes at 5000rpm and then removed the supernatant. We resuspended the bacteria in 1xPBS (phosphate buffer saline) and prepared the final infection dose of OD_{600} of 0.05 for all our infection assays.

3. *Systemic infection assay:* We used a split-vial experimental design (see ***Fig****. 2*), 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 (15-17 flies/combination/vial) and **(B)**. internal bacterial load (see ***Fig****. 2II*). With this split-vial design we were able to use replicate-matched data for both survival and bacterial load in order to estimate disease tolerance for each fly line (that is, fly survival with respect to internal bacterial load). We infected 3-5-day old male and female adult flies using a 0.14mm insect minutein needles bent at 90° angle to avoid damaging the internal tissues by dipping in *P. entomophila* bacterial solution. 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 hours for the first 48-hours following infection, then 2-3 times each day for the next 6 days (150 hours).

4. *Measuring bacterial load:* To quantify internal bacterial load after 24-hours following systemic *P. entomophila* infection first, we thoroughly washed each fly with 70% ethanol for 30 sec 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 1.5ml micro centrifuge tubes and homogenized using a motorized pestle for approximately 30-60 seconds in 100µl 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 4µL 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. Following systemic *P. entomophila* infection (24-hours), we randomly selected flies (only alive individuals) from the 2nd vial dedicated for bacterial load measurement from the split vial design (***Fig***. 2I). We did not include the mortality in this vial (for bacterial load) for assay and statistics since we had a separate vial (1st) (***Fig***. 2I) for measuring survival around the same time.

Statistics.

1. *Survival*: We analysed the survival data with a Cox mixed effects model using the R package 'coxme' (*Therneau 2015*) for different treatment groups (*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 for wild type *yw* and *Jak/Stat* mutant fly lines.
2. *Bacterial load*: We found that the bacterial load data were not normally distributed (tested with Shapiro–Wilks's test for normality). Hence, we first log transformed the data and then confirmed that the transformed residuals were still not normally distributed. Hence, we used a non-parametric one-way ANOVA Kruskal-Wallis test to test the effects of each fly lines (wild type *yw* and *Jak/Stat* mutants) on males and females following bacterial infection.
3. *Measuring disease tolerance*: Finally, to understand how the Jak/Stat signalling contributes to the phenotype disease we analysed tolerance to bacterial infection by linear reaction norm (that is, measuring the linear relationship between fly survival against bacterial load) (Raberg, Sim, and Read 2007; Ayres and Schneider 2012; Louie et al. 2016;

Oliveira, Bahia, and Vale 2020). We analysed using ANCOVA for each of the fly lines (wild type *yw* and *Jak/Stat* mutants) by fitting 'fly line' and 'sex' as categorical fixed effects and 'bacterial load' as a continuous covariate and their interactions as fixed effects. We analysed the tolerance data separately for males and females since we handled males and females of both with-type and Jak/Stat mutants on separate days due to higher replicate size. Since we were interested in identifying how the Jak/Stat mutants differ from wildtype flies during disease tolerance - we compared the estimates of the slope using pairwise comparison (f-test; w^{1118} vs. different *Jak/Stat* mutants) to find out what extent these Jak/Stat mutants significantly differed from *yw* wild type in tolerating bacterial infections.

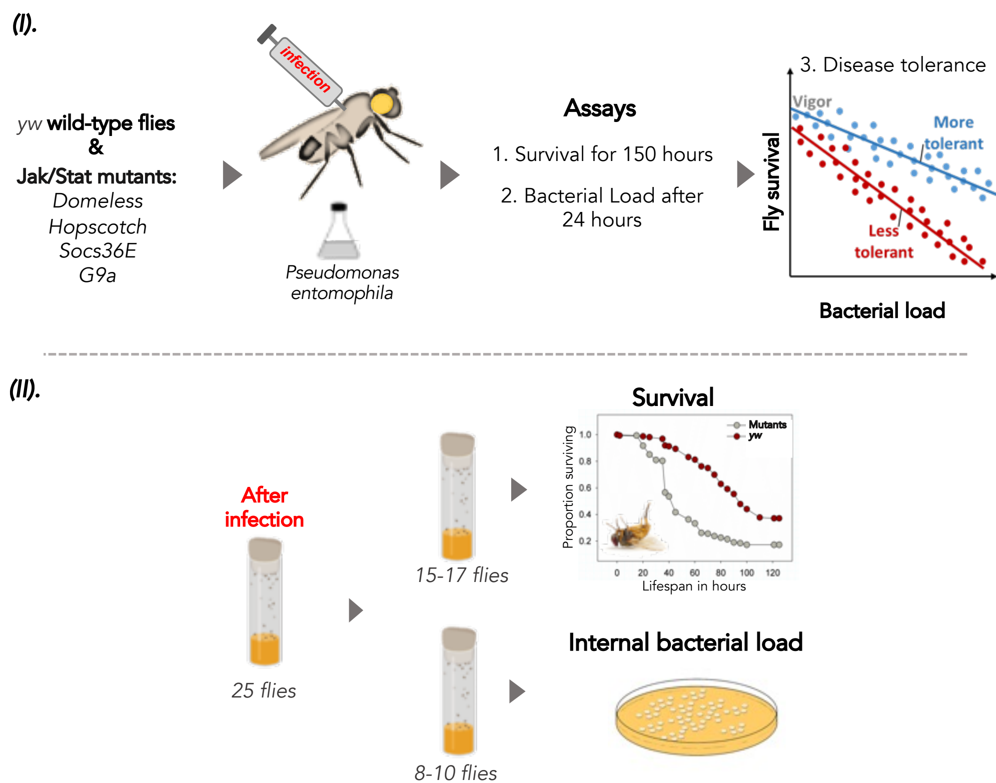

Figure 2: **(I)** Design of experiments to assay **(1)** survival (n= 15 vials of around 15-17 flies/vial/infection treatment/sex/fly line) and **(2)** internal bacterial load following systemic bacterial infection with *Pseudomonas entomophila* to test the role of the Jak/Stat pathway (n= 15 vials of around 8-10 flies/vial/infection treatment/sex/fly line) on **(3)** disease tolerance showing host vigor (good health state), more tolerant and less tolerant health state. Each point in disease tolerance (3) panel represents replicate-matched data [n= 15 vials/infection treatment/sex/fly line – with each vial containing around 25 flies] from survival (1) and bacterial load (2). **(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 (15-17 flies/combination) and **(B)**. internal bacterial load (8-10 flies/combination). Data shown in survival and disease tolerance figure is a sketch and not actual data.

4.4 *Results and discussion*

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

Overall, we found that disruption of Jak/Stat signalling affected fly survival during bacterial *P. entomophila* infections (***Fig.*** *3A* 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 wildtype (*yw*) (***Fig.*** *3A* and *B*, ***Table*** *1* and *SI-2*). However, other mutants showed improved survival relative to wild types. These included male and female flies lacking the transmembrane receptor *domeless*, males lacking the negative regulator *Soc36E* and both sexes lacking *hopscotch* (see hazard ratio in ***Fig.*** *3B*, ***Table*** *1* and *SI-2*).

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

Next, we tested whether the variation we observed between mutants in mortality could be explained by differences in their bacterial load. Given that most mortality occurred just after 24 hours for most of our fly genotypes (***Fig.*** *3A*) we quantified bacterial load at 24 hours following infection. Both wild type and Jak/Stat mutants exhibited similar levels of bacterial load around 24 hours following infection with *P. entomophila* (***Fig***. *3C*, ***Table*** *SI-3*).

Therefore, despite no substantial difference in microbe loads at 24 hours post infection, Jak/Stat mutants 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*).

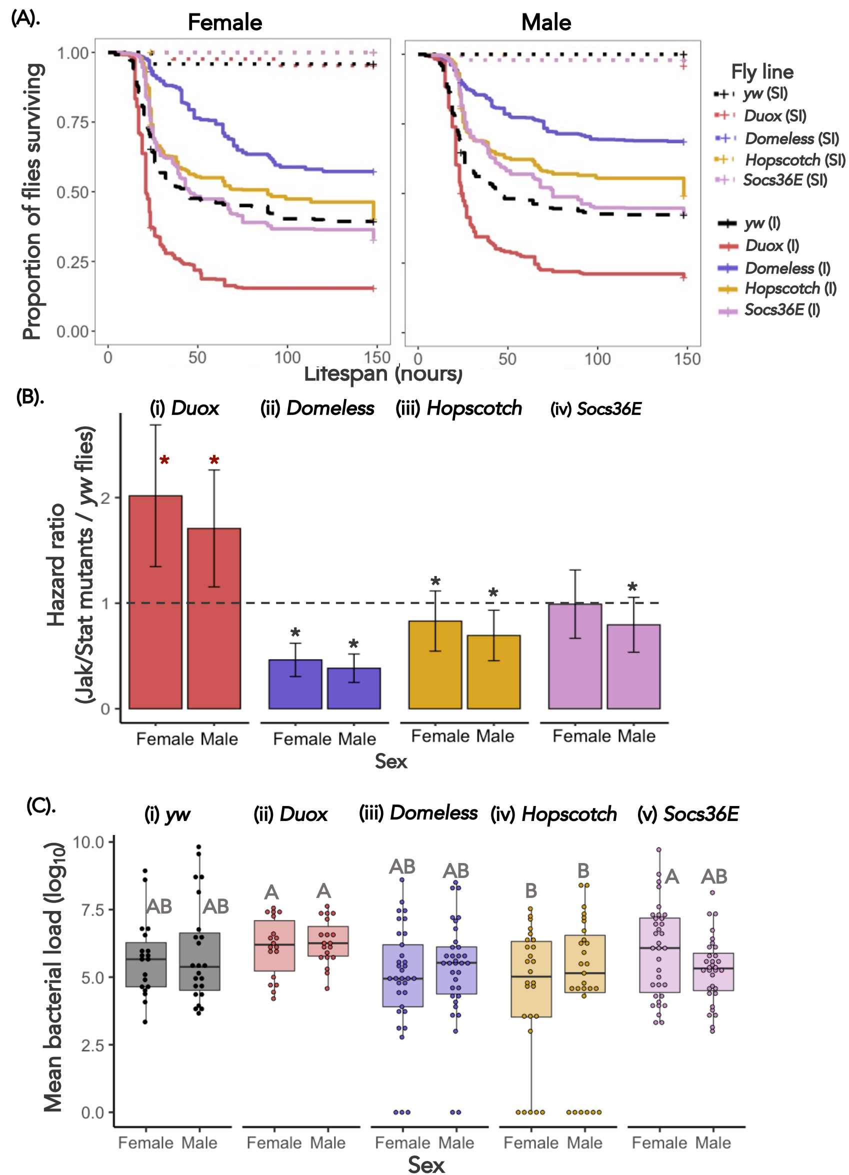

Figure 3. **(A)** Survival curves for wildtype *yw* flies and flies lacking Jak/Stat pathway components for females and males exposed to systemic *P. entomophila* of infection dose OD_{600}=0.05 (n= 15 vials with 15-17 flies each vial/fly line/treatment/sex/infection dose). ['*' indicates that the Jak/Stat mutants are significantly different from *yw* flies]. *Since sham/mock-infected controls had negligible or no mortality, the survival graph for most of the fly lines seem to be masked in the panel A* **(B)** Estimated hazard ratios calculated from the survival curves for males and female flies (y*w* and 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 log_{10}) measured around 24 hours following infection (n= 15 vials with 8-10 flies each vial/fly line/treatment/sex/infection dose). [significantly different fly lines are connected with different alphabets using Tukey's HSD as a post hoc analysis of pairwise comparisons].

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* wildtype flies while lower ratio (<1) indicates that Jak/Stat mutants have better survival than wildtype.

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

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 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 than males during viral infections (Gupta and Vale 2017). Similar examples also 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 (Kemp et al. 2013; Bang 2019; Dostert et al. 2005).

An unexpected result is that we found that female flies lacking *domeless* showed increased survival (and tolerance) relative to the *yw* wildtype (***Fig.*** *3* - survival *and* ***Fig.*** *4* - tolerance), suggesting that Jak/Stat activation may in fact be costly to flies. Immune deployment and regulation is highly energy demanding (McKean et al. 2008; Schwenke, Lazzaro, and Wolfner 2016; Nystrand and Dowling 2020). The physiological cost of specific individual immune components and pathways remains an open question for future research.

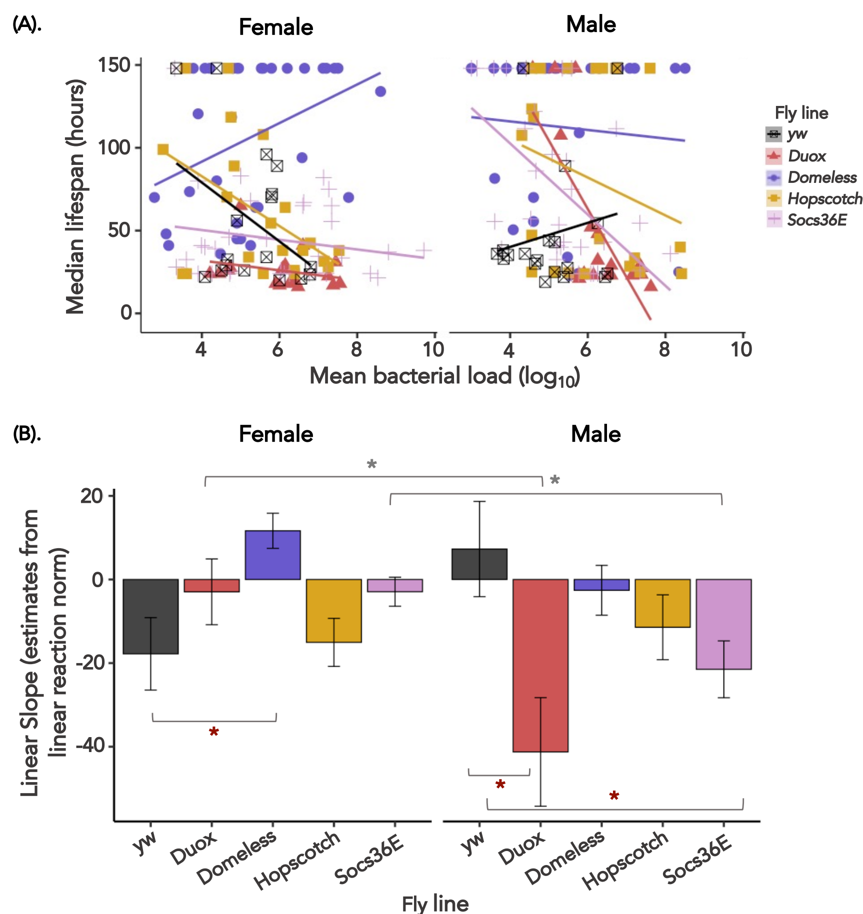

Figure 4. **(A).** The relationship between fly survival (measured as median lifespan) and bacterial load (as mean CFUs - Colony Forming Units) analysed using linear models for female and male flies (*yw* and *Jak/Stat* mutants). Each point shows data for median lifespan and mean CFUs of 15 vials (with each vial containing ~25 flies/sex/fly line combination after 24 hours

post systemic bacterial exposure. The data shown here are for the infection doses (OD_{600}=0.05). **(B).** Represents estimates of negative slope of the linear reaction norm extracted from the linear models. [Maroon asterisks '*' on the lower side of the panel B indicates that Jak/Stat mutants are significantly different from wild type *yw*, analysed using the F-test pairwise comparisons of estimates of the linear reaction norm for both males and females separately (see Table-3)]. Grey asterisks '*' on the upper side of the panel B indicates sex differences within the fly line that is, males and females significantly differ in tolerance to systemic bacterial *P. entomophila* infection.

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, around 24 hours following infection. We analysed ANCOVA and fitted 'sex' as categorical fixed effects, 'average bacterial load ($\log_{10}$)' as a continuous covariate and their interactions as fixed effects for the Jak/Stat mutants.

	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

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

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

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

The epigenetic 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 wanted to test whether the loss of function of *G9a* also affects fly survival and 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.*** *5A* for survival and ***Fig.*** *5B* 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 hours *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.***

*5C, **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 (see ***Fig.** 5D, **Table** 5,* and ***Table** 6* for comparison between estimates of tolerance slope).

Thus, 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 either sex.

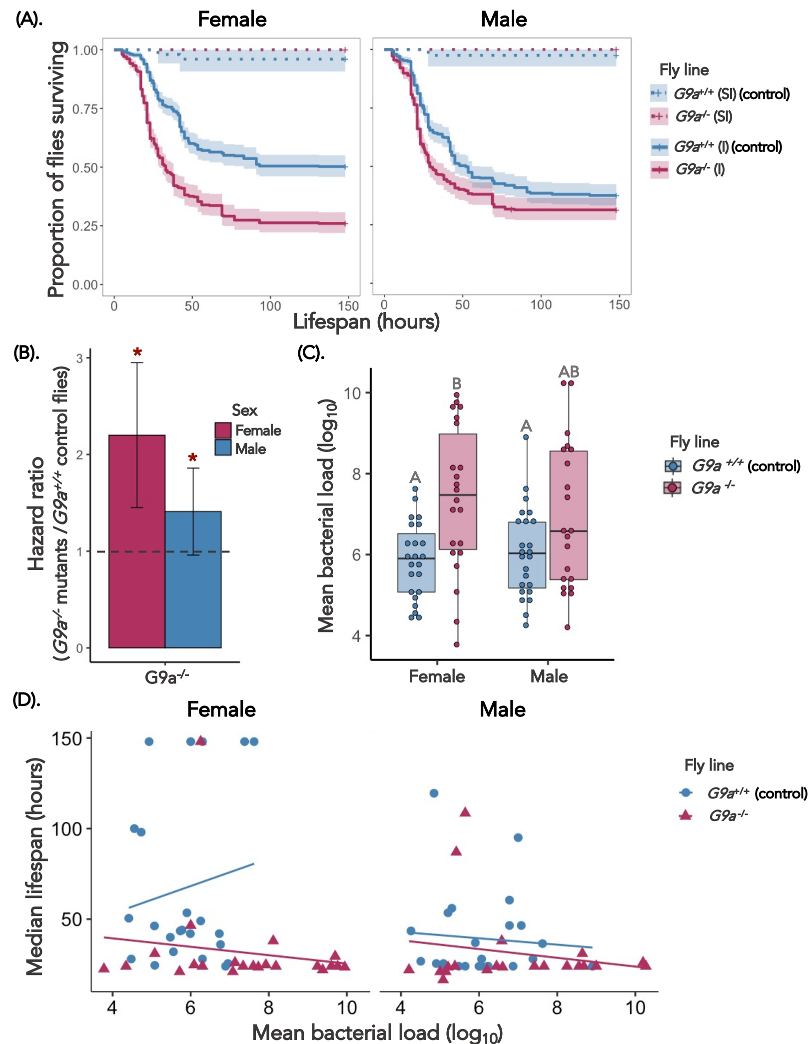

Figure 5. **(A)** Survival curves for wildtype *yw* flies and flies lacking *G9a* the epigenetic regulator of Jak/Stat for female and male flies exposed to systemic *P. entomophila* of infection dose OD_{600}=0.05 [n=15 vials with 15-17 flies in each vial/fly line/treatment/sex]. **(B)** Estimated hazard ratios calculated from the survival curves for males and female flies (wildtype y*w* 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 $\log_{10}$) measured around 24 hours following infection (n=15 vials with 8-10 flies in each vial/fly line/ treatment and sex combination). [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*$^{+/+}$).

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.

sex	*flyline*	*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 OD_{600}=0.05 infection dose, around 24 hours following infection. We analysed ANCOVA and fitted 'sex' as categorical fixed effects, 'average bacterial load (log_{10})' 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***	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 6: Summary of F-test pairwise comparisons of estimates of the linear slopes (linear reaction norm) for *G9a* $^{-/-}$ *relative to G9a* $^{+/+}$ wildtype fly lines.

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

4.5 *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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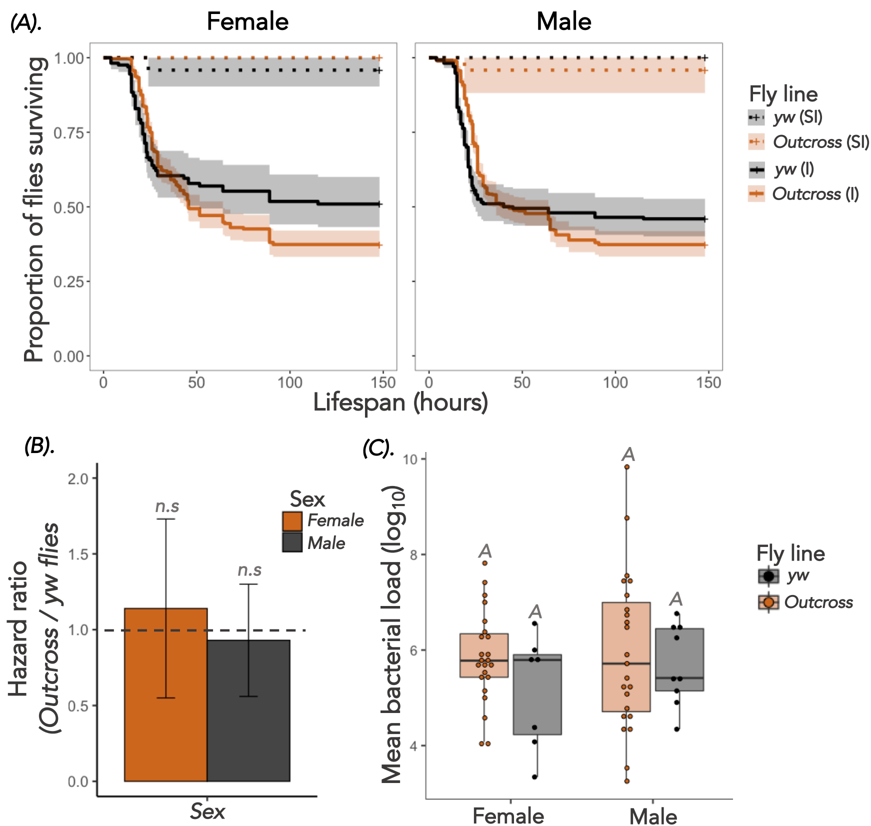
4.7 *Supplementary Information*

Figure SI-1. **(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*) and *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., 2003). 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 24-hours post-infection (***Fig***. *SI-1A* and *SI-1B,* ***Table*** *SI-1* for survival; ***Fig***. *SI-1C,* 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.

Table SI-1. Summary of mixed effects Cox model, for wildtype *yw* and flies without Jak/Stat pathway components during systemic *P. entomophila* infection. We used data from the individuals of 3-day adult males and females infected with OD_{600} = 0.05 infection dose of *P. entomophila* for each fly lines (Jak/Stat -pathway mutants) and specified the model as: survival ~ treatment x sex x fly line (1|vial), with 'treatment', 'sex' and 'fly line' 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 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*			

Table SI-2. Summary of mixed effects Cox model, for each fly line, wildtype *yw* and flies without Jak/Stat pathway mutants. We used data from the individuals of 3-day adult males and females infected with OD_{600} = 0.05 infection dose of *P. entomophila* for each fly lines (Jak/Stat -pathway mutants) and specified the model as: survival ~ treatment x sex x fly line (1|vial), with 'treatment', 'sex' and 'fly line' 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 (*yw*) and flies 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*			

Table SI-3. Summary of non-parametric one-way ANOVA (Kruskal-Wallis test) of effects on bacterial load data measures 24 hours following OD_{600}=0.05 *P. entomophila* systemic infection for wildtype (*yw)* flies and *Jak/Stat* pathway mutants. We analysed the load data (log_{10} transformed) by fitting 'fly line' (*yw* and Jak/Stat mutants) as categorical fixed effects for males and females separately.

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

Table SI-4. Summary of mixed effects Cox model, for *G9a* Jak/Stat pathway components during systemic *P. entomophila* infection. We used data from the individuals of 3-day adult males and females infected with OD_{600} = 0.05 infection dose of *P. entomophila* for each fly lines (*G9a*) and specified the model as: survival ~ treatment x sex x fly line (1|vial), with 'treatment', 'sex' and 'fly line' 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 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*			

Table SI-5. Summary of non-parametric one-way ANOVA (Kruskal-Wallis test) of effects on bacterial load data measured at 24 hours following OD_{600}=0.05 *P. entomophila* systemic infection for *G9a* mutants. We analysed the load data (log_{10} transformed) by fitting 'fly line' ($G9a^{+/+}$ *and* $G9a^{-/-}$ mutants) as categorical fixed-effects for males and females 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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Chapter 5

Regulation of the *IMD*-responsive antimicrobial peptide *Diptericin* is required for specific-immune priming

Keywords: immune priming, clearance, immune deficiency pathway; peptidoglycan recognition proteins; *Providencia rettgeri*

5.1 *Abstract*

Invertebrates lack the specialized immune-memory cells responsible for vertebrate-like acquired immunity. However, there is increasing evidence that past infection experience by the same pathogen can 'prime' the insect immune response, resulting in improved survival upon reinfection. The mechanisms underlying these phenomenological accounts of priming are diverse, and often not completely clear.

Here, we investigated the occurrence, generality and mechanistic basis of immune priming in *Drosophila melanogaster* when infected with the gram-negative bacterial pathogen *Providencia rettgeri*. By using a combination of wild-type, CRISPR/Cas knockout, loss-of-function and UAS^{-RNAi} knockdown fly lines, we find that priming in *Drosophila* is a long-lasting response, occurring in several genetic backgrounds and is particularly stronger in male flies. Mechanistically, we show that flies lacking major components of the IMD immune signalling pathway are no longer able to improve survival following initial heat-killed exposure with *P. rettgeri*. We show that the enhanced survival of individuals primed with an initial non-lethal bacterial inoculum coincides with a transient decrease in bacterial loads, and that this is likely driven by the IMD-responsive antimicrobial-peptide *Diptericin-B* in the fat body.

Further, we show that while *Diptericin* is required as the effector of bacterial clearance, it is not sufficient for immune priming, which requires the regulation by peptidoglycan recognition proteins *PGRP-LB, PGRP-LC* and *PGRP-LE*. We discuss potential explanations for the observed sex differences in priming, and implication of immune priming for pathogen transmission.

5.2 *Introduction*

Insects possess a robust innate immune response against pathogens which includes both cellular and humoral components (Hoffmann, 2003; Hultmark, 2003; Buchon et al., 2014), but lack vertebrate-like specialized immune memory cells (B and T-lymphocytes) responsible for acquired immunity. These differences in immune physiology resulted in the long-standing assumption that invertebrates should not be capable of immune memory, though this view was clearly at odds with empirical evidence from several invertebrate host-pathogen systems (Hauton and Smith, 2007; Little et al., 2008, 2005). Work in recent decades has revealed that insects also possess a form of immune memory, known generally as "immune priming", where low doses of an infectious pathogen can lead to increased survival upon reinfections (Christophides et al., 2002; Kurtz and Franz, 2003; Sadd and Schmid-Hempel, 2006; Milutinović and Kurtz, 2016).

There is now substantial evidence of diverse priming responses occurring within the same invertebrate life stage (larvae or adults), across life stages (from larvae to adults), or even between generations (parents to offspring) (Contreras-Garduño et al., 2016; Khan et al., 2016; Sheehan et al., 2020). The survival benefit of priming has been observed in a range of arthropod taxa, including Dipterans: fruit flies (Pham et al., 2007), mosquitoes (Ramirez et al., 2015); Coleopterans: flour beetles (Khan et al., 2016); Lepidopterans: greater wax-moth (Fallon et al., 2011); Hymenopterans: bumblebee (Sadd and Schmid-Hempel, 2006); Crustaceans: water fleas (Little et al., 2003) and Arachnids: spiders and scorpions (Gálvez et al., 2020). Thus, substantial experimental evidence in both lab adapted and wild-caught arthropods suggests that immune priming is a widespread phenomenon, and is predicted to have a profound impact on the outcome of host–pathogen interactions, including infection severity and pathogen transmission (Tate and Rudolf, 2012; Tidbury et al., 2012; Tate, 2016).

The widespread observation of immune priming in invertebrates underlines the importance of whole organism research in immunity in lieu of a purely mechanistic approach to immunology (Little et al., 2005). It also highlights that the same phenomenology can originate in very different mechanisms. Determining the mechanisms of invertebrate priming has often proved challenging but has generally revealed a great diversity of ways in which invertebrates may enhance their immune responses upon reinfection. In *Drosophila,* the priming response during infection with the gram-positive bacterial pathogen *S. pneumoniae* was shown to be dependent on haemocytes and phagocytosis, while the Toll-pathway - the main pathway involved in clearance of gram-positive bacteria - was shown to be insufficient for

successful priming (Pham et al., 2007). The increased phagocytic activity in primed individuals has also been shown to play a key role in priming in crustaceans (Roth and Kurtz, 2009). In other *Drosophila* work, *PGRP-LB* (peptidoglycan recognition protein *LB*, a negative regulator of the IMD-pathway in *Drosophila*) (Bozler et al., 2020) has been identified as a key mediator of transgenerational immune priming against infection with parasitoid wasps (*Leptopilina heterotoma* and *Leptopilina victoriae*), where downregulation of *PGRP-LB* is necessary to increase haemocyte proliferation, required for wasp clearance in the offspring (Bozler et al., 2020). In response to infection with *Drosophila C virus* (DCV), *Drosophila* progeny can produce a DCV-specific priming response by inheriting partial viral cDNA genome from the infected adult flies (Mondotte et al., 2018, 2020).

In other host-pathogen systems, the precise mechanisms underlying the immune priming phenotype are less clear, but the upregulation of antimicrobial peptides (AMPs) appears to be involved. Genome wide insect transcriptome studies have identified upregulation of several AMPs in primed individuals, for example, *Attacins, Defensins and Coleoptericins* in flour beetles (Ferro et al., 2019; Greenwood et al., 2017), *Cecropin, Attacin, Gloverin, Moricin* and *Lysozyme* in silkworms (Yi et al., 2019), *Gallerimycin and Galiomicin* in wax-moths (Bergin et al., 2006) and finally, *Cecropin* in tobacco moths and fruit flies (Roesel et al., 2020; Chakrabarti and Visweswariah, 2020) (reviewed in Milutinović and Kurtz, 2016; Prakash and Khan, 2022). In fruit flies, the production of AMPs during antibacterial immunity is mediated by the Immune deficiency (IMD) and Toll pathways. In both pathways, pathogens are recognised by peptidoglycan receptors (PGRPs), initiating a signalling cascade that culminates in the activation of the NF-κB-like transcription factors (*Dorsal* in Toll or *Relish* in IMD), resulting in the upregulation of AMP genes. The Toll-pathway generally recognises LYS-type peptidoglycan found in gram-positive bacteria and fungi. The IMD-pathway recognises DAP-type peptidoglycan found in gram-negative bacteria and produces AMPs such as *Diptericins, Attacins* and *Drosocin* among others (Hoffmann, 2003; Hultmark, 2003; Lemaitre and Hoffmann, 2007; Valanne et al., 2011; Myllymäki et al., 2014a). AMPs work with a high degree of specificity, so that only a small subset of the total AMP repertoire provides the most effective protection against specific pathogens (Hanson et al., 2019).

Here, we focus on immune priming in *Drosophila* when infected with the gram-negative bacterial pathogen *Providencia rettgeri*. Our interest in priming in this specific host-pathogen pair was motivated by an observation that some individual flies showed increased survival when they had previously been pricked with a heat-killed inoculum of *P. rettgeri*. While previous work

has characterised immune priming during gram-positive bacterial infection (Pham et al., 2007), the mechanisms underlying immune priming during gram-negative bacterial infection are not known.

We hypothesized that priming could be driven by the up-regulation of AMPs following the initial pathogen exposure, resulting in faster clearance upon re-infection. The IMD-responsive AMP *Diptericin* (*Dpt*) is sufficient to confer complete protection during infection with *P. rettgeri* (Hanson et al., 2019), but it is unclear whether the regulation of *Dpt* is involved in immune priming during re-infections. We therefore used several *D. melanogaster* wild type strains, as well as CRISPR/Cas9 single-gene knockouts, UAS^{RNAi} knockdown, and loss-of-function mutants, each lacking distinct immune components, to investigate the occurrence, generality, duration, and mechanistic basis of immune priming during systemic infection with *Providencia rettgeri*.

5.3 *Materials and methods*

1. Fly strains and maintenance: Several *D. melanogaster* wild type strains were selected to represent variable genetic backgrounds: w^{1118} *(Vienna Drosophila Resource Center)*, $w^{1118,iso}$ *(Bloomington Drosophila Stock Center),* Canton-S*,* Oregon-R *Wolbachia*$^{+ve}$ and Oregon-R *Wolbachia*$^{-ve}$. Transgenic flies included immune mutants Rel^{E20} (*relish - IMD* pathway regulator) and *spz (spatzle – Toll* pathway regulator) and the following CRISPR/Cas9 mutants: (a) ΔAMPs - flies lacking 13 known fly AMPs, (b) Group-B - flies lacking major IMD regulated AMPs including *Attacins* ($AttC^{Mi}$*;* $AttD^{SK1}$*), Drosocin (*Dro^{SK4}*) and Diptericins (*Dpt^{SK12}), (c) Dpt^{SK12} – flies lacking *Diptericins,* and (d) $ΔAMPs^{+Dpt}$ – flies lacking all 13 known AMPs except *Diptericins*. Since all our immune mutants are in w^{1118} background we repeated survival assays with w^{1118} fly line each time we used different sets of mutant and transgenic fly lines.

   All the CRISPR/Cas9 mutants were generated previously from the *iso-*$w^{1118,iso}$ genetic background using CRISPR/Cas9 gene editing technology to induce null mutations in the selected genes (Hanson et al., 2019), and were gifted generously by B. Lemaitre and M. Hanson (U. Lausanne). We also used tissue specific UAS-RNAi mutants. *Diptericin-B (DptB)* flies (Bloomington stock# *28975*), driving the expression of a UAS-construct in fat body ($w^{1118-iso}$*,Fb-Gal4i+(P{fat}*) and haemocytes [$w^{1118-iso}$*,Hmldelta- Gal4; He-Gal4* – a combination of two hemocyte GAL4 drivers, the Hml-GAL4.Δ (Sinenko and Mathey-Prevot, 2004) and He-GAL4.Z (Zettervall et al.,

2004)]. All fly stocks and experimental flies were maintained at 25°C ±1°C on a 12:12 hour light: dark cycle in vials containing 7ml of standard cornmeal diet or Lewis medium (Siva-Jothy et al., 2018). To control the larval density 10 females and 5 males were crossed and the females were allowed to lay eggs for 48-hours. Two weeks later, the eclosing males and females were sorted and collected and separated into group of 25 flies in each vial. Three-day aged, mated individuals were used throughout the experiments.

2. *Immune priming and infection assays: P.rettgeri* was grown at 37°C in 10ml Luria broth (Sigma Ltd) overnight to reach optical density OD_{600}=0.95 (measured at 600nm in a Cell Density Meter, Fisherbrand™). The culture was centrifuged at 5000 rpm for 5 min at 4°C, and the supernatant was removed, and the final OD was adjusted 0.2 (*except when stated in the figure legend 0.1 OD was used* for *some survival assays*) by using sterile 1xPBS (Phosphate buffer saline). To obtain heat-killed bacteria the dilution was incubated at 90°C for 20-30 mins (Khan et al., 2016). To ensure all bacteria were dead the heat-killed culture was plated and no growth was confirmed. To prime individuals, 3-day old adults were pricked with a 0.14-mm minutien pin (Fine Science Tools) dipped in either heat-killed bacteria for the primed treatment or in 1xPBS solution for the unprimed treatment (exposed to a sterile solution in the first exposure) in the mesopleuron region (the area situated under the wing and to the left of the pleural suture) (Prakash et al., 2021b).

Following this initial priming treatment, the individuals were pricked using OD_{600}=0.2 (approximately 70 cells/fly) live *P. rettgeri* bacteria. To test whether male and female adult flies show priming (enhanced survival) with increasing time intervals between the initial heat-killed exposure and later challenge with live *P. rettgeri*, we tested several time points between the two challenges 18-hours, 48-hours, 96-hours, 1-week and 2-weeks (See **Fig**. 1 for experimental design). We used a split vial experimental design to obtain replicate matched data for both survival and bacterial load, see (Prakash et al., 2021a) for details. Briefly after infection each vial containing about 25 flies (of each treatment, sex and fly line combination) were divided into 2 vials with CO_2 for measuring (i) survival following infection (see **Fig**. 1i) and (ii) internal bacterial load (see **Fig**. 1ii).

3. *Bacterial load quantification*: To test whether the host's ability to supress bacterial growth varies across primed and unprimed individuals, we quantified bacterial load at 24-hours after *P. rettgeri* infection for wild types and transgenic flies across both sexes. Flies were surface sterilized in groups of 3-5 flies per vial in 70% ethanol for 30s and washed twice with distilled water before homogenising flies individually using micro pestles. We immediately plated this homogenate in serial dilutions using 1xPBS (phosphate buffer) on to LB agar plates and cultured at 29°C. The following day, we counted the resultant bacterial colonies manually (Siva-Jothy et al., 2018).

4. *Gene expression quantification*: The expression of *Diptericin* was quantified by qRT-PCR. In parallel with the survival experiment, we randomly selected a subset of wild type w^{1118} individuals (both males and females) for RNA extraction, we included 15 flies [3 flies pooled together for each treatment (primed and unprimed) for both males and females]. We randomly sampled flies (3 flies per vial) at different time points post exposure to *P. rettgeri* (18-hours and 72-hours post-priming, 12-hours and 24-hours post-challenge). We then homogenised the whole flies using sterile micro-pestles into 1.5μl microcentrifuge tubes containing 80μl of TRIzol reagent (Invitrogen, Life Technologies). The tubes containing the haemolymph samples were kept frozen at -70°C until RNA extraction. We performed mRNA extractions using the standard phenol-chloroform method and included a DNase treatment (Ambion, Life Technologies) (Prakash et al., 2021b).

   We confirmed the purity of eluted samples using a Nanodrop 1000 Spectrophotometer (version 3.8.1) before going ahead with reverse transcription (RT). The cDNA was synthesized from 2μl of the eluted RNA using M-MLV reverse transcriptase (Promega) and random hexamer primers, and then diluted 1:1 in nuclease free water. We then performed quantitative RT-PCR (qRT-PCR) on an Applied Biosystems StepOnePlus machine using Fast SYBR Green Master Mix (Invitrogen) using a 10μl reaction containing 1.5L of 1:1 diluted cDNA, 5μl of Fast SYBR Green Master Mix an 3.5μl of a primer stock containing both forward and reverse primer at 1μM suspended in nuclease free water (final reaction concentration of each primer 0.35μM). For each cDNA sample, we performed two technical replicates for each set of primers and the average threshold cycle (Ct) was used for analysis.

We obtained the *AMP* primers from Sigma-Aldrich Ltd;

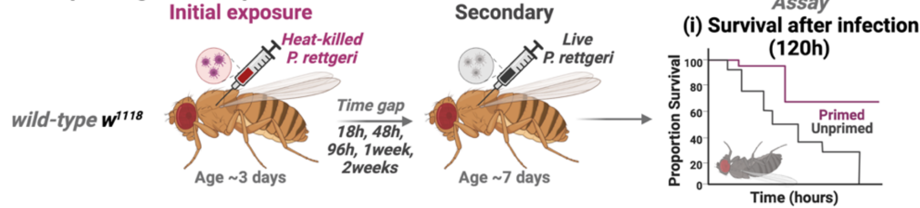
- *Dpt*_Forward: 5' GACGCCACGAGATTGGACTG 3',
- Dpt_Reverse: 5' CCCACTTTCCAGCTCGGTTC 3',
- *AttC*_Forward: TGCCCGATTGGACCTAAGC,
- *AttC*_Reverse: GCGTATGGGTTTTGGTCAGTTC,
- *Dro*_Forward: ACTGGCCATCGAGGATCACC,
- *Dro*_Reverse: TCTCCGCGGTATGCACACAT.

We used housekeeping gene *rp49* as endogenous reference gene,

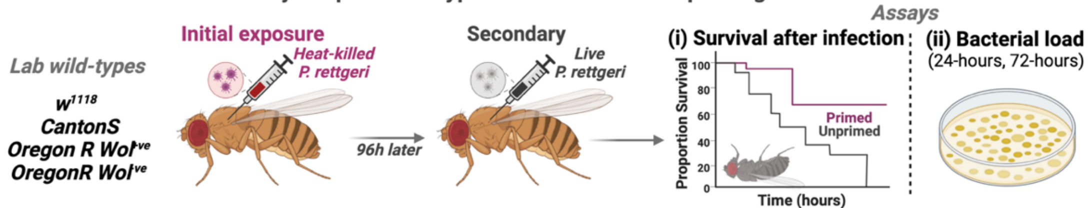
- *rp49*_Forward: 5' ATGCTAAGCTGTCGCACAAATG 3',
- *rp49*_Reverse: 5' GTTCGATCCGTAACCGATGT 3'.

We optimised the annealing temperature (T_a) and the efficiency (Eff) of the *Dpt* primer pair was calculated by 10-fold serial dilution of a target template (each dilution was assayed in duplicate); *Dpt*: T_a= 59 °C, Eff= 102%; *AttC*: T_a= 60 °C, Eff= 94%; *Dro*: T_a= 61 °C, Eff= 104%.

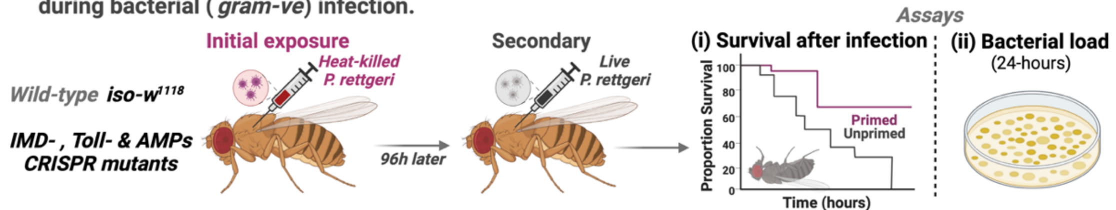
I. Does the time gap between initial (heat-killed) exposure and secondary (live bacterial) exposure affect extent of priming in *Drosophila*?



