Layers of immunity: Deconstructing the *Drosophila* effector response

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Abstract

The host innate immune response relies on the cooperation of multiple defence modules. In insects and other arthropods, which have only innate immune mechanisms, four main immune-specific modules have been described in the defence against microbial invaders: the Toll pathway, the Imd pathway, the melanization response, and phagocytosis by plasmatocytes. Our present understanding of their relative importance remains fragmented as their contribution to host defence has never been simultaneously assessed across a large panel of pathogens. Here, we have taken advantage of newly-described immune mutants in a controlled genetic background to systematically delete these four immune modules individually, in pairs, or even all four simultaneously. Surprisingly, immune deficient flies simultaneously deficient in all four immune modules are viable, homozygous fertile, and display no overt morphological defects, suggesting these immune mechanisms are not strictly required for organismal development. With this new set of lines, we assessed the contribution of each module individually and collectively to host defense against five viruses, three fungi, eight Gram-positive bacteria, and eight Gram-negative bacteria. Our findings show that these four modules largely function independently and additively in host defense, although synergistic effects can occur for select pairs of modules. Our study confirmed the importance of the Imd pathway against Gram-negative bacteria and the Toll pathway against Gram-positive bacteria and fungi, largely via the induction of effectors such as antimicrobial peptides (AMPs) and Bomanins (Bom), but also reveals the important role of melanization against viruses, and the contribution of phagocytosis against various germs. Additionally, by examining microbial load kinetics in different mutants, we provide insight into how these modules contribute to tolerance or resistance against specific microbes. Our study provides insights into the architecture of the *Drosophila* immune system, revealing differential requirements of immune modules according to each pathogen. The set of immune deficient lines provided here offer tools to better assess the role of these immune modules in host defense.

Keywords: Drosophila, innate immunity, effectors, phagocytosis, melanization, Imd, Toll

Introduction

The immune system is made up of a network of tissues and circulating cells whose primary function is to prevent and limit pathogenic infections (Danilova, 2006; Pradeu et al., 2024; Schmid-Hempel, 2021). Although immunity has been studied in many animal species, it has been extensively characterized in only a subset of them such as the nematode *Caenorhabditis elegans,* the fruit fly *Drosophila melanogaster*, zebrafish, rodents, and humans. The traditional understanding of the immune system pictures it as a specialized group of cells and tissues, but this has gradually changed over recent years to a more complex picture of highly redundant multilevel defense modules. We have learned a great deal in the last few decades on the molecular mechanisms regulating each of these immune modules from recognition and signaling to effector action (ex. phagocytosis, antimicrobial peptides) (Pradeu et al., 2024). However, an immune response encompasses the concomitant activation of several modules and there has been little investigation of how each module collectively contributes to host defense. Indeed, the immune research field has traditionally focused on one module at a time. This article aims to fill this gap by collectively analyzing the specific contribution of the main immune modules involved in the *Drosophila* systemic response.

