The immune regulation and epidemiological consequences of immune priming in *Drosophila*

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Abstract

Invertebrates lack the specialized immune-memory cells responsible for vertebrate-like acquired immunity. However, there is increasing evidence that past infection 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 generality, specificity and mechanistic basis of immune priming in the fruit fly *Drosophila melanogaster* when infected with the gram-negative bacterial pathogen *Providencia rettgeri*. We further explore the epidemiological consequences of immune priming and find it has the potential to curtail pathogen transmission by reducing pathogen shedding. We find that priming in *Drosophila* is a long-lasting, pathogen-specific response, occurring in several fly genetic backgrounds and is particularly stronger in male flies. Mechanistically, we find 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 *Diptericins* are required as the effector of bacterial clearance, it is not solely sufficient for immune priming, and requires regulation by the peptidoglycan recognition proteins *PGRP-LB, PGRP-LC* and *PGRP-LE*. We discuss potential explanations for the observed sex differences in priming, and discuss the epidemiological consequences of innate immune priming in invertebrates.

Introduction

Insects possess a robust immune response to pathogens which includes both cellular and humoral components [1–3], but lack vertebrate-like specialized immune memory cells 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 [4–6]. 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, or even an inactivated pathogen, can lead to increased survival upon reinfection [7–11].

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) [12–14]. The survival benefit of priming has been observed in a range of arthropod taxa, including Dipterans: fruit flies [15], mosquitoes [16]; Coleopterans: flour beetles [13]; Lepidopterans: greater wax-moth [17]; Hymenopterans: bumblebee [9]; Crustaceans: water fleas [18] and Arachnids: spiders and scorpions [19]. 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 [20–22].

The observation of immune priming in invertebrates underlines the importance of whole organism research in immunity in lieu of a purely mechanistic approach to immunology, highlighting that the same phenomenology can originate in very different mechanisms [6]. Determining the mechanisms of invertebrate immune 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 *Streptococcus 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 [15]. The increased phagocytic activity in primed individuals has also been shown to play a key role in priming in crustaceans [23]. In other *Drosophila* work, *PGRP-LB* (peptidoglycan recognition protein *LB*, a negative regulator of the Immune deficiency (IMD)-pathway in *Drosophila*) 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 encapsulation by haemocytes in the offspring [24]. By contrast, in response to infection with *Drosophila C virus* (DCV)*, Drosophila* progeny can produce a DCV-specific priming response by inheriting viral cDNA from the infected adult flies which is a partial copy of the virus genome [25,26].

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 [27,28], *Cecropin, Attacin, Gloverin, Moricin* and *Lysozyme* in silkworms [29], *Gallerimycin and Galiomicin* in wax-moths [30] and finally, *Cecropin* in tobacco moths and fruit flies [31,32] (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 [1,2,33–35]. 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 [36],although this specificity has been shown to be greatly reduced during aging [37].

Beyond its underlying mechanisms, an important but understudied aspect of immune priming are its potential consequences for epidemiological dynamics [21,22,38,39]. Epidemiological modelling predicts that priming should affect the likelihood of pathogen persistence, destabilise the dynamics of host–pathogen populations, and crucially, that these effects depend on the degree of protection conferred by priming [21]. Further theoretical work suggests that priming can either increase or decrease infection prevalence depending on the extent to which it affects the pathogen's colonization success and the host's ability to clear or tolerate the infection [38]. Immune priming is therefore expected to have a significant impact on the outcome of pathogen transmission by directly modifying important epidemiological parameters, but the strength and direction of these effects is not intuitive to predict. While primed individuals may live longer, thus extending the infectious period, their pathogen burden may be lower, which could lead to lower pathogen shedding and less severe

epidemics. Further, behavioural changes in primed individuals may also be likely, if immune deployment during the initial exposure leads to sickness behaviours such as lethargy or reduced social interactions [40,41].

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 our observation that 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 [15], the mechanisms underlying immune priming during gram-negative bacterial infection are poorly characterised. We hypothesized that priming could be driven by the up-regulation of AMPs following an initial pathogen exposure, resulting in faster clearance upon re-infection. The IMD-responsive AMP *Diptericins* (*DptA and DptB*) is sufficient to confer complete protection during infection with *P. rettgeri* [36], but it is unclear whether the same IMD-regulated AMP-*Dpt* might also be involved in immune priming during re-infections. We therefore used several of the most commonly used *D. melanogaster* genetic background strains (control flies), 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*.Furthermore, motivated by the theoretical predictions of the role of immune priming on epidemiological dynamics, we also designed infection experiments to enable us to test how immune priming could affect each of these behavioural and immunological components of pathogen spread.

Materials and methods

Fly strains and maintenance

Several *D. melanogaster* strains were selected to represent variable genetic backgrounds: w^{1118} (Vienna *Drosophila* Resource Center), *iso-*w^{1118} (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 (spätzle – Toll* pathway regulator) and the following CRISPR/Cas9 mutants [36]: (a) ΔAMPs - flies lacking 10 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 (DptA and DptB),* and (d) ΔAMPs^{+Dpt} – flies lacking 10 known AMPs except *Diptericins*.

All the CRISPR/Cas9 mutants were generated previously from the *iso-*w^{1118} genetic background using CRISPR/Cas9 gene editing technology to induce null mutations in the selected genes [36], and were gifted generously by the Lemaitre lab (U. Lausanne). We also used the binary GAL4/UAS system for tissue specific silencing of target genes with the RNA interference (RNAi) method. *Diptericin-B (DptB)* (Bloomington stock# *28975*) was tissue specifically knocked down from fat body ($w^{1118\text{-}iso}$*,Fb-Gal4i+(P{fat}*) and from haemocytes [$w^{1118\text{-}iso}$*,Hmldelta- Gal4; He-Gal4* – a combination of two haemocyte GAL4 drivers, the Hml-GAL4.Δ [42] and He-GAL4.Z [43]]. 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 fly medium [44]. For the experiments, we controlled larval density by placing 10 females and 5 males in each vial and the females were allowed to lay eggs for 48-hours. Fourteen days 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.

Systemic 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 to 0.1 and 0.2 by using sterile 1xPBS (Phosphate buffer saline). To obtain heat-killed bacteria the dilution was incubated at 90°C for 20-30 mins [13]. To ensure all bacteria were dead in the heat-treated culture, it 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) [45]. Following this initial priming treatment, the individuals were pricked using OD_{600}=0.1 live *P. rettgeri* bacteria (resulting in approximately 70 bacterial cells/fly). To test whether male and female adult flies show priming (measured as 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**. S1 for experimental design; n=9-13 vial treatment/sex/ fly line). We used a split vial experimental design to obtain replicate matched data for both survival and bacterial load see [46] for details. Briefly after infection each vial containing about 25 flies (of each treatment, sex and fly line combination) were divided into 2 vials for measuring (i). survival following infection (see **Fig**. S1i; 13-17 flies/combination) and (ii). internal bacterial load (see **Fig**. S1ii).

Bacterial load quantification

To test whether the host's ability to supress bacterial growth varies across primed and unprimed individuals, we quantified bacterial load as colony forming units (CFUs) at 24-hours after *P. rettgeri* infection for controls and transgenic flies in 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 performed serial dilutions of the homogenate with 1xPBS and plated them on LB agar plates and cultured at 29°C overnight. The following day, we counted the CFUs manually [47] (n=9-13 vial/sex/treatment/fly mutants).

Gene expression quantification

The expression of *Diptericins* was quantified by qRT-PCR. In parallel with the survival experiment, we randomly selected a subset of control w^{1118} individuals (both males and females) for RNA extraction, we included 15 flies [5-7 replicates of 3 flies pooled together for each treatment (primed and unprimed) for both males and females]. We randomly removed selected 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 pools of three in 80µl of TRIzol reagent (Invitrogen, Life Technologies). Homogenates 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) [45].

We confirmed the purity of eluted RNA 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 *RpL*49 as endogenous reference gene, *RpL*49_Forward: 5' ATGCTAAGCTGTCGCACAAATG 3', *RpL*49_Reverse: 5' GTTCGATCCGTAACCGATGT 3'. We optimised the annealing temperature (T□) 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□= 59 °C, Eff= 102%; *AttC*: T□= 60 °C, Eff= 94%; *Dro*: T□= 61 °C, Eff= 104%.

Oral priming and infection

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 [47]] 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 the agar on the 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 (heat-killed only, primed and unprimed treatment) by adding approximately 10 flies per vial for 18-hours and then transferred the flies onto fresh Lewis food vials. After 3-days, 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 onto fresh food vials and observed survival after oral exposure to *P. rettgeri* every 12 hours for the next 8 days (see **Fig.** S2).

Measuring locomotor activity

We measured the locomotor activity of single flies (n=52 flies for each for each sex and treatment combination) during three continuous days using a *Drosophila* Activity Monitor – DAM (v2 and v5) System [48], 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 [48]. We analysed fly activity data using three metrics [40,49–51]: total activity, the average activity during 5-min activity bouts, and proportion of 5-min bouts with zero activity (which has been defined as sleep in *Drosophila* [52]) (**Fig.** S2-III)

Measuring bacterial shedding

We measured the bacterial shedding of single flies at a single time point, 4-hours following the oral bacterial exposure. Following oral priming to either heat-killed bacterial culture (primed treatment) or 5% sucrose solution (unprimed treatment), flies were exposed to an oral infection with live infection with 80µl of live *P. rettgeri* culture (OD_{600}=25). Following 18 hours of oral exposure, flies were placed individually into 1.5ml Eppendorf tubes with approximately 50µl of Lewis medium in the bottom of the tube for 4-hours. After 4-hours, flies were removed from the tube and these were washed with 50µl of 1xPBS buffer by vortexing thoroughly for at least 5 secs. We then 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 (**Fig.** S2).

Measuring bacterial transmission

We measured transmission in groups of flies, by collecting age-matched donor and recipient w^{1118} flies, separately for each sex. To test the effect of priming on the ability of flies to transmit *P.rettgeri*, we orally exposed 3-day old w^{1118} flies (donor flies) with either 5% sucrose and heat-killed bacteria (primed) or 5% sucrose only (unprimed). After 96-hour the donor flies were exposed to OD_{600}=25 of *P. rettgeri* (*see section above*). The infected donors were marked by cutting the corner of a fly wing. We then placed one donor and five uninfected 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. After 4-hours exposure we surface-sterilised and homogenized the flies and plated the homogenate to measure the presence or absence of *P. rettgeri* infection inside the fly, as an indication of successful transmission of bacteria from the donors to the recipient [47].