II. How do different laboratory adapted wild-type flies show immune priming?



III. Dissecting the role of innate immune pathways *(IMD, Toll)* and inducible AMPs in immune priming during bacterial (*gram-ve*) infection.



IV. Deciphering mechanisms that bring about immune priming in *Drosophila* using tissue-specific mutants.

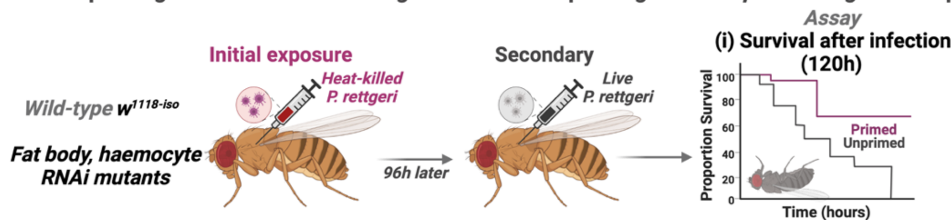


Figure 1. Schematic representation of different priming experiments aimed at (**I**) testing whether the length of the period between primary heat-killed exposure and the secondary pathogenic challenge affects the extent of priming (**II**) how different lab-adapted wild-type flies vary in priming (**III**) dissecting the role of innate immune pathways (IMD and Toll) and inducible AMPs in immune priming and (**IV**) deciphering mechanisms that bring about immune priming in *Drosophila* using tissue-specific fat body and haemocytes UAS^{RNAi} mutants. The experimental design for priming assays includes survival and internal bacterial load quantification.

5. <u>*Data analysis:*</u> We analysed the survival data following systemic *P. rettgeri* infection with a mixed effects Cox model using the R package 'coxme' (Therneau, 2015) for different treatment groups (that is, primed and unprimed) across both the sexes (males and females) fly lines (wild-types and transgenic lines). We specified the model as: survival ~ treatment x sex x (1 | vials/block), with 'treatment' and 'sex' and their interactions as

fixed effects, and 'vials' nested within each 'block' as a random effect for wild-types and transgenic fly lines.

We used ANOVA to test the impact of each fixed effect in the 'coxme' model. We analysed the bacterial load (measured as log_{10}) bacterial colony-forming units (CFUs) at 24-hours following *P. rettgeri* infection. Bacterial loads (CFUs) were non-normally distributed and hence we log-transformed the residuals and analysed using a non-parametric one-way ANOVA (Kruskal-Wallis test) to test whether the 'treatment' groups that is, primed and unprimed individuals significantly differed in internal bacterial load for males and females of each fly lines (wild-type and transgenic lines).

Finally, we analysed the gene expression data by calculating the ΔCT value (Livak and Schmittgen, 2001). We used the steadily between the treatments expressing *rp49* as reference gene. We calculated fold change in gene expression relative to the uninfected controls to calculate ΔΔCT and used ANOVA to test whether AMP expression differed significantly between primed and unprimed treatment for males and females.

5.4 *Results*

I. Immune priming in *Drosophila* is a long-lasting response, occurring in several genetic backgrounds, and is stronger in males

We examined the conditions under which the priming phenotype of increased survival following an initial non-lethal challenge could be observed in flies. We first examined if the length of time between the initial non-lethal exposure with heat-killed *P. rettgeri* and the secondary pathogenic challenge with live *P. rettgeri* affects the extent of priming. To address this, we exposed male and female wild-type flies (w^{1118}) to live *P. rettgeri* 18-hours, 48-hours, 96-hours, 1-week or 2-weeks following the initial exposure to heat-killed bacteria. Male w^{1118} flies showed increased survival after initial priming for time points 18-hours, 48-hours and 96-hours, and still showed a significant, albeit reduced, priming response 1-week and 2-weeks after the initial exposure (***Fig**. 2, right panel*). Female flies did not show a priming response when infected 18-hours following the initial challenge, but the priming response increased with 48-hours and 96-hours priming intervals before completely disappearing around a week time-interval (including 2-weeks) (***Fig**. 2, left panel*) (***Table**-SI-1*).

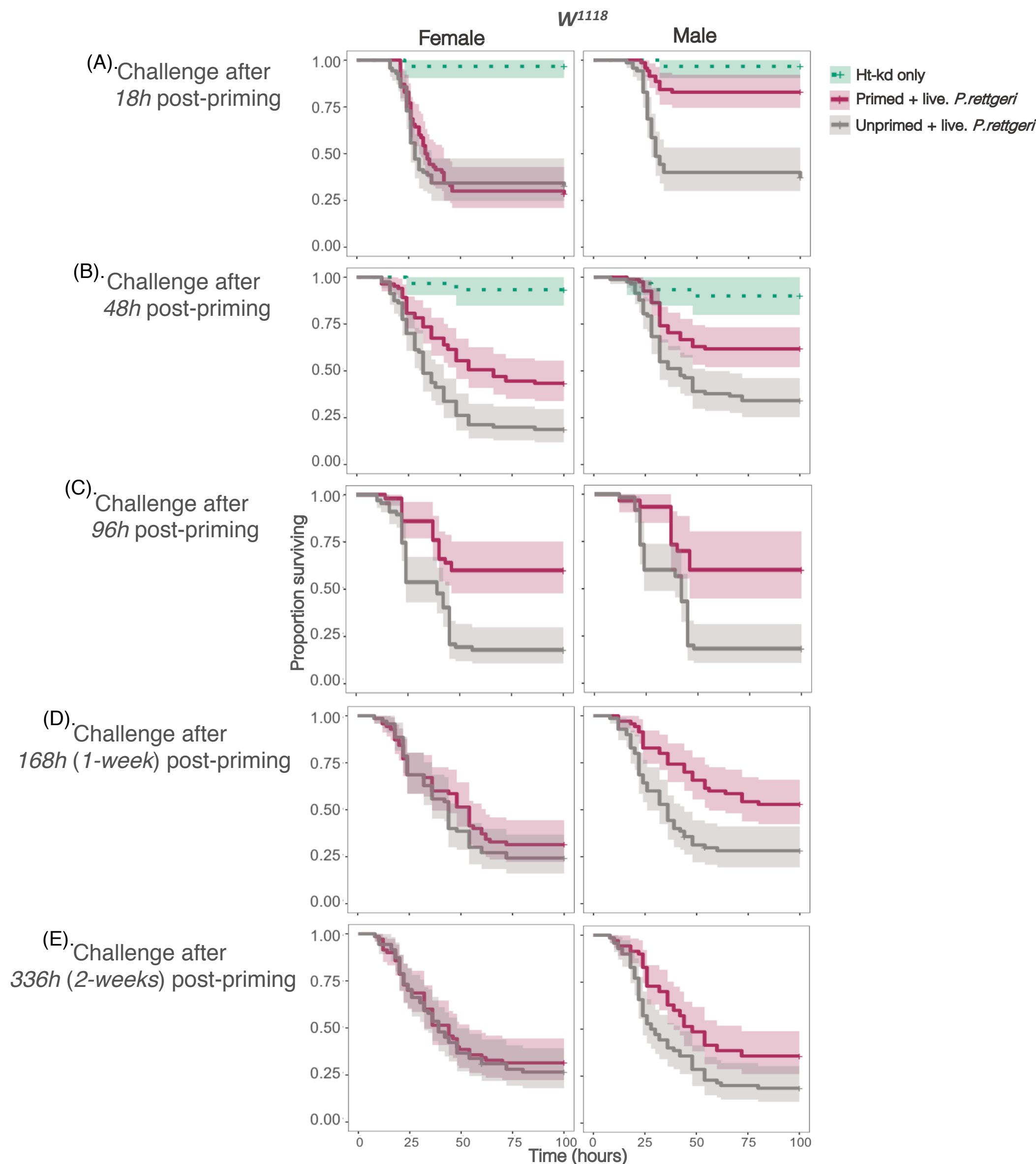


Figure 2. Survival curves of w^{1118} flies with primed (exposed to heat-killed *P. rettgeri* in the first exposure) and unprimed (exposed to a sterile solution in the first exposure) treatments challenged with live *P. rettgeri* pathogen after **(A)** 18-hours **(B)** 48-hours and **(C)** 96-hours **(D)** 168-hours/1-week and **(E)** 336-hours/2-weeks post priming that is, initial non-lethal exposure to heat-killed *P. rettgeri* (n=7-9 vial with 10-15 flies in each vial/sex/treatment/timepoint for w^{1118} wild type fly line).

Next, we asked if priming occurred in other wild-type fly lines as observed with w^{1118}. Females and males of w^{1118} and three other chosen wild type strains were treated first with heat-killed *P. rettgeri*, followed by infection with live *P. rettgeri* 96-hours after the first treatment. Since the 96-hour time gap between priming and live *P. rettgeri* treatments showed maximum priming response (difference in survival) for both males and females, we kept the 96-hours timepoint as the consistent time-gap between priming and live infection throughout the study (***Fig.*** *2C*). Canton-S flies showed increased survival following priming and we observed this survival

benefit across both sexes, although stronger in females (***Fig**. 3B,* ***Table** SI-2B*). Oregon-R males also exhibited increased survival following priming but priming had no significant effect on the females (***Fig**. 3C,* ***Table**-SI-2*).

II. *Wolbachia* infection reduces the priming response in males

Given the widespread effects of the endosymbiont *Wolbachia* on *Drosophila* immunity (Brownlie and Johnson, 2009; Teixeira et al., 2008; Gupta et al., 2017) we also tested whether the presence of *Wolbachia* had any effect on immune priming by comparing the priming response of Oregon-R (OreR), the line originally infected with *Wolbachia* strain wMel, henceforth $\mathrm{OreR}^{\mathrm{Wol+}}$ and a *Wolbachia*-free line $\mathrm{OreR}^{\mathrm{Wol-}}$ that was derived from $\mathrm{OreR}^{\mathrm{Wol+}}$ by antibiotic treatment (Gupta et al., 2017). We found that the presence of *Wolbachia* significantly improved overall survival of both males and females (***Fig**. 3C and D,* ***Table**-SI-3*). However, the immune priming observed in males in absence of *Wolbachia* ($\mathrm{OreR}^{\mathrm{Wol-}}$) was no longer present in flies carrying *Wolbachia* (***Fig.** 3D,* ***Table**-SI-3*).

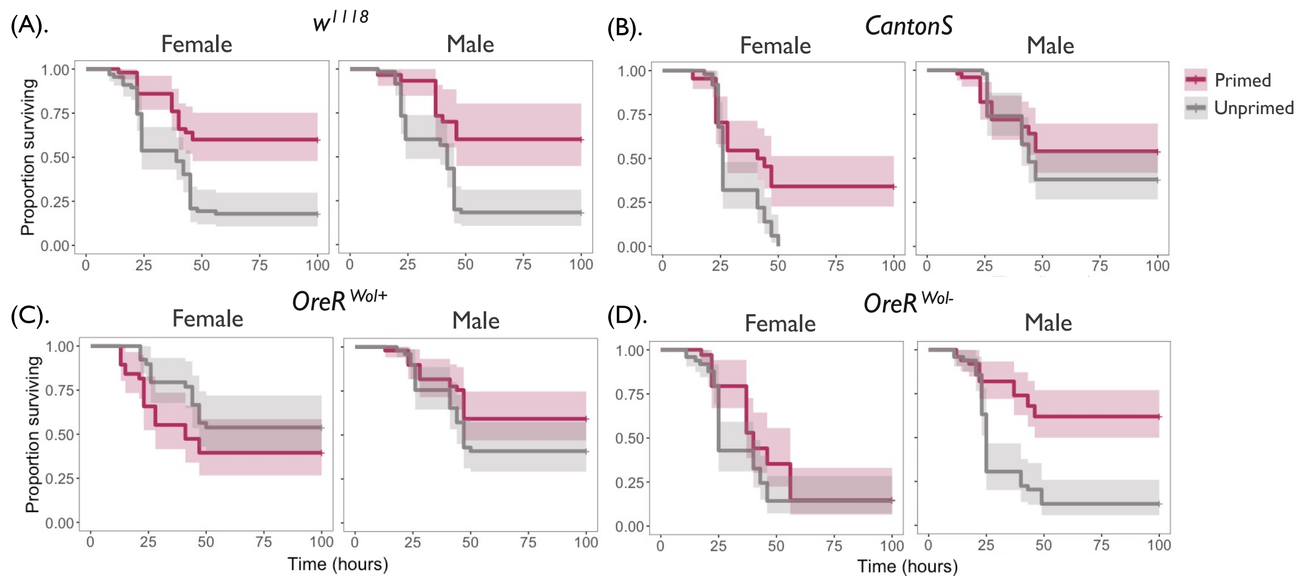


Figure 3. Survival curves for female and male flies either primed-challenged or unprimed-challenged with *P. rettgeri*. Commonly used lab wild-type flies **(A)** w^{1118} (same data/graph from Fig. 2C was used for comparison) **(B)** Canton-S **(C)** Oregon-R with endosymbiont *Wolbachia* **(D)** Oregon-R without *Wolbachia* were used in the experiment. (n=7-9 with 10-15 flies in each vial/sex/treatment/wild type fly lines).

III. Primed male w^{1118} flies exhibit a transient reduction in bacterial load

Previous studies have shown that the priming response to fungi and gram-positive bacteria can be explained by increased clearance of pathogen in primed individuals, that is, through increased resistance (Pham et al., 2007; Khan et al., 2019). We therefore examined whether the increased survival following a prior challenge we observed was a result of greater bacterial clearance in the primed individuals, or if the primed flies were simply better able to tolerate the bacterial infection. To investigate this, we repeated the priming experiment with w^{1118} as it showed the clearest priming phenotype in both sexes (***Fig***. *2C and* ***Fig***. *4A*) and measured the bacterial load at 24-hours and 72-hours post-infection for both primed and unprimed female and male flies. Again, we observed a clear priming effect in both sexes (***Fig***. *4A,* ***Table****-SI-4*). In primed males the systemic infection resulted in higher survival and also in decreased bacterial loads at 24-hours post-infection when compared to unprimed individuals (***Fig.*** *4,* ***Table****-SI-4* for survival, ***Table****-SI-5* for load). However, by 72-hours post-infection, bacterial loads had dropped in both primed and unprimed males and there was no detectable effect of priming on the bacterial loads (***Fig***. *4B,* ***Table****-SI-5*).

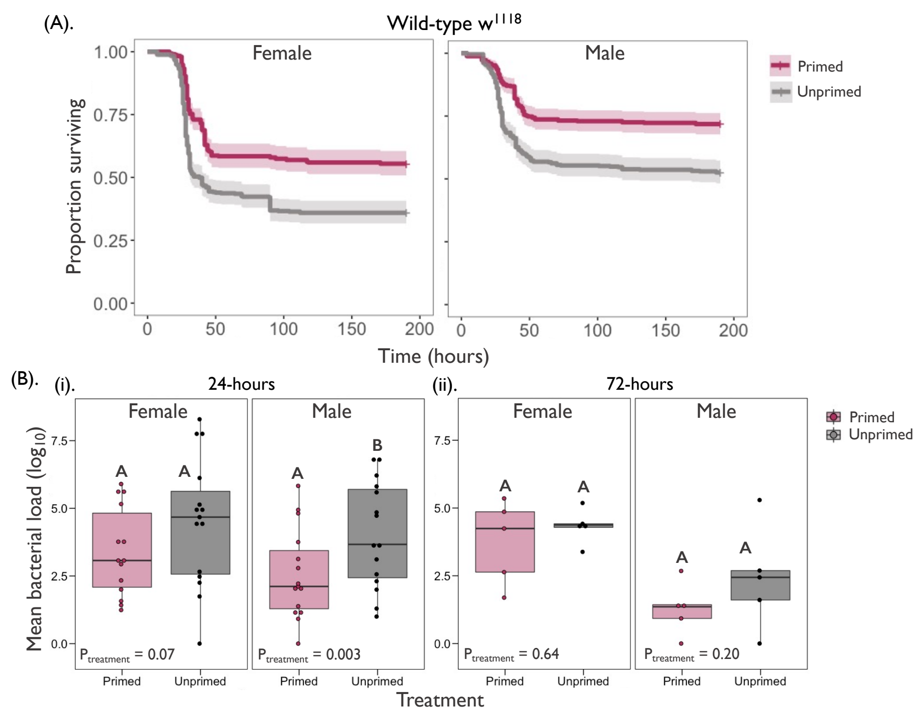


Figure 4. **(A).** Survival curves of primed and unprimed w^{1118} flies with 96-hours interval between the treatments **(B).** Internal bacterial load (n=20-22 vial with 5-7 flies in each vial/sex/treatment) after **(i)** 24-hours and **(ii)** 72-hours following *P. rettgeri* infection. Different letters in panel-B denotes primed and unprimed individuals are significantly different, tested using Tukey's HSD pairwise comparisons for each timepoint and sex combination. The error bars in panel B represent standard error.

IV. The *IMD*-pathway, but not the Toll pathway, is required for immune priming during gram-negative bacterial *P. rettgeri* infections

The inducible AMPs regulated by the IMD signalling pathway play a crucial role in resisting gram-negative bacterial infection such as *P. rettgeri*. Therefore, we wanted to investigate whether the IMD signalling pathway and IMD-responsive AMPs contribute to immune priming. To address this, we used several transgenic lines (CRISPR, loss-of-function and UAS^{RNAi}) lacking different regulatory and effector components of the IMD-signalling pathway. First, we used a *Relish* loss-of-function mutant Rel^{E20}, which lacks the transcription factor *relish*, a key regulator of the IMD immune response. As expected, we found that *Relish* mutants were not able to clear bacteria and died at faster rate, and therefore did not show any priming benefit (***Fig***. *5A-ii* for survival and ***Fig***. *5B-ii* for bacterial load, ***Table***-*SI-4* for survival and ***Table***-*SI-6* for bacterial load).

In response to gram-positive bacterial *Streptococcus pneumoniae* infection, the Toll pathway was previously shown to be required (though not sufficient) for priming (Pham et al., 2007). In other work, Toll-pathway activity differed between males and females and explained sexual dimorphism in immunity during *P. rettgeri* infection (Duneau et al., 2017). Given that we found sex differences in the priming in response to *P. rettgeri* infection (***Fig***. *2 and 3*), these previous results motivated us to investigate whether Toll-signalling also contributes to immune priming against *P. rettgeri*. To address this, we used *spz* deletion mutant which lack *spatzle*, a key regulator in the *Toll* pathway, enabling the flies to activate the Toll-signalling pathway. *Spätzle* mutants showed enhanced survival following initial heat-killed exposure, and their mortality rates were the same as in controls (w^{1118}) (***Fig***. *5A-iii,* ***Table***-*SI-4*), indicating that Toll-pathway does not contribute to priming during *P. rettgeri* infection in either females or males.

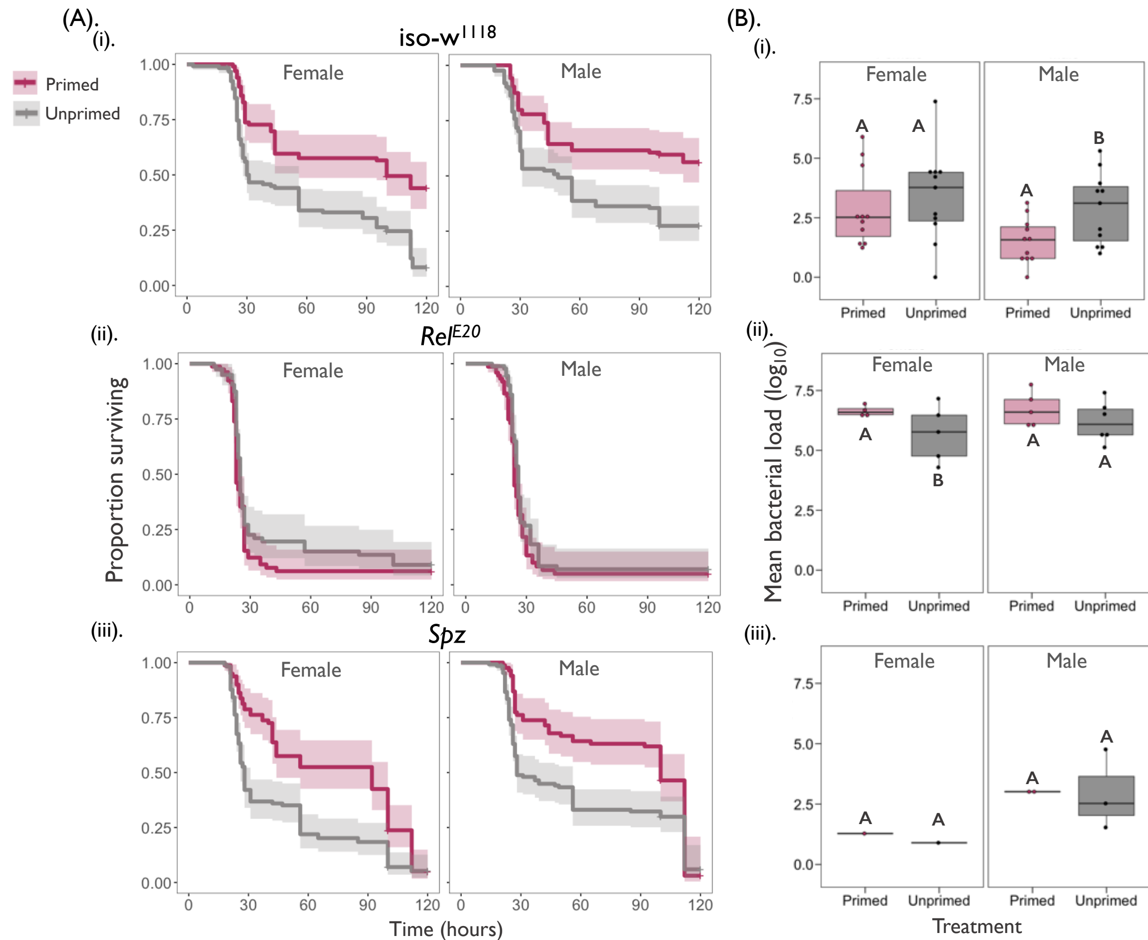


Figure 5. **(A)** Survival curves for wild-type flies and flies lacking different innate immune pathway components in females and males: **i)** wild-type *w¹¹¹⁸* **ii)** *Rel^E20^*, *IMD*-pathway transcription factor **iii)** *Spz*, Toll pathway regulator. **(B) i-iii)** bacterial load measured after 24-hours post-secondary pathogenic exposure (n=7-9 with 10-15 flies in each vial/sex/treatment/mutant fly lines). Different letters in panel-B indicate that primed and unprimed individuals are significantly different, tested using Tukey's HSD pairwise comparisons within each fly line and sex combination. The error bars represent standard error.

V. The IMD-regulated AMPs *Diptericins* (*DptA* and *DptB*) are required for priming against *P. rettgeri*

Given the important role of the IMD pathway for the priming response (***Fig.** 5A*), we next tested whether mutants with defective IMD signalling or unable to produce specific antimicrobial peptides were capable of immune priming. We first used a ΔAMP mutant fly line, which lacks most of the known *Drosophila* AMPs (13 AMPs in total). It has been previously shown that ΔAMPs flies are extremely susceptible to the majority of microbial pathogens, including gram-negative bacteria (Hanson et al., 2019). We found that both primed and unprimed ΔAMP flies succumb to death at a similar rate, and both primed and unprimed ΔAMP flies also exhibited elevated bacterial loads compared to wild type (measured 24-hours

after the secondary pathogenic exposure) (***Fig***. *6A-i* for survival, ***Fig***. *6B-i* for bacterial load, ***Table***-*SI-4* and *SI-6*, relative to wild-type w^{1118} – compare previous figures ***Fig***. *5B-i*), showing that AMPs are needed for the priming response against *P. rettgeri*. To investigate which AMPs are required for priming against *P. rettgeri* infection, we used a Group-B mutant fly line, lacking major IMD regulated AMPs (including *Diptericins* and *Attacins* and *Drosocin*) but have all upstream IMD signalling intact. Primed Group-B flies showed mortality similar to unprimed Group-B flies (***Fig***. *6A-ii,* ***Table*** *SI-4*) and exhibited increased bacterial loads compared to wild type flies, across both sexes irrespective of being primed or not (***Fig***. *6B-ii,* ***Table*** *SI-6*). Thus, despite being able to produce other AMPs, removal of IMD-regulated AMPs completely eliminated the priming effect, indicating that AMPs regulated by the IMD-pathway are required for immune priming against *P. rettgeri* infection.

Since *Diptericins* have been shown previously to play a key role in defense against *P. rettgeri*, we then used a *Dpt* (lacking *Diptericin-A* and *B*) and $AMPs^{+Dpt}$ transgenic fly lines (flies lacking all known AMPs except *Diptericin*) to test whether *Diptericins* are required and sufficient for priming in both females and males. The survival benefit of priming disappeared in flies lacking *Dpt* across both females and males and both primed and unprimed *Dpt* mutants exhibited increased bacterial load (***Fig***. *6B-iii,* ***Table*** *SI-6*). However, the priming response was recovered completely in male flies that lacked all other AMPs but possessed functional *Diptericins* ($AMPs^{+Dpt}$). However, the same effect was not seen in females (***Fig***. *6A-iv* for survival, ***Fig***. *6B-iv* for bacterial load; ***Table*** *SI-4* for survival, ***Table*** *SI-6* for bacterial load).

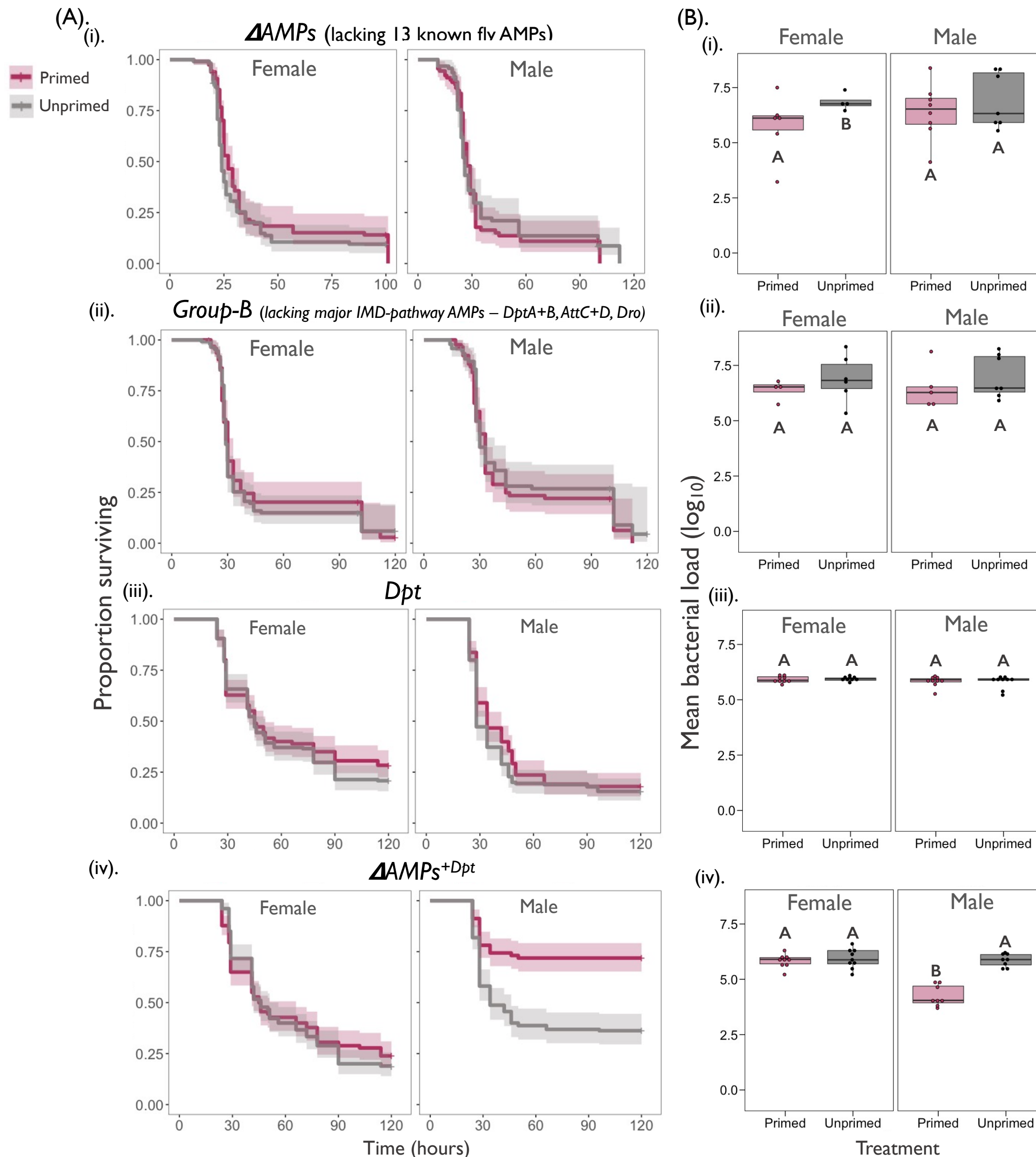


Figure 6. **(A)** Survival curves for CRISPR/Cas9 AMP mutants **i)** *ΔAMP* fly line: lacking all known 13 fly AMPs – **ii)** ***Group-B:*** lacking major IMD pathway AMPs **iii)** ***Dpt*** knockout and **iv)** ***ΔAMP*** $^{+Dpt}$: lacking all AMPs except ***Dpt***. **(B)** internal bacterial load quantified after 24-hours post-secondary exposure (n=7-9 with 10-15 flies in each vial/sex/treatment/mutant fly lines). Different letters in panel-B denotes primed and unprimed individuals are significantly different, tested using Tukey's HSD pairwise comparisons for each fly line and sex combination. The error bars in panel B represent standard error.

VI. *Diptericin B* expression in the fat body is required for priming

In response to gram-positive *S. pneumoniae* infection, previous work described the role of haemocytes in immune priming through increased phagocytosis (Pham et al., 2007). Subsequent work has also shown that ROS burst from haemocytes is important for immune priming during *Enterococcus faecalis* infection (Chakrabarti and Visweswariah, 2020). Since our results pointed to a single AMP-*Diptericin* being required for immune priming against *P. rettgeri*,

we wanted to determine if *Dipterician* expression in either the fat body or haemocytes was more important for immune priming . Using-tissue specific *Dipterecin-B* UAS^{RNAi} knock down, we found that male flies with knocked-down *DptB* in fat bodies no longer showed immune priming compared to the wildtype, while knocking down *DptB* in haemocytes resulted in a smaller but still significant increase in survival following an initial exposure (***Fig****. 7,* ***Table****-SI-7*). This would therefore support that immune priming requires *DptB* expression in the fat body, while haemocyte derived *DptB* is less important for priming. It is worth noting that it is possible that haemocytes contribute to immune priming through phagocytosis or melanisation, or via crosstalk with the IMD pathway, and this remains a question for future research.

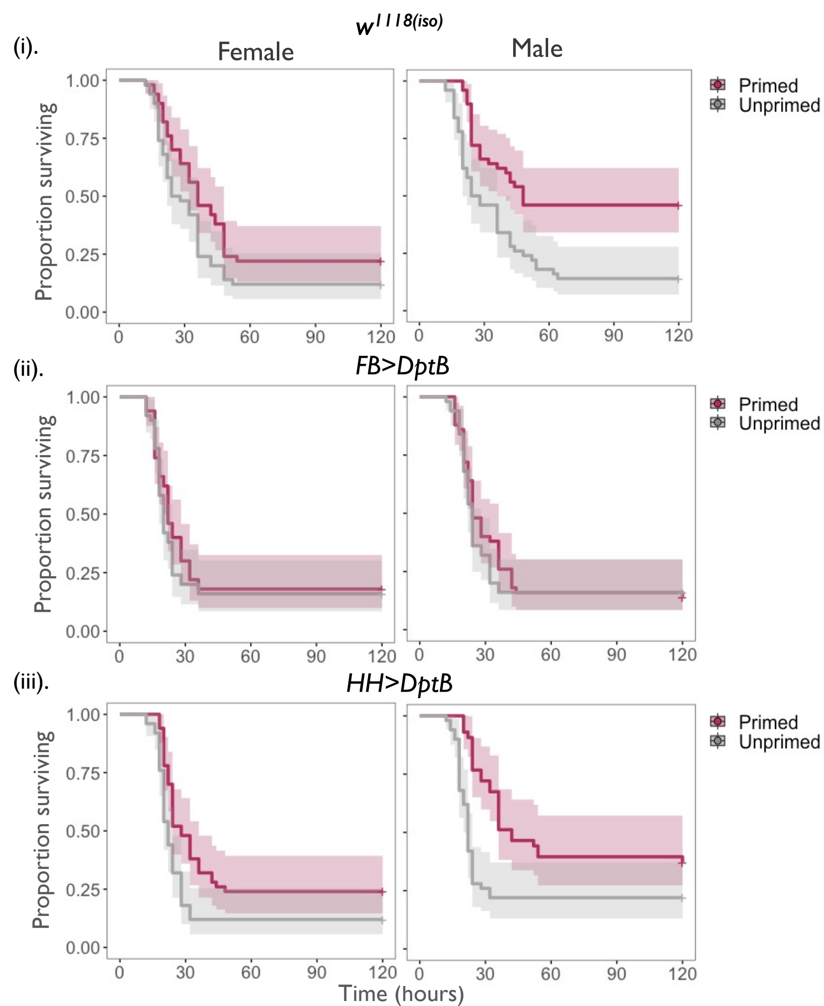
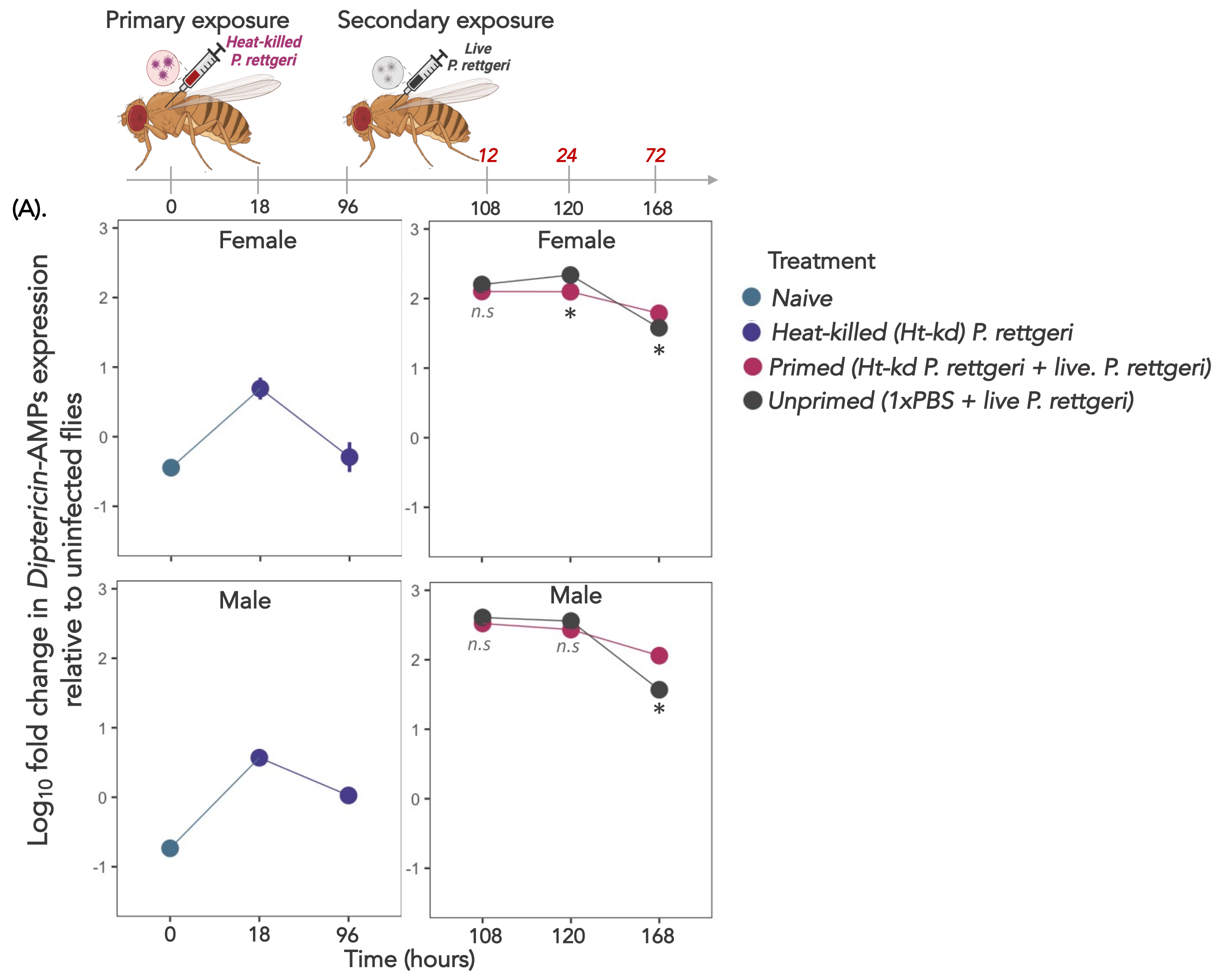


Figure 7. Survival curves for **i) wild type** $w^{1118\text{-}iso}$ and ii) ***FB>DptB*** RNAi knock down of ***DptB*** in fat body and **iii)** ***HH>DptB*** RNAi knock down of ***DptB*** in haemocytes. (n=50 flies/sex/treatment/mutant fly lines).

VII. Priming is not an outcome of constant *Dtp* upregulation following the initial challenge, but instead due to a slower resolution of the immune response upon secondary infection

As our results indicated that AMPs, especially *Diptericins* play a key role in *Drosophila* priming against *P. rettgeri*, we wanted to test if the priming effect we had observed was a result of constant upregulation of AMPs after initial heat-killed exposure allowing rapid bacterial clearance during the secondary exposure, or if AMP expression returned to a baseline level within the 96-hours between priming and the live infection. To do this, we measured *Dpt* gene expression at 18-hours and 72-hours following initial heat-killed exposure, and then 12-hours, 24-hours and 72-hours following secondary live *P. rettgeri* infection. *Diptericin* expression increased 18 hours after initial heat-killed exposure but returned to baseline levels by 72 hours (***Fig.*** *8A and 8B,* ***Table***-*SI-8*) indicating that flies did mount an immune response to the heat killed bacteria, but that this was resolved by the time they were infected with the live bacteria at 96-hours.

Further, 72-hours following a lethal secondary live *P. rettgeri* infection, we found that primed w^{1118} females and males showed increased *Dpt* levels compared to unprimed individuals (***Fig.*** *8A and 8B*). The increased *Dpt* expression was also associated with increased bacterial clearance in males (see ***Fig***. *4Bi and 5Bi*). In case of females, despite higher *Dpt* levels at 72-hours following secondary live infection in primed flies, we did not detect any difference in bacterial clearance between primed and unprimed flies (see ***Fig***. *4Bi and 5Bi*). We also tested the expression patterns of other IMD-responsive AMPs such as *Attacin-C* and *Drosocin* in females. Overall, we found that the expression of *Attacin-C* and *Drosocin* was not different between primed and unprimed females (***Fig.*** *8C for AttC and Dro,* ***Table***-*SI-8*).



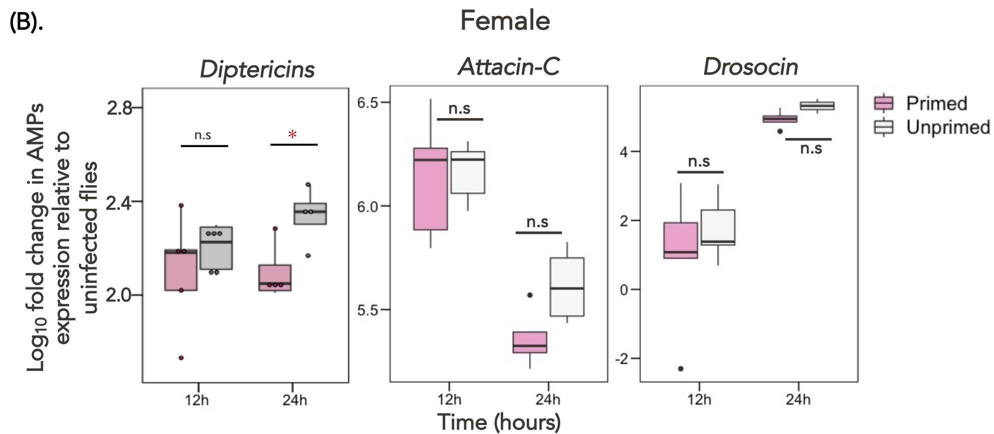


Figure 8. **(A)**. Mean±SE (standard error) of *Diptericin*-AMPs expression at different time points (hours) for females and males wild-type w^{1118} flies with treated with - naïve or unhandled flies; flies exposed to initial heat-killed *P. rettgeri*; primed flies that are initially exposed to heat-killed *P. rettgeri* followed by challenge with *P. rettgeri*; unprimed flies that receive 1xPBS during primary exposure followed by live *P. rettgeri* during secondary exposure (n=15-21 flies/sex/treatment/timepoint). **(B).** *Diptericin*$^{A+B}$*, Attacin-C* and *Drosocin* expression 12-hours and 24-hours after exposure to live *P. rettgeri* in w^{1118} female flies. Asterisks '*' indicates that primed and unprimed individuals are significantly different (p<0.05). The error bars represent standard error.

VIII. Regulation of IMD by PGRPs is required for immune priming

We found that *Diptericin* is required for immune priming and that the priming response itself relies on an increased expression of *DptB* upon reinfection. Hence, we reasoned that this change in expression must be regulated upstream of the IMD signalling cascade. Following gram-negative bacterial infection, DAP-type peptidoglycans from gram-negative bacteria are recognised by the peptidoglycan receptors *PGRP- LC* (a transmembrane receptor) and *PGRP-LE* (a secreted and an intracellular isoform*)*, which then activate the IMD intracellular signalling cascade (Myllymäki et al., 2014; Lemaitre and Hoffmann, 2007). Negative immune regulation is achieved by direct inhibition of IMD in the cytoplasm by *pirk*, or further downstream by *caudal* at the level of *Relish*. Several PGRPs also downregulate the IMD response, including *PGRP-LB*, a secreted peptidoglycan with amidase activity, that break down peptidoglycans into smaller, less immunogenetic fragments (Myllymäki et al., 2014; Lemaitre and Hoffmann, 2007). *PGRP-LB* has also been shown to be important for transgenerational immune priming in *Drosophila* against parasitoid wasp infection (Bozler et al., 2020).