The *Drosophila* systemic immune response consists of a set of humoral and cellular reactions in the hemolymph (insect blood) when flies are systemically infected by bacteria, viruses, or fungi (Liegeois and Ferrandon, 2022; Westlake et al., 2024). A first facet of the systemic immune response is the production of host-defense effectors by the fat body and hemocytes that are secreted into the hemolymph. Among the best-characterized effectors are the antimicrobial peptides (AMPs), whose expression is induced to incredibly high levels upon infection in a race to control invading microbes (Hanson et al., 2019; Imler and Bulet, 2005). AMPs are small cationic peptides that exhibit antimicrobial activity that directly participate in microbial killing. Beyond AMPs, many other proteins and host defense peptides are secreted into the hemolymph, creating a hostile environment for invading pathogens. The systemic antimicrobial response is predominantly regulated at the transcriptional level by the Toll and Imd pathways (De Gregorio et al., 2002; Lemaitre et al., 1996). In *Drosophila*, the Toll pathway is activated by cell wall components (fungal glucans and Lysine-type peptidoglycan), as well as microbial proteases (Gottar et al., 2006; Issa et al., 2018; Leulier et al., 2003; Vaz et al., 2019). Toll signaling culminates in the activation of two NF-κB transcription factors, Dif and Dorsal, which regulate a large set of immune genes (Ip, 1993; Lemaitre et al., 1996). These encode small peptides such as the antifungal peptide Drosomycin, the Toll-regulated Bomanin peptides, and many other proteins (*e.g.* serine proteases, serpins, lipases) (Clemmons et al., 2015; De Gregorio et al., 2002; Fehlbaum et al., 1994; Ligoxygakis et al., 2003). Flies lacking the Toll pathway are viable but highly susceptible to infection by Gram-positive bacteria and fungi (Buchon et al., 2009; Lemaitre et al., 1996; Rutschmann et al., 2002). The Imd pathway is activated by DAP-type peptidoglycan produced by Gram-negative bacteria and a subset of Gram-positive bacteria (*e.g. Bacillus*) (Kaneko et al., 2004; Lemaitre et al., 1995; Leulier et al., 2003). The binding of peptidoglycan to receptors of the Peptidoglycan Recognition Protein family (PGRP-SD, PGRP-LC, PGRP-LE) initiates an intracellular signaling cascade, which ultimately activates the NF-κB factor Relish (Choe et al., 2005; Iatsenko et al., 2016; Kaneko et al., 2006; Kaneko et al., 2006; Westlake et al., 2024). The Imd pathway regulates the expression of many genes encoding antibacterial peptides, and serine proteases (De Gregorio et al., 2002; Lemaitre et al., 1997). Imd deficient flies are viable but susceptible to Gram-negative bacterial infection.

In addition to humoral pathways, melanization, and phagocytosis are two complementary mechanisms that contribute to host survival upon systemic infection. Melanization is an arthropod-specific immune response resulting in the rapid deposition of the black pigment melanin at wound or infection sites and the concomitant production of microbicidal reactive oxygen species (Cerenius et al., 2008; Nappi et al., 2009; Westlake et al., 2024). The melanization and Toll pathways are co-activated at the level of the serine proteases Hayan and Persephone, which in turn activate Spatzle-processing

enzyme (SPE) of the Toll pathway and serine proteases critical to cleavage of prophenoloxidases (PPOs) (Dudzic et al., 2019; Nakano et al., 2023; Shan et al., 2023), collectively referred to as the Toll-PO SP cascade (Westlake et al., 2024). The melanization reaction itself relies on the oxidation of phenols, resulting in the polymerization of melanin. Two PPOs (PPO1 and PPO2) are the primary catalysts for the synthesis of melanin during the melanization response upon systemic infection. Flies deficient for both *PPO1* and *PPO2* are viable, but lack hemolymph phenoloxidase activity and exhibit susceptibility to certain Gram-positive bacteria and fungi (Binggeli et al., 2014; Dudzic et al., 2015). In addition, a third PPO gene (PPO3) is specifically expressed by lamellocytes, specialized hemocytes that differentiate in larvae responding to and enveloping invading parasites (Dudzic et al., 2015).

Phagocytosis by both sessile and circulating plasmatocytes (*Drosophila* equivalent of macrophages) is thought to provide a complementary and important host defense (Melcarne et al., 2019; Ulvila et al., 2011). The use of hemocyte-deficient flies, via the targeted expression of a pro-apoptotic gene in plasmatocytes only, has shown that hemocytes contribute to survival upon systemic infection to certain bacterial species (Charroux and Royet, 2009; Defaye et al., 2009; Stephenson et al., 2022). Phagocytosis of bacteria greatly relies on two transmembrane receptors, NimC1 and Eater (Bretscher et al., 2015; Kocks et al., 2005; Kurucz et al., 2007; Melcarne et al., 2019). *NimC1, Eater* double mutant larvae are viable, have elevated hemocyte numbers at the larval stage, and their hemocytes can encapsulate and melanize macroparasites; however, their hemocytes are non-sessile and nearly totally phagocytosis deficient. *NimC1, Eater* adult flies have decreased hemocyte number and fail to perform phagocytosis, providing a good tool to assess the role of cellular response (Melcarne et al., 2019; Melcarne, 2020).