Data analysis

All statistical analyses and graphics were carried out and produced in R (version 4.2.2) using the ggplot2, coxhz and lme4 packages [53–55], and all raw data and code is available at 10.5281/zenodo.7624084 [56]. We analysed the survival data following systemic *P. rettgeri* infection with a mixed effects Cox model using the R package 'coxme' [54] for different treatment groups (that is, primed and unprimed) across both the sexes and fly lines (controls and transgenic lines). We specified the model as: survival ~ treatment * sex * (1|vials/block), with 'treatment' and 'sex' and their interactions as fixed effects, and 'vials' nested within each 'block' as a random effect for control and transgenic 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. As bacterial load data was non-normally distributed, we log-transformed the data 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 (control and transgenic lines).

We analysed the gene expression data by calculating the ΔΔCT value [57]. We used *RpL32* as a reference gene as it was expressing steadily in our treatment and control conditions. 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. Data for bacterial shedding and transmission were non-normally distributed (tested using Shapiro-Wilks test for normality) hence we performed non-parametric Wilcoxon Kruskal-Wallis tests for bacterial shedding and transmission data.

We tested how immune priming affects the behavioural components of pathogen spread by calculating total locomotor activity, proportion of awake activity and sleep patterns for individual fly (16 for control, 52 flies for primed & unprimed treatment). We analysed variation in total locomotor activity, average awake activity and time asleep using a generalized linear model (GLM) by fitting a binomial distribution (best fitted model), which also fitted 'sex' and 'treatment' (i.e., heat-killed only, primed and unprimed) as categorial fixed effects separately for oral and systemic infection routes.

Results

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

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 w^{1118}male and female control flies 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**. 1 A-E). 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**. 1A-E, **Table**-S1).

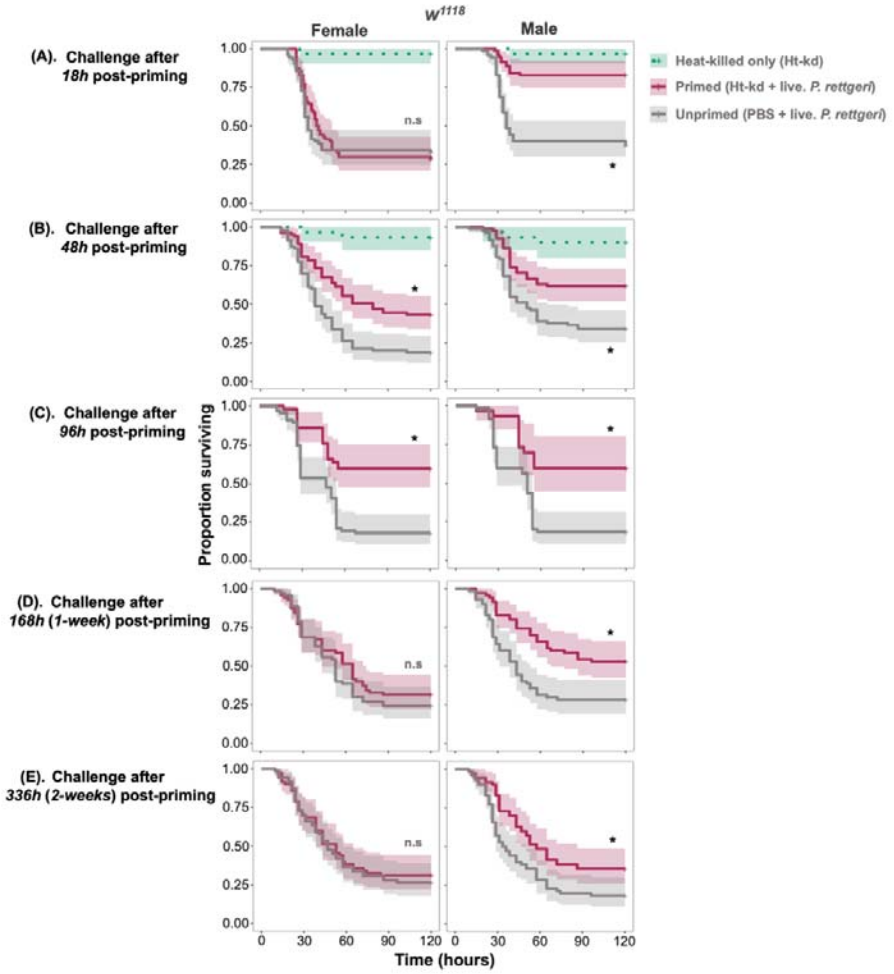

Figure 1: Survival curves of w^{1118} females and males with primed (exposed to heat-killed *P. rettgeri* in the first exposure) and unprimed (exposed to a sterile solution in

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).

Next, we asked if priming occurred in two other commonly used *Drosophila* lines, Canton-S and Oregon-R (Ore-R) as observed with w^{1118}. Given the widespread effects of the endosymbiont *Wolbachia* on *Drosophila* immunity [58–60] 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 $OreR^{Wol+}$ and a *Wolbachia*-free line $OreR^{Wol-}$ that was derived from $OreR^{Wol+}$ by antibiotic treatment [60]. Females and males of the four *Drosophila* lines 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 of the w^{1118} line (Fig. 1C), 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**. 2B, **Table** S2B). Oregon-R males also exhibited increased survival following priming, but priming had no significant effect on the females (**Fig**. 2C, **Table**-S2). We found that the presence of *Wolbachia* significantly improved overall survival of both males and females (**Fig**. 2C and D, **Table**-S3). However, the immune priming observed in males in absence of *Wolbachia* ($OreR^{Wol-}$) was no longer present in flies carrying *Wolbachia* ($OreR^{Wol+}$) (**Fig.** 2D, **Table**-S3).

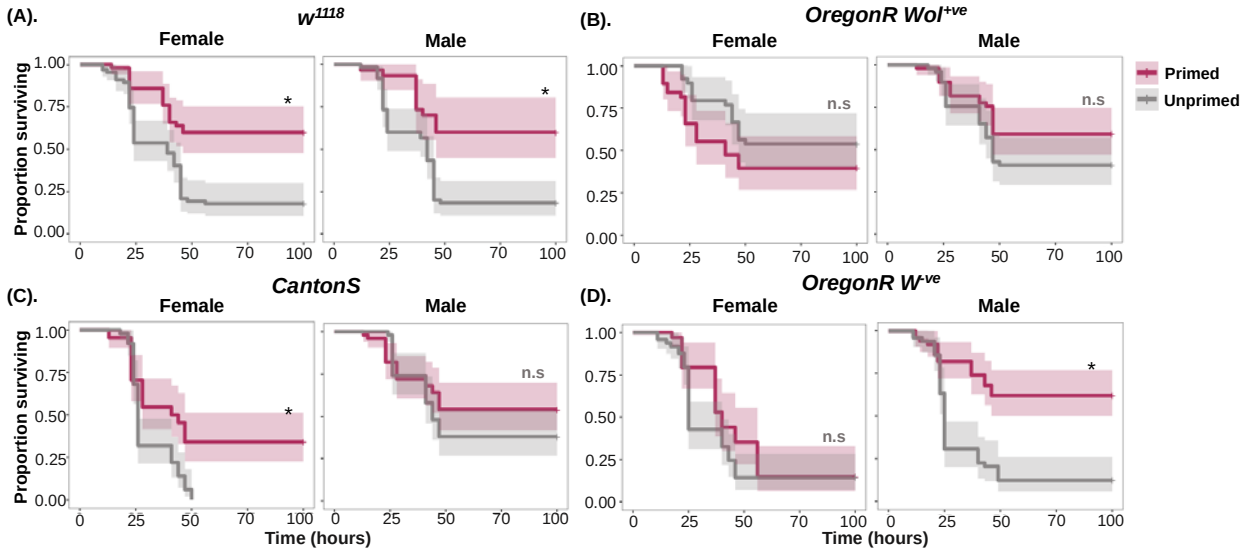

Figure 2: Survival curves of females and males of the commonly used *Drosophila* lines either primed-challenged or unprimed-challenged with *P. rettgeri*. **(A)** w^{1118} **(B)** Canton-S **(C)** Oregon-R carrying endosymbiont *Wolbachia* **(D)** Oregon-R cleared of *Wolbachia*. (n=7-9 vial with 10-15 flies in each vial/sex/treatment/line).

II. The strength of immune priming is bacterial species specific

A notable feature of immune priming is its specificity. Previous work suggests that insects show strain-specific priming against multiple B. thuringiensis strains under more frequent antigen exposure in flour beetles (Roth et al., 2009; Khan et al., 2017), suggesting an added level of functional complexity of insect immunity. We tested whether *D. melanogaster* also shows specific immune priming against *Providencia rettgeri (Pr)*. We used males of the w^{1118} line, as males were shown to have stronger priming response than females and two pathogens, one closely related species of *P. rettgeri* i.e., *Providencia burhodogranariea (Pb)* and another less closely related gram-negative bacteria i.e., *Pseudomonas entomophila (Pe)*. Male flies homologously primed and challenged with (*Pr – Pr*) species showed significantly improved survival rates compared with the heterologous treatment combinations (*Pr* – other bacteria) and corresponding control (1xPBS – challenge with live bacteria) (**Fig**. 3A-C, **Table** S4A-B). Our results thus show that flies immune system can discriminate between closely related bacterial species. This indicates a surprisingly high degree of specificity in priming.