We therefore decided to investigate the role of PGRPs in the immune priming we observed during *P. rettgeri* infection. To address this, we used fly lines with loss-of-function in *PGRP-LB*, *-LC* and *-LE*. Regardless of which PGRP was disrupted, we observed that flies were no longer able to increase their survival following an initial exposure (***Fig**. 9A,* ***Table**-SI-9*). However, unlike similar outcomes with *Relish* or *ΔAMP*, here the lack of priming wasn't driven by an inability to clear bacterial loads, as microbe loads in PGRP mutants were ~100-fold lower than in *Relish* or AMP mutants (***Fig**. 9B,* ***Table**-SI-10*; also compare ***Figs**. 5B-ii and 6B-i*). This distinction is important, because it indicates that while flies are still able to clear *P. rettgeri*, loss of any of three tested PGRPs results in a loss of priming. This suggests that *Diptericin* expression is required, but not sufficient, for successful priming, which requires adequate regulation by PGRPs. Which specific molecular signal modifies how PGRPs regulate *Diptericin* expression is unclear but must lie outside the IMD pathway and the fat body.

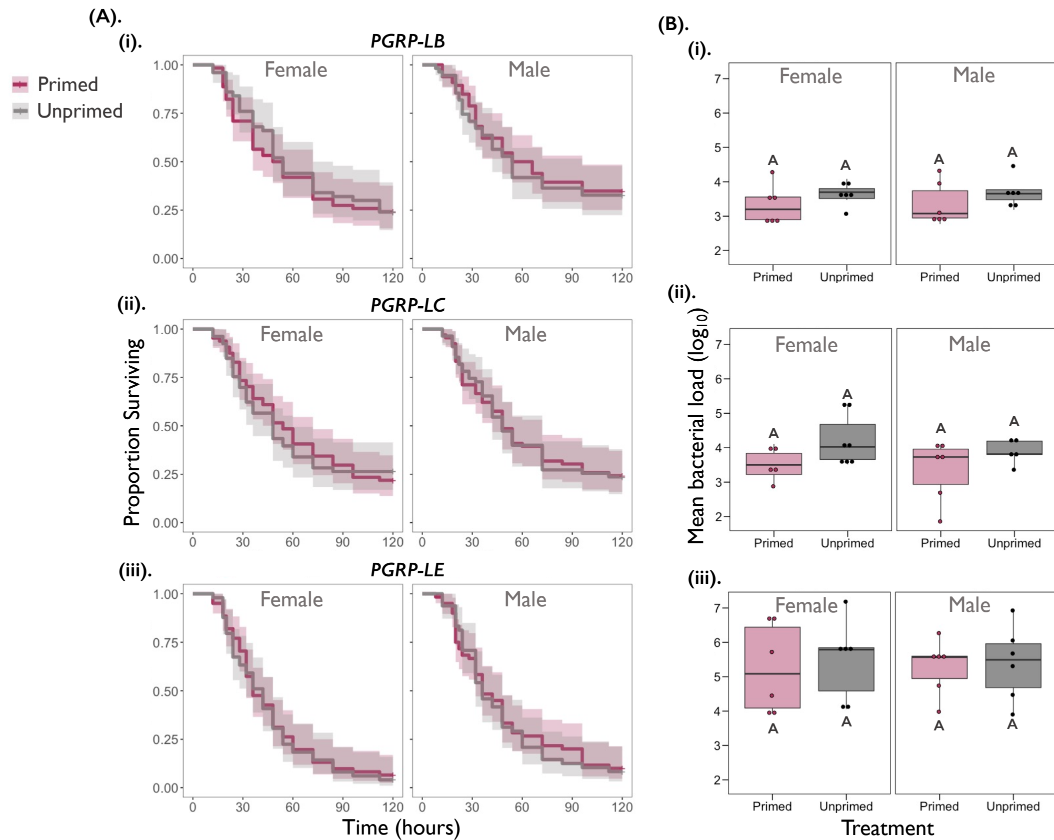


Figure 9. **A).** Survival curves for males and females of Loss-of-mutation in *PGRPs* (peptidoglycan recognition proteins) of IMD **i) *PGRP-LB* ii) *PGRP-LC*** and **iii) *PGRP-LE.* B)** Internal bacterial load quantified after 24-hours post-secondary exposure (n=7-9 with 10-15 flies in each vial/sex/treatment/mutant fly lines). Different letters in panel-B denotes primed and unprimed individuals are significantly different, tested using Tukey's HSD pairwise comparisons for each fly line and sex combination. The error bars represent standard error.

5.5 *Discussion*

In the present work, we investigated the occurrence, generality and mechanistic basis of immune priming in fruit flies when infected with the gram-negative pathogen *Providencia rettgeri*. We present evidence that priming with an initial heat-killed bacterial inoculum results an increase in survival after a secondary lethal challenge with the same live bacterial pathogen. This protective response may last at least two weeks after the initial exposure, is particularly strong in male flies, and occurs in several wild-type genetic backgrounds. We show that the increased survival of primed individuals coincides with a transient decrease in bacterial loads, and that this is likely driven by the expression of the IMD-responsive AMP *Diptericin-B* in the fat body.

Further, we show that while *Diptericin* is required as the effector of bacterial clearance, it is not sufficient for immune priming, which requires the regulation by at least three PGRPs (*PGRP-LB, PGRP-LC*, and *PGRP-LE*). Other work has shown that elimination of *PGRP-LB* results in increased expression of *Dpt* AMPs (Zaidman-Rémy et al., 2006; Myllymäki et al., 2014b). Therefore, despite having an intact IMD signalling cascade, and being able to express all AMPs including *Diptericin*, flies lacking any one of *PGRP-LB, -LC* or *-LE* were not capable of increasing survival following an initial sublethal challenge. Together, our data highlights that PGRPs are necessary for regulating immune priming against *P. rettgeri* (see ***Fig**. 10*).

In response to infection, the expression of several AMP genes in *Drosophila* increases and then drops once the infection threat is resolved or controlled. Previous studies have shown that this occurs within a period of few hours during bacterial infections (Lemaitre et al., 1997). One key aspect of our results is that immune priming was not the result of continued upregulation of *Diptericin* following the initial exposure to heat-killed bacteria. Instead, we observed that the initial immune response to heat-killed bacteria had already been resolved at 72-hours, before the secondary exposure to a lethal infection at 96-hours (see ***Fig**. 8A and B*). This second up-regulation of AMP expression was at least 10-times higher than the response to heat-killed bacteria and was initially similar between both primed and unprimed flies (exposed to a sterile solution in the first exposure). It was only at 72-hours following the second lethal exposure that we observed significantly higher expression of *Diptericin* in primed flies. This difference appears to arise because unprimed flies show a faster resolution of the immune response compared to primed flies; that is, the expression of *Diptericin* shows a faster decrease between 24-hours and 72-hours post-exposure in unprimed compared to primed flies (see ***Fig**. 8A and B*).

These patterns of gene expression provide a partial explanation for the increased survival following priming, but they do not explain why primed individuals show lower bacterial loads at 24-hours after exposure to the second lethal infection had, as at this timepoint we did not detect any differences in *Diptericin* expression between primed and unprimed flies. It is also unclear why primed females showed increased expression of *Diptericin* after 24-hours, but do not show any reduction in bacterial loads at this timepoint following priming, in the way male flies do.

The sex differences we observed in priming reflect a larger pattern of sexual dimorphism in immunity present in most species and particularly in *Drosophila* (Belmonte et al., 2020; Klein and Flanagan, 2016; Simon, 2005). For instance, the Toll-signalling pathway activity mediated sex-differences in survival following a *P. rettgeri* systemic infection (Duneau et al., 2017). Here we found that males showed better bacterial clearance after initial exposure to heat-killed *P. rettgeri* which enabled them to experience enhanced survival compared to females, who exhibited higher bacterial loads and greater mortality. One possibility for the observed sex-differences could relate to different nutritional demands and metabolic activities in female *Drosophila*. For instance, female fruit flies are often able to reallocate resources in accordance with their reproductive demands, as observed in terminal investment during infection (Camus et al., 2019; Martínez et al., 2020; Hudson et al., 2020).

Studies from other insects suggest that inducing priming responses can directly reduce the reproductive fitness in mosquitoes (Contreras-Garduño et al., 2014), wax moth (Trauer and Hilker, 2013), and mealworm beetles (Zanchi et al., 2011). There are therefore potential trade-offs between investment in reproductive effort and investment in stronger immune responses following priming. Given that all females used in this study were mated, it is possible that such trade-offs forced a reallocation of resources towards reproduction, thereby reducing the observed magnitude of the priming response in females. Future studies may consider comparing priming responses in females with different reproductive states in order to test whether immune priming is costlier for female *Drosophila*.

A subsidiary finding of this work was the effect of the endosymbiont *Wolbachia* on immune priming. There is strong evidence that *Drosophila* carrying endosymbiont *Wolbachia* are better able to survive infections, especially viral infections (Hedges et al., 2008; Teixeira et al., 2008; Martinez et al., 2014; Gupta et al., 2017). In this case we observed that the priming response that was present in male flies cleared of *Wolbachia* disappeared in males carrying the

endosymbiont. It is unclear how *Wolbachia* might induce this effect. This effect is unlikely due to a direct effect of *Wolbachia* on the ability to clear *P. rettgeri* in primed flies, as previous work has found that *Wolbachia* had no effect on the ability to suppress *P. rettgeri* during systemic infection (Rottschaefer and Lazzaro, 2012). Indeed, if priming is the result of upregulation of AMP expression, this may suggest that *Wolbachia* may actively be supressing the expression of AMPs, thereby reducing the beneficial effects of priming. However, this would appear to contradict work showing that some *Wolbachia* strains upregulate the host's immune response and result in a reduction of pathogen growth (Rancès et al., 2012; Chrostek et al., 2013; Prigot-Maurice et al., 2022). Further rigorous experimental work is therefore required to fully dissect this effect of *Wolbachia* on immune priming.

Finally, it is important to consider the implications of immune priming for disease ecology and epidemiology, and particularly how it may affect pathogen transmission. Epidemiological models that have incorporated priming into disease transmission frameworks predict that primed individuals with enhanced survival following an initial sub-lethal pathogenic exposure are less likely to become infectious upon re-infections (Tate, 2016). It remains unclear whether immune priming reduces pathogen transmissibility, varies the infectious period, or alters infection-induced behavioural changes in the host (Tate, 2016). However, if priming acts by improving bacterial clearance via increased AMP expression, as observed in the present work, we predict that priming is likely to reduce pathogen shedding at the individual level, resulting in reduced disease transmission at the population level. A better understanding of how priming contributes to host heterogeneity in infection outcomes would therefore aid our understanding of the causes and consequences of variation in infectious disease dynamics in insect-pathogen systems.

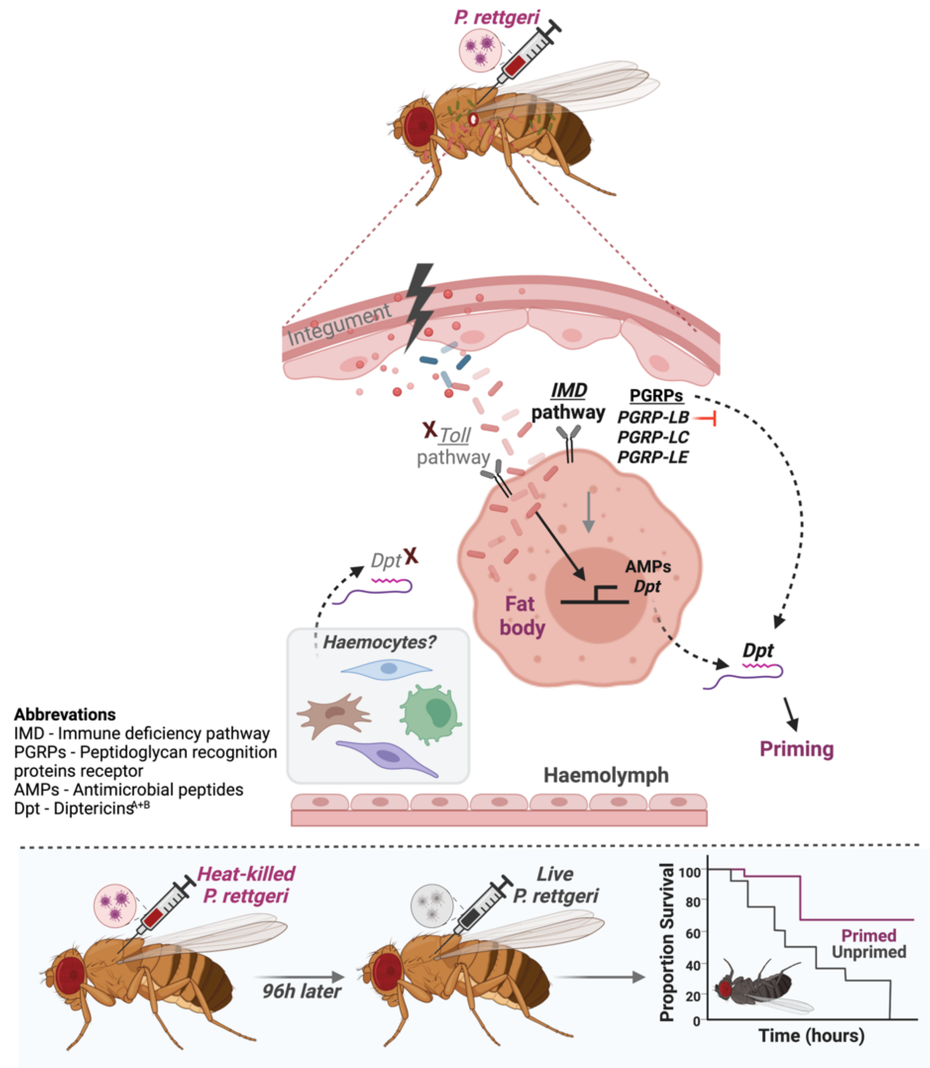


Figure 10. Features of priming response to subsequent systemic bacterial infections in male flies. Following initial priming with heat-killed *P. rettgeri* and subsequent challenge with live *P. rettgeri* the IMD-signaling cascade but not the Toll-pathway (indicated by 'X' mark) get activated through peptidoglycan receptor and a negative immune regulator *PGRP-LB*. This is followed by downstream production of inducible AMPs specifically *Diptericins* in the fat body but not from the haemocytes (indicated by 'X' mark), showing that *Diptericins* are required for priming in males and PGRPs – *LB, -LC*, and *-LE* are necessary for regulation of priming.

5.6 *References*

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5.7 *Supplementary Information*

Figure SI-1. Survival curves of Wild-type w^{1118} and iso-w^{1118} (Drosdel) flies after initial heat-killed exposure and followed by live *P. rettgeri* infection with OD_{600} = 0.1. As another control, we infected both w^{1118} and iso-w^{1118} wildtypes because the CRISPR/cas9 AMP mutants we used were on the iso-w^{1118} background, so we wanted to confirm that any changes in priming were not due to the background of the mutants, as opposed to the mutations. We found that the differences between w^{1118} and iso-w^{1118} (primed and unprimed treatments) were not significantly different.

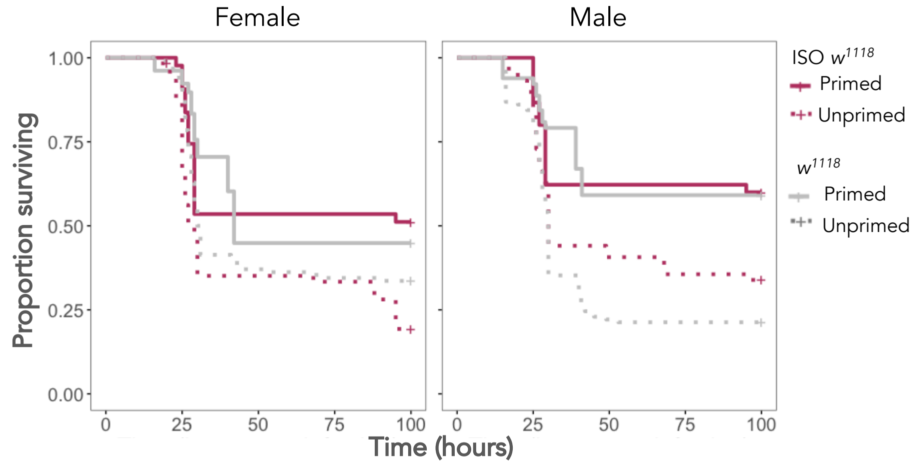

Table-SI-1. Summary of mixed effects Cox model, fitting the model to estimate time-delayed priming response in wild-type w^{1118} male and female flies. We used data from individuals exposed to live bacterial after different time intervals of initial heat-killed *P. rettgeri* exposure. We specified the model as: survival ~ treatment x sex x timepoint (1|vial/block), with treatment and sex as fixed effects, and vials nested within each block and as a random effect. The table shows model output (ANOVA) for priming in wild-type flies.

Timepoint/sex	Source	loglik	$\chi 2$	Df	P
***18-hours** post Priming overall*	Treatment	-801.96	15.92	1	<0.001
	Sex	-790.74	22.44	1	<0.001
	Sex × Treatment	- 781.47	18.54	1	<0.001
	Random effects	*Std Dev*			
	Vials/block	*0.08*			
Female	Treatment	-433.36	0.64	1	0.42
	Random effects	*Std Dev*			
	Vials/block	*0.18*			
Male	Treatment	-247.71	32.37	1	<0.001
	Random effects	*Std Dev*			
	Vials/block	*0.009*			
***48-hours** post priming overall*	Treatment	-1050.2	25.73	1	<0.001
	Sex	- 1045.7	8.94	1	0.002
	Sex × Treatment	-1045.7	0.08	1	0.76
	Random effects	*Std Dev*			
	Vials/block	*0.004*			
Female	Treatment	-315.12	21.15	1	<0.001
	Random effects	*Std Dev*			
	Vials/block	*0.23*			
Male	Treatment	-239.08	15.63	1	<0.001
	Random effects	*Std Dev*			
	Vials/block	*0.15*			

***96-hours** post Priming overall*	Treatment	-647.58	36.35	1	<0.001
	Sex	-647.51	0.14	1	0.70
	Sex × Treatment	-647.50	0.006	1	0.93
	Random effects	*Std Dev*			
	Vials/block	0.15			
Female	Treatment	-511.02	14.63	1	<0.001
	Random effects	*Std Dev*			
	Vials/block	*0.009*			
Male	Treatment	-399.48	12.69	1	<0.001
	Random effects	*Std Dev*			
	Vials/block	*0.008*			
***168-hours** post Priming overall*	Treatment	-949.67	11.712	1	0.0006
	Sex	-948.59	2.1658	1	0.14
	Sex × Treatment	-946.20	4.7705	1	0.02
	Random effects	*Std Dev*			
	Vials/block	*0.009*			
Female	Treatment	-448.02	1.1289	1	0.28
	Random effects	*Std Dev*			
	Vials/block	*0.009*			
Male	Treatment	-371.76	13.381	1	<0.001
	Random effects	*Std Dev*			
	Vials/block	*0.009*			
***336-hours** post Priming overall*	Treatment	-1035.0	5.3983	1	0.02
	Sex	-1034.9	0.1854	1	0.66
	Sex × Treatment	-1033.5	2.7814	1	0.09
	Random effects	*Std Dev*			
	Vials/block	*0.004*			
Female	Treatment	-445.91	0.2168	1	0.64
	Random effects	*Std Dev*			
	Vials/block	*0.01*			
Male	Treatment	-448.08	8.345	1	0.003
	Random effects	*Std Dev*			
	Vials/block	*0.008*			

Table-SI-2. Summary of mixed effects Cox model, fitting the model to estimate priming response in different laboratory wild type w^{1118} male and female flies. We used data from the unprimed-infected and the primed-infected treatments and specified the model as: survival ~ treatment x sex x (1|vial/block), with treatment and sex as fixed effects, and vials nested within each block and as a random effect. The table shows model output (ANOVA) for priming in wild-type flies.

Fly strain	Source	loglik	χ2	Df	P
w^{1118}	Treatment	-647.58	36.35	1	<0.001
	Sex	-647.51	0.146	1	0.70
	Sex × Treatment	-647.50	0.007	1	0.93
	Random effects	*Std Dev*			
	Vials/block	*0.15*			
Canton-S	Treatment	-633.00	11.56	1	<0.001
	Sex	-619.47	27.04	1	<0.001
	Sex × Treatment	-618.39	2.174	1	0.14
	Random effects	*Std Dev*			
	Vials/block	*0.008*			
$OreR^{Wol+}$	Treatment	-436.34	0.455	1	0.49
	Sex	-436.04	0.607	1	0.43
	Sex × Treatment	-432.80	6.482	1	0.01
	Random effects	*Std Dev*			

	Vials/block	*0.17*			
OreR^{Wol-}	Treatment	-610.99	26.18	1	<0.001
	Sex	-609.91	2.171	1	0.14
	Sex × Treatment	-604.13	11.54	1	<0.001
	Random effects	*Std Dev*			
	Vials/block	*0.02*			

Table-SI-3. Summary of mixed effects Cox model, fitting the model to estimate the impact *Wolbachia* on immune priming response using wild-type OreR male and female flies. We used data from the unprimed-infected and the primed-infected treatments and specified the model as: survival ~ treatment x sex x *Wolbachia* status (1|vial/block), with treatment and sex as fixed effects, and vials nested within each block and as a random effect. The table shows model output (ANOVA) for priming in wild-type flies.

A. Impact of *Wolbachia*

Fly strain	Source	loglik	χ2	Df	P
OreR	Treatment	-1213.1	12.88	1	<0.001
	Sex	-1210.9	4.31	1	0.037
	Wolbachia status	-1197.4	27.06	1	<0.001
	Sex × Treatment	-1189.5	15.84	1	<0.001
	Treatment x *Wol* status	-1183.8	11.36	1	<0.001
	Sex x *Wol* status	-1183.4	0.69	1	0.40
	Sex x Treatment x *Wol* status	-1183.4	0.06	1	0.79
	Random effects	*Std Dev*			
	Vials/block	*0.004*			

B. ***Wolbachia* infection on males and females separately**

Sex	Source	loglik	χ2	Df	P
Female	Treatment	-514.76	0.10	1	0.74
	Wol status	-506.29	16.94	1	<0.001
	Treatment x *Wol* status	-503.72	5.13	1	0.02
	Random effects	*Std Dev*			
	Vials/block	0.008			
Male	Treatment	-535.18	23.90	1	<0.001
	Wol status	-529.14	12.07	1	<0.001
	Treatment x *Wol* status	-525.88	6.52	1	0.01
	Random effects	*Std Dev*			
	Vials/block	0.009			

Table-SI-4. Summary of mixed effects Cox model, fitting the model to estimate priming response in male and female wild type *w^{1118}*, *IMD* and *Toll* transgenic flies. We used data from the unprimed-infected and the primed-infected treatments and specified the model as: survival ~ Treatment x sex x (1|vial/block), with treatment and sex as fixed effects, and vials within a block as a random effect for each fly line. The table shows model output (ANOVA).

Fly strain	Source	loglik	χ2	Df	P
w^{1118}	Sex	-5144.0	39.56	1	<0.001
	Treatment	-5163.8	0.351	1	<0.001
	Sex × Treatment	-5143.5	0.921	1	0.34
	Random effects	*Std Dev*			
	Vials/block	*0.453*			
iso w^{1118}	Sex	-1597.3	6.734	1	0.009
	Treatment	-1594.0	47.70	1	<0.001
	Sex × Treatment	-1594.0	0.000	1	0.994

	Random effects	*Std Dev*			
	Vials/block	*4.1*			
Rel^{E20}	Sex	-1497.2	0.048	1	0.82
	Treatment	-1497.3	3.56	1	0.058
	Sex × Treatment	-1497.1	0.20	1	0.65
	Random effects	*Std Dev*			
	Vials/block	*0.26*			
Δ *AMPs*	Sex	-1605.6	0.263	1	0.60
	Treatment	-1605.7	1.920	1	0.16
	Sex × Treatment	-1604.9	1.451	1	0.22
	Random effects	*Std Dev*			
	Vials/block	*0.21*			
Group-B	Sex	-1537.3	2.950	1	0.08
	Treatment	-1538.8	0.299	1	0.58
	Sex × Treatment	-1536.9	0.771	1	0.37
	Random effects	*Std Dev*			
	Vials/block	*0.02*			
Dpt	Sex	-3386.1	24.49	1	<0.001
	Treatment	-3398.4	3.157	1	0.08
	Sex × Treatment	-3386.1	0.105	1	0.74
	Random effects	*Std Dev*			
	Vials/block	*0.03*			
Δ *AMPs*$^{+Dpt}$	Sex	-2596.1	34.22	1	<0.001
	Treatment	-2613.2	23.92	1	<0.001
	Sex × Treatment	-2580.5	31.10	1	<0.001
	Random effects	*Std Dev*			
	Vials/block	*3.73*			
Spz	Sex	-1755.6	14.86	1	<0.001
	Treatment	-1763.0	21.61	1	<0.001
	Sex × Treatment	-1755.4	0.298	1	0.58
	Random effects	*Std Dev*			
	Vials/block	*0.08*			

Table-SI-5. Summary of log_{10} transformed bacterial load data after 0.2 OD *P. rettgeri* infection, analysed using a non-parametric test for ANOVA (Kruskal-Wallis test) by fitting 'treatment', as fixed-effects for female and male wild-type w^{1118}.

Time	Sex	Source	Chi Sq.	Df	P
24-hours	*Female*	Treatment	3.2495	1	0.07
following infection	*Male*	Treatment	8.3974	1	0.003
72-hours	*Female*	Treatment	0.2102	1	0.64
following infection	*Male*	Treatment	1.8293	1	0.17

Table-SI-6. Summary of log_{10} transformed bacterial load data after 0.2 OD *P. rettgeri* infection, analysed using non-parametric ANOVA (K-W test) by fitting 'treatment' as categorical fixed-effects for male and females of each fly lines (wild-type w^{1118} and transgenic flies).

Fly line	Sex	Source	Chi Sq.	Df	P
w^{1118}	*Female*	Treatment	1.1165	1	0.29
	Male	Treatment	7.5172	1	0.006
Rel^{E20}	*Female*	Treatment	4.0368	1	0.044
	Male	Treatment	0.2516	1	0.61
Spz	*Female*	Treatment	0.0152	1	0.90
	Male	Treatment	0.0050	1	0.94
Δ *AMPs*	*Female*	Treatment	4.9868	1	0.025

	Male	Treatment	0.3014	1	0.58
Group-B	*Female*	Treatment	0.5612	1	0.45
	Male	Treatment	3.5402	1	0.059
Dpt	*Female*	Treatment	0.2848	1	0.59
	Male	Treatment	0.0020	1	0.96
Δ AMPs^{+Dpt}	*Female*	Treatment	0.1589	1	0.69
	Male	Treatment	11.2941	1	0.0008

Table-SI-7. Summary of mixed effects Cox prop-hazard, fitting the model to estimate strength of priming response in male and female wild-type w^{1118} and UAS^{-RNAi} tissue-specific mutants. We used data from the unprimed-infected and the primed-infected treatments and specified the model as: survival ~ Treatment x sex, with treatment and sex as fixed effects for each fly line. The table shows model output (ANOVA).

Fly strain	Source	χ2	Df	P
$w^{1118\text{-}iso}$	Sex	2.306	1	0.12
	Treatment	11.95	1	0.005
	Sex × Treatment	3.226	1	0.075
FB>DptB	Sex	1.996	1	0.15
	Treatment	0.977	1	0.32
	Sex × Treatment	0.068	1	0.79
HH>DptB	Sex	3.609	1	0.057
	Treatment	17.06	1	<0.001
	Sex × Treatment	0.602	1	0.43

Table-SI-8. Summary of $\log_{10}$ transformed ***Dpt*** gene expression data in wild-type w^{1118} flies after 0.2 OD *P. rettgeri* priming and challenge, analysed using ANOVA by fitting 'treatment' and 'sex' as categorical fixed-effects.

Condition	Source	F value	Df	P
Before secondary exposure (***Dpt***)	Sex	36.96	1	<0.001
	Treatment	5.295	1	<0.001
	Sex × Treatment	0.297	1	0.13
After secondary exposure (***Dpt***)	Sex	0.487	1	0.49
	Treatment	29.48	1	<0.001
	Sex × Treatment	2.465	1	0.13
AttC and ***Dro*** AMP genes expression after secondary exposure	AMP genes	98.53	1	<0.001
	Treatment	1.704	1	0.20
	Timepoint	29.51	1	<0.001
	Treatment x genes	0.725	1	0.40
	Treatment x timepoint	0.028	1	0.86
	Gene x timepoint	60.07	1	<0.001
	Treatment x gene x timepoint	0.323	1	0.57

Table-SI-9. Summary of mixed effects Cox model, fitting the model to estimate priming response in male and female PGRP mutant flies. We used data from the unprimed-infected and the primed-infected treatments and specified the model as: survival ~ Treatment x sex x (1|vial), with treatment and sex as fixed effects, and vials within a block as a random effect for each fly line. The table shows model output (ANOVA).

Fly strain	Source	loglik	χ2	Df	P

PGRP-LB	Treatment	-818.79	-0.0048	1	1.00
	Sex	-818.13	1.3085	1	0.25
	Sex × Treatment	-817.98	0.3047	1	0.58
	Random effects	*Std Dev*			
	Vials/block	*9.011949e-03*			
PGRP-LC	Treatment	-891.65	0.0189	1	0.89
	Sex	-891.65	0.0057	1	0.96
	Sex × Treatment	-891.64	0.0068	1	0.93
	Random effects	*Std Dev*			
	Vials/block	*9.281555e-03*			
PGRP-LE	Treatment	-928.63	0.2609	1	0.60
	Sex	-928.31	0.6362	1	0.42
	Sex × Treatment	-928.31	0.0036	1	0.95
	Random effects	*Std Dev*			
	Vials/block	*0.17*			

Table-SI-10. Summary of log_{10} transformed bacterial load data after 0.2 OD *P. rettgeri* infection, analysed using non-parametric ANOVA (Kruskal-Wallis test) by fitting 'treatment' as categorical fixed-effects for male and females of PGRP mutants.

Fly line	Sex	Source	Chi Sq.	Df	P
PGRP-LB	*Female*	Treatment	0.4300	1	0.51
	Male	Treatment	0.3295	1	0.56
PGRP-LC	*Female*	Treatment	0.6063	1	0.43
	Male	Treatment	0.2020	1	0.65
PGRP-LE	*Female*	Treatment	0.0643	1	0.79
	Male	Treatment	0.0813	1	0.77

Chapter 6

Immune priming reduces pathogen transmission

Keywords: Drosophila activity monitor, immune dimorphism, immune priming, infection routes, locomotor activity, pathogen spread and transmission

6.1 *Introduction*

One of the most successful public health practices to reduce the incidence of infectious diseases is prophylactic immunization using attenuated or inactivated pathogens (Pollard and Bijker, 2021). Immunisation works because humans and other vertebrate animals have evolved a sophisticated acquired immune response capable of specific immune memory, which ensures that the response to a secondary exposure following immunization is strong, precise, and effective (Boehm and Swann, 2014). Invertebrates have not evolved the same immune mechanisms, leading to a longstanding assumption that immune memory should not be possible in invertebrates(Hauton and Smith, 2007). This view has shifted in recent years, with evidence of immune priming across a range of invertebrate taxa (Little et al., 2003; Schmid-Hempel, 2005; Sadd et al., 2005; Roth et al., 2009; Masri and Cremer, 2014; Prakash and Khan, 2022). The mechanisms underlying these responses are diverse and often specific to the type of pathogen, but all result in improved survival to a pathogenic infection after prior exposure to an attenuated, inactivated version or low doses of the same pathogen.

We have previously (in *chapter-5*) shown that *Drosophila melanogaster* fruit flies show an immune priming response to infection by the Gram-negative pathogen *Providencia rettgeri*. Flies exposed to a heat-killed inoculum of *P. rettgeri* show increased survival and a transient increase in bacterial clearance upon a secondary lethal exposure, mediated by the regulation of the IMD-responsive antimicrobial peptide *Diptericin*. While primed individuals may live longer, thus extending the infectious period, their pathogen burden may be lower, which could lead to less severe epidemics. The effects of immune priming on pathogen spread are therefore difficult to predict. Here we investigated the consequences immune for pathogen transmission. We find that priming reduces pathogen transmission by directly decreasing bacterial shedding from infectious flies.

Variation in the incidence of infectious disease can arise from individual differences in the exposure to infection and the susceptibility to infection. The likelihood of exposure will be mainly affected by host behaviours that affect the contact rate between susceptible and infectious individuals. Variation in susceptibility will affect the likelihood that hosts acquire the infection upon exposure, and also their ability to clear the pathogen. Pathogen clearance is a key parameter for transmission dynamics, as it will determine not only the duration of the infectious period, but also how much pathogen individuals shed into the environment while they are infectious, both of which can contribute to variation in pathogen exposure. We therefore designed our experimental infections to enable us to test how immune priming could

affect each of these behavioural and immunological components of pathogen spread (see ***Fig. 1***).

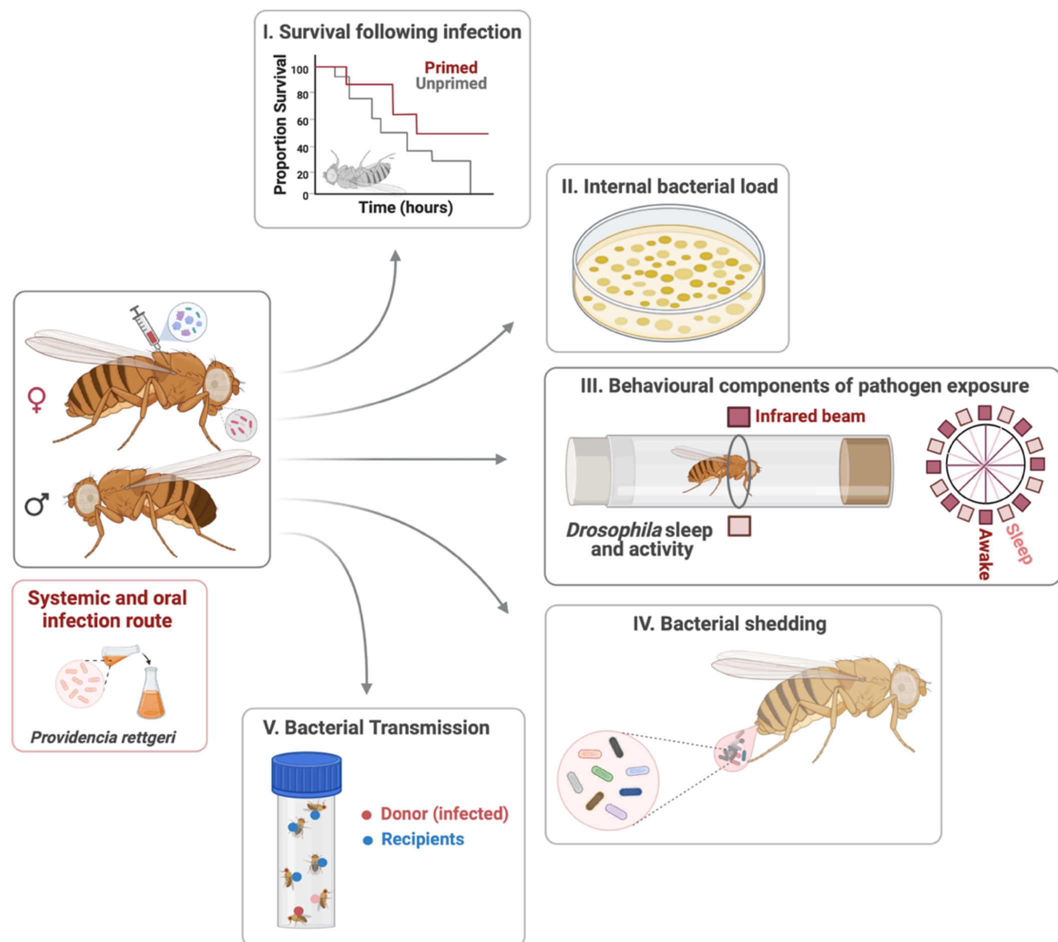

Figure 1: Experimental design to measure epidemiological components following systemic (OD_{600}=0.75) and oral (OD_{600}=25) priming and infection with initial heat-killed exposure followed by live *P. rettgeri* in male and female wildtype w^{1118} flies. The assays include (I). survival following different infection routes (II). internal bacterial load (III). behavioural components of pathogen exposure such as sleep and awake activity (IV). bacteria shedding and (V) transmission. n=6-7 vials of 8-12 flies in each vial, for each treatment and sex combination.

6.2 *Materials and methods*

1. *Fly rearing conditions:* We used males and females of wild type flies (w^{1118}) to test whether how immune priming affects epidemiological parameters such as pathogen shedding and transmission following bacterial *P. rettgeri* infection. Flies were reared in plastic vials (12 cm) on a standard diet of Lewis medium at 25±1°C with a 12 light: 12-day cycle with stocks tipped into new vials containing fresh Lewis's food every 14 days. We maintained at low density (around 10 flies per vial) for at least two generations. We used 3-5 day young adult flies for all our assays. We housed 2 males and 5 females for egg laying (48-hours) onto 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 (3-5 days).

2. <u>*Bacterial culture preparation*</u>: We used a gram-negative bacteria *Providencia rettgeri*, naturally found in soil and aquatic environments (Khalil et al., 2015) for our study. We cultured overnight using 25% glycerol stocks (-80°C) in Luria broth (LB) at 37°C and 140rpm in an orbital shaker. Upon reaching 0.95 OD_{600} we pelleted the culture at 5000rpm for 5 mins at 4°C. Then we discarded the supernatant and resuspended the bacteria in 1xPBS (phosphate buffer saline) and 5% sucrose solution adjusted the infection doses for both systemic and oral infection assays.

3. <u>*Systemic priming and live infection*</u>: For systemic priming and live infection, we prepared the final infection dose of OD_{600}=0.75 (~45 cells/fly) using sterile 1xPBS. We first obtained heat-killed bacteria by incubating the previously obtained bacterial slurry at 90°C for 20-30 mins using the heat/dry-block (Khan et al., 2016). To ensure all bacteria were dead the heat-killed culture was plated and no growth was confirmed. We initially primed the 3-5 day old adults (females and males) by pricking with a 0.14-mm minutien pin (Fine Science Tools) dipped in heat-killed bacteria and for unprimed treatment we used 1xPBS solution. After a 3-days gap to recover from the injury, we pricked (challenged) the flies, this time with live OD_{600}=0.75 *P. rettgeri* bacteria for each the sex and treatment combination. We use individuals that were only heat-killed without any live bacterial exposure as controls and scored the flies for survival (next 8 days).

4. <u>*Oral priming and live infection*</u>: For oral priming and live infection, we adjusted the final concentration to OD_{600}=25. We initially prepared vials for oral priming by pipetting 350-400 µl of standard agar [see (Siva-Jothy et al., 2018)] onto lid of a 7ml tubes (bijou vials) and allowed it to dry. Simultaneously, we starved the experimental flies on 12ml agar vials for 4 hours. Once agar on bijou (vial) lids dried, we placed a small filter disc (Whattmann-10) in the lid and pipetted 80µl of heat-killed bacterial culture (primed treatment) or 5% sucrose solution (unprimed treatment) directly onto the filter disc. Once the agar dried, we orally exposed flies (heal-killed only, primed and unprimed treatment) by adding approximately 10 flies per vial for around 18-hours and then transferred the flies on to fresh Lewis food vials. After 3-days gap (*to recover from the initial heat-killed exposure*), we again prepared the bijou vials and once the agar dried, this time we added 80µl of live bacterial culture (OD_{600}=25) and exposed flies (heat-killed

only, primed, and unprimed individuals) to live *P. rettgeri* for 18-hours. We then transferred flies on to fresh food vials and observed survival after oral exposure to *P. rettgeri* for the next 8 days.

5. *Measuring Drosophila activity:* We measured the locomotor activity of single flies (for each sex, treatment, and infection route combination) during three continuous days using a *Drosophila* Activity Monitor – DAM (v2 and v5) System (Pfeiffenberger et al., 2010), in an insect incubator maintained at 25°C ± 1°C in a 12 D: 12 L cycle. We then processed the raw activity data using the DAM System File Scan Software (Pfeiffenberger et al., 2010), and the resulting data were arranged using Microsoft Excel before analysis. We analysed the fly activity data using three metrics: total activity, the average awake activity and proportion of time spent asleep.

6. *Measuring internal bacterial load*: To test whether the host's ability to supress bacterial growth varies across primed and unprimed individuals we quantified bacterial load at 24-hours after *P. rettgeri* infection for males and females of w^{1118} wild type flies. Initially, flies were surface sterilized in groups of 3-5 flies per vial in 70% ethanol for 30 seconds and washed twice and with distilled water before homogenising flies individually using micro pestles. We immediately plated this homogenate in serial dilutions using 1xPBS on to LB agar plates and cultured at 29°C. The following day (after 18-hours), we counted the resultant bacterial colonies manually (n=9-13 vial/sex/treatment).

7. *Measuring bacterial shedding*: We measured the bacterial shedding of single flies at a single time point, 4-hours following the oral bacterial exposure (that is, 18-hours of oral exposure, immediately followed by transfer to fresh tubes for 4-hours for shedding). Following oral priming and live infection with *P. rettgeri*, single flies were placed into 1.5ml Eppendorf tubes with approximately 50µl of Lewis medium in the bottom of the tube. To measure bacterial shedding, we transferred the male and female flies (primed-infected and unprimed-infected) to tubes for 4-hours, immediately following 18 hours after oral exposure. After 4-hours, flies were removed and homogenised in 50µl of 1xPBS, tubes were also washed out with 50µl of buffer by vortexing. We plated these samples on a LB agar plates, incubated them at 29°C and counted the colonies manually after 18-hours. n = 8-12 flies per treatment and sex combination

8. *Measuring bacterial transmission*: We measured transmission by collecting age-matched donor and recipient w^{1118} flies for each sex combination. We initially exposed 3-day old

w^{1118} flies with either heat-killed (primed) or 5% sucrose only (unprimed). After 96-hour gap we divided each of the treatment groups (primed and unprimed) into donor and recipient groups for each sex combination. The donor flies were exposed to OD_{600}=25 of *P. rettgeri* (*see section 3 and 4 above*). The infected donors were marked by cutting the corner of a fly wing (*unpublished work from Vale lab*). We then placed 1-donor and 5-recipient flies in 7ml bijou vials with a small amount of Lewis food on the lid for each treatment (heat-killed bacteria only and 5% sucrose exposed without live infection) and sex-combination. It is noteworthy that previous *Drosophila* studies constituted 10-recipient and 2-donor flies in a vial or plate for measuring epidemics (Siva-Jothy and Vale, 2021). Due to logistical constraints the earlier design was modified to 1-donor and 5-recipient flies. After 4-hours exposure we measured bacterial load for both donor and recipient flies using sterile 1xPBS [see (Siva-Jothy et al., 2018)].