We have circumstantial evidence that these four immune modules are critical for defense against certain pathogens. However, how, when, and to what extent each of these four immune modules contribute to host defense against specific pathogens is fragmented as they have never been simultaneously assessed. Moreover, the extent to which these findings can be generalized to broad classes of pathogens remains unclear.

In this article, we have used a specific set of mutations principally affecting phagocytosis *(NimC1, Eater),* melanization *(PPO1, PPO2),* the Toll pathway *(spz),* and the Imd pathway *(Rel)* in a controlled genetic background to address their role in host defense. We then recombined these and other mutations to generate double and even quadruple mutants of the four immune modules. This set of tools allowed us to compare the survival of flies deficient in these responses against a broad panel of viruses, fungi, Gram-positive bacteria, and Gram-negative bacteria, to reveal the relative contribution of each immune mechanism to resistance against these pathogens.

Material and methods

Insect stocks

Precise details of *Drosophila* stocks used in this study are provided in **Table 1**. To minimize the influence of genetic background for the mutations used in this study, mutations were partially isogenized into the *DrosDel iso* w^{1118} genetic background (BDSC #5905) as described by Ferreira et al. (Ferreira et al., 2014; Ryder et al., 2004). Isogenized lines were generated and used for every mutant except the double mutant Toll and Imd *(*Rel^{E20}*,* spz^{rm7}*)* for which homozygous isogenized flies were not viable in our hands. Double mutants for $NimC1^{1}$*;* $Eater^{1}$ were poorly viable when isogenized in the *DrosDel iso* w^{1118} background, and so we used both isogenic and non-isogenic lines over the course of our study to facilitate investigation. Furthermore, the $NimC1^{1}$*;* spz^{rm7}*,* $Eater^{1}$ triple mutants were homozygous lethal isogenized or not, and so could not be included. In addition, the line Δ*AMP14* bears a knockout of 14 different AMP genes, which includes the AMP families Drosomycin, Metchnikowin,

Cecropin, Defensin, Drosocin, Attacin, and Diptericin, described previously (Carboni et al., 2022; Hanson et al., 2019). The *Bom*$^{\Delta 55C}$ mutation was characterized in Clemmons et al. (Clemmons et al., 2015) and isogenic flies generated in Hanson et al. (Hanson et al., 2019). For larval experiments, use of GFP-labelled CyO or TM6,Tb balancers were used to enable picking of homozygous mutant larvae.

Of note, the strains used in this study differ in their presence/absence of the *white*$^{+}$ gene, present in the *PPO1*$^{\Delta}$, *NimC1*^{1} and *eater*^{1} mutations. In addition to its well established function in eye pigmentation, the *white* gene can also impact host neurology and intestinal stem cell proliferation (Ferreiro et al., 2017; Sasaki et al., 2021). We did not observe any obvious correlations between *white*$^{+}$ gene status and susceptibilities in this study. Moreover, in a previous study looking at the cumulative effects of AMP mutations on lifespan, *white* gene status and fluorescent markers did not readily explain differences in longevity (Hanson and Lemaitre, 2023). We therefore believe that the extreme immune susceptibility we have created through deficiencies for pathways regulating hundreds of genes, or major immune modules, overwhelms the potential effects of *white*$^{+}$ and other transgenic markers. For additional information on which stocks bear which markers, see discussion in Supplementary file 1.

Microorganisms culture

Microbe strain information, microorganism classifications, and culture conditions are listed in **Table S1**. Bacterial cultures and *C. albicans* were grown overnight shaking at 200rpm. *Aspergillus fumigatus* fungus was grown on Malt Agar at room temperature until sporulation. The entomopathogenic fungus *Beauveria bassiana* strain R444 was provided by Andermatt AG as spore preparations (BB-PROTEC) which were directly used in natural infections or dissolved in PBS for septic injuries. Viruses DCV and FHV were kindly provided by Carla Saleh (Pasteur Institute, Paris), produced in S2 cells. The supernatant of S2 cells was titrated before being used for fly infections. Viruses SINV, DXV and IIV-6 were kindly provided by Ronald van Rij (Radboud University Medical Center, Nijmegen).