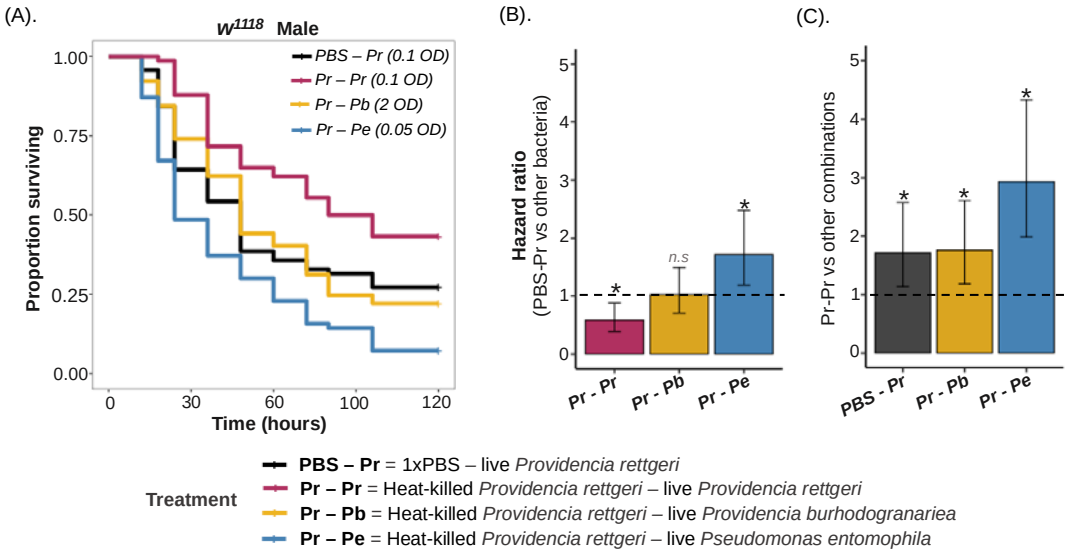

Figure 3: Bacterial species-specific immune priming response in w^{1118} males. Two closely related species of Providencia bacteria *Providencia rettgeri* (Pr) and *Providencia burhodogranariea* (Pb) and another gram-negative bacteria *Pseudomonas entomophila* were used for testing the immune priming specificity

in *Drosophila*. (A) survival curves (B) hazard ratio of unprimed males with 1x PBS followed by live *P. rettgeri* against males with primed *P. rettgeri* followed by live infection with other bacterial species *Pb* and *Pe*. (C). hazard ratio of primed males with heat-killed *P. rettgeri* followed by live *P. rettgeri* against males with primed *P. rettgeri* followed by live infection with other bacterial species *Pb* and *Pe*.

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 bacterial pathogens in primed individuals [15,61]. 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 strongest priming phenotype in both sexes (**Fig**. 1C 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**-S5). 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**-S5 for survival, **Table**-S6 for load). However, by 72-hours post-infection, bacterial loads had dropped in both primed and unprimed male flies and there was no detectable effect of priming on the bacterial loads (**Fig**. 4B, **Table**-S6).

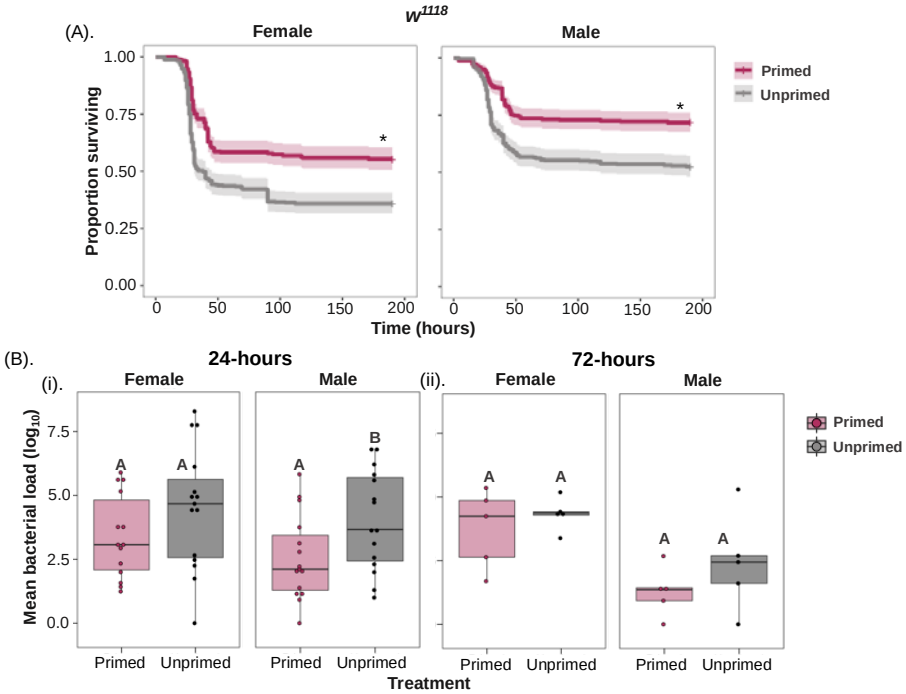

Figure 4: Bacterial loads of primed and unprimed w^{1118} flies. **(A).** Survival curves of primed and unprimed control w^{1118} flies **(B).** Internal bacterial load (n=20-22 vial with 5-7 flies in each vial/sex/treatment) after **(i)** 24-hours and **(ii)** 72-hours following *P. rettgeri* infection.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

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

Apart from increasing survival during infections, immune priming may also have consequences to pathogen spread and transmission [22]. Despite having enhanced survival, the primed individuals may also extend the infectious period or their pathogen burden may be lower, which would lead to less severe epidemics [22]. To investigate how immune priming affects some of these epidemiological parameters we tested whether primed individuals have reduced bacterial spread in w^{1118} flies. However given he oral-faecal nature of bacterial transmission and that our previous results all related to systemic infections, we decided to also investigate immune priming under oral 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 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. 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.** 5A, **Table**-S7).

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.** 5B, **Table**-S8). 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.

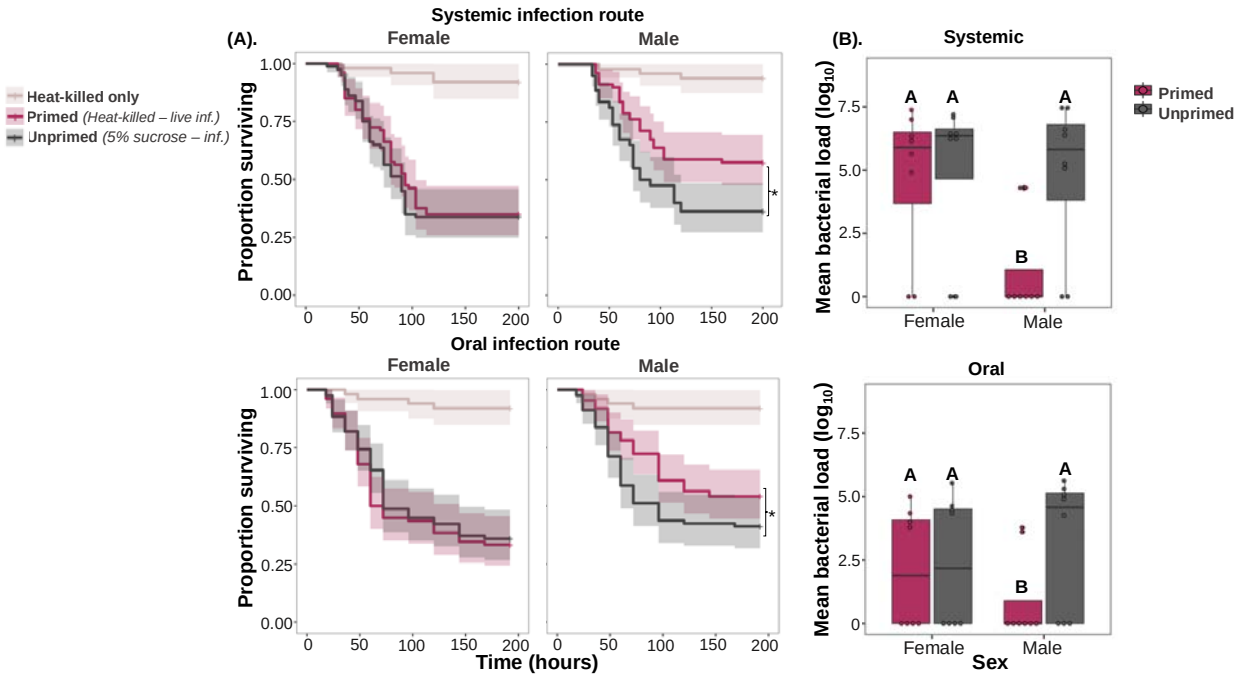

Figure 5: Priming occurs during both systemic and oral infections. **(A)** Survival curves of male and female wildtype (w^{1118}) flies with (i) systemic (ii) oral priming where flies initially exposed to heat-killed *P. rettgeri* or unprimed flies exposed to a sterile solution in the first exposure, 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/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.

V. Immune priming does not affect fly locomotor activity

While the duration of the infectious period is determined to some extent by the infected lifespan of a fly, pathogen transmission is also strongly influenced by host behaviours that impact the contact rates between infected and uninfected individuals [62]. One of the simplest behavioural readouts that is relevant for how frequently individuals may come into contact is locomotor activity[40,41,63]. 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[40,51]. A crucial point is that host behaviours are often modified by infection, or even by immune stimulation that mimics infection[62,64,65]. 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.** 6, **Table**-S9). 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.