9. *Statistical analysis*: All our survival, bacterial load, bacterial shedding, and transmission data were non-normally distributed (tested using Shapiro-Wilks test for normality) hence we performed non-parametric tests. We performed Cox-proportional hazard for survival, and non-parametric Wilcoxon Kruskal-Wallis tests for bacterial load measurement, bacterial shedding, and transmission data. We tested how immune priming could affect each of these behavioural and immunological components of pathogen spread survival after priming, locomotor activity and pathogen shedding in males and female w^{1118} flies. We used a full factorial three-way interaction between infection status (heat-killed only/primed/unprimed), sex (male/female) for wild type w^{1118} flies, all modelled as fixed effects using a generalized linear model (GLM). To assess locomotor activity, we analysed all three response variables separately for systemic and oral infection routes (total locomotor activity, proportion of awake activity and time asleep). All statistical analyses and graphics were carried out and produced in R using the ggplot2, coxhz and lme4 packages (Wickham, 2016; Therneau, 2015; Bates et al., 2014).

6.3 Results and Discussion

1. *Immune priming affects the duration of infection and pathogen clearance during systemic and oral bacterial infection*

Following an initial 'priming' treatment consisting of an exposure to a heat-killed *P. rettgeri* culture, after a 72-hour period we exposed female and male wildtype w^{1118} flies to a lethal

dose of live *P. rettgeri*. This time period was chosen because we had previously observed successful immune priming within 72-96 hours following the initial 'priming' treatment, and that in males this response was still strong even a week after the initial exposure. While these previous results all related to systemic infections, the oral-faecal nature of bacterial transmission required that we also investigate immune priming under oral infection. We found that primed w^{1118} males, but not females, showed increased survival after initial priming to both systemic and oral route of infection. (***Fig.*** *2A,* ***Table 1***).

To test whether the increased survival after priming was due to increased ability for pathogen clearance, we measured internal bacterial loads around 24-hours following infection for in both systemic and oral infections. Consistent with the observed pattern of survival, primed males showed decreased internal bacterial loads in both infection routes but priming in female flies did not result in any difference in bacterial loads (***Fig.*** *2B,* ***Table*** *2*). These results are generally consistent with our previous observations of immune priming during systemic infection, and also occur during oral infection. In the context of disease transmission, our results during oral infection suggest that immune priming can affect the duration of infection, and the ability to clear infection.

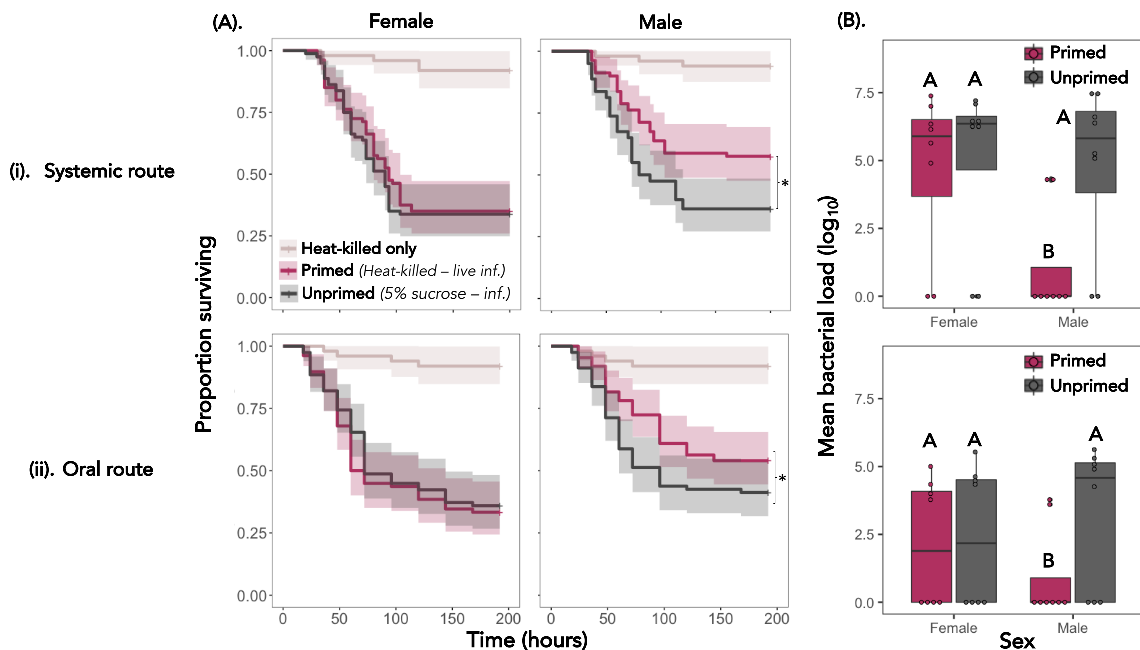

Figure 2: **(A)** Survival curves of male and female wildtype (w^{1118}) flies with (i) systemic (ii) oral priming where flies initially exposed to heat-killed *P. rettgeri* or unprimed flies exposed to a sterile solution in the first exposure, this is followed by live *P. rettgeri* pathogen after 72-hour time gap [systemic infection dose = 0.75 OD (~45 cells/fly) and oral *P. rettgeri* infection dose = 25 OD_{600}]. (n= 7-9 vials of 8-12 flies in each vial/sex/treatment/infection route) **(B).** Mean bacterial load measured as colony forming units around 24 hours following (i) systemic and

(ii) oral priming, followed by live *P. rettgeri* infection for male and female w^{1118}. Different letters in panel-B denotes primed and unprimed individuals are significantly different, tested using Tukey's HSD pairwise comparisons.

Table 1: Summary of Cox prop-hazard model, for female and male flies of wild type w1118. We used data from 3 to 7-day adult male and females infected with OD_{600} = 0.75 dose for systemic and dose OD_{600} = 25 for oral priming and infection with *P. rettegeri*. We specified the models as survival ~ sex x treatment with 'treatment', and 'sex' as fixed effects. Table shows model output (ANOVA) for survival post-infection for male and female w^{1118} flies.

Response	*Predictor*	*df*	*Chi sq*	*p*
Systemic Infection route	Sex	1	1.383	0.23
	Treatment	2	100.7	<0.001
	Sex x Treatment	2	3.103	0.21
Oral Infection route	Sex	1	0.728	0.39
	Treatment	2	85.76	<0.001
	Sex x Treatment	2	3.421	0.180

***Table 2*:** Summary of log transformed bacterial load data after OD_{600}=0.75 systemic and OD_{600}=25 oral *P. rettgeri* systemic infection for male and female w^{1118} flies analysed using a non-parametric Wilcoxon (Kruskal-Wallis) test by fitting 'Sex' as categorical fixed-effects after 24-hours post systemic and oral priming and infection.

Response	*Sex*	*Predictor*	*Chi sq*	*df*	*p*
Systemic route	Female	Treatment	0.403	1	0.52
	Male	Treatment	6.090	1	0.01
Oral route	Female	Treatment	0.255	1	0.61
	Male	Treatment	3.870	1	0.04

2. *Immune priming does not affect behavioural components of pathogen exposure*

One of the simplest behavioural readouts that is relevant for how frequently individuals may come into contact is locomotor activity. Highly active individuals are more likely to contact conspecifics and therefore are more likely to spread and to acquire new infections. How much an individual moves throughout the day is determined not only by its level of activity while awake, but also by the fraction of the day it is not moving for example, while asleep. A crucial point is that host behaviours are often modified by infection, or even by immune stimulation that mimics infection.

We therefore tested how immune priming might alter host behaviours such locomotor activity and sleep that may contribute to the rate of contact between individuals. Overall, we found clear effects of the route of infection on the total locomotor activity, average awake activity the proportion of time spent asleep. Orally infected flies did not show any change in

their behavioural phenotypes when exposed to a heat-killed bacterial inoculum only, or even when exposed to a lethal inoculum. In systemically infected flies, exposure to a lethal bacterial inoculum resulted a reduction in activity and increase in the time spent sleeping, but these effects were not different between primed and unprimed individuals (***Fig.*** *3*, ***Table*** *3*). In the context of the effect of priming on disease transmission, immune priming during oral infection would appear to not be a substantial source of variation in a fly's behavioural competence for pathogen transmission.

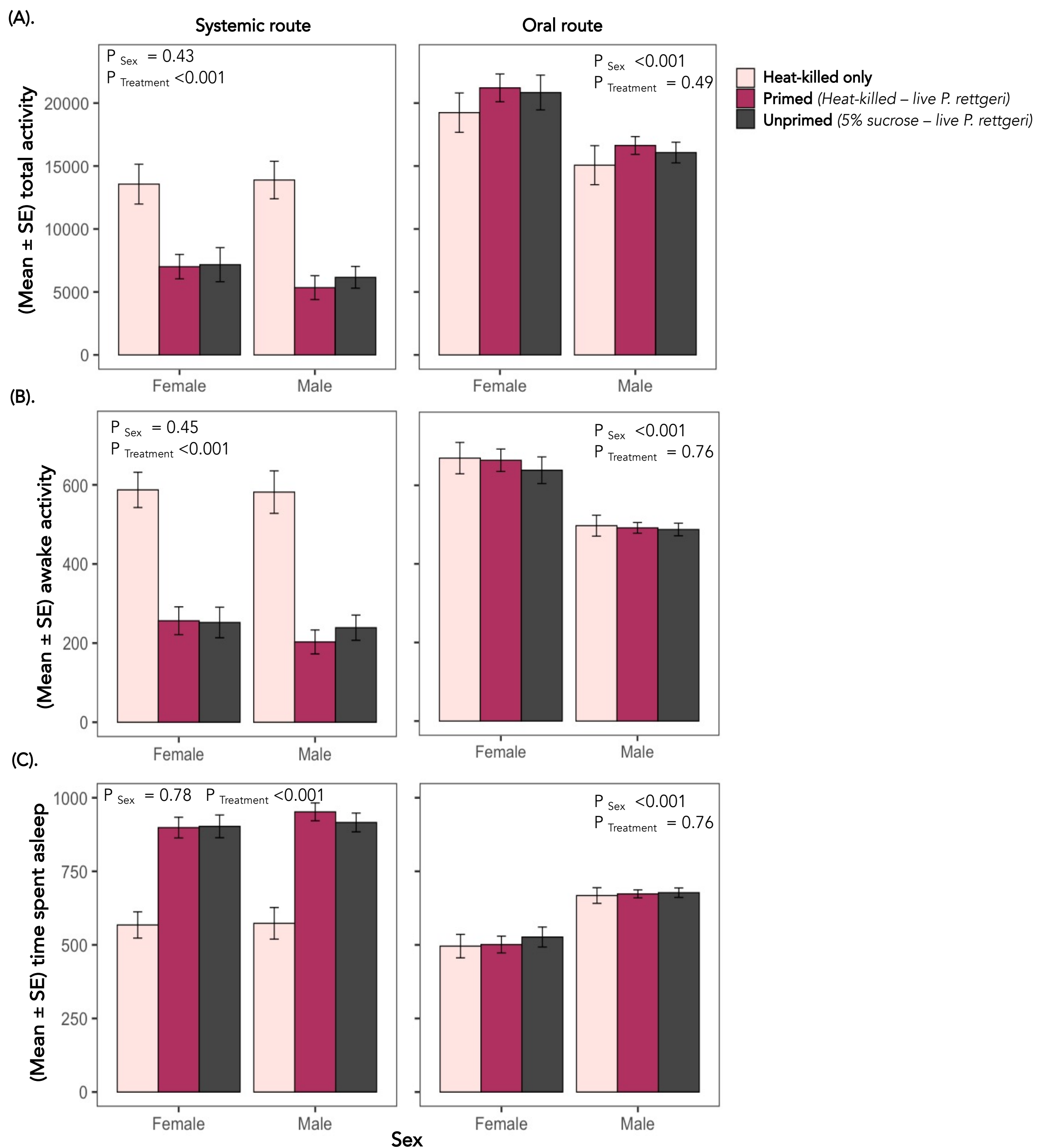

Figure 3: Mean ±SE total locomotor activity for males and females, during first 72-hours following systemic and oral infection with live *P. rettgeri*. **(A)** average total locomotor activity **(B)** average awake activity and **(C)** proportion of flies spent sleeping (n= 36-64 flies per treatment and sex combinations).

Table 3: Model outputs for statistical test (GLM) performed on host activity data, that is, locomotor activity, sleep patterns and average awake activity in males and females of w^{1118} during systemic and oral priming and infection respectively.

Infection	*Response*	*Predictor*	*df*	*F ratio*	*p*
Oral	*Total activity*	Sex	1	16.82	<0.001
		Treatment	2	0.711	0.49
		Sex x Treat	2	0.020	0.97
	Awake activity	Sex	1	40.86	<0.001
		Treatment	2	0.269	0.76
		Sex x Treat	2	0.112	0.89
	Time asleep	Sex	1	40.86	<0.001
		Treatment	2	0.269	0.76
		Sex x Treat	2	0.112	0.89
Systemic	*Total activity*	Sex	1	0.612	0.43
		Treatment	2	19.18	<0.001
		Sex x Treat	2	0.294	0.74
	Awake activity	Sex	1	0.555	0.45
		Treatment	2	41.03	<0.001
		Sex x Treat	2	0.238	0.78
	Time asleep	Sex	1	0.555	0.45
		Treatment	2	41.03	<0.001
		Sex x Treat	2	0.238	0.78

3. *Priming during oral exposure reduces bacterial shedding and transmission in males*

We proceeded with our examination of - apart from increasing survival during infections, immune priming may also have consequences for pathogen spread and transmission (Tate, 2016). Despite having enhanced survival, the primed individuals may also extend the infectious period, their pathogen burden may be lower, which would lead to less severe epidemics (Tate, 2016). To investigate how immune priming affects some of these epidemiological parameters we tested whether primed individuals have reduced bacterial spread. We measured the shedding of single flies at a single time point, 4-hours after infection (that is, initial heat-killed exposure followed by live *P. rettgeri* exposure). We found that primed male flies shed less bacteria than unprimed flies. However, both primed and unprimed females showed increased bacterial shedding following oral *P. rettgeri* infection compared primed males (***Fig.** 4A,* ***Table** 4*). Another possibility is that priming might lead to more severe epidemics – especially if a lower level of shedding is extended over a sufficiently long period of time. Then, the net outcome could also depend on factors, such as exposure patterns (various time points) and dose-response relationships (infection doses). Future studies should consider these parameters

in the study design. However, we only measured bacterial shedding at a single infection dose and time point - we chose this based on our previous unpublished results in Vale the lab.

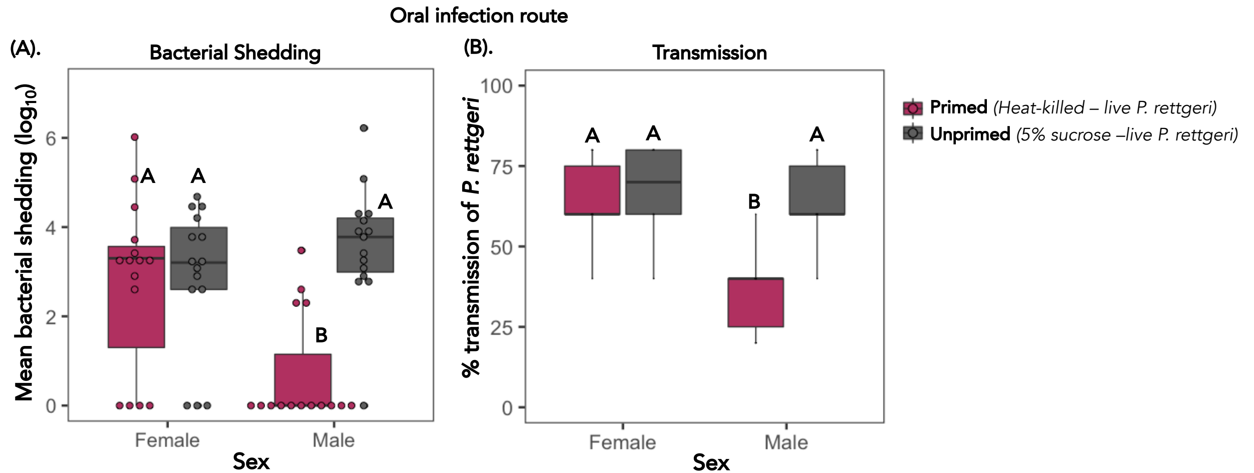

Figure 4: (A). Bacterial shedding after 4-hours following oral priming (initial heat-killed exposure) and infection with live *P. rettgeri* (OD_{600}=25) for males and females. (B) percentage transmission of measurable bacterial loads for male and female w^{1118} flies (recipient-flies) over the first 4-hours following exposure with infected donor flies. Different letters in panel-B denotes primed and unprimed individuals are significantly different, tested using Tukey's HSD pairwise comparisons.

Table 4: Summary of non-parametric Wilcoxon (Kruskal-Wallis) test for oral bacterial shedding (log transformed bacterial load) after 4-hours of oral priming and infection with *P. rettgeri* OD_{600}=25. Bacterial transmission (percentage of recipient flies with measurable bacterial load) after 4-hours of exposure with infection (donor) w^{1118} after oral priming and infection.

Response	*Sex*	*Predictor*	*Chi sq*	*df*	*p*
Bacterial Shedding	Female	Treatment	0.003	1	0.95
	Male	Treatment	17.29	1	<0.001
Transmission	Female	Treatment	0.187	1	0.66
	Male	Treatment	5.444	1	0.019

Our bacterial shedding results indicates that primed males with initial non-lethal exposure shed very little bacteria after re-infection with oral exposure. More recently, it has been shown that during viral infection, both host's genetic background and sex-specific variation can affect different components of disease transmission (Siva-Jothy and Vale, 2021). The authors showed that most infected individuals produced fewer secondary infection cases of while non-infectious individuals were at high risk of disease out-break (Siva-Jothy and Vale, 2021). This result motivated us to measure pathogen transmission potential during oral bacterial infection, primed males spread or transmit very little pathogen upon re-infection with oral *P. rettgeri* (***Fig.*** *4B,* ***Table*** *4*). However, both primed and unprimed females showed increased bacterial spread following oral *P. rettgeri* infection compared to primed males (***Fig.***

4B, ***Table** 4*). Although we did not measure directly measure pathogen transmission rate our results provide substantial evidence that immune priming reduces pathogen transmission by directly decreasing bacterial shedding from infectious flies.

6.5 *Conclusion*

In summary, we addressed important implications of immune priming for disease ecology and epidemiology and particularly tested how priming may affect pathogen transmission. Our results indicate that immune priming reduces pathogen transmission by directly decreasing bacterial shedding from infectious flies. We provide experimental evidence how immune priming reduces pathogen shedding at the individual level, which in turn may result in reducing disease transmission at the population level. Lastly, a better understanding of how immune priming contributes to host heterogeneity in disease outcomes would aid our understanding of the causes and consequences of variation in infectious disease dynamics.

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Chapter 7

General discussion

7.1 ***Overview***

The fruit fly, *Drosophila melanogaster*, is a powerful and well-established model organism to study several fundamental processes of innate immunity (Buchon et al., 2014; Mirzoyan et al., 2019; Troha and Buchon, 2019). Over the last few decades, fruit flies have been instrumental in the characterization of major hosts immune responses and defense strategies to infections including resistance, tolerance, damage repair processes, immunopathology etc. While mechanisms of pathogen elimination such as resistance to infections, have been well documented, mechanisms of disease tolerance and damage repair processes remain poorly elucidated. This thesis has been centred on discovering novel aspects of innate immune regulation of disease tolerance and damage limitation mechanisms in males and females of the fruit fly *Drosophila melanogaster*. In the following section, in general, we discuss our key findings, and implications of the thesis (see ***section*** *7.2*). We follow this up with some of the caveats associated with this thesis in a separate ***section*** (*7.3*). Finally, we provide some of the exciting possibilities in the form of fundamental open questions in ***section*** (*7.4*) and future prospects (***section*** *7.5*), in the light of recently published literature.

In the introductory ***chapter 1***, we first present, why understanding health promoting mechanisms such as disease tolerance and damage repair processes is important. Using several *Drosophila* fly lines, their natural bacterial pathogens and by employing candidate gene approach, we show that in results ***chapter 2***, the negative immune regulators (*necessary for dampening the immune responses when infection threat is reduced*) of the *Drosophila* IMD-pathway (*caudal* and *pirk*), are important regulators not only for the previously described pathogen clearance, but also important for disease tolerance during systemic bacterial infection. In ***chapter 3***, we show how the damage prevention and repair mechanism in gut mediate disease tolerance during enteric bacterial infections. The experiments of ***chapter 4***, suggest that during systemic bacterial infections the *Jak/Stat* pathway mediates disease tolerance phenotype. Overall, our study uncovers substantial sexual dimorphism in damage sensing and repair processes during disease tolerance to bacterial infections.

In ***chapter 5***, we find that priming in *Drosophila* is a long-lasting response, occurring in several genetic backgrounds and is particularly stronger in male flies. Mechanistically, we show that the enhanced survival of individuals that are primed with initial heat-killed inoculum is likely driven by the IMD-responsive antimicrobial-peptide *Diptericin-B* in the fat body through the regulation of *PGRPs* (*LB, LC* and *LE* -peptidoglycan receptor proteins). In the final results

chapter 6, we further show that immune priming has the potential to reduce disease transmission by affecting pathogen shedding in *Drosophila*.

7.2 *Key findings and implications*

In the section below, we discuss some of the major findings of this thesis and their broader implications on our understanding of disease tolerance during bacterial infections. While genetic variations to resistance mechanisms that eliminate pathogens have been well documented in several insect species, very few studies have addressed the mechanisms that prevent or repair tissue damage arising from infection. We currently know less about how the damage limitation mechanisms vary among individuals of different genotypes and sexes. This thesis has demonstrated the potential importance of damage-repair mechanisms, and negative immune regulators in disease tolerance and limiting the tissue damage. Our work also contributes to the emerging work on sexually dimorphic immune responses in the context of disease tolerance and damage limitation mechanisms against bacterial pathogens. In addition, we also present the evidence that the enhanced survival after priming with heat-killed exposure in *Drosophila* is likely driven by the IMD-responsive antimicrobial-peptide *Diptericin-B* in the fat body through PGRP (*LB, LC* and *LE*) regulation. We further show the potential implication of immune priming for pathogen transmission against bacterial diseases.

This thesis has following major implications: -

1. *Immune signalling pathways contribute to disease tolerance*: Previous studies on disease tolerance have used global profiling such as Transcriptomics, GWAS (genome wide association studies), and microarrays to identify the candidate genes associated with disease tolerance phenotypes and immune priming. In this thesis, we took a more targeted approach to specifically investigate the mechanistic basis of disease tolerance and priming through candidate gene approach. This distinction is important because the previously identified several genes and candidates of disease tolerance do not seem to be directly linked with traditional or canonical immune responses. Though these studies provide early evidence on how some of these candidates may impact diseases tolerance, the extent to which they affect disease tolerance in hosts remain unclear. By taking the target specific approach we provide evidence for negative immune regulators of the best-studied innate immunological signal and control pathway in *Drosophila* the IMD-pathway apart from the previously known role in pathogen clearance, some of these negative immune regulators also contribute to disease tolerance.

2. *Potential therapeutic target to enhance host disease tolerance*: By highlighting that some of the mechanisms of tissue damage sensing, prevention, repair process and negative immune regulators of well-defined immune signalling pathways are important to disease tolerance, it may therefore be possible to use these as potential candidates for drug targets and treatments to improve host tolerance of infection (Vale et al., 2016). For instance, the observations from human infection studies suggest that the drugs that target the cytokine-dependent pathways, are effective in treating several autoimmune diseases and also promising candidates in the context of cancer therapy (Salas et al., 2020). Our results highlight that the mechanisms of damage sensing, control, prevention and renewal mediate disease tolerance phenotype and provide a more refined understanding of tolerance mechanisms. Perhaps, , it may therefore be possible to use these target mechanisms of tissue-damage prevention and repair, to repurpose some of the existing drugs and promote health.
3. *Pathogen transmission*: Our priming results suggest some of the important implications of priming in disease epidemiology. Particularly, how immune priming may affect pathogen transmission and disease spread. Primed with enhanced survival after the initial sub-lethal or heat-killed exposure are likely to become less infectious upon subsequent infections that is, likely to shed or transmit less pathogens. Apart from increasing survival following infection, immune priming may also have consequences for pathogen transmission. The epidemiological models have incorporated immune priming interactions with disease transmission (Tate, 2016). How does immune priming act on various epidemiological parameters? Do primed individuals (i) simply reduce pathogen susceptibility or have enhanced pathogen killing ability? (ii) induce reduced pathology with a lower transmission rate? (Tate, 2016). Apart from increasing survival following infection, priming may have consequence for pathogen spread and transmission. While primed individuals live longer, thus extending the infectious period, on the other hand, we show that priming reduces microbial loads and shedding, which would affect disease transmission and lead to less severe epidemics.
4. The results from this thesis provide evidence that – in fruit flies, during gram negative bacterial infections, negative immune regulation of IMD, Jak/Stat signalling, and damage prevention and repair mechanisms all affect disease

tolerance. On the other immune priming that is, increased survival in fruit flies following gram negative bacterial infection, is due to a transient increase in bacterial clearance upon a secondary lethal exposure, mediated by the regulation of the IMD-responsive antimicrobial peptide *Diptericin*. Further, priming reduces pathogen transmission by directly decreasing bacterial shedding from infectious fruit flies. However, we still need further experimental evidence especially during other pathogens (for instance gram-negative bacteria, viral and fungal infections) to draw general conclusions.

7.3 *Limitations*

The work presented in this thesis provides a number of useful insights to understanding of disease tolerance and damage limitation mechanisms, however it is important to recognise some of the caveats associated with this thesis work. Here, I summarise the main limitations that should be addressed by future research.

7.3.1 *Leakiness of the immune mutants*: The targeted gene expression technology in *Drosophila* such as the GA4-upstream activator sequence (UAS) system (adapted from yeasts; (Brand and Perrimon, 1993)) is a powerful approach to understanding the function of several specific genes, cells, and tissues (McGuire et al., 2004). This system utilizes a specific promoter (also known as endogenous enhancer) to direct the expression of the yeast transcriptional activator GAL4 in a spatially restricted fashion (or temperature sensitive GAL80). The GAL4 trans activator then drives the expression of the gene of interest that has been downstream of a UAS binding site. Also known as the "GAL4 driver".

Both the "driver" (that is, transcriptional activator which can be ubiquitous or tissue specific) and the "target" (transgene) are usually carried in different parental lines. This ensures the viability (gene of interest) and enabling a combinatorial approach with different driver and target lines. Once generated, a line expressing GAL4 in each spatial pattern can be crossed with any UAS target line, allowing the GAL4 line to be used as a general resource. Similarly, when a given UAS target line is generated, the target gene can be transcribed anywhere in the fly by crossing it to the appropriate GAL4 line. One drawback of the GAL4-UAS system is the lack of defined temporal control over the expression of the target transgene (leakiness). The UAS-RNAi fly lines used in *Chapter-2* had about 32-40% leakiness (*data not shown*). Another caveat associated with using the temperature sensitive UAS-GAL80 system in ***chapter-2***, to address how the negative IMD pathway regulators affect disease tolerance is that we found 15-20% mortality in mutant fly lines before systemic bacterial infections, because flies need to

be incubated at 29°C for few hours for the target gene to be transcribed under GAL80 system. A future solution to this issue would be to employ newer alternative gene expression systems, including the Gene-switch system using the hormone-inducible tetracycline drug with minimal leakiness (McGuire et al., 2004) among others.

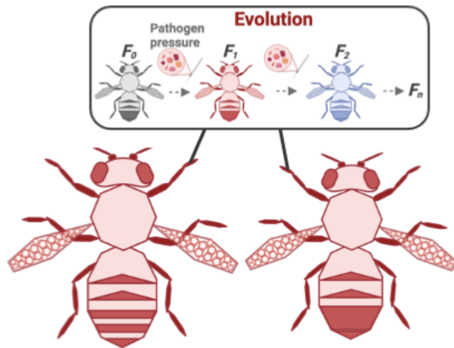
7.3.2 *Life-history fitness trade-offs*: From infection and immunity context, fitness costs can be detrimental to life-history traits, such as reproductive success, growth/development rate etc., since the energy allocations required for the major physiological processes are being diverted towards the immune system (Schwenke et al., 2016). However, these costs can be categorised into two kinds: maintenance costs (energy required for the constitutive maintenance of the pathways) and deployment costs (that is, costs arising from the activation of signalling cascades and production of immune effectors such as AMPs) (McKean et al., 2008; Schwenke et al., 2016; Nystrand and Dowling, 2020). We hope future *Drosophila* studies addresses how fitness costs, associated with not just with innate immune signalling pathways such as IMD and Toll-activation, but also their downstream inducible AMPs and damage-repair process etc. The results from addressing these fitness costs would deepen our understanding of life-history trade-offs in *Drosophila melanogaster*.

7.3.3 *Ecological factors such as age and mating status:* In most animals, ageing is associated with a decline in immune function (also known as immunosenescence) (DeVeale et al., 2004). Several studies have addressed how age-related inflammation and immune function decline make individuals more susceptible to infections and the response varies between sexes (Hamilton, 1966; Rera et al., 2012; Regan et al., 2016; Badinloo et al., 2018). In several animals, mating typically decreases immune function (Fowler and Partridge, 1989; Rolff and Siva-Jothy, 2002). The immune system is energy demanding, therefore relies on reallocation of resources for combating pathogens (Schwenke et al., 2016). However, we only considered young and mated individuals throughout out study. While mating reduces immune function, from our preliminary experiments (*data not shown*) we did not find any difference in survival post-infection (for the infection doses we used).

7.4 *Fundamental open questions*

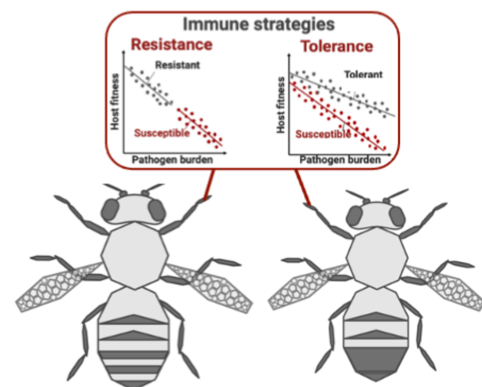
Mechanisms that improve health such as disease tolerance and damage repair processes that are aimed at resolution of infection-induced inflammation may offer potentially novel therapeutic targets to treat diseases (Sears et al., 2011; Soares et al., 2014; Vale et al., 2014, 2016; Soares et al., 2017; Martins et al., 2019). Although, some of these damage limiting

mechanisms could also be linked directly with other host defense mechanisms such as resistance, leading to unpredictable disease outcomes. Therefore, we are emphasising the need for careful blending of evolutionary basis and mechanistic details while looking at across infection outcomes. In this section we highlight the unprecedented diversity and flexibility of using *Drosophila* system while addressing some of the immune adaptations (*resistance, tolerance, priming and other damage limitation processes*) against pathogens. We underscore the importance of a combinatorial approach in future research. For instance, tracking the traditional innate immune regulatory networks with various other sources of cellular components and immune modulations such as metabolic, epigenetic reprogramming, and host-microbiota. Here we suggest few exciting and interrelated approach that can be addressed in the future.



I. *<u>Pathogen evolution under variable host tolerance</u>*:

Evolution under frequent exposure to pathogens has been reported in earlier studies with majority studies primarily focusing on the mechanisms that resist infections (Kraaijeveld and Godfray, 2008; Faria et al., 2015; Gupta et al., 2016; Khan et al., 2017; Ferro et al., 2019). While recent experiments have addressed disease tolerance (Raberg et al., 2007; Ayres and Schneider, 2012; Medzhitov et al., 2012; Louie et al., 2016; Gupta and Vale, 2017; McCarville and Ayres, 2018). The in-depth analyses of their selective conditions, fitness effects and various life-history trade-offs are still posing several questions. Several theoretical models suggest that tolerance is consistently beneficial (Roy and Kirchner, 2000) and hence, under selection tolerance-related alleles can rapidly be fixed in a population (Miller et al., 2005). In contrast, apart from pathogen elimination, resistance mechanisms often involves costly immune activation which would lead to various life-history and other fitness trade-offs (Schwenke et al., 2016). However, despite these distinct evolutionary trajectories of host defense strategies (resistance, tolerance, immune priming, and damage limitation mechanisms), we are still unclear about how immune signalling networks constrain pathogen evolution.

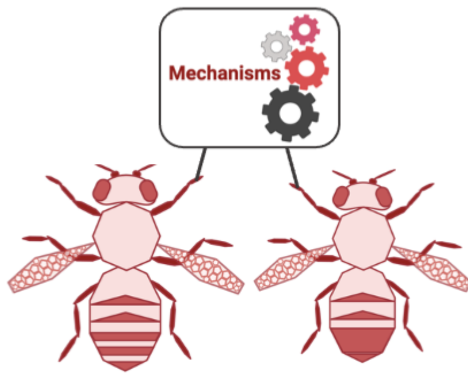


The future studies might include: -

- Does pathogen virulence evolve under different immune defense strategies?
- What are the overall fitness costs associated with the evolved immune responses?

II. *Mechanisms*:

There are interesting possibilities of intimate crosstalk between the immune system and other physiological and metabolic processes, which may ultimately influence host's responses against infecting pathogens. In fact, several transcriptome studies to infection responses (resistance, tolerance, priming) have reported upregulated genes related to metabolic activity across species [that is, in mosquitoes *Anopheles albimanus* (Maya-Maldonado et al., 2021), fruit flies *Drosophila melanogaster* (Martínez et al., 2020), flour beetles *Tribolium castaneum* (Ferro et al., 2019)]. In fruit flies for example, to neutralize pathogenic invasion, flies undergo a rapid metabolic switch which explicitly triggers lipid metabolism to compensate for the AMP synthesis (Martínez et al., 2020). Additionally, several mutations in mitochondria including mitochondrial DNA (mtDNA) are also emerging as key mediators of insect innate immunity (Riley and Tait, 2019; Salminen and Vale, 2020). Together, these results would suggest that future studies might open newer avenues to explore the energetics associated with various immune strategies against pathogens.



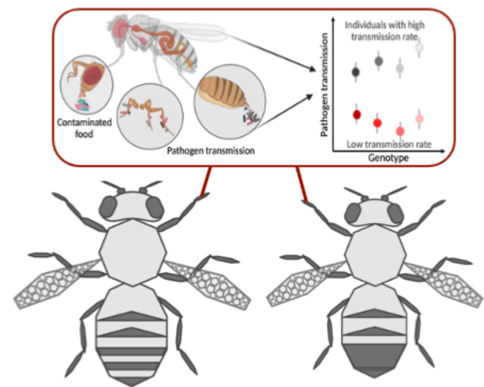
Some of the outstanding questions include: -

- Whether energetics including metabolic switch can explain the variable immune responses?
- How are the cross-talks between overlapping sets of immune signalling pathways and other immune components mediated?

III. *Disease epidemiology*:

Finally, there are some exciting possibilities of addressing how some of the damage limitation mechanisms affect various disease epidemiology. Several factors such as host's heterogeneity and behavioural changes (following infection) can determine the host's ability to

spread and transmit pathogens (Keiser et al., 2018; Siva-Jothy and Vale, 2021). For example, certain individuals (also known as 'super-spreaders') contribute disproportionately to the disease spread. This would obviously make outbreak predictions difficult and unreliable. The fact that, host's infections responses can vary on multiple levels including immunological, physiological, and behavioural aspects, all of which can affect their transmission potential. Until recently, we lack direct empirical evidence on some of the sources of variations (multiple host traits) affecting individual transmission potential (Siva-Jothy and Vale, 2021). However, we still lack information on how some of the host as well as pathogen drivers affect modes of disease transmission. For instance, addressing the role of damage limitation mechanisms (that is, gut damage repair and immune priming), vary in their ability to shed and transmit bacterial pathogens. It remains unknown whether these mechanisms reduce or increase pathogen transmissibility, vary infectious period, and manipulate sickness behaviour and overall disease outcomes.



- How does loss-of-functions in gut-epithelial damage-repair processes impact pathogen transmissibility (ability to shed and transmit bacterial diseases)?
- How does immune priming affect various epidemiological parameters?

7.5 *Future directions*

Over the past 2 decades, research on the fundamental processes governing innate immune responses using the fruit-fly *Drosophila melanogaster* as a model have made several important contributions - including the host-pathogen interactions, antibacterial- and antiviral defense, ageing and immunosenescence. While early infection and immunity studies mostly focused on deciphering the molecular components of canonical immune networks such as the IMD-, Toll-, and Jak/Stat-pathways and distinguishing the genetic variation between immune defense (resistance vs. tolerance) processes.

The field is now shifting towards more global approach that integrates traditional immune regulatory networks with the underlying physiology, their metabolic basis, the role of epigenetic reprogramming and finally, the plausible immune modulations associated with the host-microbiota. It is noteworthy that including the additional parameters, such as the

infection route, incorporating both males and females, pathogen pressure, have recently gained importance as they seem to affect disease outcomes.

Together, all these features, combined with a powerful and genetically tractable system, the fruit-fly *Drosophila* is now proposed as ideally suited model to dissect mitochondrial immunity, sex-specific dimorphisms, neurodegeneration, and to study cancer, and other infectious human diseases. Finally, we hope that characterization of novel role of the negative immune regulators, disease tissue damage prevention, repair and limitation mechanisms and immune priming in the light of promoting disease tolerance will stay in research focus on both insects and mammals as well. This is crucial for predicting, controlling and efficient management of emerging diseases and improve global health.

7.6 *References*

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Appendix

Video Article

Oral Bacterial Infection and Shedding in *Drosophila melanogaster*

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Abstract

The fruit fly *Drosophila melanogaster* is one of the best developed model systems of infection and innate immunity. While most work has focused on systemic infections, there has been a recent increase of interest in the mechanisms of gut immunocompetence to pathogens, which require methods to orally infect flies. Here we present a protocol to orally expose individual flies to an opportunistic bacterial pathogen (*Pseudomonas aeruginosa*) and a natural bacterial pathogen of *D. melanogaster* (*Pseudomonas entomophila*). The goal of this protocol is to provide a robust method to expose male and female flies to these pathogens. We provide representative results showing survival phenotypes, microbe loads, and bacterial shedding, which is relevant for the study of heterogeneity in pathogen transmission. Finally, we confirm that *Dcy* mutants (lacking the protective peritrophic matrix in the gut epithelium) and Relish mutants (lacking a functional immune deficiency (IMD) pathway), show increased susceptibility to bacterial oral infection. This protocol, therefore, describes a robust method to infect flies using the oral route of infection, which can be extended to the study of a variety genetic and environmental sources of variation in gut infection outcomes and bacterial transmission.

Video Link

The video component of this article can be found at https://www.jove.com/video/57676/

Introduction

The fruit fly (also known as the vinegar fly), *D. melanogaster*, has been extensively used as a model organism for infection and immunity against a variety of pathogens[1,2]. This work has offered fundamental insights into the physiological consequences of infection and was also pioneering in unraveling the molecular pathways underlying the host immune response against parasitoid, bacterial, fungal, and viral infections. This knowledge is not only useful to understand the innate immune response of insects and other invertebrates, but because many of the immune mechanisms are evolutionarily conserved between insects and mammals, *Drosophila* has also spurred the discovery of major immune mechanisms in mammals, including humans[3].

Most work on *Drosophila* infection and immunity has focused on systemic infections, using inoculation methods that deliver pathogens directly into the body of the insect by pricking or injection[4,5,6]. The advantage of these methods in allowing the delivery of a controlled infectious dose is clear and supported by a large body of work on systemic infections. However, many naturally occurring bacterial pathogens of *D. melanogaster* are acquired through feeding on decomposing organic matter where gut immunocompetence plays a significant role in host defence[7,8,9,10,11,12,13,14,15]. Experiments that employ systemic infections bypass these defenses, and, therefore, provide an altogether different picture of how insects mount defenses against natural pathogens. This is especially relevant if the aim of the work is to test predictions about the ecology and evolution of infection, where the use of natural pathogens and routes of infection is important[16,17]. Recent work has highlighted how the route taken by pathogens significantly affects disease outcome[18,19], elicits distinct immune pathways[20,21], can determine the protective effect of inherited endosymbionts[16], and may even play an important role in the evolution of host defenses[17].

Another reason to employ oral routes of infection is that it allows the investigation of the variation in pathogen transmission by measuring bacterial shedding during fecal excretion following oral infection[22,23,24]. Understanding the sources of host heterogeneity in disease transmission is challenging in natural populations[25,26], but measuring components of transmission, such as pathogen shedding, under controlled laboratory conditions offers a useful alternative approach[27]. By feeding flies bacteria and measuring bacterial shedding under a variety of genetic and environmental contexts in controlled experimental conditions, it is possible to identify sources of variation in transmission among hosts.