Heat-killed microbes were prepared by two repeats of boiling at 95°C for 30 minutes then freezing at -20°C for 30 minutes, before storage long term at -20°C. Microbe preparations were streaked onto agar plates to check for full efficiency of heat-killing before use in experiments.

Gene expression

Flies were inoculated by pricking in the junction of thoracic pleura with a needle dipped in a mixed pellet containing a 1:1 mixture of OD600 = 200 heat-killed *E. coli* and *M. luteus* (final OD600 = 100 for each), and frozen at -20˚C 6h, 12h and 24h post-infection. Total RNA was then extracted from pooled samples of five flies using TRIzol reagent per manufacturer's protocol and resuspended in MilliQ dH2O. Reverse transcription was performed using PrimeScript RT (Takara) with random hexamer and oligo dT primers. Quantitative PCR was performed on a LightCycler 480 (Roche) in 96-well plates using Applied Biosystems PowerUP SYBR green Master Mix (Applied Biosystems). Data points represent pooled samples from three replicate experiments. Error bars represent one standard deviation from the mean. Statistical analyses were performed using one-way ANOVA with Holms-Sidak multiple test correction. Primers used in this study were:

Drs-F 5'- CGTGAGAACCTTTTCCAATATGAT-3'
Drs-R 5'- TCCCAGGACCACCAGCAT-3'
DptA-F: 5'-GCTGCGCAATCGCTTCTACT-3'
DptA-R: 5'-TGGTGGAGTGGGCTTCATG-3'
RPL32-F: 5'-GACGCTTCAAGGGACAGTATCTG-3'
RPL32-R: 5'-AAACGCGGTTCTGCATGAG-3'

186 **Ex vivo larval hemocyte phagocytosis assays**

187 Ex vivo phagocytosis assays were performed using a mix of equal volumes of *E. coli* and *S. aureus*
188 AlexaFluorTM488 BioParticlesTM (Invitrogen), following manufacturer's instructions; and see
189 (Melcarne et al., 2019). Five L3 wandering larvae from our newly generated mutant lines, or carrying
190 the *Hml-Gal4, UAS-GFP* hemocytes marker as a control, were bled into 150µL of Schneider's insect
191 medium (Sigma-Aldrich) containing 1µM phenylthiourea (PTU; Sigma-Aldrich). The hemocyte
192 suspension was then transferred to 1.5 mL low binding tubes (Eppendorf, SigmaAldrich), and
193 AlexaFluorTM488 bacteria BioParticlesTM were added. The samples were vortexed, incubated at room
194 temperature for 2 hours to allow phagocytosis and then placed on ice to stop the reaction. The
195 fluorescence of extracellular particles was quenched by adding 0.4% trypan blue (Sigma-Aldrich)
196 diluted to 1/3 concentration. Phagocytosis was quantified using a flow cytometer (BD Accuri C6) to
197 measure the fraction of cells phagocytosing, and their fluorescence intensity. Isogenic wild-type iso
198 w^{1118} larvae and *Hml-Gal4, UAS-GFP* larvae with or without bacterial particles were used to define the
199 gates for hemocyte counting and the thresholds for phagocytosed particle emission. The phagocytic
200 index was calculated as follows:

$$\text{Fraction of haemocytes phagocytosing(f)} = \frac{[\text{number of haemocytes in fluorescence positive gate}]}{[\text{total number of haemocytes}]}$$

$$\text{Phagocyticindex}(PI) = [\text{Mean fluorescence intensity of haemocytes in fluorescence positive gate}] \times \text{f}$$

201

202 Finally, due to experimenter and experiment batch differences in total hemocytes collected and knock-
203 on effects to calculating phagocytic index, phagocytic index was normalised with the wild-type as a
204 reference set to 100% within experiment batch.