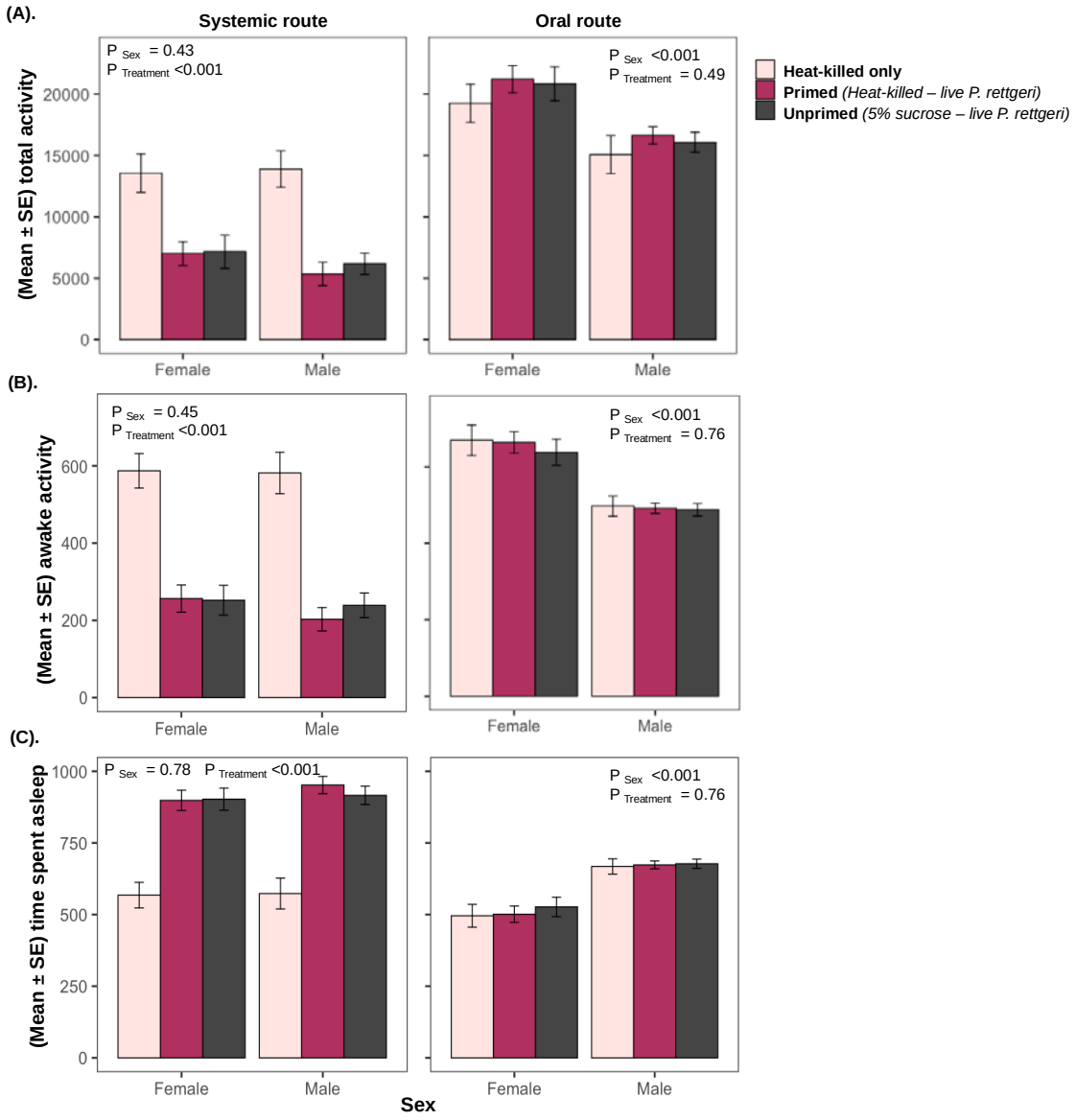

Figure 6: The effect of priming on fly locomotor activity. Mean ±SE total locomotor activity for males and females (n= 52 individual flies per treatment), during first 72-hours following systemic and oral priming and infection. **(A)** average total locomotor activity **(B)** average awake activity and **(C)** proportion of flies spent sleeping.

VI. Priming following oral infection reduces bacterial shedding and transmission in males

Given that primed male w^{1118} flies showed a reduction in internal bacterial loads (Fig 5B) we hypothesised that priming could therefore directly impact the amount of bacterial shedding, and therefore have a direct impact on pathogen transmission. 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 (**Fig.** 7A, **Table-**S10).

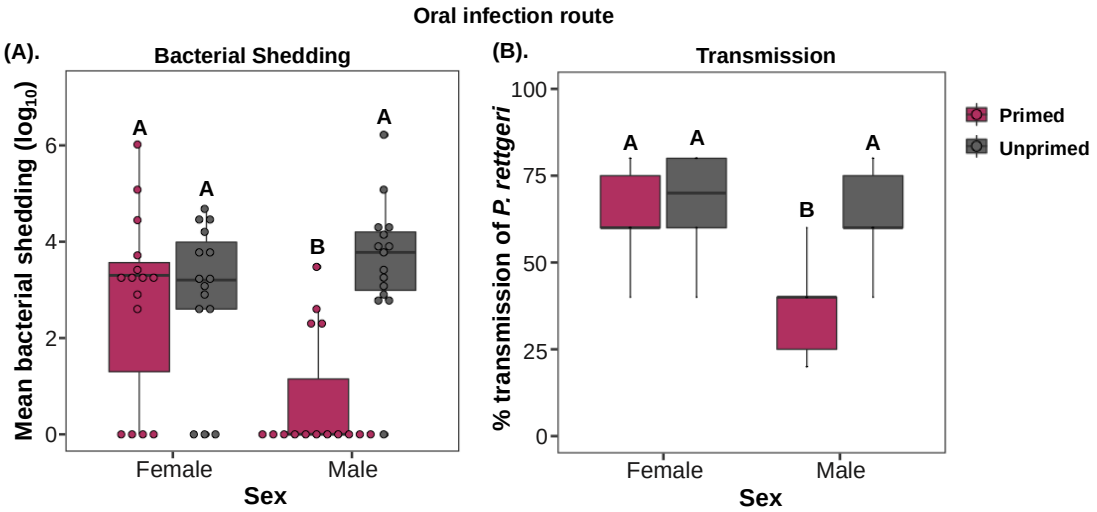

Figure 7: The effects of priming on bacterial shedding and transmission (A). Bacterial shedding after 4-hours following oral priming (initial heat-killed exposure) and infection with live *P. rettgeri* (OD_{600}=25) for males and females. (B) percentage transmission of measurable bacterial loads for 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.

Primed males also spread or transmit very little pathogen upon re-infection with oral *P. rettgeri* (**Fig.** 7B, **Table-**S10). However, both primed and unprimed females showed increased bacterial spread following oral *P. rettgeri* infection (**Fig.** 7B, **Table-**S10). Our

results therefore provide evidence that, at least in male flies, immune priming reduces pathogen transmission by directly decreasing bacterial shedding from infectious flies.

VII. The *IMD*-pathway, but not the Toll pathway, is required for immune priming during *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* [33]. 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 fly lines (CRISPR knockouts and UAS-RNAi knockdowns) lacking or knockdown of 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 did not show any bacterial clearance, died at faster rate, and therefore did not show any priming benefit (**Fig**. 8A-ii for survival and **Fig**. 8B-ii for bacterial load, **Table**-S11 for survival and **Table**-S12 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 [15]. In other work, the Toll-pathway activity differed between males and females and explained sexual dimorphism in immunity during *P. rettgeri* infection [66]. Given that we found sex differences in the priming in response to *P. rettgeri* infection (**Fig**. 1 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 mutants which lack *spätzle*, a key regulator in the *Toll* pathway, and these flies are not able 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**. 8A-iii, **Table**-S11) indicating that Toll-pathway does not contribute to priming during *P. rettgeri* infection in either males or females (**Fig**. 6A-iii, **Table**-S11).

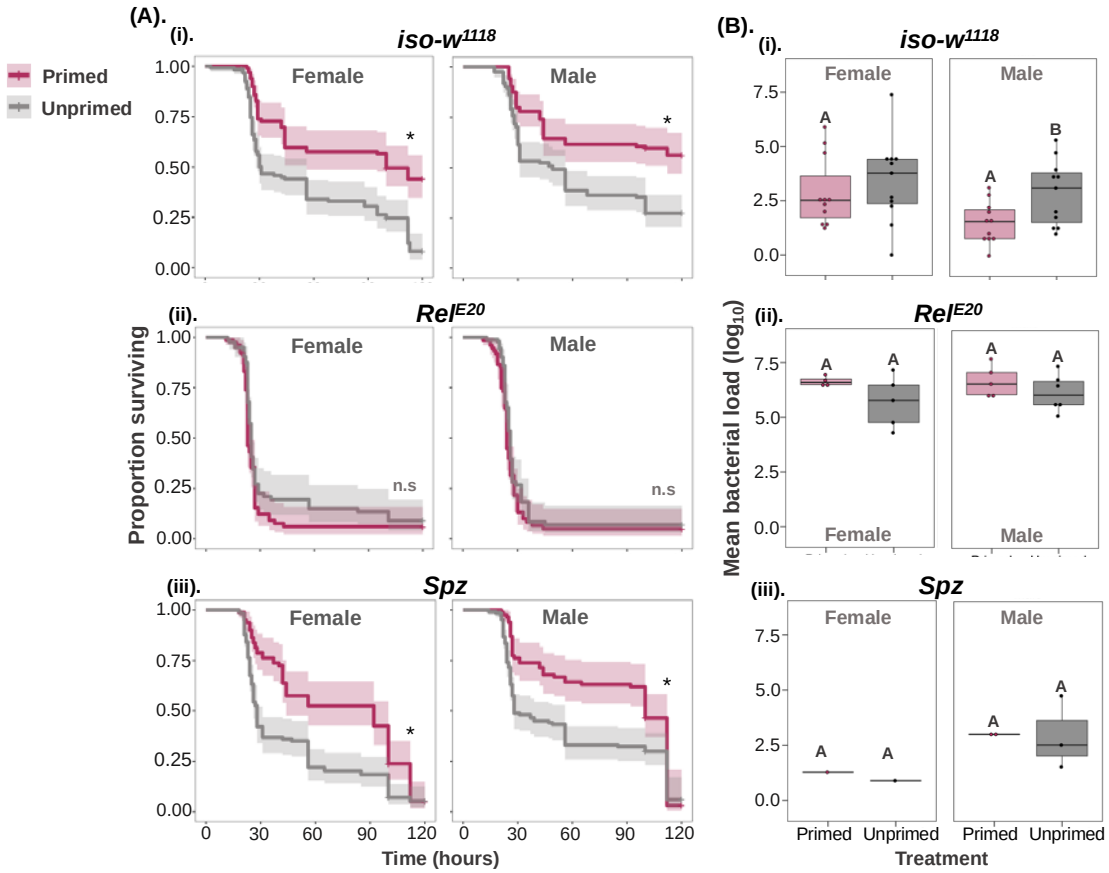

Figure 8. Priming requires the IMD but not the Toll pathway (A): Survival curves for control flies and flies lacking different innate immune pathway components in females and males **(i)** control *iso-w*^{1118} **(ii)** *Rel*^{E20}- IMD-pathway transcription factor **(iii)** *Spz*- Toll pathway regulator in both males and females **(B) (i-iii)** bacterial load measured after 24-hours post-secondary pathogenic exposure (n=7-9 vial/sex/treatment/transgenic lines). Different letters in panel-B indicate that primed and unprimed individuals are significantly different, tested using Tukey's HSD pairwise comparisons, analysed separately for each timepoint and sex combination. The error bars in panel B represent standard error.