Here, we describe a protocol for orally infecting *D. melanogaster* with bacterial pathogens, and for quantifying the bacterial growth and shedding that follows (**Figure 1**). We describe this protocol on two *Pseudomonas* bacteria: a virulent strain of the opportunistic pathogen *P. aeruginosa* (PA14), and a less virulent strain of the natural fly pathogen *P. entomophila*. Pseudomonads are common gram-negative bacteria with a broad host range, infecting insects, nematodes, plants, and vertebrates, and are found in most environments[4,6]. Enteric infection of *Drosophila* by *P. aeruginosa* and *P. entomophila* results in pathology to intestinal epithelia[12,13,14,15,28]. While we focus on these two bacterial pathogens, the methods described here can in principle be applied to any bacterial pathogen of interest with minor modifications. Following oral exposure, we measure post-infection survival, and measure the microbe load within individual flies and the viable microbes shed into the environment,

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expressed in colony forming units (CFUs). Finally, because gut immunocompetence results from a combination of epithelial barrier and humoral responses, we also measure the survival of fly lines where these defenses are disrupted. Specifically, Drosocrystallin (*Dcy)* mutants have been previously shown to be more susceptible to oral bacterial infection due to a depleted peritrophic matrix in the gut[29]. We also measure survival in a Relish (*Rel*) mutant which is impeded from producing antimicrobial peptides against Gram-negative bacteria via the IMD pathway[30].

Protocol

1. Maintain Flies

1. Maintain flies in 23 mL plastic vials containing 7 mL of freshly made Lewis medium (modified from reference[31]; 1 L triple distilled H_2O, 6.1 g agar, 93.6 g brown sugar, 68 g maize, 18.7 g instant yeast, 15 mL Tegosept anti-fungal agent) in incubators at 25 ± 1 °C, in a 12 h:12 h light:dark cycle with ~60% humidity. Plug the vials with non-absorbent cotton wool.
2. After every 14 days, transfer 20–30 adults to a new food vial, with instant, dry yeast added to the surface, for 2–3 days to allow egg-laying to occur. After this time period, ensure that the eggs are visible on the surface of the food. Remove adult flies.
   NOTE: This keeps flies in the vials as single generation, age-matched populations.
3. Leave the eggs to develop.
   NOTE: At 25 °C, adult flies start to eclose from pupae on day 11 and continue over days 12–14.

2. Prepare Experimental Flies

1. Collect the eggs of the parent generation in a population/embryo collection cage on a 75 mL apple-agar plate (1 L triple distilled H_2O, 30 g agar, 33 g sucrose, 330 mL apple juice, 7 mL Tegosept anti-fungal agent) with a yeast paste spread (mix dry yeast with water to a peanut butter-like consistency). Add water-soaked cotton wool to the cage to provide moisture.
   NOTE: To avoid confounding effects caused by differences in larval rearing density, it is important that experimental flies in different vials are reared in similar densities. The above step is performed to avoid confounding effects.
2. Incubate for 24 h at 25 °C in a 12 h:12 h light:dark cycle until egg-laying has occurred. If there are too few eggs after 24 h, provide a longer habituation period. Replace apple-agar plates and allow egg-laying to occur for a further 24 h.
3. Take egg-laden apple-agar plates from the population cage. Remove the remaining yeast paste and any dead flies from the agar's surface.
4. Submerge the agar in 20 mL of 1x phosphate-buffered saline (PBS) and gently dislodge the eggs from the apple-agar with a fine paintbrush. While suspended in PBS, transfer the eggs to a 50 mL centrifuge tube and leave for 5 min so the eggs sink to the bottom.
   NOTE: Most eggs are found on the outer edge of the agar.
5. Remove by cutting the bottom 4 mm of a p1000 filtered pipette tip and use the pipette tip to draw 1 mL of solution, taken from the bottom of the 50 mL centrifuge tube. Transfer this to a 1.5 mL microcentrifuge tube and allow it to settle.
   NOTE: When pipetting up eggs, snap-releasing the plunger is more efficient than a gentle release.
6. Remove by cutting the bottom 4 mm of a p20 filtered pipette tip. Set the pipette to a desired volume and draw from the bottom of the microcentrifuge tube.
   NOTE: With practice, a volume of 5 µL contains roughly 100 eggs.
7. Dispense the collected eggs onto the food and leave them to develop for the required amount of time.

3. Bacterial Culture

1. To grow *P. entomophila* and *P. aeruginosa* cultures, inoculate 10 mL of Luria-Bertani (LB) broth with 100 µL of a frozen bacterial stock at 30 °C (*P. entomophila)* and 37 °C (*P. aeruginosa*), respectively. Shake at 150 rpm overnight. Ensure that the bacterial culture reaches the saturation phase.
2. To ensure the bacteria used for inoculating the flies are in the exponential phase and rapidly replicating, inoculate the overnight culture into a new subculture, of a desired volume, the following morning. Ensure that the pre-inoculum is 10% of the total volume of the subculture culture.
   NOTE: Oral infection requires high . It is therefore necessary to grow a substantial volume of bacterial culture so that enough inoculation culture can be produced for the desired dose and experimental size. Calculate how much subculture is needed to produce the required infectious doses using the equation $M_sV_s = M_iV_i$, where M represents a culture's optical density measured at 600 nm (OD_{600}) value and V represents its volume. Subscript letters refer to whether the culture is used as a subculture (s) or an infectious dose (i).
3. Grow this subculture in a 2 L conical flask in a volume such that the subculture's surface falls (at most) just above the beginning of the flask's slope. Do not fill above this mark as it will stunt the growth of bacteria.
4. Ensure the bacteria in this subculture are in the exponential growth phase by measuring the OD every 30 min.
   NOTE: This occurs after 3–5 h, where the subculture reaches an OD_{600} between 0.6–0.8.
5. Pour equal volumes of this subculture across 50 mL centrifuge tubes and spin the subculture at 2,500 x g for 15 min at 4 °C to pellet the bacteria. Once pelleted, remove and then spin the supernatant again at the above conditions to confirm the removal of the vast majority of bacteria.
   NOTE: A pellet of negligible size (smaller than 1 mm in height) confirms this.
6. Combine the bacterial pellets of the separate tubes by re-suspending them in 5 mL of subculture supernatant and recombining these solutions in a single 50 mL tube. Spin this concentrated culture at 2,500 x g for 15 min at 4 °C to pellet the bacteria.
7. Remove the supernatant and re-suspend the final bacteria pellet in 5% sucrose water solution. Check the OD and adjust to the desired infectious dose (OD_{600} = 100 for *P. entomophila*[8] and OD_{600} = 25 for *P. aeruginosa*[16,28]), by re-suspending the pellets in 5% sucrose water solution to the required volume.
   NOTE: The amount of 5% sucrose water solution to be added can be calculated using the equation in step 3.2.1 ($M_sV_s = M_iV_i$).

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4. Orally Infecting Flies

1. To ensure oral infection, starve the flies for 2–4 h before exposure to bacteria by transferring the flies to standard agar vials (1 L triple distilled H_2O, 20 g agar, 84 g brown sugar, 7 mL Tegosept anti-fungal agent).
2. Prepare infection vials while flies are being starved. Make a *Pseudomonas* infection vial by pipetting 500 µL of standard sugar agar into the lid of a 7 mL sample tube and leave it to dry. Place a disc of filter paper in the lid and pipette 100 µL of bacterial culture directly onto the filter disc. For control infections, replace the bacterial culture with the same volume of 5% sucrose water solution on the filter paper.
3. Add single flies to the sample tube and leave for 18–24 h.
4. To confirm oral infection, first surface-sterilize the flies immediately after bacterial exposure, by placing them in 100 µL of 70% ethanol for 20–30 s. Remove the ethanol and add 100 µL of triple distilled water for 20–30 s before removing the water. Add 100 µL of 1x PBS and homogenize the fly.
5. Transfer the homogenate to the top row of a 96-well plate and add 90 µL of 1x PBS to every well below.
6. Serially dilute this sample to distinguish a range of CFU values. Take 10 µL of the homogenate in the top well and add this to the well below. Repeat this step with the second well, transferring 10 µL to the third well, and so on, for as many serial dilutions as required.
   NOTE: It important that new pipette tips are used for each set of dilutions.
7. Plate the serial dilutions on an LB nutrient agar plate in 5 µL droplets, to ensure all droplets remain discrete.
8. Incubate the LB Agar plates overnight at 30 °C and 37 °C for *P. entomophila* and *P. aeruginosa*, respectively and count visible CFUs.
   NOTE: While *Drosophila* gut microbes require distinct anaerobic growth conditions, selective medium, for example *Pseudomonas* Isolation Medium (PIM), may be used to make sure only *Pseudomonas* CFUs are counted.
9. Calculate the number of CFUs per fly by counting the number of colonies present at the serial dilution where 10–60 CFUs are clearly visible. Then multiply by the dilution factor present to calculate the number of bacteria per fly.
10. Perform statistical analysis. Where necessary, transform the CFUs per fly to a normal distribution. Do this by log-transformation. Once transformed, use Generalized Linear Models (GLMs)[30,31,32] to test how treatment groups differ in CFUs per fly (using commonly available statistical software packages such as R[33]).
   NOTE: The remaining fly homogenate can be used for measuring gene expression through quantitative reverse transcription PCR (RT-qPCR) analysis. Fix the homogenate in 50 µL of RNA isolation reagent, extract RNA, and quantify specific immune gene titers by RT-qPCR (see *e.g.*, Gupta and Vale[16] for a detailed protocol). The expression of specific immune gene transcripts should be normalized to the transcript levels of a housekeeping gene (*i.e.*, rp49) and expressed as a fold change relative to control flies using the $2^{-\Delta\Delta Ct}$ method[31,32,33,34].

5. Recording Survivorship Following Infection

1. Infect flies orally as described in step 4.2.
2. Transfer the infected or control flies from their respective infection vials into standard Lewis vials and keep in an incubator at 25 °C in a 12 h:12 h light-dark cycle (or desired conditions). Keep flies until they are dead.
3. Count the number of living or dead flies in each vial every day, or as often as required.
4. Transfer the flies to new vials every 5 days to avoid the flies getting stuck in the food.
5. Present these data as Kaplan-Meier (KM) survival curves or Mean ± SE proportional survival plots. To analyze the effect of several factors and/or their respective interactions with one another use a statistical package (for example, the package "survival" in R[33]) to run a survival analysis such as the Cox Proportional Hazards model[35].

6. Measuring Bacterial Load

1. At the desired time point, transfer a single infected fly to a sterile 1.5 mL microcentrifuge tube.
2. Surface sterilize the flies as described in step 4.4.
3. Homogenize the fly and quantify the bacterial load using the protocol described in steps 4.5–4.10.

7. Measure Bacterial Shedding

1. Measure the shedding alongside the internal load.
2. After infection, transfer single flies to 1.5 mL microcentrifuge tubes containing ~50 µL of Lewis medium for 24 h.
3. Remove the flies for the internal load measurement (see step 6) and wash the tubes with 100 µL of 1x PBS by vortexing heavily for 3 s.
4. Measure the CFUs in this wash by plating on LB nutrient agar using the same protocol as described in steps 4.6–4.8.
5. After infection, transfer single flies to 1.5 mL microcentrifuge tubes containing ~50 µL of Lewis medium for 24 h.
6. Transfer the flies to new microcentrifuge tubes containing ~50 µL of Lewis medium for a further 24 h. Wash the contaminated tubes with 100 µL of 1x PBS by vortexing heavily for 3 s.
7. Measure the CFUs in this wash by plating on LB nutrient agar using the same protocol described in steps 4.6–4.8.
8. Repeat steps 7.2 and 7.3 and record fly mortality at every transfer.

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Representative Results

Here, we present illustrative results from experiments where *D. melanogaster* was orally infected with *P. aeruginosa* or *P. entomophila*. **Figure 2** demonstrates the successful oral infection of flies following a 12 h or 24 h exposure period to bacterial cultures of OD_{600} = 25 and 100 for *P. aeruginosa* (**Figure 2A**) and *P. entomophila* (**Figure 2B, C**), respectively. **Figure 2B** illustrates the importance of using a more concentrated culture of *P. entomophila,* shown by the increase in bacterial load when flies are exposed to bacterial cultures of greater optical density. Male and female Oregon R (OreR) flies clear *P. aeruginosa* infection at the same rate (**Figure 3**) and shed the same number of *P. aeruginosa* CFUs (**Figure 4A**). When infected with *P. entomophila* however, male and female OreR flies differ in the number of bacteria shed, in a manner that changes over time (**Figure 4B**). Males and females die from *P. aeruginosa* (**Figure 5A**) and *P. entomophila* (**Figure 5B**) at different rates. We also see that *Dcy* mutants (which lack the protective peritrophic matrix in the gut epithelium) and Relish mutants (which lack a functional IMD immune pathway), show decreased survival following *P. entomophila* and *P. aeruginosa* oral infection (**Figure 5C**).

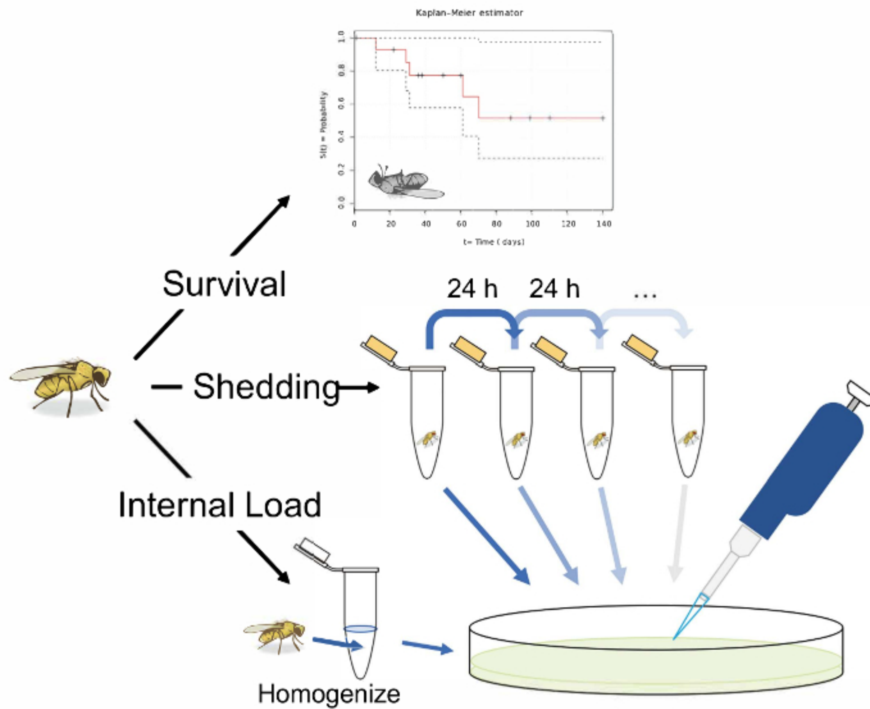

Figure 1: Schematic overview of protocols for measuring survival, shedding, and internal bacterial load following oral infection in *Drosophila melanogaster*. An illustration of 3 potential experiments following the oral infection of *D. melanogaster*. Measure the 'survival' by transferring single flies to vials and recording their infected lifespan. Measure 'shedding' by transferring single flies to 1.5 mL microcentrifuge tubes with 50 µL of Lewis medium in the cap. After 24 h in the tube, remove the fly and vortex the tube with 100 µL of 1x PBS. Remove and plate this solution on LB nutrient agar to calculate the bacterial shedding. Measure the shedding in the same fly longitudinally, by transferring flies to fresh tubes with Lewis medium in the cap after 24 h, and washing and plating the now contaminated tube. A fly's 'internal load' can be measured by taking an infected fly, surface sterilizing it, and homogenizing it before finally plating the homogenate on LB nutrient agar. This can be performed after shedding has been measured to calculate how the 'internal load' and shedding correlate. The fly illustration used in this figure was originally drawn by B. Nuhanen[36]. The authors have modified it to accompany the example Kaplan-Meier curve which is taken from Wikimedia Commons[37]. All other illustrations are original. Please click here to view a larger version of this figure.

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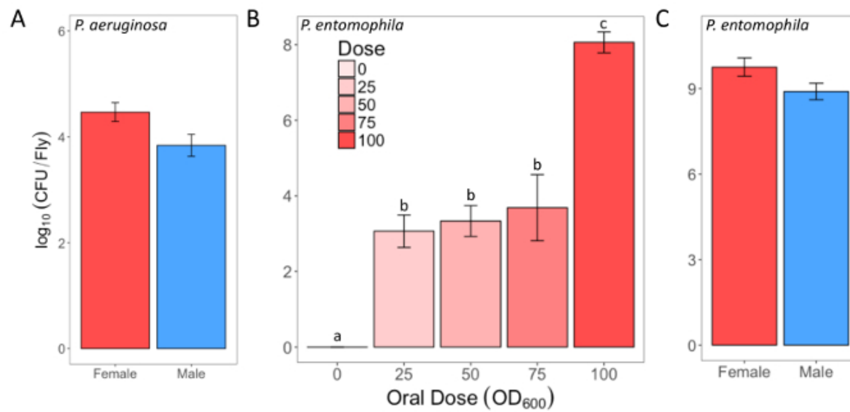

Figure 2: Infectious dose of bacteria following oral infection. (A) Infectious dose of male and female Oregon-R flies following exposure to a *P. aeruginosa* culture (OD_{600} = 25) for 12 h. The mean and SE were calculated from 3 males and 3 females. **(B)** Infectious dose of outcrossed wild-type females following exposure to one of four *P. entomophila* cultures (OD_{600} = 100, 75, 50, and 25) or control 5% sucrose solution for 24 h. The statistical difference of ($F_{3,76}$ = 18.567, p <0.001) in the infectious dose between exposure treatments is denoted by differing letters above bars. The means were calculated from 5 flies for the OD_{600} = 0 dose, and 18-20 for all other doses. **(C)** The infectious dose of male and female Oregon-R flies following exposure to *P. entomophila* culture (OD_{600} = 100) for 24 h. The mean and SE were calculated from 20 males and 20 females. Please click here to view a larger version of this figure.

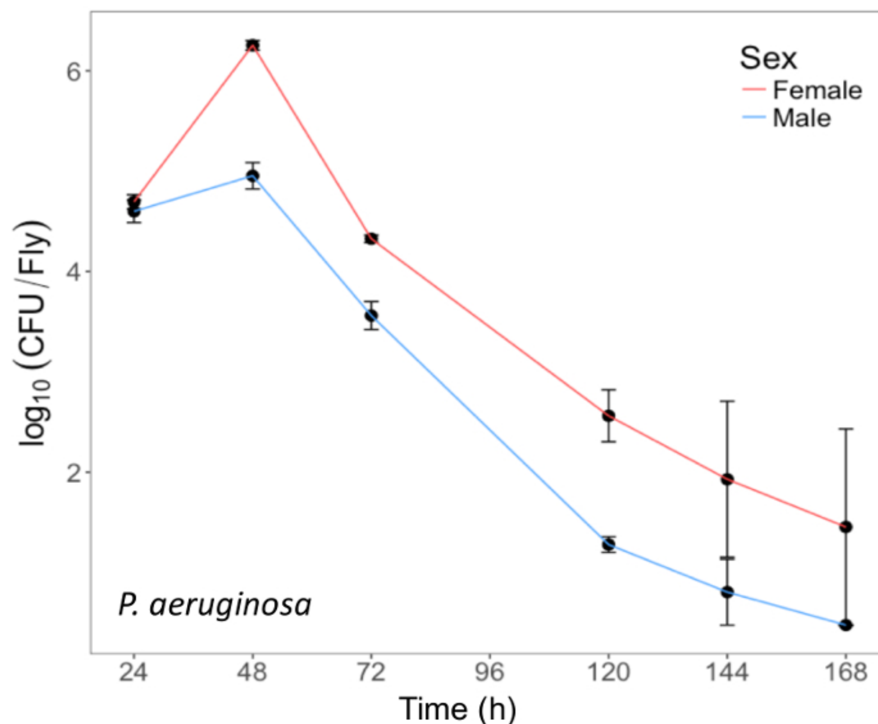

Figure 3: Internal *P. aeruginosa* load in flies after oral infection. Mean ± SE bacterial load of male and female Oregon-R flies following oral infection with *P. aeruginosa* (OD_{600} = 25) up to 168 h post-infection. The mean and SE of each time point are calculated from 3 individuals. A fly's internal bacterial load significantly changes over time (p <0.001). Please click here to view a larger version of this figure.

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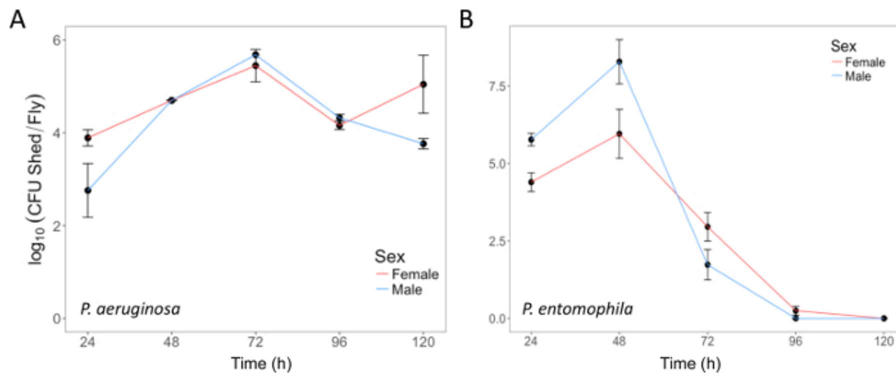

Figure 4: Bacterial shedding following oral infection. (A) *P. aeruginosa* shed by the same flies used in **Figure 3**, up to 120 h post-infection. The mean and SE were calculated from 3 males and 3 females. **(B)** *P. entomophila* shed by male and female Oregon-R flies following oral infection with *P. entomophila* (OD_{600} = 100) up to 120 h post-infection. The mean and SE were calculated from 34 males and 38 females. For both *P. aeruginosa* and *P. entomophila*, the number of CFUs shed by a fly significantly changes over time (p <0.001). Please click here to view a larger version of this figure.

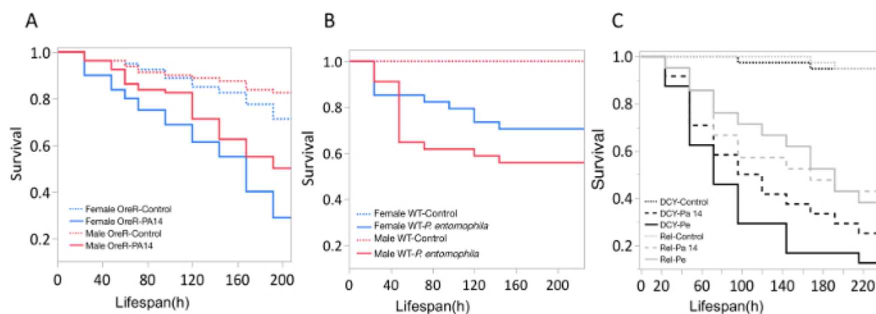

Figure 5: Survival of flies following bacterial oral infection. Kaplan-Meier (KM) survival curves of **(A)** Oregon-R male and female flies following oral infection with *P. aeruginosa* (OD_{600} = 25) or control 5% sucrose solution. The KM survival curve was calculated from 4 vials of 20 flies per treatment group. **(B)** OreR male and female flies after oral infection with *P. entomophila* (OD_{600} = 100). The KM survival curve was calculated from 4 single control flies and 34 infected flies for both males and females. **(C)** Immune mutants: *Dcy* (Drosocrystallin-peritrophic matrix mutant) and *Rel* (Relish-IMD mutant), exposed to *P. entomophila* (Pe), *P. aeruginosa* (Pa14), or a control 5% sucrose solution. All infected groups die significantly faster than the control flies (p <0.001). Please click here to view a larger version of this figure.

Discussion

We present a protocol for reliably orally infecting *D. melanogaster* with bacterial pathogens. We focus on *P. aeruginosa* and *P. entomophila*, but this protocol can easily be adapted to enable infection of other bacterial species, *e.g.*, *Serratia marcescens*[7]. Key aspects of this protocol will vary between bacterial species. Accordingly, the most efficient infectious dose, corresponding virulence, and host genotype susceptibility should all be considered and ideally tested in pilot studies. Exposing flies to bacterial cultures of a range of optical densities and measuring their infectious dose and survival is an appropriate starting point when working with new bacterial species or fly lines.

Protocol steps such as fly starvation prior to feeding and re-suspending bacterial pellets in 5% sucrose solution are commonplace in oral infection and increase the reliability of bacterial infection during exposure[7,8,9,10]. However, it is important to note that during exposure, flies essentially live on a surface of bacterial culture. In the process of walking on this culture, bacteria will become lodged on the fly's surface, especially on the cuticle or around the bristles[24]. These epicuticular bacteria, do not reflect a successful enteric infection but would still be detected by the fly homogenization and plating. To reduce the potential for false positives, it is essential to surface sterilize flies through immersion in 70% ethanol for up to 1 min.

When considering bacterial shedding rates, oral infection is essential. The number of pathogens a host releases into the environment is often difficult to measure and the internal load is often taken as a proxy for the severity of infection and therefore transmission[26,27]. Measuring bacterial load alongside bacterial shedding allows an examination of the relationship between these two important components of disease severity and spread[38]. One limitation of the method presented is that assaying the internal bacterial load of flies requires destructive sampling. This makes it difficult to investigate longitudinal trends of pathogen growth and clearance within the same individual. However, it is possible to overcome this limitation by destructively sampling cohorts of individuals at different stages of infection, under the assumption that the average microbe load in each cohort reflects the longitudinal pathogen dynamics within any given individual. Bacterial shedding does not suffer from the same limitations, and we offer examples of how shedding can be quantified in a cross-sectional sample, or longitudinally to investigate how shedding changes within an individual over time.

Many host and pathogen traits jointly determine an individual's propensity to transmit disease[25,26,39]. While the significance of these traits likely varies between host-pathogen systems, shedding is likely a major determinant of fecal-oral transmission. The ability to measure bacterial shedding opens the opportunity to test this assumption. Having characterized host-pathogen dynamics in a desired panel of fly lines,

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experimenters could orally infect individuals, and place them alongside uninfected, susceptible hosts during their infectious periods. These 'recipient' flies could then be assayed for internal bacterial load at various time points as a way of directly measuring transmission.

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RESEARCH ARTICLE

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Pathogen susceptibility and fitness costs explain variation in immune priming across natural populations of flour beetles

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Abstract

1. In many insects, individuals primed with low doses of pathogens early in life have higher survival after exposure to the same pathogen later in life. Yet, our understanding of the evolutionary and ecological history of priming of immune response in natural insect populations is limited. Previous work demonstrated population-, sex- and stage-specific variation in the survival benefit of priming response in flour beetles (*Tribolium castaneum*) infected with their natural pathogen *Bacillus thuringiensis*. However, the evolutionary forces responsible for this natural variation remained unclear.
2. In the present work, we tested whether the strength of the priming response (measured as the survival benefit after priming and subsequent infection, relative to unprimed controls) was associated with multiple fitness parameters and immune components across 10 flour beetle populations collected from different locations in India.
3. Our results suggest two major selective pressures that may explain the observed inter-population variation in priming: (a) Basal pathogen susceptibility – populations that were more susceptible to infection produced a stronger priming response, and (b) Short-term early reproductive success – populations where primed females produced more offspring early in life (measured over 2 days) had lower survival benefit (measured over 120 days), suggesting a potential trade-off between early reproduction and priming response. However, the negative association between survival and reproduction is limited to priming and infection in adults, but not in larvae. While other components of beetle fitness (starvation resistance and larval development) and immune function (haemolymph antibacterial activity and antimicrobial quinone secretion) also varied widely across populations, none of them was correlated with the variation in priming responses across populations.
4. Our work is the first systematic empirical demonstration of multiple selective pressures that may govern the evolution of immune priming in the wild. We hope that this motivates further experiments to establish the role of pathogen-imposed selection and fitness costs in the evolution of priming in natural insect populations.

KEYWORDS

insect immunity, natural populations, priming response, reproductive benefit, survival benefit

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1 | INTRODUCTION

Immune priming is now regarded as an integral feature of insect immunity, whereby exposure to low doses of a pathogen can prime the immune response and confer increased protection against subsequent challenge by the same pathogen (reviewed in Little & Kraaijeveld, 2004; Milutinović, Peuß, Ferro, & Kurtz, 2015; Contreras-Garduño et al., 2016). Mathematical models predict that the strength of the priming response can have major implications for infection prevalence, epidemiology and population stability in the wild (Tate, 2017; Tidbury, Best, & Boots, 2012). Thus, it is important to understand how priming ability evolves in insects and what selective forces drive its evolution in natural populations. In a previous study, we reported considerable variation in the priming response (from a 13-fold survival benefit to no detectable effect) among wild-caught populations of the red flour beetle *Tribolium castaneum* (Khan, Prakash, & Agashe, 2016a). However, a major question remained unanswered – what evolutionary forces generate such large inter-population divergence in immune priming?

In general, host immune function can vary due to spatial variation in the strength of pathogen pressure (Corby-Harris & Promislow, 2008; Linhart & Grant, 1996; Mayer, Mora, Rivoire, & Walczak, 2015; Reznick & Ghalambor, 2001). Classic examples of immune investment shaped by parasite-mediated selection include migratory shore birds (Mendes, Piersma, Hasselquist, Matson, & Ricklefs, 2006) and island populations of Darwin's finches (Lindström, Foufopoulos, Pärn, & Wikelski, 2004), in which investment in immune defence (e.g. increased production of antibodies) is correlated with local infection prevalence. In insects, encapsulation ability increases with the virulence of the pathogen (Kraaijeveld & Van Alphen, 1994), and natural populations of *Drosophila melanogaster* exposed to diverse pathogen communities show an increased ability to clear bacterial infection (Corby-Harris & Promislow, 2008). Strong pathogen selection may similarly play a direct role in the evolution of the insect priming response (Best, Tidbury, White, & Boots, 2013), as we recently demonstrated: immune priming evolved rapidly in laboratory-selected flour beetle populations exposed to a lethal dose of bacterial infection (Khan, Prakash, & Agashe, 2017). Does strong pathogen pressure also select for priming in natural conditions? Wild populations inhabiting pathogen-rich environments have an increased likelihood of reinfection by the same pathogen and should invest more in specific protection against those pathogens (discussed in Corby-Harris & Promislow, 2008). We thus reasoned that the strength of the priming response in natural populations may be determined by the severity of the infection, such that populations with increased pathogen susceptibility should face selection for a stronger priming response that is specific to the pathogen.

On the other hand, if mounting a priming response is metabolically and energetically costly (Norris & Evans, 2000; Sheldon & Verhulst, 1996), trade-offs with other fitness components may constrain the strength of immune priming such that natural populations invest differentially in the immune system. Mathematical models already highlight the importance of the fitness costs of priming (Best et al., 2013; Tate & Rudolf, 2012; Tidbury et al., 2012), although only a few studies have experimentally demonstrated the costs of priming. For instance, primed female mosquitoes (Contreras-Garduño, Rodríguez, Rodríguez, Alvarado-Delgado, & Lanz-Mendoza, 2014) and offspring of primed tobacco hornworms (Trauer & Hilker, 2013) lay fewer eggs, suggesting a trade-off between priming and reproduction. Maternal immune priming also prolonged offspring development time in mealworm beetles (Zanchi, Troussard, & Martinaud, 2011), compromising their competitive ability at high density (Koella & Boete, 2016). Based on these results, we speculated that variable fitness costs could also determine the occurrence and maintenance of priming ability in natural populations. For instance, if the relative cost of priming exceeds its fitness benefits, priming may not evolve in a population despite its high susceptibility to infection.

To elucidate the selective parameters underlying variation in immune priming, we analysed the response of 10 wild-collected populations of the red flour beetle *Tribolium castaneum* primed with a naturally occurring insect pathogen *Bacillus thuringiensis* (previously described in Khan et al., 2016a, 2016b,2016b, 2017). For each of these populations, we measured the within-generation priming response, basal pathogen susceptibility (without priming) and various fitness and immune components (see Figure 1). First, we asked whether the benefit of priming increases with susceptibility to infection. Second, we tested whether the potential fitness costs of immune priming trade off with its survival benefit. Finally, we also tested whether trade-offs with other immune responses may explain the observed variation in priming responses. Our experiments were thus explicitly designed to detect relationships between various life-history and ecological parameters that may drive population divergence in priming ability.

2 | MATERIALS AND METHODS

2.1 | Generating experimental beetles

We collected 10 natural populations (abbreviated AG, AL, B1, B2, AM, CB, GO, HD, ND and NG) of *Tribolium castaneum* from grain warehouses at 9 different geographical locations in India (B1 and B2 were collected from different grain warehouses within the same city, whereas others were collected from 8 different cities; described in Khan et al., 2016a). We reasoned that multiple factors such as individual age, mating, migration history, nutrient and local environmental factors are likely to influence variability in immune responses across populations. Since it was impossible to account for all these factors, we did not measure immune priming responses of individuals that were directly collected from the grain warehouses. Instead, we maintained them in the laboratory on whole-wheat flour at 34°C for 10 non-overlapping generations before commencing the experiments (Khan et al., 2016a). We did not test for naturally occurring microbes already persisting in the populations, which could interfere with their priming response during the assay.

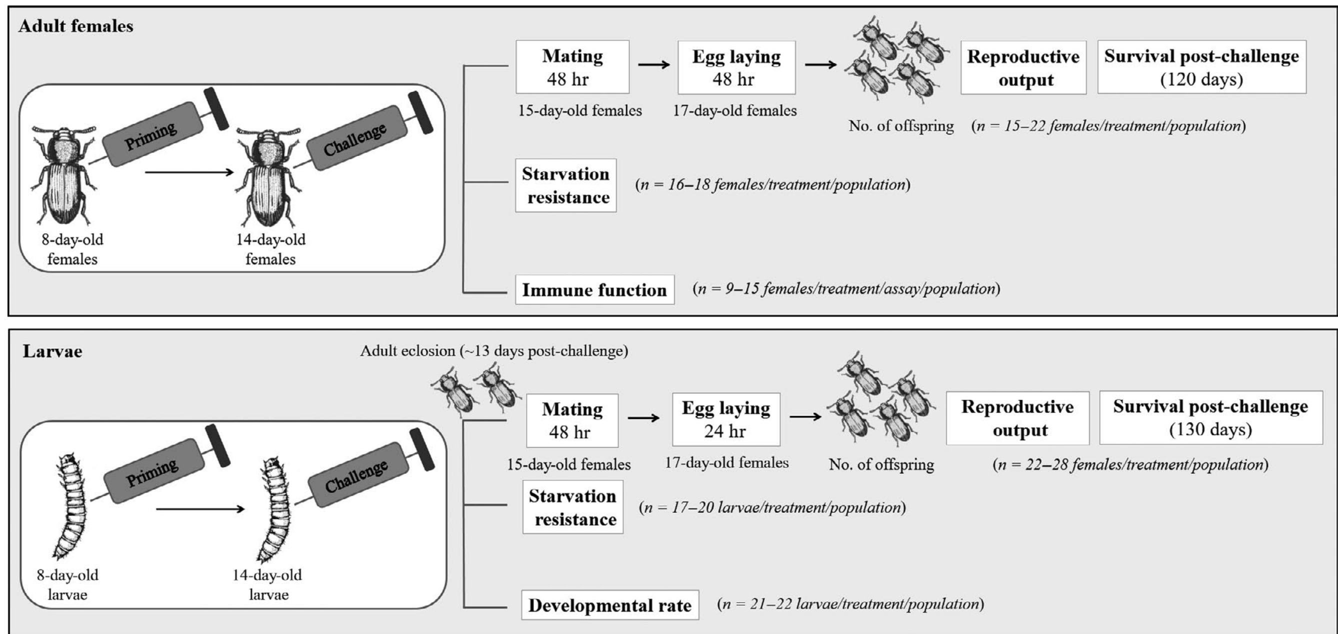

FIGURE 1 Experimental design: Our joint assay of basal pathogen susceptibility and survival benefits of priming involved three treatment groups namely primed beetles (priming with heat-killed bacteria followed by live infection), unprimed beetles (mock priming followed by a live infection) & control beetles (mock priming followed by a mock infection). Other experiments (reproductive output, survival under starvation, development rate and immune components) only compared primed and unprimed beetles

To generate experimental individuals for each population, we allowed 800–1,000 individuals to oviposit in 350 g of organic wheat flour for 48 hr. We then removed the adults and collected offspring at the larval stage (after ~2 weeks). A week later, we sexed individuals at the pupal stage where we only retained females, but discarded males since handling both sexes simultaneously was logistically challenging. We housed individual pupae in 48-well microplates containing ~0.3 g flour for 12 days. Separately, we also collected larval offspring after 10 days. Since the pupal stage lasts for 3–4 days and eggs usually hatch in 2 days, we obtained 8-day-old adults and 8-day-old larvae for all our experiments.

2.2 | Immune priming and challenge

We used a strain of the natural insect pathogen *Bacillus thuringiensis* (Strain no. DSM 2046, originally isolated from a Mediterranean flour moth) to measure within-generation priming for all populations as described in Khan et al. (2016a). Our previous experiments show that flour beetles produce priming responses that are highly specific to the strain of *B. thuringiensis* (Bt) used in this study (Khan et al., 2017). Briefly, we pricked adults between the head and thorax, and larvae between the last and last but one segment, using a 0.1-mm insect pin (FST, CA) dipped in heat-killed bacterial slurry (i.e. priming) or insect Ringer (i.e. sham priming control). We plated the heat-killed culture on an agar plate and incubated overnight to confirm that no live bacteria were present. Similarly, we also checked the sterility of the Ringer solution at the end of each experiment to confirm that control beetles were never primed or infected with live bacteria. We note that pricking alone can lead to wounding that can activate non-specific immune responses (Behrens et al., 2014). Yet, we think that this is insufficient to provide survival benefits of priming since other experiments with laboratory-adapted outbred populations (described in Khan et al., 2017) suggest that naïve beetles or beetles injected with Ringer solution are equally susceptible to live infection later in life (see Figure S1). However, we did not test whether such wounding affects other traits such as reproduction, immune components or development. We made bacterial slurry from 10 ml freshly grown overnight culture of *B. thuringiensis* at 30°C (optical density of 0.95; adjusted to ~10^{11} cells in 1 ml insect Ringer solution). We used heat-killed cells to prime a subset of beetles (n = 15–22 females or 22–28 larvae) from each population, because this would induce an immune response without a direct cost of virulence. We note the possibility that Bt-secreted virulence factors may survive during the heat treatment, mimicking the effects of a live infection after priming (Milutinović, Fritzlar, & Kurtz, 2014). However, since we did not observe any mortality in the period between the priming and live pathogen challenge treatments (as explained next), we conclude that the levels of such virulent factors have little effect on immediate beetle survival. After this, we isolated experimental beetles in wells of 96-well microplates (Corning) containing wheat flour. Six days later, we checked for mortality and again challenged with a live bacterial culture adjusted to ~10^{10} ml^{-1} (delivering ~900 bacterial cells per beetle). We did not perform mock challenge (injecting Ringer solution) with primed beetles. The challenge did not cause significant mortality in beetles until a week, providing us a window of opportunity to measure post-infection reproductive output (described below). The remaining beetles

only received mock priming followed by either a live infection (unprimed beetles) or mock challenge with insect Ringer (control beetles) (n = 15–22 females or 22–28 larvae/ treatment/ population). After the immune challenge, we immediately returned all three types of experimental beetles (primed, unprimed and control) from each population to wells of fresh 96-well microplates and measured various traits as described below (also see Figure 1). Since we used a low dose of infection (compare with Khan et al., 2016a), we observed a late onset of post-infection mortality. For instance, while a few infected larvae (<1%) died before pupation in some populations, there were no deaths during their adult stage until 23 days post-eclosion. We also did not observe any mortality in adults until a week after infection.

We note that we did not control for body size variation across beetles. As a result, it is possible that our effective priming or infection dose varied significantly across populations. Nonetheless, we could ensure that we assayed larvae at equivalent developmental stage since previous experiments in the laboratory showed that larvae from all populations develop at a similar rate (Khan et al., 2016a). We also found that adults have similar dry body weight (a proxy for body size; Imroze & Prasad, 2011) across 5 tested populations (see Figure S2). We thus conclude that the effective adult infection dose would not vary substantially across populations.

2.3 | Joint assay of basal pathogen susceptibility, survival benefit of priming and changes in reproductive output after priming

One day after the immune challenge, we paired primed and unprimed adult females with uninfected, 8-day-old virgin males from the respective population for 48 hr in a 1.5-ml microcentrifuge tube containing 1 g flour (one pair of beetles per tube). We then separated females to measure the total number of offspring of all life-stages (larvae, pupae and adults) produced by each female (48 hr of oviposition; eggs allowed to develop in 6 g flour for 3 weeks). Following this, we returned mated females to 96-well microplates with flour and noted their survival every 3–5 days for another ~117 days (total 120 days post-challenge), transferring them to fresh microplates with food every 5 days to minimize the interaction between females and new offspring (n = 15–22 females/treatment/population). For larvae, we isolated primed and challenged individuals in their respective wells until they pupated. Subsequently, we identified and retained only female pupae. Fifteen days post-eclosion, we paired each adult female with a virgin male as described above. We allowed females to oviposit for 24 hr and recorded their mortality every 3–5 days for another 100 days (total ~130 days post-challenge) as described above (n = 22–28 females/treatment/population). This procedure allowed us to obtain a correlated dataset for early reproductive success and survival of each experimental female after priming and challenge. A few replicate plates for reproductive output after adult priming were accidentally lost during the experiment. Hence, the sample size used for estimating the reproductive output for each population was lower than expected in adult females (See Table S1). Also, we could not record the number of offspring produced by the control beetles due to logistical reasons (Figure 1).

We analysed post-immune challenge survival data for each population and life stage separately using Cox proportional hazard survival analysis with priming as a fixed factor and life span in days as the response variable. We considered beetles that were still alive at the end of the experiment as censored values. For each population and life stage, we calculated pathogen susceptibility as the estimated hazard ratio of unprimed vs. control groups (Rate of deaths occurring in unprimed group/Rate of deaths occurring in full control group). A hazard ratio significantly greater than one indicates an enhanced risk of mortality in unprimed groups compared to control individuals. To estimate the strength of the priming response, we calculated the survival benefit to the host after infection, with vs. without previous exposure to the same pathogen (Rate of deaths occurring in unprimed group/Rate of deaths occurring in primed group). A hazard ratio significantly greater than one indicates an enhanced risk of mortality in unprimed groups compared to primed individuals.

The residuals for reproductive success were not normally distributed (tested with Shapiro–Wilks test). We thus used nonparametric Wilcoxon rank-sum tests to test the impact of larval and adult priming on reproductive success. We quantified the impact of priming on reproductive output as: Mean number of offspring produced by primed females/Mean number of offspring produced by unprimed females. To test for a correlation between reproductive vs. survival benefits of priming across populations, we analysed the same survival data used previously to calculate the strength of priming responses (described above).

2.4 | Separate assays to measure the impact of priming on development rate, life span under starvation and immune components

In these assays, we did not re-measure the immune priming response in terms of survival benefit after infection, since this was already measured for each population as described above. Instead, we directly measured the impact of priming (i.e. compared primed vs. unprimed groups) for the following fitness and immune components and then tested for a correlation with priming strength (calculated as hazard ratios) using the survival data described earlier.

1. Survival under starvation: We first tested whether priming affects survival under starvation in different populations. To do this, we isolated a subset of virgin females and larvae individually in 96-well microplates without food (n = 16–20/life stage/ treatment/population) immediately after immune challenge. We noted their mortality every 12 hr (10 a.m. & 10 p.m. ± 1 hr) for the next 11 days. We quantified the impact of priming (primed vs. unprimed) on survival under starvation using hazard ratios, as described above. A hazard ratio significantly greater than

one would suggest a higher risk of mortality in primed groups compared to unprimed individuals. Due to logistical reasons, we could only measure starvation resistance of larvae from 7 populations (except B1, AM and ND; described in Khan et al., 2016a).

2. Immune components: To measure aspects of immune function, we first primed and challenged adult females from each population (described above). After 24 hr, we used a subset of females to quantify the antibacterial activity of beetle haemolymph, a proxy for internal innate immunity (n = 9–15/treatment/population) (see Khan, Prakash, & Agashe, 2016b for detailed methods). Briefly, we measured the zone of inhibition produced by beetle homogenates on a lawn of *B. thuringiensis* growing on nutrient agar medium. Flour beetles also secrete defensive quinone compounds that inhibit the microbial growth in their surroundings, acting as an external immune defence (Joop, Roth, Schmid-Hempel, & Kurtz, 2014; Khan et al., 2016b). To quantify this immune response, we measured the zone of inhibition produced by cold-shocked females (−80°C for 20 min) embedded vertically in a lawn produced by *B. thuringiensis* growth on nutrient agar plates (n = 9–10 females/treatment/population). A cold shock triggers the release of abdominal and thoracic stink gland contents with antimicrobial quinones (Khan et al., 2016b). We analysed the non-normally distributed immune response data using nonparametric Wilcoxon rank-sum tests and estimated the impact of priming on immune components as: Mean zone of inhibition produced by primed females/Mean zone of inhibition produced by unprimed females.
3. Developmental rate: To measure the effect of priming on larval development, we placed immune-challenged experimental larvae individually in 96-well microplates (n = 21–22 larvae/treatment/population). We observed larvae every 12 hr (11 a.m. & 11 p.m. ± 1 hr) and noted the time to pupation for each larva. We analysed the data (non-normally distributed) using nonparametric Wilcoxon rank-sum tests and calculated the impact of priming on larval development as: Mean time to pupation of primed larvae/Mean time to pupation of unprimed larvae.

3 | RESULTS

The strength of immune priming is usually quantified as the survival benefit to the host after infection, with vs. without previous exposure to the same pathogen (Roth *et al.*, 2010; Khan et al., 2016a). Hence, we quantified the strength of the priming response as the proportional hazard ratio estimated from survival data for unprimed vs. primed individuals, all subsequently infected with live bacteria. In most populations, both larval and adult survival increased significantly after priming (adults: 9/10 populations, larvae: 8/10 populations; Figure 2a,b; Table S2). In some populations (5/10 populations), beetles primed and infected as larvae also appeared to have higher survival than uninfected control groups (Figure 2a), but the difference was not statistically significant (see Table S3). As reported earlier (Khan et al., 2016a), the priming response varied substantially across populations, ranging from no detectable response to a 10-fold increase in larval post-infection survival relative to the unprimed control (Figure 3a). This variation was strongly associated with susceptibility to infection, measured as the hazard ratio for infected vs. uninfected groups (Figure 3a,b). Note that larvae and adults from population ND were most susceptible to infection relative to other populations (ND: 10-fold vs. Others: ~2- to 4-fold difference in survival), potentially driving the association between priming and susceptibility. However, the positive correlation between priming and susceptibility was robust to removing data from the ND population (adult: R^2 = 0.5, p = 0.031; larvae: R^2 = 0.44; p = 0.04).

We note that in our experiments we used a mild infection dose that induced late mortality in beetles (Figure 2). Hence, it is unclear whether the observed mortality is caused by susceptibility to infection alone, or arises from other confounding effects such as late-life physiological costs of early infection, effects of bacterial toxins or persisting low level of infection. We thus reanalysed our previous data on these same populations infected with a higher infection dose (~8,000 live bacterial cells per beetle) that killed 20%–40% beetles within the first 48 hr, capturing a more direct and immediate impact of infection on mortality rate (unprimed infected vs. primed infected beetles; Khan et al., 2016a). We added unpublished data on infection susceptibility at this high dose (uninfected control vs. unprimed infected beetles; Figure S3; Table S4). Even with this high dose of infection, we found stronger priming ability in populations that were more susceptible to infection (Figure S4). Next, to verify that beetle mortality is actually caused by infection, we directly compared bacterial load in beetles from two populations with significantly different responses to the high Bt dose (susceptibility with respect to uninfected control: AM 10-fold vs. AL 4-fold; see supplementary methods; Siva-Jothy, Prakash, Vasanthakrishnan, Monteith, and Vale (2018)). As expected, we found that the highly susceptible AM beetles also harboured higher bacterial load than AL beetles (Figure S5; Table S5). Further, priming significantly reduced the bacterial load of AM beetles, with a concurrent increase in post-infection survival; these effects were not detected in the AL population (Figure S5; Table S5). Together, these results provide strong evidence that mortality caused by bacterial infection is an important correlate of priming ability.

The expression of priming may also be limited by the costs of mounting a priming response, which may vary across natural populations. To test this hypothesis, we first analysed the impact of priming on reproduction. Contrary to the cost hypothesis, we found that only two populations (B1 and ND) showed a significant decrease in reproductive fitness after adult priming (i.e. significant reproductive impact <1, Figure 3b). In most populations, females either produced more offspring after priming (5/10) or showed no detectable change in reproduction (3/10) (Figure 4a,b; Table S6). Similarly, for larval priming, none of the populations showed a significant cost of reproduction (Figure 4a,b; Table S6). Interestingly, the strength of adult

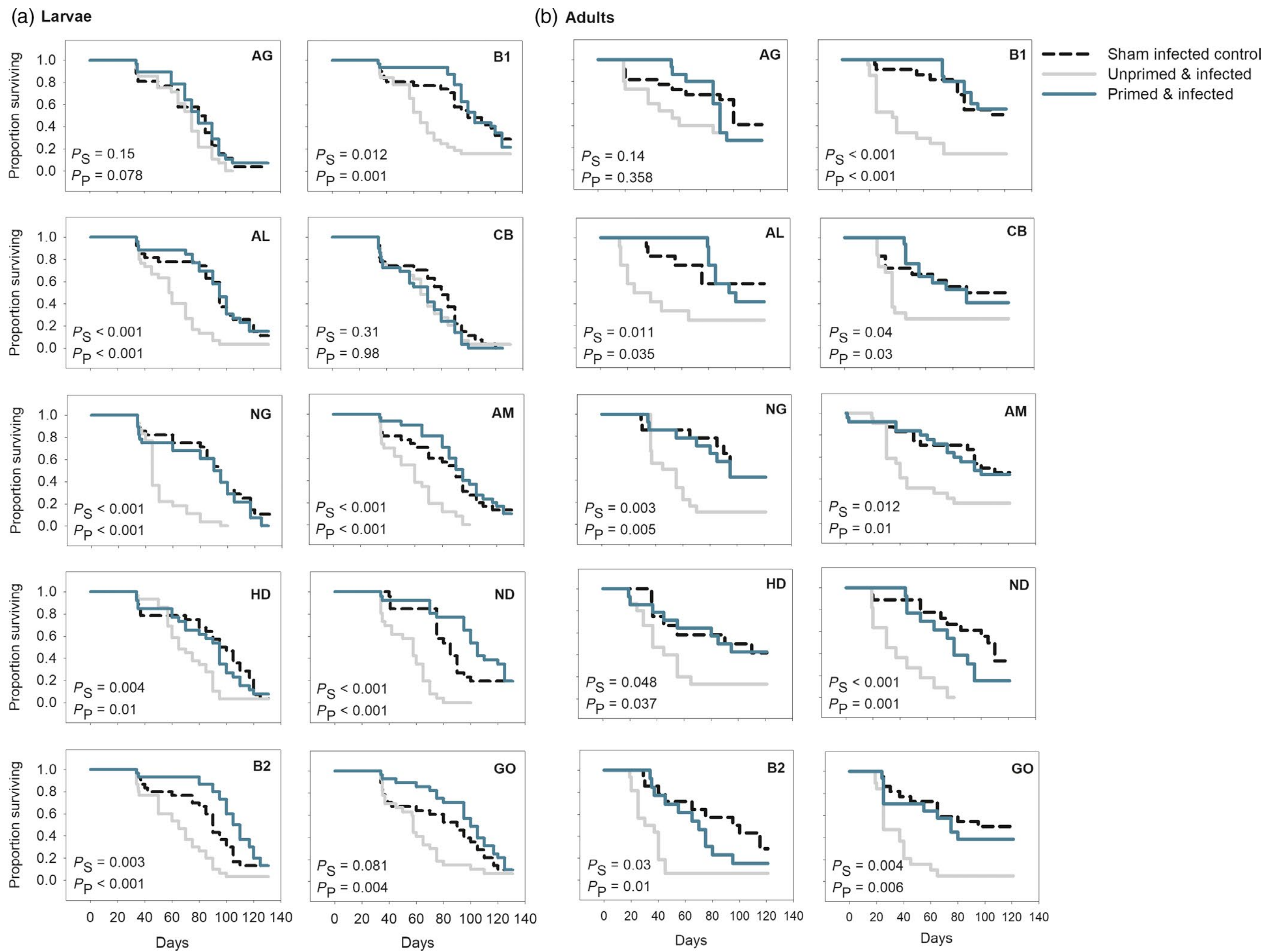

FIGURE 2 Survival curves for (a) larvae and (b) adult females from each population, after within-life stage immune priming. *P* values for the impact of infection (P_S: unprimed infected vs. sham infected control beetles) and immune priming (P_P: unprimed infected beetles vs. primed and infected beetles) treatment are reported in each panel

immune priming and subsequent change in reproductive fitness was negatively correlated, though there was no such association for larvae (Figure 3b). Together, these results suggest that although priming generally does not impose a direct reproductive cost, there may be a stage-specific trade-off between its survival vs. reproductive benefits.

In separate experiments, we also quantified the impact of priming on additional fitness traits and immune components in each population and tested whether these traits were correlated with the priming response, as follows.

3.1 | Survival under starvation

Overall, priming did not affect survival under starvation, except for a few populations where it either prolonged (larvae: 2/7 populations; adults: 1/10 populations) or reduced survival (larvae: 1/7 populations; adults: 1/10 populations) (Figure S6a,b; Table S7). Although larval priming and survival under starvation were not consistently associated, our data suggest a potential relationship between adult investment in priming vs. starvation life span. Individuals from the population (AG) that lacked a priming response lived twice as long under starvation, whereas adult beetles from the population showing the strongest priming response (ND: ~5-fold survival benefit) died faster under starvation (Figure 3c). These results suggest a possible cost of priming in terms of depleting energy reserves, in turn reducing life span under starvation.

3.2 | Immune components

We found that immune priming had a contrasting effect on two components of adult immunity. While priming significantly reduced external immunity in several populations (5/10), its impact on antibacterial activity was highly variable (Figure 4c,d; Table S8). Priming had no impact on antibacterial activity in most populations (7/10), except a few cases where primed females produced either larger (2/10) or smaller zones of inhibition (1/10) than unprimed controls (Figure 4c). None of the immune components were correlated with the observed variation in priming response across populations

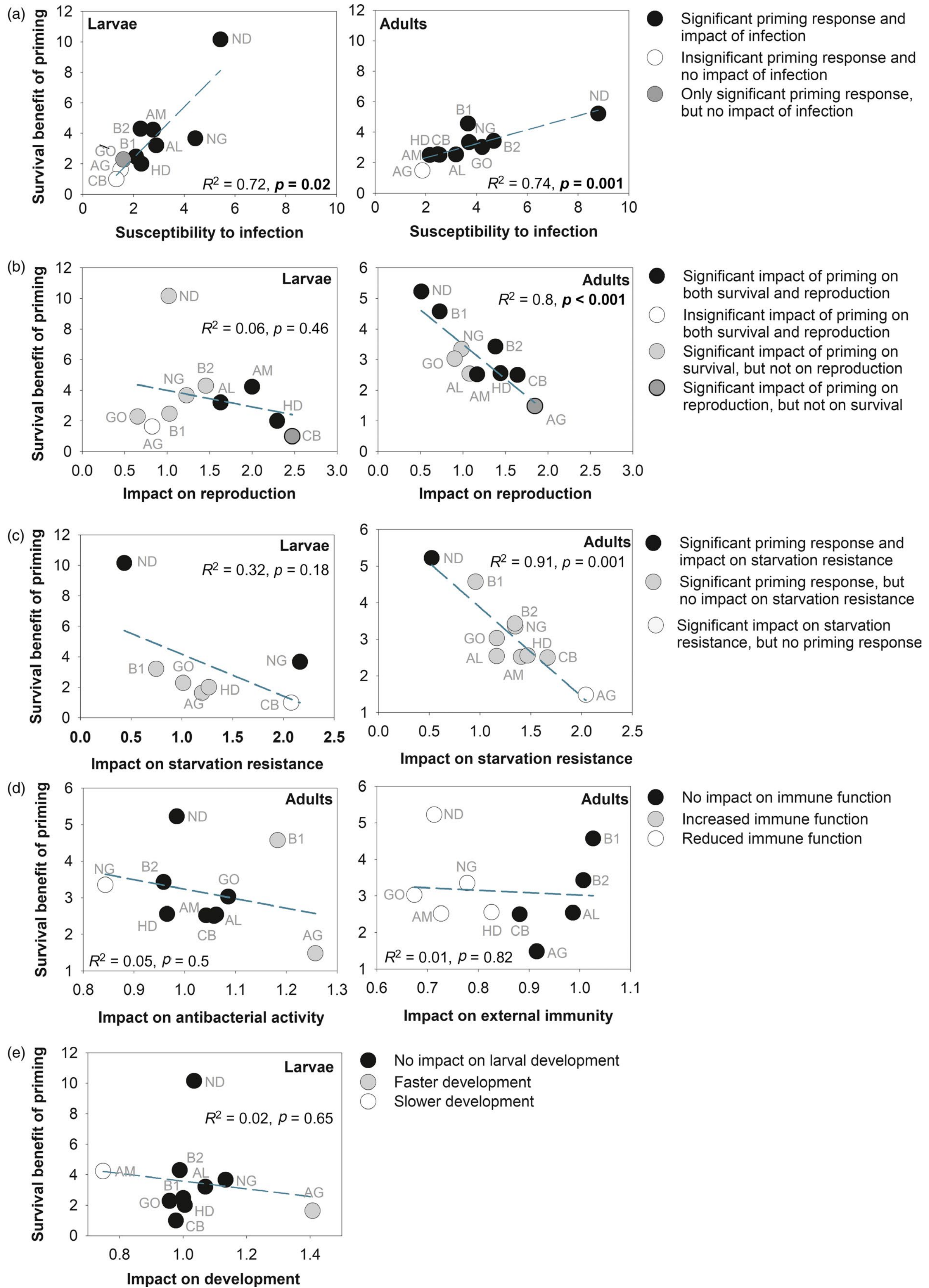

FIGURE 3 (a,b) Correlation between the survival benefit of immune priming and (a) basal susceptibility to infection (b) reproductive benefit of priming. (c–e) Correlation between the survival benefit of priming and the impact of priming on (c) starvation resistance (d) antibacterial ("AB") activity and external immunity and (e) larval developmental rate

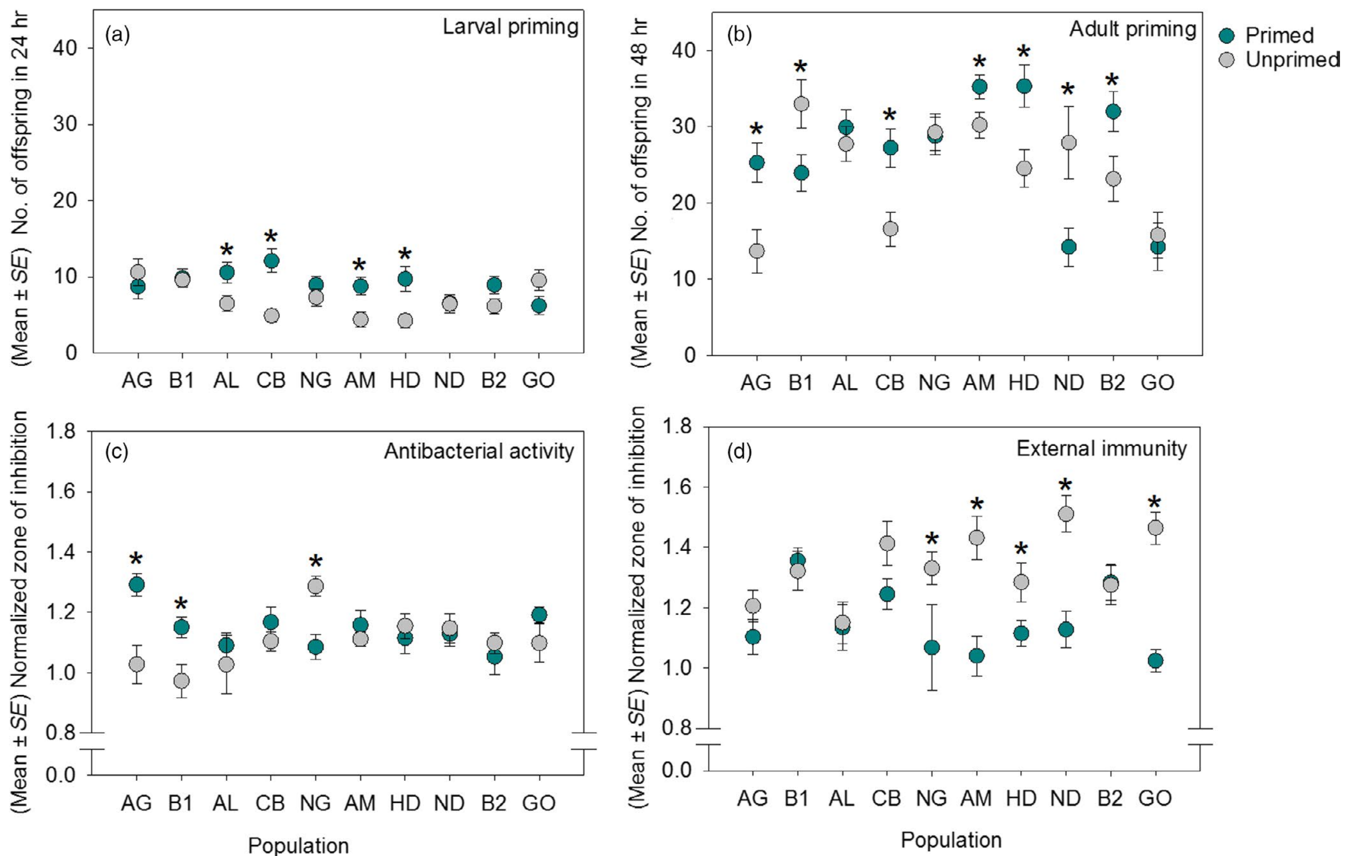

FIGURE 4 (a,b) Impact of (a) larval and (b) adult immune priming on reproduction. (c,d) Impact of adult priming on (c) antibacterial activity and (d) external immunity. Populations where priming altered reproduction or immune traits significantly (i.e. $p \le 0.05$) are highlighted with as asterisk

(Figure 3d). Although previous results suggest a trade-off between external and internal innate immunity (Cotter, Littlefair, Grantham, & Kilner, 2013), we did not find any correlation when analysed separately in primed (R^2 = 0.1, p = 0.35) or unprimed beetles (R^2 = 0.11, p = 0.33). Priming-induced changes in immune components were also uncorrelated with each other (primed beetles/unprimed beetles: R^2 = 0.03, p = 0.6).

3.3 | Larval development

Finally, we tested whether larval immune priming traded off with larval development rate across populations. We found that immune priming did not alter larval development except in two populations, where larvae either developed faster (AG) or showed delayed development (ND) compared to controls (Figure S7; Table S8). Thus, developmental rate of primed larvae cannot explain population-level variation in the larval priming response (Figure 3e).

4 | DISCUSSION

We present the first systematic test of multiple factors that may determine the evolution of the strength of immune priming in natural insect populations. Previously, we documented large variation in the priming response among wild-collected flour beetle populations (Khan et al., 2016a), ranging from no detectable response to a 13-fold survival benefit in some populations. Although this work provided an empirical framework to understand whether and to what degree priming responses vary in natural populations, the selective forces responsible for this variability remained unclear. Previous theoretical work suggests that the strength of the priming response may depend on the strength of selection imposed by pathogens, as well as constraints imposed by the cost of immune priming via trade-offs with other fitness components (Best et al., 2013; Tate & Rudolf, 2012). Our results are consistent with both types of selection. First, we show that the bacterial pathogen *B. thuringiensis* has a variable impact on different beetle populations, suggesting that the same pathogen can impose divergent selection pressures across populations of a single host species. Subsequently, we find that increased susceptibility to *B. thuringiensis* is positively correlated with an increased survival benefit of immune priming, such that priming is most beneficial for populations (e.g. AM) that are highly susceptible to infection. Thus, priming perhaps rescues post-infection by reducing the pathogen load. Prior studies also show that priming alters bacterial load, though the timing (Tate, Andolfatto, Demuth, & Graham, 2017) and direction of the impact varies (compare Pham, Dionne,

Shirasu-hiza, & Schneider, 2007; Kutzer, Kurtz, & Armitage, 2019). Together, these results indicate that pathogen-mediated reduction in life span may impose strong selection for a priming response in natural populations, as predicted by theoretical models (Best et al., 2013) and observed in laboratory-evolved populations (Khan et al., 2017). Second, we found a life stage-specific negative relationship (a possible trade-off) with early reproductive success (measured over 2 days) that may constrain the strength of priming in adults, but not in larvae. When primed and infected adult females produced more offspring than unprimed controls (i.e. priming increased reproduction), they showed a reduced survival benefit of priming. Hence, the most important implication of our work is that both specific fitness costs and the fitness impact of infection may underlie the evolution of stronger priming.

We note that some of our results differ from our previous experiments with the same beetle populations, where only 4 of 10 populations showed priming responses at both larval and adult stages (Khan et al., 2016a). However, the previous experiment differed from our present work in two key features. First, we previously used a substantially higher infection dose to infect beetles (10-fold higher than the current study), inducing >50% mortality within a week (early mortality rate compared to the present study). Second, we had earlier recorded mortality until 10 days post-infection, whereas in the present work we monitored survival until 90 days post-infection. Both factors could decrease the likelihood of observing a significant impact of priming in our prior study. However, despite this difference, we found that priming and susceptibility showed a positive correlation in both experiments, suggesting that this correlation is repeatable regardless of the infection dose (high vs. low) or the timing of mortality (immediate vs. delayed). We thus stress that while most natural populations possess the mechanism to show a priming response to infection, its expression and fitness benefits may often depend on the infection intensity. Our recent work with experimentally evolved beetle populations supports this inference: basal priming ability against a low infection dose evolved to improve survival against a higher infection dose only under strong pathogen pressure for multiple generations (Khan et al., 2017).

A notable strength of our study is the use of multiple natural populations to gain deeper insights into the evolutionary and ecological history of priming in an insect. Our results indicate a general adaptive role of immune priming in some populations (larvae: 4/10 populations vs. adults: 5/10 populations) where priming improved long-term life span as well as immediate reproductive effort. This seems to contradict a mathematical model that predicts large reproductive costs associated with priming (Best et al., 2013). However, a careful comparison across populations revealed that immune priming does not improve survival and reproduction of adults equally in a population. Instead, greater benefits of reproduction come at the cost of reduced survival, suggesting a broadly distributed hidden trade-off between these traits. Similarly, primed female mosquitoes that invested more in egg production showed reduced pathogen clearance and greater susceptibility to infection (Contreras-Garduño et al., 2014). These parallel results suggest the existence of general trade-offs between the survival and reproductive benefits of priming in adult insects. However, we note that we could only measure a small fraction of the reproductive period of *Tribolium* females (48 hr post-infection in adults). Hence, we cannot ignore the possibility of terminal investment in some populations (Reaney & Knell, 2009) where primed beetles increase their reproduction immediately after infection, whereas in other populations beetles suppress immediate reproduction in favour of survival and somatic maintenance later in life (Luu & Tate, 2017). To explicitly test these possibilities, we suggest experiments to measure the long-term effects of priming on reproduction and other life-history traits. In contrast to adult priming, reproduction and survival benefits were not correlated when individuals were primed and challenged as larvae. While the exact reasons for such stage-specific effects are unclear, it is plausible that the stage-specific results arise from different time frames between infection and mating (larvae: 24 days vs. adults: 1 day; see experimental plan), or the oviposition period (larval priming: 24 hr vs. adult priming: 48 hr).

Our data also suggest a weak negative association between priming and starvation resistance, although only two populations (AG and ND) showed a significant impact of priming on adult starvation resistance. Previous studies have documented trade-offs between immune investment and life span during starvation in fruit flies (Valtonen, Kleino, Rämet, & Rantala, 2010) and bumblebee workers (Moret & Schmid-Hempel, 2000). In fruit flies, the trade-off between immunity and starvation resistance may also have a genetic basis: genotypes that invest more in immunity have lower survival under starvation and vice versa (Hoang, 2001). It is currently unclear whether such phenotypic or genetic trade-offs are widespread with respect to priming ability and life span during starvation; this is an exciting possibility that requires further work. Another interesting finding from our study is that although priming altered larval development in some populations, it was uncorrelated with population-level variation in priming response. This contrasts previous studies showing between-individual correlations: larval immune activation accelerated development (Roth & Kurtz, 2008) and maternal priming either accelerated (Tate & Graham, 2015) or reduced (Zanchi et al., 2011) offspring development. Therefore, an additional implication of our work is that studies using a single population are insufficient to generalize the costs or benefits of priming, because priming has variable consequences across populations.

The lack of associations between priming and different immune components was surprising, given prior work with other insects showing that priming is beneficial because it induces more efficient immune responses (Barribeau, Schmid-Hempel, & Sadd, 2016, but also see Greenwood et al., 2017; Tate & Graham, 2017) and that components of innate and external immunity (quinone secretion outside the body; Joop et al., 2014) trade off (Cotter et al., 2013). The contrast with our results may perhaps reflect species-specific trade-offs. Another possibility is that since immune responses are dynamic and known to depend strongly on the time point selected for analysis (Tate & Graham, 2017), our single measurement of immune response may not capture earlier or longer-term impacts of priming on different immune components. Finally, the reduction

of external immunity after priming in multiple beetle population could still suggest the extent of shared underlying immune pathways (e.g. innate immune activation vs. quinone production pathways) across species or across diverse immune components measured in different experiments (Cotter et al., 2013; Khan et al., 2016b). These are intriguing possibilities that underscore the need for more detailed experiments with a diverse set of insects to understand processes driving the evolution of insect immunity.

Overall, our data highlight the importance of explicitly testing the impact of pathogen selection and fitness costs on the immune system of multiple populations, with possible implications for natural host–pathogen systems in the wild. For example, a previous study suggested that *Drosophila* populations were more resistant to *Lactococcus lactis* infection if they were previously exposed to multiple pathogens or other *Lactococcus* species (Corby-Harris & Promislow, 2008). Similarly, it is possible that our beetle populations had encountered variable selection imposed by widely distributed natural pathogens such as *B. thuringiensis* in their natural habitat. Therefore, the observed responses against experimental manipulations (e.g. priming and/or live pathogen exposure) can also be influenced by previously experienced pathogen selection. Although we do not have information on local pathogen pressure that our beetles experienced before we brought them into the laboratory, we previously showed that strong pathogen selection is indeed necessary to evolve immune priming in laboratory populations of flour beetles (Khan et al., 2017). Hence, our work serves as an important first step towards understanding whether and why natural populations of insects differ in their immune priming response. We suggest that future experiments allow multiple susceptible wild populations to evolve under a range of pathogen-imposed selection, to directly determine causal relationships between the strength of selection, the nature of the immune response, and the associated fitness costs.

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AUTHORS' CONTRIBUTIONS

I.K. conceived the experiments; I.K., D.A. and A.P. designed the experiments; A.P. and I.K. carried out the experiments; I.K. analysed the data; I.K. and D.A. wrote the manuscript with inputs from A.P. All authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.344032g (Khan, Prakash, & Agashe, 2019).

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SUPPORTING INFORMATION

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Why do insects evolve immune priming? A search for crossroads

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ABSTRACT

Until recently, it was assumed that insects lack immune memory since they do not have vertebrate-like specialized memory cells. Therefore, their most well studied evolutionary response against pathogens was increased basal immunity. However, growing evidence suggests that many insects also exhibit a form of immune memory (immune priming), where prior exposure to a low dose of infection confers protection against subsequent infection by the same pathogen that acts both within and across generations. Most strikingly, they can rapidly evolve as a highly parallel and mutually exclusive strategy from basal immunity, under different selective conditions and with divergent evolutionary trade-offs. However, the relative importance of priming as an optimal immune strategy also has contradictions, primarily because supporting mechanisms are still unclear. In this review, we adopt a comparative approach to highlight several emerging evolutionary, ecological and mechanistic features of priming vs basal immune responses that warrant immediate attention for future research.

1. Introduction

In recent decades, the discovery of immune memory-like responses in insects, whereby previous sublethal exposure to a pathogen confers subsequent protection against the same pathogen (i.e., immune priming), remains as one of the most important conceptual advancements in invertebrate immunology. The phenomenon was initially puzzling because insects apparently lack the legacy of immune cells or antibodies functionally equivalent to vertebrate lymphocytes (B and T-memory cells; components of adaptive immunity) that form the mechanistic basis of immune memory. Therefore, the most well studied evolutionary response against a virulent pathogen in insects was their increased basal immune function activated upon live pathogen exposure (Rolff and Siva-Jothy 2003; Corby-Harris and Promislow, 2008). However, this long-standing notion is now being replaced by a large number of evidence for diverse insect priming forms where their effects are detected within the same developmental stage, across life stages, or even to offspring (Contreras-Garduño et al., 2016; Khan et al., 2016; Tetreau et al., 2019; Sheehan et al., 2020). They are abundantly found in a wide range of insects species as diverse as dipterans (Pham et al., 2007; Ramirez et al., 2015), coleopterans (Khan et al., 2019), lepidopterans (Fallon et al., 2015), hymenopterans (Sadd and Schmid-Hempel, 2006) and arachnids (Gálvez et al., 2020). Recent studies document the widespread presence of various priming forms in wild-caught insect populations as well, providing the much-needed boost to justify their natural relevance (Tate and Graham, 2015; Khan et al., 2016). Mathematical models propose that immune priming or memory-like responses might have large impacts in reducing the infection severity and disease spread in natural populations (Tate and Rudolf, 2012; Best et al., 2013; Tate, 2016), thereby highlighting their adaptive values. Priming has thus rapidly emerged as an integral feature of insect immunity. Discovery of insect immune priming also implies that although vertebrate immune memory is achieved via mechanistically distinct immune responses (Netea et al., 2020), it is perhaps functionally not as unique as perceived traditionally.

2. Evolving immune priming: where, when and how?

Despite the surge of phenomenological evidence, the evolutionary basis of priming remained obscured until recently (Khan et al., 2017a). It was unclear whether selective parameters underlying the evolution of priming and their potential evolutionary trade-offs were different from basal immune responses (which function without any prior exposure to pathogens). Also, we lack estimates of how much genetic variation exists for priming in natural populations (Khan et al., 2016), thereby restricting our ability to predict its evolvability. Unlike basal immunity

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which positively correlates with infection prevalence and strength of pathogen pressure (Linhart and Grant, 1996; Reznick and Ghalambor, 2001; Corby-Harris and Promislow, 2008; Mayer et al., 2015), it was unclear whether the same parameters play a similar role for priming as well. Wild populations inhabiting pathogen-rich environments certainly have a higher risk of reinfection by the same pathogen (see Corby-Harris and Promislow, 2008), but the link between the strength of priming and infection severity was never tested. This was confirmed very recently in wild-caught flour beetle populations, where the benefit of priming was determined by the infection severity, with increased pathogen susceptibility imposing selection for stronger priming responses (Khan et al., 2019). However, although these results could establish the ubiquitous role of pathogen pressure behind both priming and basal immunity, a source of major confusion is— how does pathogen selection distinguish the evolutionary trajectories between priming vs basal immunity? From an evolutionary perspective, this certainly needs to be clarified before we could analyse the relative importance of priming vs basal immunity as effective immune responses in insects.

While experiments were lacking initially, a general mathematical model was able to offer some critical insights (Mayer et al., 2015). Although not specific to insects, this model, for the first time, brought more detailed features of the pathogenic environment, such as frequency and duration of pathogen exposure, at the forefront. It was also coupled with critical analyses of relative costs vs benefits of different immune components to explain why organisms choose to evolve divergent immune strategies. For example, when pathogen exposure is relatively rare, inducible immune responses can be adaptively favoured to minimise the costs of constitutively expressed innate immunity, suggesting complex interactions between pathogenic conditions, pathogen exposure statistics and fitness effects of counteractive immune components behind evolving optimal immune responses (Mayer et al., 2015). Subsequently, the first experimental evidence came from Khan and co-workers where they manipulated the frequency of pathogen exposure (e.g., once vs twice every generation) in flour beetle *Tribolium castaneum* populations, evolving against a lethal dose of their natural pathogen *Bacillus thuringiensis* (Khan et al., 2017a). Within 14 generations of experimental evolution, they found populations that were exposed to a single large dose of infection every generation evolved both within- and across-generation priming (Khan et al., 2017a; Prakash et al., 2019). In contrast, populations that were exposed repeatedly to the same pathogen (Khan et al., 2017a; Prakash et al., 2019), evolved inherently higher post-infection survival, without any additional effects of priming (henceforth, basal infection response). The study also made an implicit assumption that such increase in post-infection survival was possibly achieved by improving the basal immune function or resistance, but this causal link has not been established yet (also discussed later). Nonetheless, these results on evolving diverse immune strategies in insect models as a function of pathogen exposure statistics closely mirrored the predictions of Mayer et al. (2015), highlighting the possible implications for the general evolution of immune responses.

Around the same time, another study by Ferro and co-workers (Ferro et al., 2019) also reported the evolution of priming in flour beetles against *B. thuringiensis*, using a different experimental set-up from Khan et al. (2017a). Instead of using a specific strain of a single pathogen, they used combinations of different bacteria, including multiple *B. thuringiensis* strains, to prime and infect beetles for 14 generations. However, priming only evolved against a focal *B. thuringiensis* strain that primed and infected beetles at every three generations (e.g., generation 1, 4, 7 and so on) (Ferro et al., 2019), suggesting that the selection for priming doesn't have to be continuous across generations. Interestingly, the selective conditions for evolving inherently high basal infection response remained comparable between Ferro et al. (2019) and Khan et al. (2017a)— i.e., in both the studies, beetles evolving high basal infection response were exposed to the focal *B. thuringiensis* antigen twice (priming with heat-killed cells followed by the live infection) at every generation. Hence, despite some differences in the experimental design, comparable results from separate studies with independently evolving beetle populations unequivocally supported the role of lower pathogen exposure frequency in priming evolution, whereas basal immune function increasing survival is favoured in populations exposed to the same pathogen more frequently (Khan et al., 2017a; Ferro et al., 2019). Together, these two studies also established priming as a rapidly evolving independent strategy from basal infection responses, ending all the prior speculations on whether they have distinct evolutionary origins. However, since both studies addressed the evolution of priming in flour beetles, more studies are needed to verify whether similar selective conditions are also relevant for other insect species. Till then, they can serve as an important benchmark for future studies, revealing several previously unknown features of priming vs basal immunity and infection responses. Below, we outline how our current understanding based on these studies might provide the impetus for further research on insect immune priming, using an integrated framework of evolutionary, ecological and mechanistic insights.

3. Emerging features of priming

While recent data clearly underscore the adaptive significance of priming independently of basal infection responses (Ferro et al., 2019; Khan et al., 2017a), they also attract newer complications vis-à-vis diverse fitness effects, unexpected evolutionary trade-offs and intertwined mechanisms. For example, it is surprising that evolution of priming does not increase the basal infection response in flour beetles at all (Khan et al., 2017a)—i.e., without a primary antigen exposure, beetles evolving priming can still be highly susceptible to infection, suggesting an intrinsic difference in how priming vs basal immunity might respond to infections. However, one of the most striking aspect of these results is the consistently higher survival benefits of increased basal infection response relative to priming (80% vs 50% survival after pathogen exposure), raising further questions on their actual adaptive potential. If evolving basal infection response and immunity can confer better survival, it is surprising why it does not evolve in all the populations facing the pathogen pressure? One plausible explanation for this observation could be that basal infection response is also associated with other fitness costs, but contrary to such expectations, later experiments revealed that evolving basal infection response is also beneficial for multiple other reasons. For example, these beetles have increased reproduction and longer lifespan (Prakash et al., 2019). They also seem to have better body condition as they have extended lifespan under starvation (Prakash et al., 2019). Together, these results might strongly contradict the traditional view of immunity-fitness trade-offs, but lack of any measurable fitness costs of pathogen resistance has been experimentally proven in other studies as well (see Ye et al., 2009; Ma et al., 2012).

In contrast, both maintenance and activation of evolved priming responses can impose diverse suits of fitness costs such as reduced offspring survival, developmental time and reproduction (Ferro et al., 2019; Prakash et al., 2019). These results also corroborate outcomes from other single-generation experiments, where inducing priming responses can directly reduce the reproductive fitness in flour beetles (Contreras-Garduño et al., 2014), mosquitoes (Ramirez et al., 2015) and wax moth (Trauer and Hilker, 2013) or delayed development in mealworm beetles (Zanchi et al., 2011). Together, these pervasive costs might limit the survival benefits of priming responses at a much lower level than consistently beneficial basal immunity. Yet, it remains a mystery why putative alleles for basal immunity and infection responses did not outcompete priming alleles in all populations (Khan et al., 2017a, 2019; Ferro et al., 2019). Can the answer be hidden underneath mechanistic constraints? For example, selective conditions favouring the evolution of priming (e.g., lower pathogen exposure frequency, Khan et al., 2017a) might also produce inhibitory signals that can mechanistically prevent the alleles for more beneficial basal infection response to appear and fix in the host population. Clearly, more experiments

combining both organismal biology and molecular insights are necessary to test these possibilities, systematically pinpointing various distinctive features of priming relative to basal immunity. To facilitate some of these initiatives, below, we have discussed several emerging features of insect immunity in the context of priming vs basal immunity, combining both organismal studies as well as mechanistic information.

3.1. Multifaceted insect immune responses: where and how?

Insects are widely known to possess a robust immune system against various infections that includes both cellular and humoral components (Hoffmann, 2003; Stofanko et al., 2010, Buchon et al., 2014; Parsons et al., 2016; Hanson et al., 2019). While some of them are inducible and slow-acting against persistent infections, such as anti-microbial peptides (henceforth, AMPs), there are also fast-acting immune effectors, such as phenoloxidase response (henceforth, PO) which is constitutively expressed (Haine et al., 2008). However, despite knowing how they might broadly function, assigning these immune responses to priming vs basal immunity and their fitness consequences might not be straightforward. It can be more complicated when a relatively limited repertoire of immune cells is stretched into playing diverse roles across species and pathogens. For example, while PO is required to prevent pathogen growth and increase survival in *Drosophila melanogaster* during *Salmonella typhimurium* and *Listeria monocytogenes* infections (Ayres and Schneider, 2008), it might not be needed against other pathogens, such as *Providencia rettgeri* (Duneau et al., 2017). The same PO activity is also cytotoxic to *Drosophila* cell lines (Zhao et al., 2011), and can produce immunopathological effects in mealworm beetle *Tenebrio molitor*, causing a drastic reduction in their lifespan by damaging key organs such as Malpighian tubules (Khan et al., 2017b). It becomes even more intriguing when PO activity which usually responds nonspecifically to invading pathogens (Cerenius and Söderhäll, 2004; Khan et al., 2017b), might also play a key role in evolving pathogen-specific priming responses (Ferro et al., 2019). Together, these information underscore the astounding level of functional complexity of molecules involved in a so-called simple insect immune system. More recently, another interesting example emerged from fruit flies, where reactive oxygen species with pronounced cytotoxic effects (Lennicke and Cocheme, 2020) such as H_2O_2, produced by Toll-and JAK/STAT pathway activation, and damage associated signalling molecules (Draper) in haemocytes, could also signal the priming of haemocytes against future infections (Chakrabarti and Visweswariah, 2020). The absence of ROS response in haemocytes can lead to loss of priming, indicating its causal role in training innate immunity. Although this study only used a single pathogen *Enterococcus faecalis*, it exemplifies how immune-related molecules should be analysed using diverse lenses of their functionalities.

Importantly, exploring candidate genes and immune molecules for priming vs basal immunity should be integrated with their functional verifications and phenotypic outcomes (A graphical summary combining some of the currently discussed immune priming mechanisms across species is provided in Fig. 1). For example, the only study that tracked mechanisms underlying the evolution of priming, using flour beetle populations infected with *B. thuringiensis*, found a positive correlation with the over-expression of dopa decarboxylase (ddc), a gene involved in PO response (Ferro et al., 2019). AMPs such as attacins, defensins and coleoptericin were also upregulated, but priming ability was more consistently associated with higher expression of ddc, indicating greater importance of PO response than AMPs. This is corroborated by other single-generation or short-term studies as well, including higher PO activity in primed greater wax moths *Galleria mellonella* (Vertyporokh and Wojda, 2020), or a transgenerational study with flour beetles, where paternal priming caused a dramatic increase in offspring's haemolymph PO activity (Roth et al., 2010; Eggert et al., 2014). However, fitness benefits of priming responses that are primarily dependent on PO activity (Nappi and Ottaviani, 2000; Khan et al., 2017b) should also be interpreted with a caution. This is because the net fitness gain can be constrained by an upper limit to reduce the immunopathological costs of a hyper-active PO response (Khan et al., 2017b). This is possibly also the reason why evolved priming always showed lower survival benefits against lethal infections, relative to evolved basal infection response, in earlier experiments with flour beetles (see Khan et al., 2017a). It could have been interesting to compare mechanistic changes in these beetles parallelly evolving both stronger priming and basal immunity, but unfortunately such a comparative framework is missing.