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

Given the important role of the IMD pathway for the priming response (Fig. 5A), next, we tested whether mutants with defective IMD signalling or unable to produce specific antimicrobial peptides were capable of immune priming. We first used a ΔAMP transgenic line, which lacks most of the known *Drosophila* AMPs (10 AMPs in total). It has been previously shown that ΔAMPs flies are extremely susceptible to the majority of microbial pathogens, including gram-negative bacteria [36]. 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 (measured 24-hours after the secondary pathogenic exposure) (**Fig**. 9A-i for survival, **Fig**. 9B-i for bacterial load, **Table**-S11 and S12, relative to control w^{1118} flies – compare with previous figure Fig. 8B-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 transgenic 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**. 9A-ii, **Table** S11) and exhibited increased bacterial loads across both the sexes irrespective of being primed or not (**Fig**. 9B-ii, **Table** S12). 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* mutant (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**. 9B-iii, **Table** S12). 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**. 9A-iv for survival, **Fig**. 9B-iv for bacterial load; **Table** S11 for survival, **Table** S12 for bacterial load).

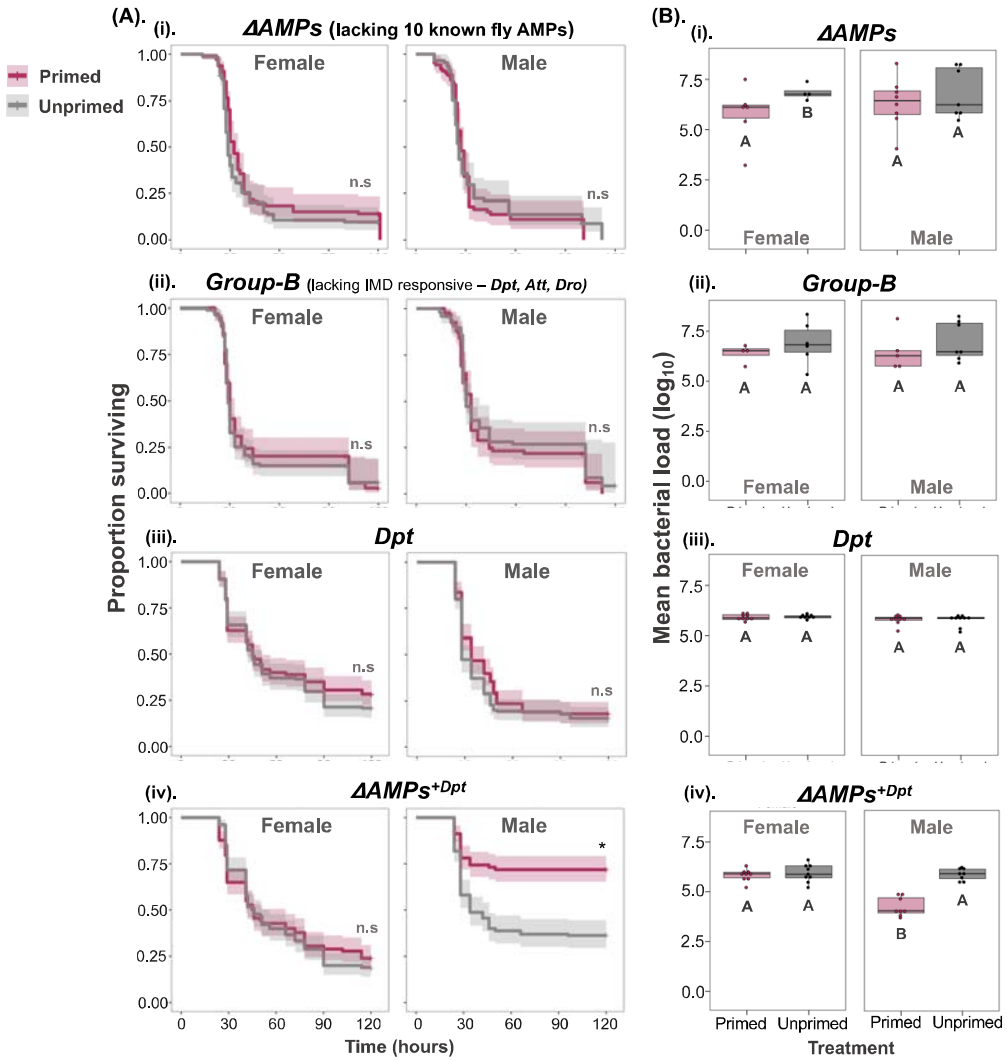

Figure 9: Priming requires specific AMP expression (A). Survival curves for CRISPR/Cas9 AMP mutants **(i)** *ΔAMP* fly line: lacking all known 10 fly AMPs – **(ii)** ***Group-B:*** lacking major IMD pathway AMPs **(iii)** ***Dpt*** knockout and **(iv)** ***ΔAMP^{+Dpt}*:** lacking all AMPs except ***Dpt***.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

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

In response to gram-positive *Streptococcus pneumoniae* infection, previous work described the role of haemocytes in immune priming through increased phagocytosis [15]. Subsequent work has also shown that reactive oxygen species (ROS) burst from haemocytes is important for immune priming during *Enterococcus faecalis* infection [32]. Since our results pointed to a *Diptericins* being required for immune priming against *P. rettgeri*, we wanted to

determine if *Diptericin* expression in either the fat body or haemocytes was more important for immune priming. Using-tissue specific *Diptericin-B* UAS-RNAi knockdown, we found that male flies with knocked-down *DptB* in fat bodies no longer showed immune priming compared to the background or control iso-w^{1118}, while knocking down *DptB* in haemocytes resulted in a smaller but still significant increase in survival following an initial exposure (**Fig**. 10, **Table**-S13). This would therefore support that immune priming requires *DptB* expression in the fat body, while haemocyte-derived *DptB* is not important for priming. It is worth noting that it is possible that haemocytes contribute to immune priming through phagocytosis or melanisation, or via cross-talk with the IMD pathway, and this remains a question for future research.

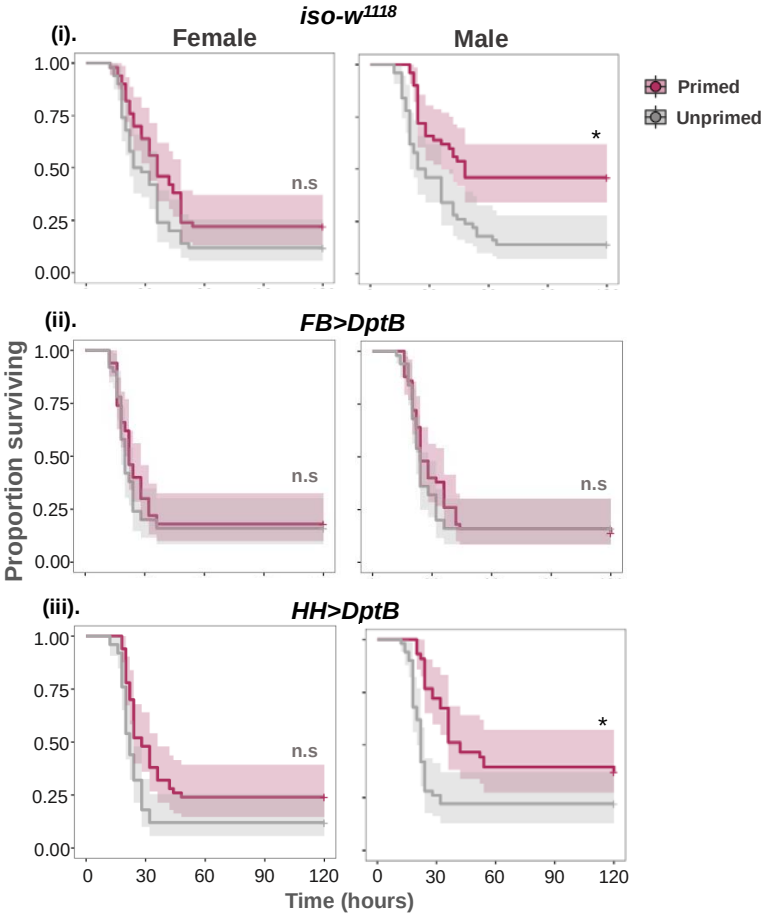

Figure 10: Survival curves for **(i)** control *iso-w*^{1118} and *UAS-RNAi* mutants - **(ii)** ***FB>DptB*** RNAi knock down of *DptB* in fat body and **(iii)** ***FB>DptB*** RNAi knock down of *DptB* in haemocytes. (n=~50 flies/sex/treatment/fly lines).

X. Priming is not an outcome of constant *Dpt* 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. *DptB* expression increased 18-hours after initial heat-killed exposure but returned to baseline levels by 72-hours (**Fig.** 11A and 11B, **Table**-S14) 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 *DptB* levels compared to unprimed individuals (**Fig.** 12A and 12B). The increased *DptB* expression also resulted in increased bacterial clearance in males (see **Fig**. 4Bi and 8Bi). 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 8Bi). We also tested the expression pattern 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.** 11C for *AttC and Dro*, **Table**-S14).