Finally, there are also exciting possibilities of intimate cross-talk between the host's immune system and other physiological processes such as metabolic changes which may influence the host's evolutionary response against pathogen attacks. Indeed, in addition to traditional immune effectors, recent experiments found tight correlations between downregulated metabolism-associated genes such as hexokinase type 2 and sedoheptulokinase with increased priming in flour beetles (Ferro et al., 2019). These results draw close comparisons with the metabolic basis of macrophage polarisation (Kelly and O'Neill, 2015) and trained immunity in vertebrates (Cheng et al., 2014). More recently, another study analysing transcriptome from the midgut of primed mosquitoes *Anopheles albimanus* also reported upregulated genes related to metabolic activities, including trehalose transporter, GDP-D-glucose phosphorylase and fatty acid hydroxylase (Maya-Maldonado et al., 2021), suggesting the importance of metabolic variations in priming across species. In addition, several mutations in mitochondrial DNA are also emerging as key mediators of both humoral and cellular immunity in insects (Riley and Tait, 2020; Salminen and Vale, 2020). In *Drosophila*, Toll-signalling induces a rapid metabolic switch that triggers anabolic lipid metabolism, facilitating more phospholipid synthesis and endoplasmic reticulum expansion, which in turn compensates for the increasing demand of antimicrobial peptide synthesis to neutralize pathogens (Martínez et al., 2020). It thus appears that future studies might open up new promising avenues to explore whether and how energetics can explain the divergent mechanisms of evolving priming vs basal immunity (Fig. 1).

3.2. Role of competing immune strategies: tolerance vs resistance

Although most studies on pathogen defense primarily focus on the mechanisms that hosts employ to resist infections, recent experiments also provide ample evidence for tolerance to pathogenic infections— i.e., hosts can coexist with pathogens and withstand their negative fitness effects by reducing pathogen- or immune-mediated damage (McCarville and Ayres, 2018; Råberg et al., 2009; Råberg et al., 2007; Schneider and Ayres, 2008; Medzhitov et al., 2012; Seal et al., 2021). Theoretical models suggest that tolerance is consistently beneficial and hence, can be rapidly fixed in the population under directional selection, reducing the level of genetic variation (Miller et al., 2005; Roy and Kirchner, 2000). In contrast, pathogen resistance by inducing immune responses, often involve costly immune components and life-history trade-offs (Khan et al., 2017b). Consequently, resistance trait might converge to an intermediate optimum under stabilizing selection (Råberg, 2014). Besides, it might also have features of balancing selection, maintaining highly polymorphic infection outcomes within the population (Lefevre et al., 2011; Råberg, 2014). However, despite these distinct evolutionary trajectories of pathogen resistance vs tolerance, their relevance to diverse forms of insect immunity and infection responses has never been analysed systematically— e.g., can choice of immune strategies (i.e., resistance vs tolerance) determine the evolutionary potential of priming vs basal immunity (see Fig. 2 for the conceptual link)? Previous studies suggest that the link between priming and tolerance can be species-specific. For instance, in fruit flies, larval priming with the *Drosophila* C virus increases tolerance against the subsequent exposure to the same virus both in adults (Mondotte et al., 2018), as well as in their progeny (Mondotte et al., 2020). In contrast, transgenerational priming of crustacean *Daphnia magna with bacterial pathogen Pasteuria*

ramosa increases antibacterial activity in their offspring (Little et al., 2003) increases antibacterial activity to inhibit the growth of *Pasteuria ramosa* in their offspring. More recently, flour beetles primed with *B. thuringiensis* also exhibit enhanced survival by suppressing the pathogen burden (Khan et al., 2019), suggesting a form of pathogen resistance. However, as implicated earlier, such priming-induced pathogen clearance in flour beetles may be mediated via reactive and cytotoxic PO response (Zhao et al., 2011; Khan et al., 2017b; Ferro et al., 2019), increasing the overall physiological costs associated with priming (Ferro et al., 2019; Prakash et al., 2019). This can also offer a more detailed and composite (evolutionary & mechanistic) explanation for limited fitness benefits of evolving priming, as described earlier in Khan et al. (2017a). If priming indeed relies on immunopathological PO responses (Zhao et al., 2011) to resist pathogen growth (Khan et al., 2019), it can only evolve an intermediate trait value under stabilizing selection (also see above, Miller et al., 2005; Roy and Kirchner, 2000) and hence, is unlikely to be fixed in the population due to fitness constraints.

On the contrary, evolution under frequent exposure to the same pathogen, as reported in Khan et al. (2017a) and Ferro et al. (2019), might hint at a tolerance-like response to infections. Notably, both the studies interpreted the improved basal infection response primarily as resistance, but ~80% post-infection survival (Khan et al., 2017a),

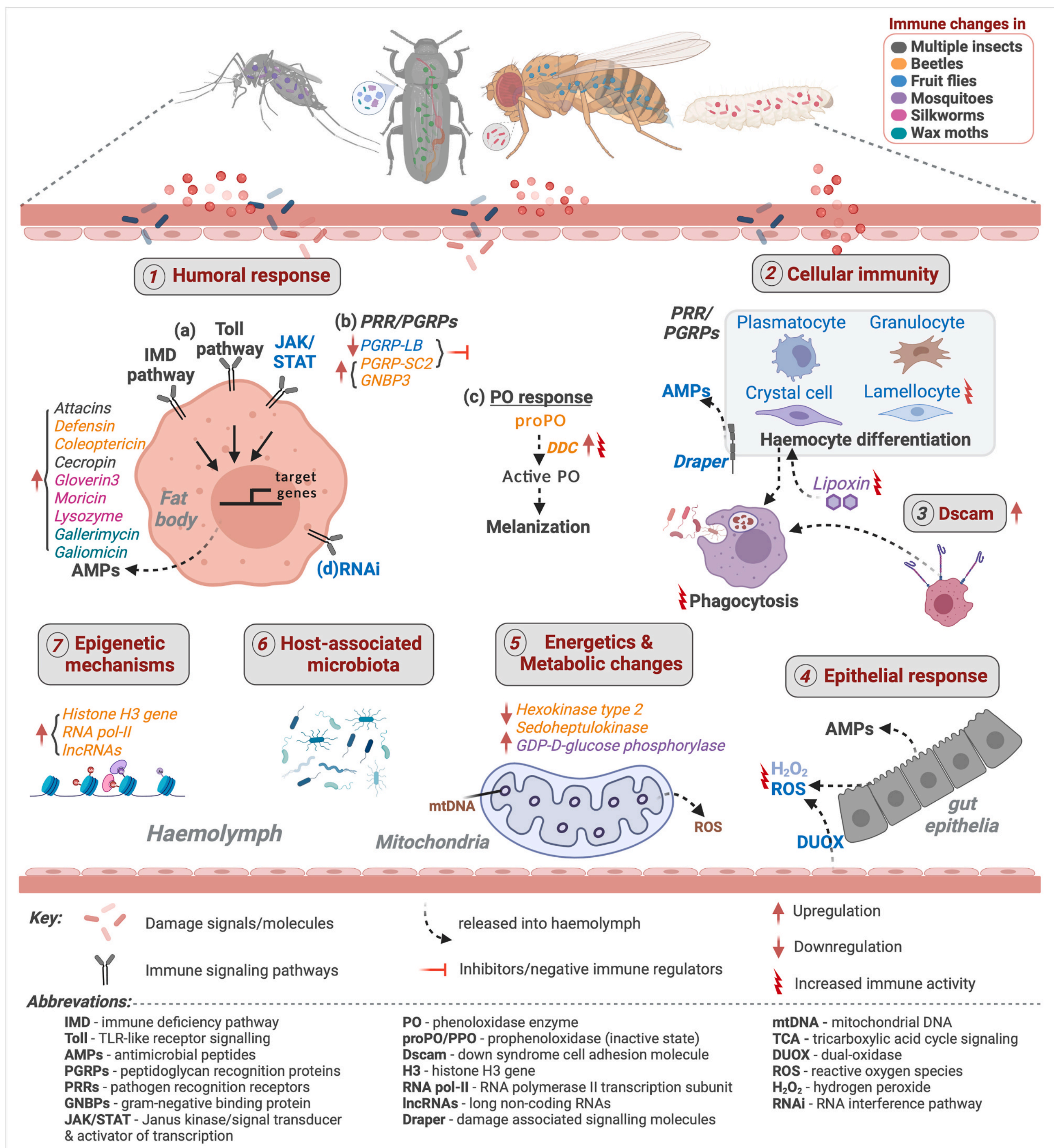

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Fig. 1. A brief summary of known (1–3) vs proposed (4–7) priming mechanisms from recent studies (1) *Humoral response*: (a) Activation of IMD- (immune deficiency), Toll- (TLR-like receptor), and JAK/STAT (Janus kinase signal transducer and activator of transcription) signalling pathways leading to synthesis & production of inducible AMPs (Kuraishi et al., 2013; Milutinović et al., 2016) such as Attacins, Defensins & Coleoptericins in flour beetles (Ferro et al., 2019; Greenwood et al., 2017); Cecropins in fruit flies (Chakrabarti and Visweswariah, 2020); Cecropin, Attacin, Gloverin, Moricin & Lysozyme in silkworms (Yi et al., 2019); Gallerimycin & Galiomicin in wax-moths (Bergin et al., 2006); Cecropin in tobacco moths (Roesel et al., 2020). (b) Upregulated PGRP2 (peptidoglycan recognition proteins - receptor for IMD pathway) and GNBPs (gram-negative binding proteins - receptor for Toll-pathway) in flour beetles (Ferro et al., 2019); Downregulated PGRP-LB (negative regulator of IMD-pathway) in fruit flies (Bozler et al., 2019). (c) Phenoloxidase (PO) response: Increased PO activity in flour beetles and mealworm beetles (Tetreau et al., 2019); Increased DDC (dopa-decarboxylase – a gene involved in PO cascade) in flour beetles (Ferro et al., 2019). (d) RNAi-pathway mediated specific priming against viral pathogens in fruit flies (Mondotte et al., 2018). (2) *Cellular immunity*: Increased phagocytosis activity (Pham et al., 2007; Weavers et al., 2016) and production of lamellocytes (Bozler et al., 2020) in fruit flies; Haemocyte differentiation induced by lipoxin (a lipid carrier) in mosquitoes (Rodrigues et al., 2010; Ramirez et al., 2015). (3) Down syndrome cell adhesion molecule (Dscam): Suspected role in specific immunity of flour beetles, fruit flies and other insects (Armitage et al., 2015); Acts as a receptor during phagocytosis in crabs (Li et al., 2018); Upregulated in primed silkworms (Yi et al., 2019). (4) Epithelial response: Activation of haemocytes via intracellular accumulation of H_2O_2 (hydrogen peroxide) produced by DUOX (Reactive oxygen species producing dual-oxidase) through Toll & JAK/STAT pathway activation & Draper (damage associated signalling molecules) in fruit flies (Chakrabarti and Visweswariah, 2020). (5) Metabolism and energetics: Downregulated metabolism-associated genes - hexokinase type 2 and sedoheptulokinase in flour beetles (Ferro et al., 2019); Upregulated trehalose transporter, GDP-D-glucose phosphorylase in mosquito *Anopheles albimanus* (Maya-Maldonado et al., 2021). (6) Host-associated microbiota: Loss of priming in flour beetles (Futo et al., 2017) and *Anopheles. gambiae* mosquitoes (Rodrigues et al., 2010) after depleting microbiota. (7) Epigenetic mechanisms and reprogramming: Upregulated histone H3 gene, RNA polymerase II (transcription subunit 15) & exosome complex exonuclease (RRP6-like) (Ferro et al., 2019); Upregulated lncRNAs (long non-coding RNAs – necessary for regulating-metabolic, immune signalling and epigenetic processes in flour beetles (Ali and Abd El Halim, 2020).

coupled with increased reproduction, better body condition and no other measurable fitness costs (Prakash et al., 2019) perhaps warrant alternative explanations. For example, the rapid increase from 50% to ~80% post-infection survival within <5 generations (see Khan et al., 2017a) can also be achieved by the rapid spread of tolerance alleles under directional selection (Miller et al., 2005), increasing the survival by reducing the cost of infection or immune responses, rather than by employing an efficient pathogen killing mechanism (Ayres and Schneider, 2012). Future experiments directly estimating changes in fitness with increasing immune function or pathogen clearance (slope of fitness-by-pathogen load curve, see Fig. 2II) might resolve the ambiguity here.

Also, it remains to be seen what happens when immune strategies (i.e., pathogen clearance vs infection tolerance) are swapped across priming vs basal infection responses— e.g., if priming evolution is mediated by increased tolerance (see Mondotte et al., 2018; for fruit flies against viral infections), would it be able to increase the fitness gain more than basal immunity (compare with Khan et al., 2017a)? Certainly, more studies are needed to understand whether or to what extent fitness trajectories of priming vs basal immune function and infection responses across diverse insect host-pathogen systems depend on the choice of immune strategies. They might serve as a critical resource for mechanistic studies where the role of transcriptionally and translationally active immune molecules and pathways can be analysed and compared across species to impart a deeper holistic understanding of diverse insect immune strategies.

We also speculate that the negative regulators of major insect immune responses e.g., IMD, Toll or JAK/STAT pathway (Ali et al., 2020; Frank and Schmid-Hempel, 2019; Kuraishi et al., 2013) might hold the key for understanding the relative impacts of resistance vs tolerance, thereby influencing the outcome of basal infection response vs priming. For instance, in fruit flies, G9a (an epigenetic negative regulator of the damage sensing JAK/STAT pathway) has been previously identified as an important molecule in reducing immunopathology by tolerating viral infections (Gupta and Vale, 2017; Merkling et al., 2015). Recently, a negative regulator PGRP-LB (peptidoglycan recognition protein LB) from the IMD pathway has also been identified as a key candidate during transgenerational immune priming in *Drosophila* against parasitoid wasps (*Leptopilina heterotoma* and *L. victoriae*) (Bozler et al., 2020)— i.e., global downregulation of PGRP-LB is necessary to increase lamellocytes for pathogen clearance in the offspring. In another instance, the absence of PGRP-LB renders flies more resistant to *Eschericia coli*, whereas its normal activation leads to greater tolerance (Vincent and Dionne, 2021). Overall, we have just begun to uncover the potential role of tolerance via various regulatory mechanisms during infections. We certainly need more experiments to explore how targeted modulation of various receptors, signal transduction and immune effectors can be combined with functional characterization and phenotypic responses across insect species. They might offer important clues about how negative feedback mechanisms can evolve to support diverse priming types vs basal infection response and immunity (Khan et al., 2017a; Ferro et al., 2019). Lastly, another indirect possibility, though not mutually exclusive, is that selection acts on the composition and function of the host-associated microbiota which functions as a signalling hub to regulate pathways underlying inflammatory responses (Thaiss et al., 2016) (also see Fig. 1). This may significantly contribute towards maintaining tolerance and reducing the damage caused by misregulated inflammatory responses. Such a balanced immunity-microbiota alliance is also known to reduce oxidative damage to DNA and its repair machinery, whereas an imbalance leads to the development of health hazards in humans (Ray and Kidane, 2016; Belkaid and Hand, 2014). Based on these facts, future studies should also explore whether, or to what extent, the microbiome might evolve in insects under pathogenic conditions to act as an additional regulatory switch between immune strategies, thereby influencing priming vs basal infection responses (also see Rodrigues et al., 2010; Futo et al., 2017; Martínez et al., 2020).

3.3. Remarkable specificity of priming: why and how?

An interesting feature of insect immune priming is their ability to differentiate between strains of the same pathogen (Roth et al., 2009; Wu et al., 2015; Futo et al., 2017), revealing an unexpectedly high degree of specificity, a feature that is previously thought to be restricted to only vertebrate adaptive immunity. Recent flour beetle studies have not only reported phenomenological evidence of such exquisite specificity (Roth et al., 2009; Khan et al., 2016), rather they could also experimentally evolve priming responses specific to a single *B. thuringiensis* strain that was used to infect beetles across generations (Khan et al., 2017a). Surprisingly, priming with another strain of the same pathogen could not confer any survival advantage in the evolved beetles (Khan et al., 2017a). More recently, the evolution of specific priming is corroborated by another study where priming could evolve only against the most virulent *B. thuringiensis* strain, whereas other less virulent strains or non-natural pathogens failed to evoke sufficient selection pressure (Ferro et al., 2019). While these results indicate that priming evolution is perhaps effective only against natural pathogens, they also suggest that even within natural pathogens, priming response is strongly contingent upon their specific strain information which could actually impose the selection pressure.

How do insects mechanistically exercise such specific priming responses? Till date, the answer to this question is conceptually challenging because of our incomplete understanding of insect immunity

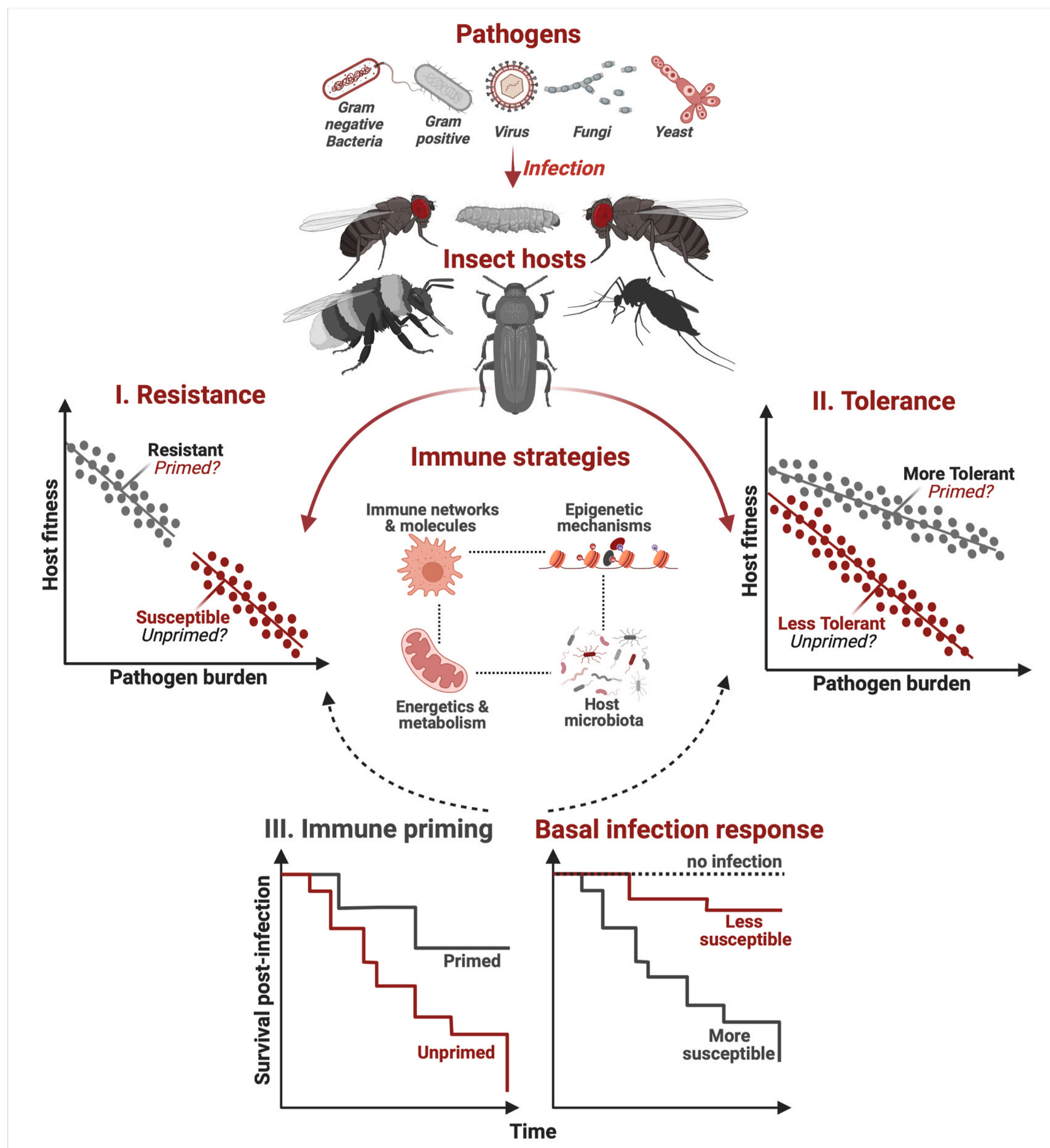

Fig. 2. A diagrammatic representation linking hosts ability to **(I)** either resist pathogen growth, by immune activation, or **(II)** tolerate pathogen burden, by reducing the fitness costs of infection or immune activation without directly reducing the pathogen numbers to **(III)** the outcome of immune priming (i.e., post-infection survival benefits after priming relative to unprimed controls) or basal infection response (i.e., survival response to infection without prior priming relative to uninfected control). At the mechanistic level, a complex interplay between immune pathways & molecules, host metabolism, epigenetic reprogramming and host-associated microbiota might be collectively responsible for evolving priming vs basal infection responses. Figures on tolerance and resistance are adapted from Råberg et al., 2007).

and its full potentials related to priming. For example, a longstanding notion was that the insect immunity has limited ability to distinguish between pathogens (Cooper and Eleftherianos, 2017). Untill recently, antimicrobial peptides (AMPs), the closest candidate for imparting specific immunity in insects, was known only to differentiate between broadly classified microbial families such as Gram-positive vs. Gram-negative bacteria or fungi, but functional distinctions between individual AMPs and their specific effects on pathogens were largely overlooked (Rolff and Schmid-Hempel, 2016; Unckless and Lazzaro, 2016). Newer evidence, however, contradicts this conservative view (Unckless et al., 2016; Hanson et al., 2019), by revealing an unexpectedly high degree of non-redundancy and specificity: Even a single AMP can be wholly responsible to prevent infections caused by a specific pathogen. For example, in fruit flies, AMPs Diptericins or Drosocin alone can confer complete protection against *Providencia rettgeri* or *Enterobacter cloacae* infection respectively (Hanson et al., 2019). Although experiments are lacking, such targeted AMP actions might have a role in specific priming too.

In recent years, several other candidates/ immune genes have also been implicated in pathogen-specific priming. For example, increased phagocytosis (Pham et al., 2007; Weavers et al., 2016) or blood cell differentiation into granulocytes (Rodrigues et al., 2010) plays a role during pathogen-specific immune priming in fruit flies and mosquito *Anopheles gambiae* respectively. Flour beetles that experimentally evolved specific priming have increased dopa-decarboxylase gene expression, which in addition to activating the PO response, can also promote nodulation and phagocytosis (Ferro et al., 2019; Sideri et al., 2008), although the causal links are yet to be tested. Fruit flies can also use RNA interference pathways to produce virus-specific priming responses, using information from the remnants of viral DNA (Mondotte

et al., 2018, 2020; also see Maori et al., 2007 for honeybee *Apis melifera*). Yet, these examples collectively can only explain pathogen-specific responses in diverse insect species, but the mechanisms underlying the evolution of pathogen strain-specific priming (Khan et al., 2017a) is still unknown. For instance, it is difficult to imagine how nonspecific insect immune responses such as increased PO activity (Cerenius and Söderhäll, 2004), which has been previously implicated in evolving specific priming (Ferro et al., 2019), can also distinguish between multiple *B. thuringiensis* strains in flour beetles (Khan et al., 2017a). Thus, future studies can also expand their horizon by exploring (i) the production of receptor diversity via alternative splicing of Down syndrome cell adhesion molecule (Dscam) to discriminate between different pathogen strains (Kurtz and Armitage, 2006; Armitage et al., 2015), or mechanisms equivalent to (ii) functional reprogramming of vertebrate myeloid cells or (iii) cytolytic innate immune effectors such as natural killer cells which can produce long-lasting and antigen-specific immune memory independent of B- and T cells (see O'Leary et al., 2006; Vivier et al., 2011; Netea et al., 2020). Moreover, recent studies suggest the role of genes involved in the epigenetic reprogramming of immune cells, such as histone H3 gene, that are upregulated during the evolution of specific priming responses (Ferro et al., 2019; also see Netea et al., 2020). It is quite possible that the plasticity in immune responses evolve due to quantitative (rather than qualitative) changes in gene expression patterns and downstream pathways, regulated via epigenetic processes (Morandini et al., 2016), producing nuanced differences across both pathogen- and their strain-specific responses. In fact, epigenetic mechanisms such as DNA methylation, histone acetylation and microRNA expression can be particularly relevant for the transgenerational effects of paternal priming (Vilcinskas, 2021).

Finally, another striking feature is that the same insect populations that evolve strain-specific priming can also favour the evolution of generalized protection (i.e. without priming) against multiple Bt strains under more frequent antigen exposure (in flour beetles, Khan et al., 2017a), suggesting an added level of functional complexity of insect immunity vis-à-vis its specificity vs non-specificity. While further work is needed to understand how they can be mechanistically supported in such situations, distinctions perhaps reside in what immune strategies they choose to evolve with. For example, if priming is achieved via pathogen resistance (Khan et al., 2019), it might favour specific modulation of immune responses that are just sufficient to clear infections, minimising the cost of general immune activation as much as possible. By contrast, increased basal infection response by plausible evolution of tolerance under frequent antigen exposure might operate via improving the overall body condition (Khan et al., 2017a), with reduced inflammatory condition or more investment in antioxidant mechanisms to compensate the immune-mediated cytotoxicity (Ha et al., 2005). The overall physiological effects of such tolerogenic response are perhaps more likely to be generalized and non-specific across pathogens and their strains.

3.4. Priming during multiple infections

Under natural conditions, hosts are frequently exposed to infections caused by multiple pathogens, ranging from closely-related strains and pathogens to very diverse phyla, imposing complex selection pressure on their immune systems (Tate, 2019). In such cases, spatial and temporal constraints on immune system development, maturation or expression might play critical roles in disease outcomes (Tate and Graham, 2015). Previous infections might leave an immunological imprint and create a historical contingency upon exposure to the second pathogen, as found in lab mice where prior infection with nematodes increases resistance against later infections caused by *Plasmodium* parasites (Griffiths et al., 2015). In contrast, co-infection by gut protozoa (*Gregarine* sp.) and *B. thuringiensis* in flour beetles can severely curtail their progeny survival against *B. thuringiensis* infection (Tate and Graham, 2015). Depending on pathogen species, coinfection might thus induce myriad effects on host immunity. One possibility is that it can activate shared immune pathways against pathogens, exemplified by *Plasmodium* parasites coexisting with several bacterial species inside the mosquito midgut (Pumpuni et al., 1996; Contreras-Garduño et al., 2015). Alternatively, coinfection can also lead to the expansion of immune repertoires catering to divergent immunological pursuits against invading pathogens, increasing the overall costs of immune activation (Viney and Graham, 2013). Similar situations in insects might arise when they need the activation of both (i) IMD and Toll pathways against co-infecting Gram-negative and -positive bacteria (Lemaitre and Hoffmann, 2007); or (ii) rapid-acting PO vs inducible AMPs against fast- and slow-growing pathogens (Haine et al., 2008).

How do such mixed pathogenic environments affect the evolution of priming? Although direct experiments are lacking, answer to this question also has important implications while evaluating the natural relevance of previously reported strain-specific priming evolution in flour beetles (Khan et al., 2017a). It is unclear whether and how such strain-specificity is affordable in the wild, particularly when there is a high chance of experiencing multiple co-infecting *B. thuringiensis* strains (Abdel-Razek et al., 1999). Unless there is a large difference in the virulence level across strains, it is perhaps more favourable to evolve more generalized responses against the pathogen, disregarding its strain identity. The closest evidence for the evolution of priming under coinfection comes from Ferro et al. (2019), where selection treatments consisted of either exposing beetle to the same type of bacterium for both priming and challenge (specific treatment) or different ones (unspecific selection treatment) every generation. Although the study design does not include simultaneous infections by different pathogens, thereby limiting their direct interactions, it tests the effects of selection for vs. against the ability to mount specific priming responses. It also mimics the effects of mixed pathogen selection in natural conditions where specific adaptation to one particular pathogen might not be an optimal strategy for protection against the next set of pathogens. Despite experiencing mixed selection by multiple pathogen species (*Pseudomonas fluorescens, Lactococcus lactis*, and 4 strains of *B. thuringiensis*) for 14 generations, priming could evolve only against the most virulent strain of *B. thuringiensis* (i.e., DSM 2046; also used in Khan et al., 2017a), reemphasizing how strength of priming selection might be most strongly determined by the virulence level of specific strains of a natural pathogen (see Khan et al., 2019). However, further studies are needed to test situations where multiple pathogenic strains with similar virulence levels (e.g., *B. thuringiensis* strain DSM 2046 vs MTCC 6905 described in Khan et al., 2017a; also see Bose and Schulte 2014) are coinfecting, followed by probing their evolutionary effects on the level of priming specificity. Perhaps, such a competing situation between strains with comparable virulence might prevent the evolution of strain-specific priming.

4. Summary and future perspectives

In closing, we want to highlight that recent studies have established different immune priming types as distinct strategies in insects, evolving separately from their basal immunity and infection responses (Khan et al., 2019; Ferro et al., 2019). However, in-depth analyses of their selective conditions, fitness effects and various life-history trade-offs are posing several open questions that are paradoxical. For example, a stronger basal infection response is consistently more beneficial than priming (Prakash et al., 2019). Both, early survival vs reproductive costs of priming can constrain its evolution and yet, priming evolves in insect populations as a mutually exclusive independent strategy. It is puzzling what prevents alleles responsible for evolving consistently beneficial basal infection response from sweeping across all the populations? Can certain selective conditions demarcate the non-overlapping evolutionary as well as mechanistic space for these divergent immune features? Evolutionarily, it is perhaps expected, because, in both the studies that successfully evolved priming (Khan et al., 2017a; Ferro et al., 2019),

there is a clear connection between pathogen exposure statistics and evolved infection responses (see Mayer et al., 2015). However, the underlying mechanistic constraints that prevent the evolution of more beneficial basal immune function or infection responses during the low frequency of pathogen exposure remain unknown. Certainly, the first hurdle here is to ascertain how evolved priming vs basal infection response are linked to different host immune strategies— e.g., do they increase pathogen resistance by immune activation or show tolerance? This might not only reflect in their relative fitness impacts but also determine the evolution of the underlying mechanisms (see Miller et al., 2005). Do they involve different or overlapping sets of immune pathways? How do they cross-talk? At present, there are multiple immune mechanisms known to influence immune priming in several insect models (reviewed in Milutinović et al., 2016; Contreras-Garduño et al., 2016; Sheehan et al., 2020), but there is a serious lack of consensus on how these mechanisms might explain the divergent adaptive potential of priming vs basal immune function and infection responses across species. In this perspective, while we have highlighted the importance of considering the role of remarkable diversity and flexibility of insect innate immune adaptation against infections, we also suggest that future studies should carefully identify the source of diverse complexities in characterizing modalities of these immune responses- e.g., various regulatory networks underlying immune strategies; the energetics and metabolic basis; the role of epigenetic reprogramming and finally, plausible immune modulations by host-associated microbiota. Such a multifaceted approach is crucial to identify and validate various potential trade-offs between immune components, and constraints underlying immune strategies that are responsible for a mutually exclusive evolutionary space of basal infection response vs priming. It is also required to demystify the emergent properties of insect immunity such as strain-specificity which appears counterintuitive without a functional adaptive immune system.

Finally, we caution that our experimental understanding of priming evolution in insect populations is mostly limited to a few studies on flour beetles (Khan et al., 2017a; Ferro et al., 2019). While they certainly represent the first step uncovering the evolutionary basis of diverse immune strategies in response to selection imposed by the same pathogen, more studies are required to reproduce these effects in other insects too and validate the related hypotheses (described above) on fitness effects and mechanistic changes. For example, does priming always confer lower fitness benefits than basal immune function? It may not be true in a species like *Drosophila* infected with *Drosophila* C virus where, unlike in flour beetles (Khan et al., 2019), prior priming increases tolerance (Mondotte et al., 2018) and hence, can be fixed in a population more rapidly than basal immunity (Miller et al., 2005). We want to end by emphasising that an integrated evolutionary understanding of divergent immune strategies and infection outcomes, as well as decoding the underlying mechanistic basis, are not any more insect-specific problems. By all means, this is a pressing issue in higher animals as well, where the evolution of diverse immune types and respective fitness landscapes lack thorough experimental validation (Mayer et al., 2015; Netea et al., 2020). We suggest that insects, owing to their rapidly emerging complex immune forms and functions analogous to vertebrate immunity, can be a powerful system to model the evolution and mechanistic basis of divergent animal immune responses.

Author contributions

IK and AP developed the idea; IK and AP wrote the manuscript.

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Declaration of competing interest

We have no competing interests.

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The costs and benefits of basal infection resistance vs immune priming responses in an insect

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ABSTRACT

In insects, basal pathogen resistance and immune priming can evolve as mutually exclusive strategies, with distinct infection outcomes. However, the evolutionary drivers of such diverse immune functions remain poorly understood. Here, we addressed this key issue by systematically analyzing the differential fitness costs and benefits of priming vs resistance evolution in *Tribolium* beetle populations infected with *Bacillus thuringiensis*. Surprisingly, resistant beetles had increased post-infection reproduction and a longer lifespan under both starving as well as fed conditions, with no other measurable costs. In contrast, priming reduced offspring early survival, development rate and reproduction. Priming did improve post-infection survival of offspring, but this added trans-generational benefit of immune priming might not compensate for its pervasive costs. Resistance was thus consistently more beneficial. Overall, our work demonstrates the evolutionary change in trans-generational priming response, and provides a detailed comparison of the complex fitness consequences of evolved priming vs resistance.

1. Introduction

Until recently, it was assumed that insects have nonspecific immunity and cannot build immune memory against previously encountered pathogens, since they lack the immune cells responsible for adaptive immunity in vertebrates (Cooper and Eleftherianos, 2017). Now, growing evidence contradicts this traditional view: in addition to basal resistance mechanisms against infections (Rolff and Siva-Jothy, 2003), priming with a sub-lethal exposure to a pathogen often protects against subsequent exposure to the same pathogen. This survival benefit of priming is observed both in later life stages of primed individuals ("within-generation immune priming"; henceforth WGIP), and in their offspring ("trans-generational immune priming"; henceforth TGIP), in a range of insect species (reviewed in Milutinović et al., 2016) including Dipterans (Pham et al., 2007; Ramirez et al., 2015, 2017), Coleopterans (Roth et al., 2009, 2010; Khan et al., 2016), Lepidopterans (Fallon et al., 2011) and Hymenopterans (Sadd and Schmid-Hempel, 2006). Theoretical studies also highlight the importance of priming in reducing infection prevalence and regulating population size, stability and age structure during infection (Tate and Rudolf, 2012; Best et al., 2013). Thus, it appears that under pathogen pressure, priming responses should be selectively favoured. Additionally, our recent experiments directly demonstrated the adaptive value of within-generation priming and showed that it is a distinct immune strategy that can evolve independently of basal resistance to infection susceptibility in the flour beetle *Tribolium castaneum* (Khan et al., 2017a). Another important result from these experiments was that the net survival benefit of evolved priming was considerably lower than that of resistance (50% vs 80% survival after infection; Khan et al., 2017a), suggesting their inherently different effects on overall infection outcomes. However, the selective forces influencing the evolution of these diverse insect immune strategies and infection outcomes remain largely unclear (Prakash and Khan, 2022).

Do different evolutionary forces shape insect priming vs basal resistance to infection? The potentially divergent costs of maintenance and deployment of various immune responses can shed some light here (Sheldon and Verhulst, 1996; Mckean et al., 2008; Schwenke et al., 2016). Several studies suggest that resistance is associated with overexpression of fast-acting immune responses that impose large physiological costs (e.g. Sadd and Siva-Jothy, 2006; Khan et al., 2017b). A general mathematical model predicts that such costs of constitutively

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expressed basal resistance can be outweighed by its benefit only under frequent lethal pathogenic infections, maximising the population's growth rate (Mayer et al., 2016). However, the cost of resistance may be larger when pathogens are encountered infrequently. This is perhaps one reason why our beetle populations infected with a single large dose of infection every generation evolved priming (Khan et al., 2017a). In contrast, resistance could evolve only in populations that were exposed repeatedly to the pathogen within the same generation (primary exposure with heat-killed bacteria followed by live bacterial infection) (Khan et al., 2017a). However, few studies have actually measured the fitness consequences of evolved resistance in insects, and these were equivocal: e.g. while some found significant costs in terms of reduced longevity, poor larval competitive ability and delayed development (Kraaijeveld and Godfray, 1997; Ye et al., 2009; Ma et al., 2012), several others did not (Faria et al., 2015; Gupta et al., 2016). In general, costs of pathogen resistance may manifest as widespread tradeoffs with other life-history parameters as well, including reproduction (Reviewed in Sheldon and Verhulst, 1996; Rolff and Siva-Jothy, 2003; Schwenke et al., 2016). In contrast, the impact of immune priming-mediated protection on various fitness parameters has only recently been tested: e.g., primed mosquitoes (Contreras-Garduño et al., 2014), tobacco hornworms (Trauer and Hilker, 2013) and flour beetles (Khan et al., 2019) show reduced fecundity or primed mealworm beetle mothers produced progeny that developed slowly (Zanchi et al., 2011). Although these experiments revealed diverse fitness impacts of mounting priming responses using phenotypic manipulations, the costs of evolving, maintaining, and deploying priming responses in populations while facing persistent pathogen-imposed selection have never been measured in detail (also see Ferro et al., 2019).

We also considered the potential added fitness benefits of evolved priming, where survival benefits manifest not only in the primed individuals (i.e., WGIP) but also across generations (i.e., TGIP). Besides enhancing the net fitness impact of priming via WGIP, TGIP might also facilitate the evolution and spread of priming ability in populations. Although no direct experiments have tested whether such transgenerational benefits evolve simultaneously with WGIP, theory offers some important clues. A model by Tidbury and coworkers suggests that since TGIP has a lower ability to reduce infection prevalence, selection should favour WGIP more (Tidbury et al., 2012). On the other hand, Tate and Rudolf suggested that the stage-specific effects of infection are important: TGIP is more beneficial when an infection affects juvenile stages, whereas WGIP is more effective if adults incur higher infection costs than larvae (Tate and Rudolf, 2012). The model also predicts that selection can strongly favour both WGIP and TGIP when the pathogen affects larvae and adults equally (Tate and Rudolf, 2012). Our previous experimental results suggest that this hypothesis might be relevant at least for flour beetles: both WGIP and TGIP were equally beneficial in beetles infected with their natural bacterial pathogen *Bacillus thuringiensis* (Bt), which imposed similar infection costs across life stages (Khan et al., 2016). Although these results represent an interesting correlation, the causal link between the pathogen's impact on the host and its role in determining relative investment in different priming responses is not yet confirmed.

To understand the selective pressures and fitness effects that directly impact the evolution of diverse priming responses vs basal infection resistance, we used previously described, evolved replicate populations of the red flour beetle *T. castaneum* that were infected in each generation with Bt (DSM, 2046), either with or without exposure to priming with heat-killed Bt cells (described in Khan et al., 2017a; also see Fig. 1A and Supplementary methods). Earlier, we had analysed evolved immune features of these populations after 11 generations of evolution (Khan et al., 2017a): (i) post-infection survival benefits of primed individuals (relative to their unprimed counterparts) as effects of immune priming, and (ii) the inherent ability to survive after infection (without prior priming with heat-killed cells) with respect to sham infected controls, as a proxy for basal resistance against infections. In the present study, we re-tested the same populations after a further 3 generations of experimental evolution to analyse the underlying fitness costs and benefits of their evolved immune features. We measured a range of critical fitness-related traits such as offspring development, early reproduction, and early survival, as well as adult lifespan under starvation and normal conditions. We also tested whether the evolution of priming ability was associated with transgenerational survival benefits during infection.

Note that we did not measure the direct changes in immune function or pathogen clearance in our experiments. Hence, the resistance, estimated from post-infection survival data alone, can be confounded by the potential evolution of tolerance, whereby survival might increase by reducing the cost of infection or immune responses, not because of more efficient pathogen killing mechanisms (Ayres and Schneider, 2012). Nonetheless, our work provides a systematic analysis of the evolutionary cost and benefit structure influencing parallelly evolved, potentially divergent immune responses to pathogenic infections. We also show

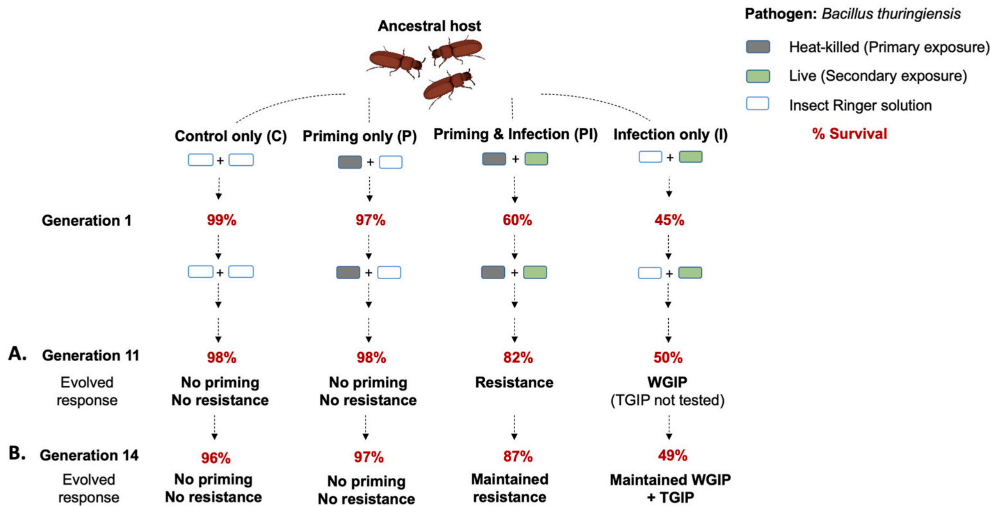

Fig. 1. Summary of the design and outcome of experimental evolution of *Tribolium castaneum* flour beetles against the bacterial pathogen *Bacillus thuringiensis*. The schematic indicates beetle survival under the respective conditions of selection as well as evolved immune strategies (resistance or different priming forms) observed in all populations of each regime, after **(A)** 11 and **(B)** 14 generations of experimental evolution, described in Khan et al. (2017a) and the present study respectively. C, P, PI and I populations assayed at 11th generation (Khan et al., 2017a). Every generation, 10-day-old virgin beetles were either injected with heat-killed bacterial slurry (P & PI beetles) or sterile insect Ringer solution (C & I beetles) (primary exposure) (See **SI** methods). After six days, individuals from I and PI regimes were challenged with live Bt, whereas C and P beetles were pricked with sterile insect ringer solution (secondary exposure) (See **SI** methods). In the current study, we analysed 3 replicate populations from each regime. WGIP = Within-generational immune priming; TGIP = Trans-generational immune priming.

evolutionary change in TGIP in insects.