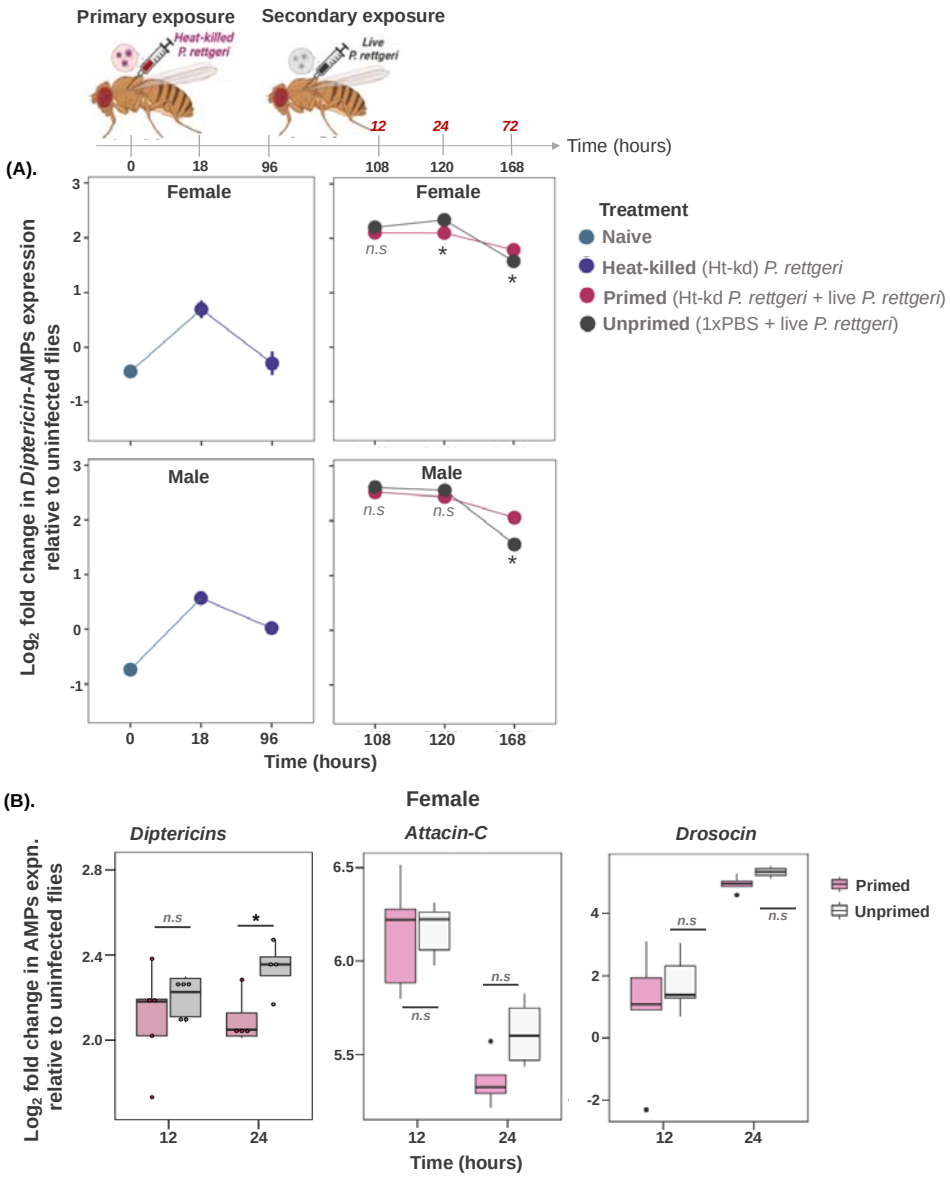

Figure 11: **Antimicrobial gene expression following priming** (A) Mean±SE (standard error) of *Diptericin*-AMPs expression at different time points (hours) for females and males wild-type w^{1118} flies with treated with - naïve or unhandled flies; flies exposed to initial heat-killed *P. rettgeri*; 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 (3 flies pooled) /sex/treatment/timepoint]. **(B).** *Diptericin (A+B), Attacin-C* and *Drosocin* AMPs 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.

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

Although 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 isoform and an intracellular isoform), which then activate the IMD intracellular signalling cascade [33,34,67]. 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[33,34,67]. *PGRP-LB* has also been shown to be important for transgenerational immune priming in *Drosophila* against parasitoid wasp infection [24].

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**. 12A, **Table**-S15). However, unlike similar outcomes with *Relish* or *ΔAMP*, here the lack of priming was not 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**. 12B, **Table**-S16; also compare **Figs**. 8B-ii and 9B-i. Further Dpt expression compared to the w^{1118} control was either similar (PGRP-LC, PGRP-LE) or higher (PGRP-LB), as expected, in both primed and unprimed flies (**Fig.** 13, **Table**-S17). This suggests that *Diptericin* expression is required, but not sufficient, for successful priming, which requires adequate regulation by *PGRPs*. Which specific molecular signal modifies *PGRP-mediated* regulation of *Diptericin* expression following an initial priming challenge is unclear, but must lie upstream of the IMD pathway and the fat body.

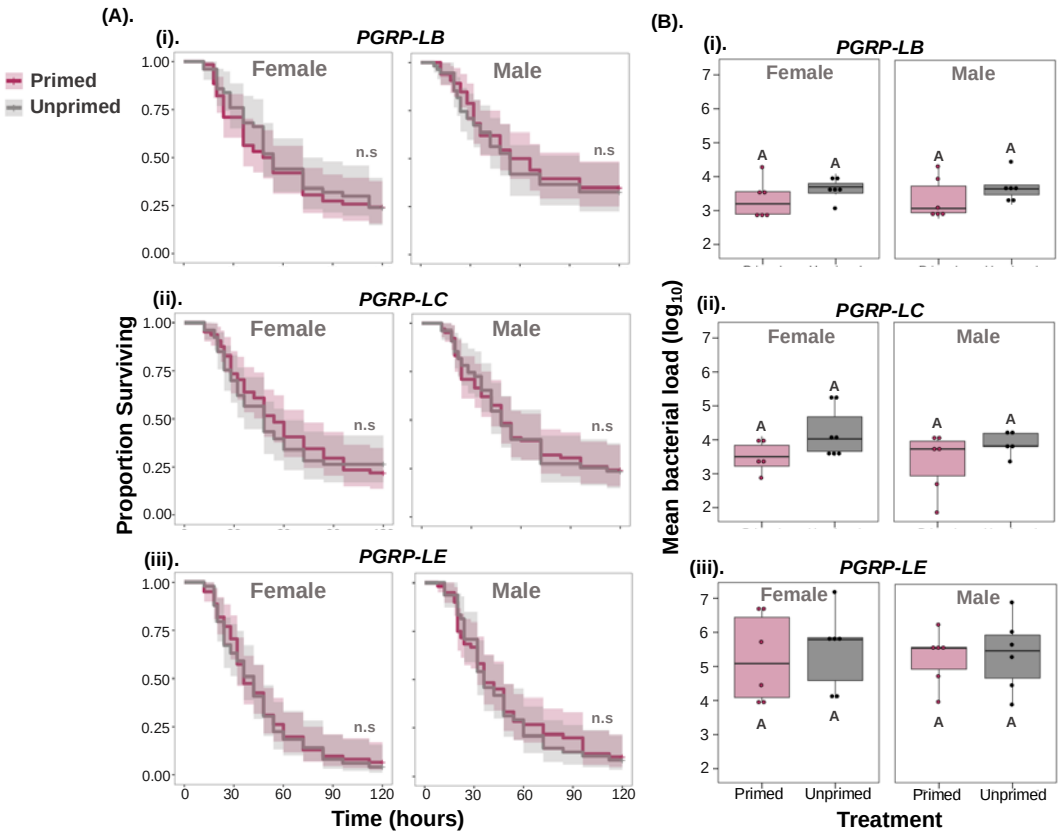

Figure 12: (A). Survival curves for males and females of Loss-of-function in *PGRPs* (peptidoglycan recognition proteins) of IMD **(i)** *PGRP-LB* **(ii)** *PGRP-LC* and **(iii)** *PGRP-LE* **(B).** internal bacterial load quantified after 24-hours post-secondary exposure in female and male flies (n=7-9 vials with 10-15 flies in each vial/sex/treatment/ 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.

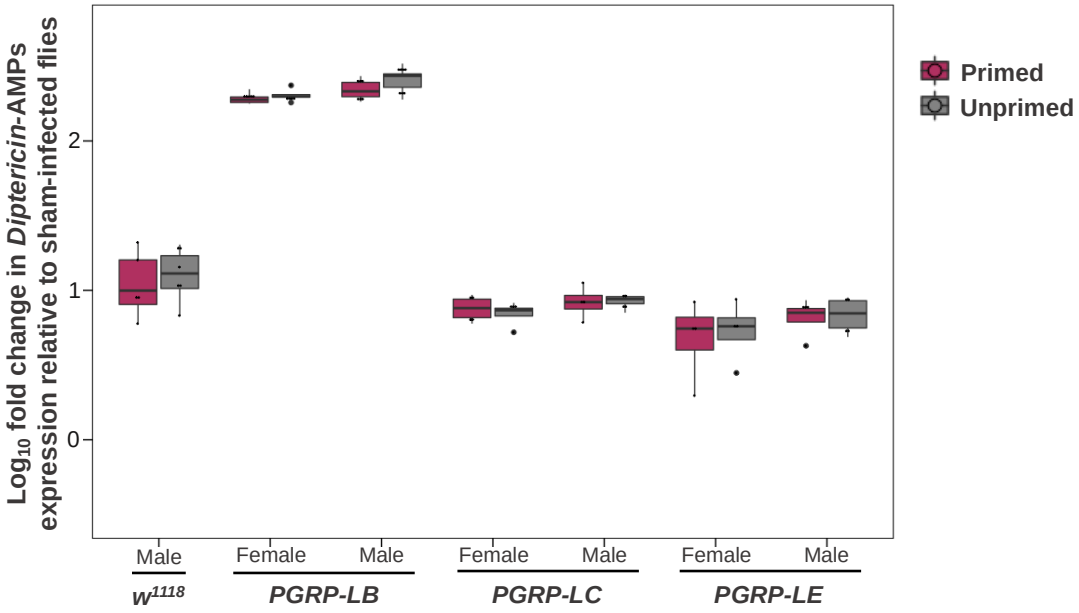

Figure 13: *Diptericin*-AMPs expression 24-hours after exposure to live *P. rettgeri* in male and female control w^{1118} flies and flies with loss-of-function in different *PGRPs*, *PGRP-LB, PGRP-LC & -LE*.

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 non-lethal 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 genetic backgrouns. 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 [68,69]. 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 indicates that PGRPs are necessary for regulating immune priming against *P. rettgeri* (see **Fig**. 14).

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 [70]. 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**. 9A 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**. 9A 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 had lower bacterial loads at 24-hours after exposure to the second lethal infection, 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* [71–73]. For instance, the Toll-signalling pathway activity mediated sex-differences in survival following a *P. rettgeri* systemic infection [66]. 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 [74–76]. Studies from other insects suggest that inducing priming responses can directly reduce the reproductive fitness in mosquitoes [77], wax moth [78], and mealworm beetles [79]. 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 [80,59,81,60]. 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 [82]. 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 [83–85]. 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 [22]. It remains unclear whether immune priming reduces pathogen transmissibility, varies the infectious period, or alters infection-induced behavioural changes in the host [22]. 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 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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Data accessibility: All raw data and R code used to produce the analyses and figures is available at 10.5281/zenodo.7624084 and is citable as: Prakash A, Vale P. Data and code for "The immune regulation and epidemiological consequences of specific immune priming in Drosophila." Zenodo; 2023. doi:10.5281/zenodo.7624085 [56].

Author contribution:

AP and PFV conceived and designed the experiments. AP, FF, BS, KMM, TSS performed the experiments. AP analysed the data with help from FF and PFV. AP and PFV wrote the paper with additional inputs from IK, TSS, FF, BS & KMM.

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Conflicts of interests

The authors declare no conflict of interests.

References

1. Hoffmann JA. The immune response of Drosophila. Nature. 2003;426: 33–38. doi:10.1038/nature02021

2. Hultmark D. Drosophila immunity: paths and patterns. Curr Opin Immunol. 2003;15: 12–19. doi:10.1016/S0952-7915(02)00005-5

3. Buchon N, Silverman N, Cherry S. Immunity in Drosophila melanogaster — from microbial recognition to whole-organism physiology. Nat Rev Immunol. 2014;14: 796–810. doi:10.1038/nri3763

4. Hauton C, Smith VJ. Adaptive immunity in invertebrates: A straw house without a mechanistic foundation. BioEssays. 2007;29: 1138–1146. doi:10.1002/bies.20650

5. Little TJ, Colegrave N, Sadd BM, Schmid-Hempel P. Studying immunity at the whole organism level. BioEssays News Rev Mol Cell Dev Biol. 2008;30: 404–405; author reply 406. doi:10.1002/bies.20738

6. Little TJ, Hultmark D, Read AF. Invertebrate immunity and the limits of mechanistic immunology. Nat Immunol. 2005;6: 651–654. doi:10.1038/ni1219

7. Christophides GK, Zdobnov E, Barillas-Mury C, Birney E, Blandin S, Blass C, et al. Immunity-Related Genes and Gene Families in Anopheles gambiae. Science. 2002;298: 159–165. doi:10.1126/science.1077136

8. Kurtz J, Franz K. Evidence for memory in invertebrate immunity. Nature. 2003;425: 37–38. doi:10.1038/425037a

9. Sadd BM, Schmid-Hempel P. Insect Immunity Shows Specificity in Protection upon Secondary Pathogen Exposure. Curr Biol. 2006;16: 1206–1210. doi:10.1016/j.cub.2006.04.047

10. Milutinović B, Kurtz J. Immune memory in invertebrates. Semin Immunol. 2016;28: 328–342. doi:10.1016/j.smim.2016.05.004

11. Prakash A, Khan I. Why do insects evolve immune priming? A search for crossroads. Dev Comp Immunol. 2022;126: 104246. doi:10.1016/j.dci.2021.104246

12. Contreras-Garduño J, Lanz-Mendoza H, Franco B, Nava A, Pedraza-Reyes M, Canales-Lazcano J. Insect immune priming: ecology and experimental evidences. Ecol Entomol. 2016;41: 351–366. doi:10.1111/een.12300

13. Khan I, Prakash A, Agashe D. Divergent immune priming responses across flour beetle life stages and populations. Ecol Evol. 2016;6: 7847–7855. doi:10.1002/ece3.2532

14. Sheehan G, Farrell G, Kavanagh K. Immune priming: the secret weapon of the insect world. Virulence. 2020;11: 238–246. doi:10.1080/21505594.2020.1731137

15. Pham LN, Dionne MS, Shirasu-Hiza M, Schneider DS. A Specific Primed Immune Response in Drosophila Is Dependent on Phagocytes. Vernick K, editor. PLoS Pathog. 2007;3: e26. doi:10.1371/journal.ppat.0030026

16. Ramirez JL, de Almeida Oliveira G, Calvo E, Dalli J, Colas RA, Serhan CN, et al. A mosquito lipoxin/lipocalin complex mediates innate immune priming in Anopheles gambiae. Nat Commun. 2015;6: 7403. doi:10.1038/ncomms8403

17. Fallon JP, Troy N, Kavanagh K. Pre-exposure of Galleria mellonella larvae to different doses of Aspergillus fumigatus conidia causes differential activation of cellular and humoral immune responses. Virulence. 2011;2: 413–421. doi:10.4161/viru.2.5.17811

18. Little TJ, O'Connor B, Colegrave N, Watt K, Read AF. Maternal Transfer of Strain-Specific Immunity in an Invertebrate. Curr Biol. 2003;13: 489–492. doi:10.1016/S0960-9822(03)00163-5

19. Gálvez D, Añino Y, Vega C, Bonilla E. Immune priming against bacteria in spiders and scorpions? PeerJ. 2020 [cited 2 Oct 2021]. doi:http://doi.org/10.7717/peerj.9285

20. Tate AT, Rudolf VHW. Impact of life stage specific immune priming on invertebrate disease dynamics. Oikos. 2012;121: 1083–1092. doi:10.1111/j.1600-0706.2011.19725.x

21. Tidbury HJ, Best A, Boots M. The epidemiological consequences of immune priming. Proc R Soc B Biol Sci. 2012;279: 4505–4512. doi:10.1098/rspb.2012.1841

22. Tate AT. The Interaction of Immune Priming with Different Modes of Disease Transmission. Front Microbiol. 2016;7: 1102. doi:10.3389/fmicb.2016.01102

23. Roth O, Kurtz J. Phagocytosis mediates specificity in the immune defence of an invertebrate, the woodlouse Porcellio scaber (Crustacea: Isopoda). Dev Comp Immunol. 2009;33: 1151–1155. doi:10.1016/j.dci.2009.04.005

24. Bozler J, Kacsoh BZ, Bosco G. Maternal Priming of Offspring Immune System in *Drosophila*. G3 GenesGenomesGenetics. 2020;10: 165–175. doi:10.1534/g3.119.400852

25. Mondotte JA, Gausson V, Frangeul L, Blanc H, Lambrechts L, Saleh M-C. Immune priming and clearance of orally acquired RNA viruses in Drosophila. Nat Microbiol. 2018;3: 1394–1403. doi:10.1038/s41564-018-0265-9

26. Mondotte JA, Gausson V, Frangeul L, Suzuki Y, Vazeille M, Mongelli V, et al. Evidence For Long-Lasting Transgenerational Antiviral Immunity in Insects. Cell Rep. 2020;33: 108506. doi:10.1016/j.celrep.2020.108506

27. Ferro K, Peuß R, Yang W, Rosenstiel P, Schulenburg H, Kurtz J. Experimental evolution of immunological specificity. Proc Natl Acad Sci. 2019;116: 20598–20604. doi:10.1073/pnas.1904828116

28. Greenwood JM, Milutinović B, Peuß R, Behrens S, Esser D, Rosenstiel P, et al. Oral immune priming with Bacillus thuringiensis induces a shift in the gene expression of Tribolium castaneum larvae. BMC Genomics. 2017;18: 329. doi:10.1186/s12864-017-3705-7

29. Yi Y, Xu H, Li M, Wu G. RNA-seq profiles of putative genes involved in specific immune priming in Bombyx mori haemocytes. Infect Genet Evol. 2019;74: 103921. doi:10.1016/j.meegid.2019.103921

30. Bergin D, Murphy L, Keenan J, Clynes M, Kavanagh K. Pre-exposure to yeast protects larvae of Galleria mellonella from a subsequent lethal infection by Candida albicans and is mediated by the increased expression of antimicrobial peptides. Microbes Infect. 2006;8: 2105–2112. doi:10.1016/j.micinf.2006.03.005

31. Roesel CL, Rosengaus RB, Smith W, Vollmer SV. Transcriptomics reveals specific molecular mechanisms underlying transgenerational immunity in Manduca sexta. Ecol Evol. 2020;10: 11251–11261. doi:10.1002/ece3.6764

32. Chakrabarti S, Visweswariah SS. Intramacrophage ROS Primes the Innate Immune System via JAK/STAT and Toll Activation. Cell Rep. 2020;33: 108368. doi:10.1016/j.celrep.2020.108368

33. Lemaitre B, Hoffmann J. The Host Defense of Drosophila melanogaster. Annu Rev Immunol. 2007;25: 697–743. doi:10.1146/annurev.immunol.25.022106.141615

34. Valanne S, Wang J-H, Rämet M. The Drosophila Toll Signaling Pathway. J Immunol. 2011;186: 649–656. doi:10.4049/jimmunol.1002302

35. Myllymäki H, Valanne S, Rämet M. The Drosophila Imd Signaling Pathway. J Immunol. 2014;192: 3455–3462. doi:10.4049/jimmunol.1303309

36. Hanson MA, Dostálová A, Ceroni C, Poidevin M, Kondo S, Lemaitre B. Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. eLife. 2019;8: e44341. doi:10.7554/eLife.44341

37. Shit B, Prakash A, Sarkar S, Vale PF, Khan I. Ageing leads to reduced specificity of antimicrobial peptide responses in Drosophila melanogaster. Proc R Soc B. 2022; 2022.06.25.497570. doi:10.1101/2022.06.25.497570

38. Tate AT. A general model for the influence of immune priming on disease prevalence. Oikos. 2017;126: 350–360. doi:10.1111/oik.03274

39. Prakash A, Khan I. Why do insects evolve immune priming? A search for crossroads. Dev Comp Immunol. 2022;126: 104246. doi:10.1016/j.dci.2021.104246

40. Siva-Jothy JA, Vale PF. Viral infection causes sex-specific changes in fruit fly social aggregation behaviour. Biol Lett. 2019;15: 20190344. doi:10.1098/rsbl.2019.0344

41. Romano V, Lussiana A, Monteith KM, MacIntosh AJ, Vale PF. Host genetics and pathogen species modulate infection-induced changes in social aggregation behaviour. Biol Lett. 2022 [cited 20 May 2022]. doi:10.1098/rsbl.2022.0233