2. Methods

2.1. Experimental evolution

We used laboratory-adapted populations of *T. castaneum* to initiate four distinct selection regimes, described in (Khan et al., 2017a): control (C; no priming or infection), priming only (P), primed and infected (PI) and infection only (I), each with originally 4 independent replicate populations (also outlined in Fig. 1). In the present study, for logistical reasons, we could only analyse three replicates from each selection regime (C 1, 2 & 4; P 1, 2 & 4; PI 1, 2 & 4; I 1, 2 & 4). On different days, we handled one replicate population from each selection regime together. The detailed protocol for the experimental evolution is described in (Khan et al., 2017a) (also see Supplementary methods), where we quantified evolved immune features after 11 generations of selection (Fig. 1A). In the current study, we reanalyzed these evolved lines after three further generations of selection (Fig. 1B). Thus, after 14 generations of continuous selection, we isolated a subset of individuals from each replicate population and propagated them without any selection for two generations, i.e., without priming or infection (unhandled). This relaxing of selection is expected to generate standardised experimental beetles with minimum non-genetic parental effects.

2.2. Joint assays of evolved priming and resistance, and their impacts on reproduction

We designed our experimental framework to jointly compare survival benefits and reproductive effects of evolved priming vs resistance against Bt infection (see Fig. 2A for experimental design). Besides measuring survival after priming and infection, we measured female reproductive output both before and after infection. This allowed us to estimate the direct impact of experimental evolution with pathogens vs the actual impact of inducing each type of immune response. Simultaneously, we also tested for the evolution of trans-generational immune priming (TGIP), to compare relative survival and reproductive effects of different priming responses. We followed a full factorial design, so that beetles from each population were tested under all priming and infection conditions.

2.2.1. Confirmation of evolved immune features, and measurement of their reproductive effects (see Fig. 2A for experimental outline)

We first collected pupae from each standardised population and isolated them into 96-well microplate wells with ~0.2g wheat flour, for eclosion. We randomly assigned 10-day old virgin males and virgin females from each population to one of the following primary exposure treatments: (a) naïve (or unhandled) (b) primed (injected with heat-killed Bt) and (c) unprimed (i.e., injected with insect Ringer) (see **SI** methods for various priming and infection treatments). After 24 hours of primary exposure, we formed mating pairs using males and females within the same primary exposure treatment combination from each population in 1.5 ml micro-centrifuge tubes with 1g of wheat flour (n = 12 mating-pairs/priming treatment/replicate population/selection regime). We allowed them to mate for 48 hours and then isolated the 12-day-old females to oviposit for another 48 hours in 5 g whole wheat flour (oviposition plate), whereas males were returned to 96-well microplates. After oviposition, we also returned the 14-day-old females to 96-well microplates. Two days later (i.e., total six days after primary exposure), we infected males and females with live Bt. We recorded male survival every 6 hours for 1 day and then every 24 hours for 7 days post-infection (same as the selection window during experimental evolution; Khan et al., 2017a). We tracked female survival similarly, except that a day after infection, we again allowed 48-hour oviposition to estimate the

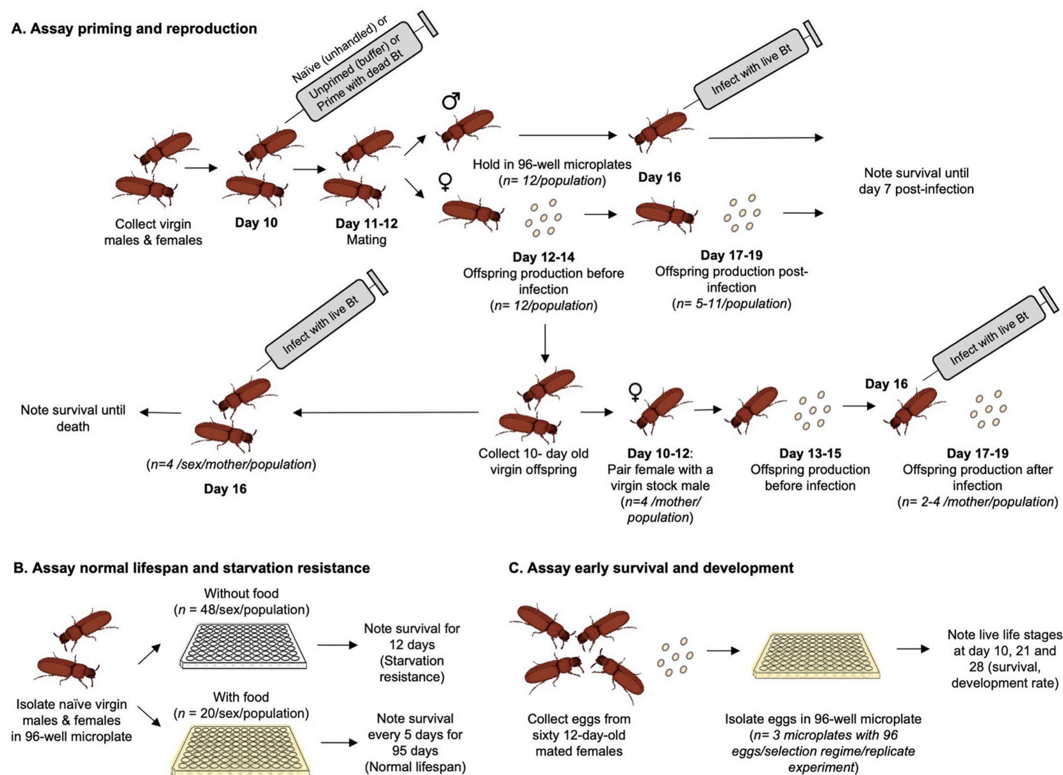

Fig. 2. Design of full factorial experiments to assay **(A)** evolved immune features and their impacts on beetle reproduction **(B)** survival under normal condition and starvation **(C)** early survival and development. Beetles from each population were allowed to develop for two generations under relaxed selection, before each assay. For (A), each population was tested under all priming and infection conditions (i.e., naïve, unprimed or primed). For (B, C), beetles from each population were tested only in the naïve unhandled condition.

reproductive impact of infection and induction of any priming responses. Here, we caution that since bacterial infection imposed significant mortality across regimes, the replicate size for our fitness assays was lower than expected (n = 5–12 females/priming treatment/replicate population/selection regime; Table S1). Resulting analyses can thus be relatively underpowered compared to the reproductive assays performed before infection. We also conducted a mock challenge for a subset of unprimed beetles as a procedural control for the survival assay, but not for reproductive output. We did not find any mortality in uninfected beetles within the experimental window of 7 days.

To test whether reproductive costs of evolved resistance manifest under stressful conditions, we compared the reproductive output of naïve unhandled PI beetles with C beetles reared in a poor-quality diet, corn (Agashe et al., 2011). To generate experimental adults, we allowed females from standardized C and PI populations (replicate populations 1 and 2) to oviposit in cornflour and collected their offspring to measure reproductive output as described above (n = 36 females/replicate population/selection regime).

2.2.2. Generation of beetles (progeny) for trans-generational priming (TGIP) assay

We used eggs laid by naïve, unprimed, and primed females (assayed to estimate the effects of WGIP, described above) to also measure TGIP. We allowed the eggs to develop for 21 days at 34 °C and counted the total number of progeny (mostly pupae). Particularly, we retained the offspring from the first round of oviposition (without infection) (See Fig. 2A). At this time, most offspring were pupae, and the few adults we observed had pale body coloration indicating that they were not sexually mature and hence, unlikely to be mated (Sokoloff, 1977). We isolated these pupae and adults in 96-well plates with ~0.2g flour, to obtain virgin beetles for assays to measure trans-generational priming and offspring reproduction. We included offspring from mothers that produced more than 8 female and 4 male offspring (n = 8–10 mothers/priming treatment/replicate population/selection regime), enabling us to sample enough beetles to jointly test offspring post-infection survival (a proxy of trans-generational priming; described below) and reproduction of each parental pair.

2.2.3. Assaying transgenerational priming response and its reproductive effects (see Fig. 2A for experimental outline)

After 10 days, we combined a subset of female offspring from each parental pair (n = 4 offspring/parental pair/priming treatment/replicate population/selection regime) with 10-day old virgin males from standard laboratory stock population into a single mating pair for 48 hours and then allowed them to oviposit. This enabled us to measure the impact of parental priming on offspring reproductive output across populations. On day 16, we infected females and then again assayed their reproductive output as described above. We could only compare the post-infection reproduction of primed and unprimed females, but not naïve females as we lost them accidentally. On the same day, we also infected the remaining 16-day old virgin male and female offspring from each parental pair with live Bt (n = 4 offspring/sex/parental pair/priming treatment/replicate population/selection regime) and noted their survival every 6 hours for 2 days and then every 24 hours until all of them were dead. This experimental design not only allowed us to jointly estimate the survival and reproductive effects of WGIP vs TGIP for each parental pair, but also to analyse the impact of each type of immune response relative to evolved resistance. We did not find any mortality in sham infected offspring within the experimental window.

2.3. Quantifying development and survival under starvation and with food, in evolved lines

In separate experiments, we measured the direct impacts of maintaining evolved priming responses and resistance on other fitness components such as early survival, development, starvation resistance and lifespan, using naïve unhandled beetles (i.e., without priming or challenge) across selection regimes.

2.3.1. Impact on lifespan under starvation and with food (See **Fig.** 2B for experimental outline)

We first isolated 10-day old naïve virgin males and females from each population in 96-well microplate wells without food (*n* = 20 beetles/sex/replicate population/selection regime). We noted mortality every 12 hours (10 a.m. & 10 p.m. ± 1 hour) for the next 12 days until all beetles died (Fig. 2B). In a separate experiment, we similarly distributed naïve virgin females into 96-well microplates, but with access to food (*n* = 48 females/replicate population/selection regime). We noted their survival every 5 days for 95 days to estimate the long-term survival costs of evolved immune features. We could not assay males for long-term survival costs due to logistical challenges.

2.3.2. Quantifying early survival, development, and viability costs in evolved lines (See **Fig.** 2C for experimental outline)

We next estimated the impact of evolved immune features on aspects of early survival and development. We allowed 12-day old, mated females from each population (n = 60/replicate population/selection regime) to oviposit in 150g of doubly sifted flour (using sieves with pore size of 50μ to remove large flour particles; Diager USA) for 24 hours. We had 3 replicates for each population. We discarded the females, and isolated 96 randomly chosen eggs into 96-well microplate wells with ~0.2 g flour (n = 3 microplates/replicate population/selection regime). This method was designed to detect intrinsic patterns of several developmental features across populations, without the confounding effects of density-dependent competition at the juvenile stage. After 10 days, we sifted the flour from each microplate to count live larvae and measure egg hatchability. Following this, we again returned the live larvae to 96-well plates and provided fresh flour. In our standard stock beetle populations, pupation and adult emergence begins around 3–4 weeks after oviposition. Therefore, we estimated the proportion of pupae and adults after 3- and 4-weeks post-egg collection respectively, as proxies for time to pupation and adult emergence. Finally, we calculated the percentage of surviving larvae, pupae, and adults at week 4, as a proxy for overall viability. Since P beetles did not evolve priming or resistance, we excluded them from the assay.

2.4. Data analysis

2.4.1. Within generation priming response and resistance in parents

We first analysed survival data for all selection regimes using a mixed effects Cox model using the R package ‘coxme’ (Therneau, 2015). We fit separate models for females and males since they were assayed on different days. In each case, we specified the model as survival ~ selection regime × priming treatment + (1|selection regime/replicate population), with selection regime and priming treatment as fixed effects, and replicate populations nested within the selection regime as a random effect. A significant interaction between priming treatment and selection regime would indicate that the effects of priming varied across selection regimes. We note that although this analysis provides an overall estimate of each effect, complex interactions can prevent us from making meaningful comparisons between selection regimes. To understand the differences across priming treatments and selection regimes in detail, we therefore separately analysed survival data for each standardised replicate population, using Cox Proportional Hazard survival analysis. We noted individuals that were still alive at the end of the survival experiment as censored values and estimated the survival benefit of priming as the hazard ratio of unprimed versus primed groups. A hazard ratio significantly greater than one indicates a higher risk of mortality in the unprimed group relative to primed individuals; hence, a significant survival benefit of WGIP.

Separately, we also estimated the hazard ratio of naïve infected beetles from the C regime against naïve infected beetles from all other

regimes, to quantify evolved resistance against Bt infection. While a hazard ratio significantly greater than one indicates a higher risk of mortality in C beetles, it also implies increased evolved resistance in selected regimes relative to C beetles.

2.4.2. *Transgenerational priming response in offspring*

To measure TGIP, we recorded the survival of 4 male and 4 female replicate offspring from each parental mating pair assayed earlier for within-generation priming. We calculated the mean lifespan for both the sexes as the unit of analysis but noted that residuals of lifespan data were not normally distributed (verified with Shapiro-Wilk tests; $p < 0.01$). Therefore, we transformed the data into square root values that fit a normal distribution, and then compared group means of male and female offspring survival using a linear mixed effects model (R package 'lme4', Bates et al., 2012) with selection regime, parental priming status and offspring sex as fixed factors and replicate populations as a random effect. We tested for pairwise differences between selection regime and treatment after correcting for multiple comparisons using Tukey's HSD, using the R package 'lsmeans' (Lenth, 2017). We performed model reduction through stepwise removal of nonsignificant terms.

In addition to detecting the overall TGIP response in I beetles, we also wanted to compare their relative survival benefit with that of WGIP. To this end, for each replicate population, we analysed the lifespan of male and female offspring from each parental pair, using a mixed effects Cox model to calculate the estimated hazard ratio of offspring from unprimed parents versus primed parents. Since we recorded the survival of 4 male and 4 female offspring from each parental pair, we specified the model as: survival ~ parental priming status + (1| parental mating pair/replicate offspring), with parental priming status as a fixed effect and male (or female) replicate offspring nested within each parental mating pair as a random effect. Since all beetles died within the experimental window, we did not have any censored beetles in the analysis.

Subsequently, we used non-parametric Wilcoxon Rank Sum tests to compare hazard ratios calculated from TGIP versus WGIP for each population.

2.4.3. *Reproductive effects*

We found that the residuals of pre-infection reproductive output data of both parents and offspring were non-normally distributed and could not be transformed to a normal distribution. We, therefore, used non-parametric Wilcoxon Rank Sum tests to analyse the impact of selection regime and priming treatment (for replicate populations of C, P, PI and I that were handled together). We also used Wilcoxon tests to analyse the impact of bacterial infection on the reproductive output of parents and offspring, separately for each replicate population across selection regimes and priming treatments.

We analysed post-infection reproductive fitness data using a linear mixed effects model with selection regime and priming treatment as fixed factors and replicate populations as a random effect [Model: No. of offspring ~ Priming treatment × Selection regime + (1 | Replicate population); fitted separately for parents and offspring]. To understand the complex interactions between treatment and selection regime and to disentangle the effects of each type of evolved immune features (i.e., priming in I or resistance in PI), we also compared post-infection reproductive data of parents from each selection regime separately with that of control beetles (C), wherever needed. We used Tukey's HSD to test for pairwise differences between selection regimes and treatments. As described above, we removed the nonsignificant effects stepwise to determine the best reduced model.

2.4.4. *Survival under starvation and fed conditions*

We analysed survival data under starvation and with food, using mixed effects Cox models as described below (best reduced model, wherever needed):

I. Lifespan under starvation: survival ~ selection regime + sex + (1| selection regime/replicate population), with selection regime and sex as fixed effects, and replicate populations nested within selection regimes as a random effect.
II. Lifespan with food: survival ~ selection regime + (1| selection regime/replicate population), with selection regime as a fixed effect, and replicate populations nested within selection regimes as a random effect.

2.4.5. *Early survival, development, and viability costs*

We analysed data using a linear mixed effects model with selection regime as fixed factor and replicate population as a random effect. In each case, we described the model as: Trait ~ Selection regime + (1| Replicate population). We tested for pairwise differences using Tukey's HSD.

3. Results

3.1. *Beetles retained evolved immune features after 14 generations of experimental evolution*

A mixed-effects Cox model fitted separately to male and female survival data revealed a significant interaction between selection regime and treatment, suggesting that effects of priming and infection varied across selection regimes (Figs. S1–2, Tables S2–3). To understand these complex effects in detail, we analysed survival data for each population with a Cox proportional hazard analysis. As seen earlier (Khan et al., 2017a), we found evolved priming responses only in males and females from I populations (~3-fold increase in their survival relative to control beetles) (Figs. S1–2, Tables S2–S3); whereas PI beetles had higher basal resistance (3–28-fold increase in the survival of naïve PI beetles relative to control beetles) (Figs. S1–2, Table S4). We also found that while the survival of I beetles after Bt infection was 50%, PI beetles showed ~80% survival (Fig. S3) (compare with ancestral population where only 40% beetles survived the infection, Khan et al., 2017a). As expected, populations from the C or P regimes (where beetles were not exposed to live infection) did not evolve any priming ability or higher resistance to infection.

3.2. *Evolved immune features do not incur reproductive costs under experimental conditions*

We measured the impact of evolved immune features on beetle reproduction and found complex fitness effects that varied substantially with priming type and infection status. Evolved priming or resistance had no significant impact on the reproduction of uninfected beetles (Fig. 3, Table S5) (also see Fig. S4 for comparison across replicate populations handled together). Also, there were no general effects of priming treatment on reproduction (Table S5). Thus, the maintenance of evolved priming or resistance does not impose a reproductive cost. However, we noticed that females infected with live pathogen later in their life (see methods) had reduced reproductive output in most populations, except those from PI regime where the effect was relatively mild (compare across selection regimes and treatments; Fig. 3, Table S6) — i.e., most PI beetles, regardless of their priming status, did not exhibit significant reproductive costs of infection (7/9 treatments, $P > 0.05$; Table S6). Also, the average post-infection reproductive cost of evolved priming appeared higher than that of evolved resistance (compare beetles from PI vs I populations in Fig. 3, Table S6), highlighting contrasting outcomes of live infection across evolved regimes. These patterns were further confirmed in subsequent analyses where we separately compared the reproductive fitness of naïve and unprimed females after infection, using a Wilcoxon rank sum test (data was not normally distributed; Shapiro-Wilk test: $P < 0.05$). We did not include primed beetles in these analyses, because we wanted to measure the effect of live infection without any interference from previous antigen

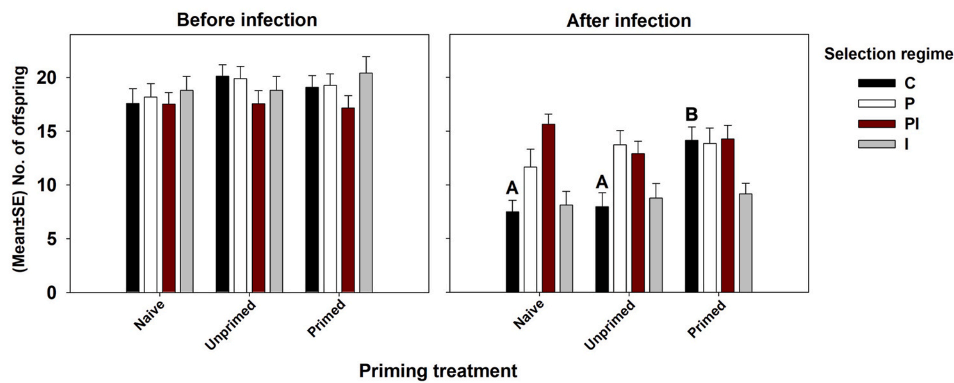

Fig. 3. Impact of evolved within-generation immune priming (WGIP) and resistance on female reproductive output, both before (n = 12 females/priming treatment/replicate population/selection regime) and after bacterial infection (n = 5–11 females/priming treatment/replicate population/selection regime). Different letters denote significant differences across experimental treatments, for a given selection regime. E.g., post-infection reproduction of C beetles after mounting a within-generation priming response was significantly higher ($P < 0.05$; tested with Tukey's HSD). We did not assign letters to treatments within P, PI & I as they were not statistically significant ($p > 0.05$; Tukey's HSD). Error bars denote standard errors.

exposure (i.e., heat-killed bacteria during priming). Overall, there was no effect of the experimental treatment (naïve vs unprimed females after infection: df = 1, $\chi 2 = 0.021$, $P = 0.87$), but overall infected PI beetles produced significantly more offspring than C (df = 3, $\chi 2 = 12.458$, $P < 0.001$) and I beetles (df = 3, $\chi 2 = 14.837$, $P < 0.001$) beetles.

Next, we analysed the full dataset (including naïve, unprimed & primed females) to study the impact of experimental priming on the reproductive output of infected beetles. A linear mixed effects model with reproductive output data from all regimes revealed significant main effects of both selection regime and treatment as well as their interaction (Table S7 I). Beetles from the PI ($P = 0.04$) and P ($P = 0.02$) regimes appeared to produce more offspring than C beetles, though these effects were perhaps confounded by their priming status. Since our experiments were primarily designed to disentangle the reproductive effects of separately evolved responses with respect to control C beetles, we also compared each selection regime individually with C beetles (e.g., I vs C, or PI vs C) (Table S7 II-IV). In contrast to results obtained from the full dataset (Table S7 I), these comparisons did not show consistent effects of the selection regime, but revealed significant effects of the priming treatment and its interaction with selection regime in each case (Table S7 III-IV). In both analyses (i.e., full dataset and pairwise comparisons), experimental priming induced a large increase in reproduction only in C regime after infection (primed vs unprimed C; Tukey's HSD: $P = 0.005$), but not in beetles from other selection regimes (Tukey's HSD: $p > 0.9$) (Figs. 3 and S4 Surprisingly, primed I beetles failed to increase their reproduction despite increased post-infection survival benefits (after mounting WGIP), suggesting a fitness cost. This was in contrast to PI lines that could apparently alleviate this reproductive cost by maintaining higher reproduction than C beetles regardless of their priming or infection status (discussed above). Thus, evolved resistance is better than priming not only in terms of its survival benefit, but also in terms of reproduction. We also verified these significant differences by calculating confidence intervals (2.5%, 97.5%) [C: naïve (4.633, 10.366), unprimed (5.528, 10.391), primed (11.595, 16.665) vs I: naïve (5.595, 10.665), unprimed (6.108, 11.415), primed (6.977, 11.345) vs PI: naïve (13.269, 18.038), unprimed (10.719, 15.087), primed (12.046, 16.486)].

Finally, in a separate experiment, we demonstrated that PI beetles reared in poor quality diet corn produced an equal number of offspring to that of C beetles (Fig. S5; Table S8: $p = 0.34$), indicating that evolved resistance might not impose reproductive costs even under stressful conditions.

3.3. Evolved resistance increases survival under both starvation and fed conditions

We analysed the direct impacts of evolved priming and resistance on survival under starvation or fed conditions, using a mixed effects Cox model. In both cases, survival was significantly higher in PI beetles compared to C beetles ($P < 0.04$ in each assay), but not in I or P beetles ($P > 0.05$ in each assay) (Figs. S6–7; Tables S9–10). Males and females from all selection regimes had similar lifespan under starvation (Fig. S6, Table S9), suggesting no sex-specificity in starvation resistance.

3.4. Evolved priming reduces early survival and extends the development time

We found significant effects of selection regime on egg hatchability, the total number of viable offspring and proportion of adult offspring at week 4, but not on the proportion of pupae at week 3 (Fig. 4). The number of viable offspring at week 4 was drastically reduced in beetles from the I regime (Fig. 4D, Table S11). This was perhaps due to significant early mortality during egg to larval development in I beetles: while ~75% C, P and PI eggs hatched into larvae, only 55% I eggs survived (Fig. 4A, Table S11). Besides, the proportion of adults at week 4 was lowest in the I regime, suggesting delayed development (Fig. 4C, Table S11). Overall, these results indicate that maintenance of priming imposed considerable costs in terms of reduced early survival and slower development in I beetles. In contrast, evolved basal resistance did not appear to impose a significant cost with respect to these traits.

3.5. Evolved WGIP is also associated with TGIP

Finally, we asked whether evolved priming conferred added trans-generational benefits, increasing its overall fitness impacts. We used a linear mixed effects model to analyse the mean post-infection survival of offspring from beetles assayed above as a function of selection regime, parental priming status and offspring sex. The selection regime and parental priming status had significant main effects as well as an interaction effect, whereas offspring sex had no impact ($P > 0.05$) (Fig. 5A, Table S12). Here too, we found that overall, offspring of PI beetles had the highest survival (compared with C beetles across parental priming status, all comparisons with Tukey's HSD: $p < 0.001$), though they did not show effects of parental priming ($P > 0.05$). In contrast, parental priming increased offspring survival in the I regime (Tukey's HSD: $p < 0.001$), suggesting that TGIP benefits are restricted to I beetles. In addition, we found that the relative survival benefits of TGIP were equal to that of WGIP (see supplementary results, Fig. S8, Table S13).

3.6. Evolved trans-generational priming (TGIP) does not impose a separate reproductive cost

As found with mothers (above), evolved priming and resistance did not consistently affect the reproductive output of naïve or uninfected offspring (Fig. 5B, Table S14) (also see Fig. S9 for comparison across replicate populations handled together); but infection generally reduced offspring reproductive output in all selection regimes except PI (Table S15). A linear mixed effects model only revealed significant main

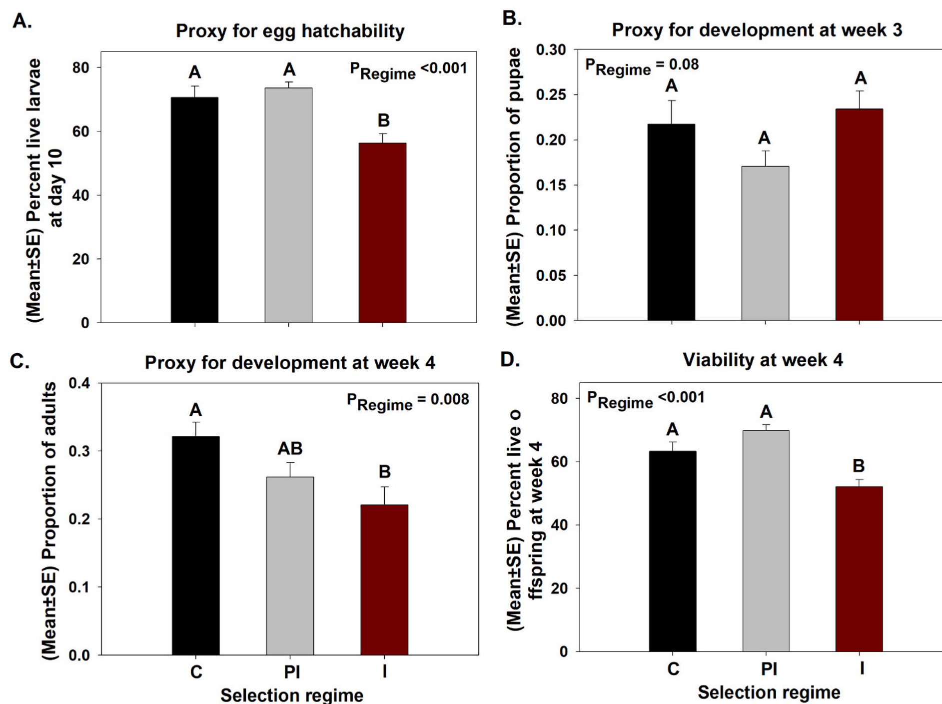

Fig. 4. Impact of evolved immune features on **(A)** total number of eggs laid by naive females (i.e., without infection) across selection regimes that hatched into larvae (egg hatchability); proportion of **(B)** pupae at week 3 and **(C)** adults at week 4 as proxies for developmental rate; **(D)** the total number of viable offspring (larvae, pupae and adults) at week 4 ($n = 3 \times 96$ well microplates/replicate population/selection regime). P values for the impact of the selection regime are reported in each panel. Significantly different selection regimes are indicated by distinct letters (based on Tukey's HSD). Error bars denote standard errors.

effects of selection regime and parental priming status (Table S16). PI beetles produced more offspring than control beetles (C vs PI: $P = 0.009$, Tukey's HSD) (Figs. 5B and S9); but the reproductive output of P and I beetles did not differ from control (all $P > 0.05$, Tukey's HSD). Interestingly, parental priming reduced reproductive output in all beetles across selection regimes (Table S16). However, since there was no significant interaction effect between selection regime and parental priming (Table S16), we inferred that transgenerational survival benefits in I beetles did not impose any added reproductive costs, relative to C beetles (Figs. 5B and S9).

4. Discussion

Previously, we showed that priming response and basal resistance against *Bt* infection evolve as divergent mutually exclusive strategies in flour beetles (Khan et al., 2017a). However, it was unclear if selective parameters underlying their evolution, and potential trade-offs, were also different. Here, we revisited the same beetle lines to identify whether these evolved immune features incurred divergent fitness costs. Concomitantly, we also asked whether priming confers additional, trans-generational fitness benefits. To our surprise, we did not find any evidence for a cost of evolved resistance. It did not impact development and reproduction, and even increased survival during both starvation and normal conditions, contradicting the traditional view of immunity-fitness trade-offs (Ye et al., 2009; Ma et al., 2012). Instead, our results are consistent with previous studies that suggest only a weak role for life-history trade-offs during the evolution of pathogen resistance (Faria et al., 2015; Gupta et al., 2016). In contrast to the missing costs of resistance, evolved priming imposed diverse fitness costs such as reduced offspring early survival, development rate and reproduction. Interestingly, we found rare evidence of rapid evolutionary change in both within- and trans-generational priming responses. However, the combined benefit of these two forms of priming (~50% survival after Bt infection) was still lower than that conferred by increased basal resistance to Bt (~80% survival).

Interestingly, we found that although infection reduced reproduction in all regimes, the effect was less pronounced in PI beetles; hence, evolved basal resistance was also associated with a relative reproductive advantage. P (priming only) beetles also had higher reproduction than control beetles after infection, which is counterintuitive because these beetles never experienced live infection during experimental evolution. How do we interpret these reproductive fitness benefits in PI and P beetles? First, as described earlier, the reduced cost of infection might represent evolved tolerance (Ayres and Schneider, 2012), enabling PI and P beetles to make a greater reproductive investment during infection. Second, our results could also reflect a trade-off between early vs late reproduction. Higher reproduction might represent a terminal investment in P and PI populations, whereas C and I populations instead suppress immediate reproduction after infection to maintain survival and somatic maintenance later in life (Luu and Tate, 2017). However, note that long-term reproductive effects are not meaningful in the present context, because during experimental evolution our beetles were allowed to reproduce for only 7 days after infection.

We explored most of the plausible contexts in which immunity-fitness trade-offs could be expressed in our evolved PI populations. Yet it is possible that some underlying fitness trade-offs remained hidden because PI beetles lived in a relatively stable environment, with *ad libitum* food (Mckean et al., 2008). In contrast, suboptimal living conditions such as limited access to food and nutrition (Mckean et al., 2008) could have revealed fitness costs. To test this possibility, we compared the reproductive output of beetles from PI lines vs C control lines when reared in a poor-quality corn diet (Agashe et al., 2011). Surprisingly, beetles that had evolved resistance to Bt still produced as many offspring as control beetles, indicating that evolved resistance did not impose reproductive costs even under stressful conditions.

Our results contradict our prior hypothesis that at a low pathogen frequency (experienced by I beetles), priming may be more favourable than resistance due to its low maintenance costs (Khan et al., 2017a). We found that the overall maintenance of priming responses is costly (e.g., reduced early survival and developmental rate in naïve beetles). These results broadly corroborate results from another beetle study by Ferro and coworkers showing that Bt-driven evolution of priming in larvae delayed their development (Ferro et al., 2019). However, they observed such responses only after mounting a priming response, suggesting a deployment cost, whereas basal maintenance costs in naïve beetles were not analysed (Ferro et al., 2019). In our study, priming also had variable

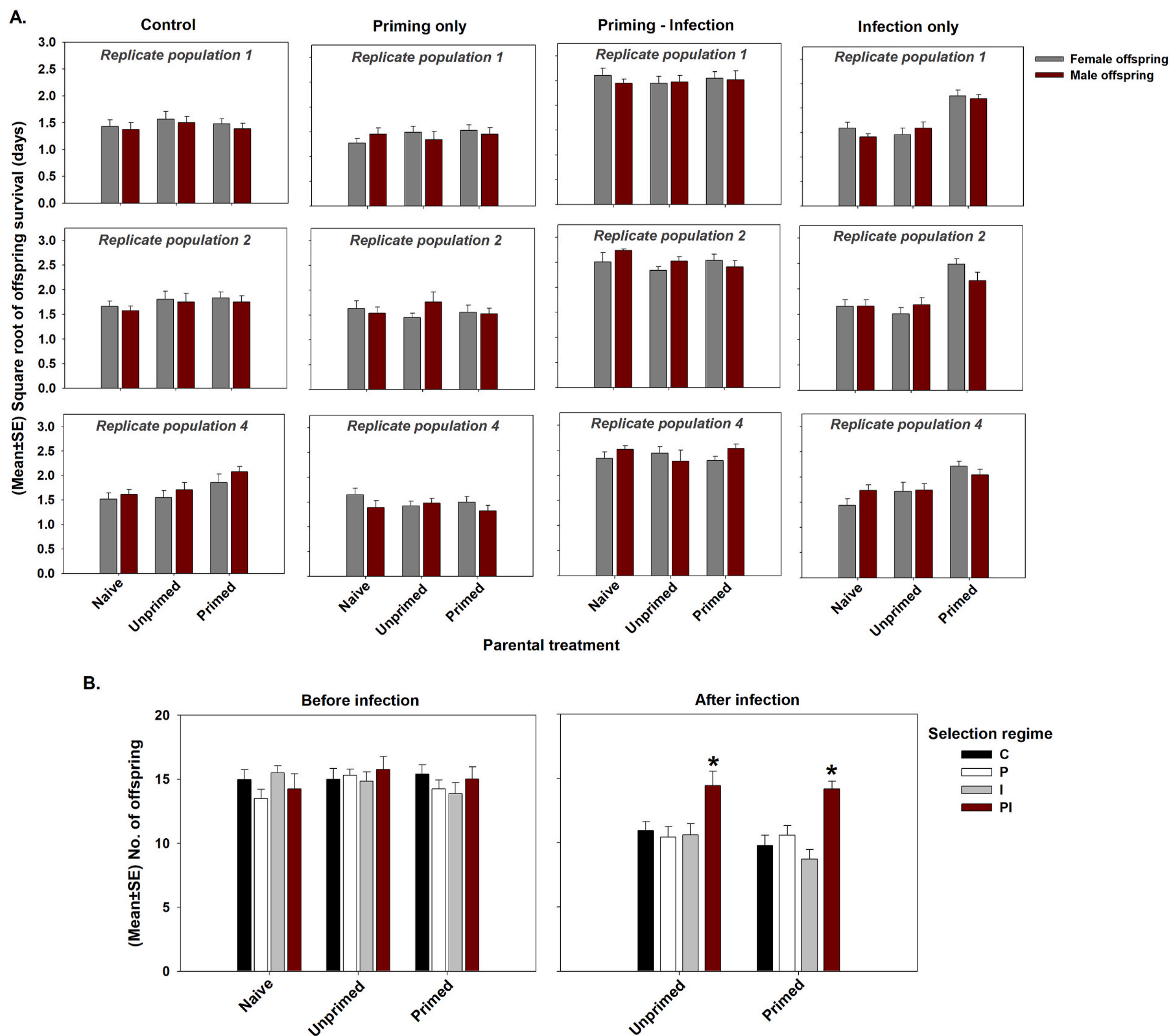

Fig. 5. **(A)** Offspring survival after trans-generational immune priming (TGIP) and infection (group mean survival of 4 offspring/sex from each of the 8–11 parental pairs/parental priming status/replicate population/selection regime). **(B)** Impact of evolved trans-generational priming on offspring's reproductive output, both with and without infection (group mean reproduction of 4 female offspring from each of the 8–11 parental pair/parental priming status/replicate population/selection regime). Asterisks in panel B for PI beetles after infection denote that PI beetles produced significantly more offspring than C, P & I beetles ($p < 0.05$). Error bars denote standard error.

effects on reproduction across selection regimes. For instance, mounting a within-generation priming response helped C beetles to increase their reproduction after infection; whereas infected I beetles, despite increasing survival benefits, could not improve their reproduction. These results mirror our recent observations with wild-caught beetle populations, where primed and infected females with increased post-infection lifespan produced fewer offspring (Khan et al., 2019) and vice versa. We thus speculate that a hidden trade-off with post-infection reproduction might constrain the survival benefits of within-generation priming responses at a much lower level than resistance (see Fig. 1B).

We also speculate that the evolution of costly priming requires reactive and self-damaging immune responses (e.g., phenoloxidase; see Ferro et al., 2019) to clear pathogens (Khan et al., 2017b, 2019) and hence physiologically constrains the extent of post-infection fitness benefits. In contrast, PI beetles either used less toxic immune responses to clear pathogens or evolved tolerance, reducing immunopathological costs and maximising fitness benefits (Khan et al., 2017b). Mounting trans-generational priming responses, on the other hand, did not affect offspring reproduction, suggesting that fitness effects are not uniform across different types of evolved priming responses. Nevertheless, these results broadly corroborate other work showing the negative effects of priming on various fitness parameters (Trauer and Hilker, 2013; Contreras-Garduño et al., 2014). However, unlike these studies that primarily used phenotypic manipulations within a single generation, ours is a detailed study that directly measured the complex fitness costs associated with experimentally evolved priming across multiple generations of pathogen exposure.

Our experiments also provide empirical evidence that insects can evolve multiple priming responses simultaneously. Interestingly, both trans-generational and within-generation priming provided almost equivalent fitness benefits, corroborating our prior work with natural beetle populations (Khan et al., 2016). Such parallel results from natural and laboratory-evolved populations might indicate that natural pathogens such as Bt may serve as a potent source of selection favouring the evolution of diverse immune responses in insects. As discussed earlier, Bt reduces the survival of flour beetle larvae and adults equally (Khan et al., 2016), which should favour the simultaneous evolution of WGIP and TGIP (Tate and Rudolf, 2012). However, during experimental evolution, we only infected adult beetles, which should have restricted host-pathogen interaction to adults. It is still possible that infected adults

directly transmitted Bt to eggs, imposing selection on juveniles and thereby, favouring TGIP (see Tate and Rudolf, 2012). Alternatively, infected adults could have transmitted Bt (or antigen) to larvae via the flour, either through infected beetle cadavers (~10–15% mortality during oviposition period in I beetles) or excreta (Argôlo-filho and Loguercio, 2014). Another possibility is that ancestral beetle populations may have already coevolved with Bt in their natural habitat before they were brought into the lab. Consequently, despite being infected only as adults during experimental evolution, the beetle immune system could readily recognise Bt as a risk across life stages. Finally, if WGIP and TGIP involve shared molecular pathways, direct pathogen pressure on adults could result in simultaneous evolution of both types of priming. Although the molecular details responsible for immune priming are still unclear (Cooper and Eleftherianos, 2017) and perhaps confounded by diverse processes such as metabolic changes (Ferro et al., 2019) and epigenetic reprogramming of immune cells (Tate et al., 2017), recent data indeed hint at shared immune pathways and components between different priming types (Prakash and Khan, 2022). For instance, both within-generationally primed fruit flies (Pham et al., 2007) and trans-generationally primed bumble bees (Barribeau et al., 2016) require Toll pathway activation for priming-induced protection (also see flour beetles; Ferro et al., 2019). Further experiments comparing the molecular basis of different immune priming responses can help to distinguish between the above hypotheses.

5. Conclusion

In closing, we note that the relative importance of priming vs general resistance has long been debated, primarily because it was unclear whether (a) diverse priming types (within- vs trans-generational) together constitute distinct strategies, separate from basal resistance (b) their costs vs benefits differ substantially, and (c) they involve different or overlapping sets of immune pathways. Our work represents one of the first steps to address the first two problems, demonstrating distinct costs and benefits of multiple priming responses vs resistance evolving simultaneously in response to selection imposed by the same pathogen. While these results highlight the remarkable diversity and flexibility of insect innate immune adaptation against infections, they also suggest that the early survival vs reproductive costs of priming can constrain its evolution, much more so than resistance. Finally, we end by posing an interesting unanswered question— why are priming responses, despite being so costly, still observed in experimental (I) and natural populations, rather than constitutive resistance? We note the tendency of uninfected PI beetles (with higher resistance) to produce fewer offspring (See Fig. 3) with slower development (Fig. 4B). However, it is unclear if these apparent costs of resistance are sufficient to explain the persistence of priming when infections are rare, since the trends are weak and statistically non-significant. Nonetheless, we hope that our results will motivate further experiments to understand this intriguing problem. Specifically, we look forward to detailed mechanistic studies to test how host-pathogen interactions at a low frequency of infection only favour the evolution of priming, mechanistically precluding more beneficial resistance alleles from fixing in host populations.

Declaration of competing interest

We have no competing interests.

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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.2021.104261.

Author contributions

IK conceived of the experiments; IK, AP and DA designed the experiments; AP carried out the experiments; IK and DA analysed the data; acquired the funding; wrote the manuscript with inputs from AP. All authors gave final approval for publication.

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