42. Sinenko SA, Mathey-Prevot B. Increased expression of Drosophila tetraspanin, Tsp68C, suppresses the abnormal proliferation of ytr-deficient and Ras/Raf-activated hemocytes. Oncogene. 2004;23: 9120–9128. doi:10.1038/sj.onc.1208156

43. Zettervall C-J, Anderl I, Williams MJ, Palmer R, Kurucz E, Ando I, et al. A directed screen for genes involved in Drosophila blood cell activation. Proc Natl Acad Sci. 2004;101: 14192–14197. doi:10.1073/pnas.0403789101

44. Lewis E. A new standard food medium. 1960 Drosophila Information Service. in. Cold Spring Harb Protoc. 2014;2014: pdb.rec081414. doi:10.1101/pdb.rec081414

45. Prakash A, Monteith KM, Vale PF. Negative regulation of IMD contributes to disease tolerance during systemic bacterial infection in Drosophila. Evolutionary Biology; 2021 Sep. doi:10.1101/2021.09.23.461574

46. Prakash A, Bonnet M, Monteith KM, Vale PF. The Jak/Stat pathway mediates disease tolerance during systemic bacterial infection in Drosophila. Evolutionary Biology; 2021 Sep. doi:10.1101/2021.09.23.461578

47. Siva-Jothy JA, Prakash A, Vasanthakrishnan RB, Monteith KM, Vale PF. Oral Bacterial Infection and Shedding in Drosophila melanogaster. J Vis Exp. 2018; 57676. doi:10.3791/57676

48. Pfeiffenberger C, Lear BC, Keegan KP, Allada R. Processing Sleep Data Created with the Drosophila Activity Monitoring (DAM) System. Cold Spring Harb Protoc. 2010;2010: pdb.prot5520. doi:10.1101/pdb.prot5520

49. Anderson L, Camus MF, Monteith KM, Salminen TS, Vale PF. Variation in mitochondrial DNA affects locomotor activity and sleep in Drosophila melanogaster. Heredity. 2022; in press. doi:10.1101/2021.10.30.464953

50. Gupta V, Stewart CO, Rund SSC, Monteith K, Vale PF. Costs and benefits of sublethal Drosophila C virus infection. J Evol Biol. 2017;30: 1325–1335. doi:10.1111/jeb.13096

51. Vale PF, Jardine MD. Sex-specific behavioural symptoms of viral gut infection and Wolbachia in Drosophila melanogaster. J Insect Physiol. 2015;82: 28–32. doi:10.1016/j.jinsphys.2015.08.005

52. Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. Correlates of sleep and waking in Drosophila melanogaster. Science. 2000;287: 1834–7. doi:10.1126/science.287.5459.1834

53. Wickham H. Ggplot2: elegant graphics for dataanalysis. New York, NY: Springer.; 2016. Available: https://mran.microsoft.com/snapshot/2015-02-16/web/packages/ggplot2/ggplot2.pdf

54. Therneau T. Mixed Effects Cox Models. Mixed Effects Cox Models. CRAN repository; 2015. Available: https://repo.bppt.go.id/cran/web/packages/coxme/vignettes/coxme.pdf

55. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models using lme4. ArXiv14065823 Stat. 2014 [cited 25 Sep 2021]. Available: http://arxiv.org/abs/1406.5823

56. Prakash A, Vale P. Data and code for “The immune regulation and epidemiological consequences of specific immune priming in Drosophila.” Zenodo; 2023. doi:10.5281/zenodo.7624085

57. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods. 2001;25: 402–408. doi:10.1006/meth.2001.1262

58. Brownlie JC, Johnson KN. Symbiont-mediated protection in insect hosts. Trends Microbiol. 2009;17: 348–354. doi:10.1016/j.tim.2009.05.005

59. Teixeira L, Ferreira Á, Ashburner M. The Bacterial Symbiont Wolbachia Induces Resistance to RNA Viral Infections in Drosophila melanogaster. PLOS Biol. 2008;6: e1000002. doi:10.1371/journal.pbio.1000002

60. Gupta V, Vasanthakrishnan RB, Siva-Jothy J, Monteith KM, Brown SP, Vale PF. The route of infection determines Wolbachia antibacterial protection in Drosophila. Proc R Soc B Biol Sci. 2017;284: 20170809. doi:10.1098/rspb.2017.0809

61. Khan I, Prakash A, Agashe D. Pathogen susceptibility and fitness costs explain variation in immune priming across natural populations of flour beetles. Cotter S, editor. J Anim Ecol. 2019;88: 1332–1342. doi:10.1111/1365-2656.13030

62. Stockmaier S, Stroeymeyt N, Shattuck EC, Hawley DM, Meyers LA, Bolnick DI. Infectious diseases and social distancing in nature. Science. 2021;371: eabc8881. doi:10.1126/science.abc8881

63. Craft ME. Infectious disease transmission and contact networks in wildlife and livestock. Philos Trans R Soc B Biol Sci. 2015;370: 20140107. doi:10.1098/rstb.2014.0107

64. Lopes PC, Block P, König B. Infection-induced behavioural changes reduce connectivity and the potential for disease spread in wild mice contact networks. Sci Rep. 2016;6: 31790. doi:10.1038/srep31790

65. Lopes PC, French SS, Woodhams DC, Binning SA. Sickness behaviors across vertebrate taxa: proximate and ultimate mechanisms. J Exp Biol. 2021;224: jeb225847. doi:10.1242/jeb.225847

66. Duneau DF, Kondolf HC, Im JH, Ortiz GA, Chow C, Fox MA, et al. The Toll pathway underlies host sexual dimorphism in resistance to both Gram-negative and Gram-positive bacteria in mated Drosophila. BMC Biol. 2017;15: 124. doi:10.1186/s12915-017-0466-3

67. Valanne S, Vesala L, Maasdorp MK, Salminen TS, Rämet M. The Drosophila Toll Pathway in Innate Immunity: from the Core Pathway toward Effector Functions. J Immunol. 2022;209: 1817–1825. doi:10.4049/jimmunol.2200476

68. Zaidman-Rémy A, Hervé M, Poidevin M, Pili-Floury S, Kim M-S, Blanot D, et al. The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection. Immunity. 2006;24: 463–473. doi:10.1016/j.immuni.2006.02.012

69. Myllymäki H, Valanne S, Rämet M. The Drosophila Imd Signaling Pathway. J Immunol. 2014;192: 3455–3462. doi:10.4049/jimmunol.1303309

70. Lemaitre B, Reichhart J-M, Hoffmann JA. Drosophila host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc Natl Acad Sci. 1997;94: 14614–14619. doi:10.1073/pnas.94.26.14614

71. Belmonte RL, Corbally M-K, Duneau DF, Regan JC. Sexual Dimorphisms in Innate Immunity and Responses to Infection in Drosophila melanogaster. Front Immunol. 2020;10: 3075. doi:10.3389/fimmu.2019.03075

72. Klein SL, Flanagan KL. Sex differences in immune responses. Nat Rev Immunol. 2016;16: 626–638. doi:10.1038/nri.2016.90

73. Simon V. Wanted: women in clinical trials. Science. 2005;308: 1517–1518. Available: https://go.gale.com/ps/i.do?p=AONE&sw=w&issn=00368075&v=2.1&it=r&id=GALE%7CA133569531&sid=googleScholar&linkaccess=abs

74. Camus MF, Piper MD, Reuter M. Sex-specific transcriptomic responses to changes in the nutritional environment. Keller L, Eisen MB, Zajitschek F, editors. eLife. 2019;8: e47262. doi:10.7554/eLife.47262

75. Martínez BA, Hoyle RG, Yeudall S, Granade ME, Harris TE, Castle JD, et al. Innate immune signaling in Drosophila shifts anabolic lipid metabolism from triglyceride storage to phospholipid synthesis to support immune function. Hietakangas V, editor. PLOS Genet. 2020;16: e1009192. doi:10.1371/journal.pgen.1009192

76. Hudson AL, Moatt JP, Vale PF. Terminal investment strategies following infection are dependent on diet. J Evol Biol. 2020;33: 309–317. doi:10.1111/jeb.13566

77. Contreras-Garduño J, Rodríguez MC, Rodríguez MH, Alvarado-Delgado A, Lanz-Mendoza H. Cost of immune priming within generations: trade-off between infection and reproduction. Microbes Infect. 2014;16: 261–267. doi:10.1016/j.micinf.2013.11.010

78. Trauer U, Hilker M. Parental Legacy in Insects: Variation of Transgenerational Immune Priming during Offspring Development. PLOS ONE. 2013;8: e63392. doi:10.1371/journal.pone.0063392

79. Zanchi C, Troussard J-P, Martinaud G, Moreau J, Moret Y. Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect. J Anim Ecol. 2011;80: 1174–1183. doi:10.1111/j.1365-2656.2011.01872.x

80. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. Wolbachia and Virus Protection in Insects. Science. 2008;322: 702–702. doi:10.1126/science.1162418

81. Martinez J, Longdon B, Bauer S, Chan Y-S, Miller WJ, Bourtzis K, et al. Symbionts Commonly Provide Broad Spectrum Resistance to Viruses in Insects: A Comparative Analysis of Wolbachia Strains. PLOS Pathog. 2014;10: e1004369. doi:10.1371/journal.ppat.1004369

82. Rottschaefer SM, Lazzaro BP. No Effect of Wolbachia on Resistance to Intracellular Infection by Pathogenic Bacteria in Drosophila melanogaster. PLOS ONE. 2012;7: e40500. doi:10.1371/journal.pone.0040500

83. Rancès E, Ye YH, Woolfit M, McGraw EA, O'Neill SL. The Relative Importance of Innate Immune Priming in Wolbachia-Mediated Dengue Interference. PLOS Pathog. 2012;8: e1002548. doi:10.1371/journal.ppat.1002548

84. Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, Jiggins FM, et al. Wolbachia Variants Induce Differential Protection to Viruses in Drosophila melanogaster: A Phenotypic and Phylogenomic Analysis. PLOS Genet. 2013;9: e1003896. doi:10.1371/journal.pgen.1003896

85. Prigot-Maurice C, Beltran-Bech S, Braquart-Varnier C. Why and how do protective symbionts impact immune priming with pathogens in invertebrates? Dev Comp Immunol. 2022;126: 104245. doi:10.1016/j.dci.2021.104245