205 **Wounding experiment**

206 Clean injury was performed with a needle sterilized with an EtOH and PBS wash. For imaging of the
207 melanization reaction upon pricking, the thorax of 3-8 days old flies was pricked using a sterile needle
208 (diameter ~0.1mm). Pictures were taken 24h post-pricking and categorized into normal, weak, or no
209 melanization blackening seen at the injury site per Dudzic et al. (Dudzic et al., 2019).
210

211 **Systemic infections and survival**

212 Systemic infections were performed by pricking 3- to 5-day-old adult males in the thorax with a 0.1mm-
213 thick needle dipped into a concentrated pellet of bacteria, yeast, or fungal spores. For natural infections
214 with *A. fumigatus,* flies were anesthetized and then shaken on a sporulating plate of fungi for 30 seconds.
215 For *B. bassiana*, flies were shaken for 30s in a tube containing an excess of commercial spores sufficient
216 to coat all flies uniformly, of which the flies remove the vast majority later through grooming. At least
217 two replicate survival experiments were performed for each infection, though in specific cases some
218 genotypes may have been included in only one experiment. 20-35 flies were included per vial when
219 possible, kept on cornmeal fly media containing (per 1L): 6.2g agar, 58.8g cornmeal, 58.8g yeast, 60mL
220 grape fruit juice, 4.83mL propionic acid, and 26.5mL 100g/L moldex in pure ethanol. Survivals were
221 scored daily, and flies were flipped to new food vials 3 times per week. Due to challenges working with
222 genotypes of different viability, we could not include all genotypes in all experimental replicates.
223 Moreover, comparisons across genotypes required multiple levels of consideration. Given the
224 complexity of genotype-vs-reference-by-pathogen comparisons in our study, needing to compare
225 infections both to clean injury within genotype, and to wild-type or *ΔITPM* across treatments, we chose
226 to focus on major differences apparent in summary statistics, highlighting survival differences only

227 when they were: **i)** consistent across experimental replicates; **ii)** of a consistent logic across comparable
228 genotypes; for instance, compound mutants containing *ΔMel* (e.g. *ΔIMD*, *ΔMel*) should be as or more
229 susceptible to infections as *ΔMel* alone if melanization is truly critical for defence; and **iii)** of a mean
230 lifespan difference ≥1.0 days after accounting for comparisons with unchallenged or clean-injury data.
231 Total experiments (N exp) and total flies per genotype are reported to provide summary statistics per
232 pathogen-by-genotype interaction. The mean lifespan reported in survival summary data has a maximum
233 of 7 or 10 days depending on experimental conditions. Kaplan-Meier survival curves for all experiments
234 are provided in the main text or supplementary information.
235
236 **Quantification of microbial load for growth kinetics**
237 3-8 days old flies were infected with the indicated microbe and concentration per OD600 as described
238 in **Table S1**, and allowed to recover. At the indicated time post-infection, flies were anesthetized using
239 CO2 and surface sterilized by washing them in 70% ethanol. Ethanol was removed, and then flies were
240 homogenized using a Precellys bead beater at 6500 rpm for 30 s in LB broth (*Pr. rettgeri*), BHI (*S.*
241 *aureus*), or YPG (*C. albicans*) with 500 μL as pools of 5 flies. These homogenates were serially diluted
242 and 100μL was plated on LB, BHI or YPG agar. Plates were incubated overnight, and colony-forming
243 units (CFUs) were counted using a Interscience Scan 500 plate scanning colony counter, and validated
244 independently by manual counts for a subset of plates to ensure accuracy. Statistical differences were
245 tested using Brown-Forsythe and Welch ANOVA tests with Dunnett's multiple test correction, which
246 consider unequal variance.
247
248 # Results
249 **A set of immune-deficient lines in the iso Drosdel background**
250 Mutations affecting only one of the four main modules of the *Drosophila* systemic immune
251 response have been described. These include $Relish^{E20}$ (Rel^{E20}, deficient for the Imd pathway, referred
252 to as Δ*IMD*), $spatzle^{rm7}$ (spz^{rm7}, deficient for the Toll pathway, referred to as Δ*TOLL*), $PPO1^{\Delta}$, $PPO2^{\Delta}$
253 (no melanization, referred to as Δ*Mel*), and $NimC1^{1}$; $eater^{1}$ (reduced phagocytosis, referred to as
254 Δ*Phag*). In addition, to blocking phagocytosis, Δ*Phag* flies have a defect in hemocyte sessility, an
255 increased total number of larval hemocytes, and a decreased number of hemocytes at the adult stage
256 (Bretscher et al., 2015; Melcarne et al., 2019; Melcarne, 2020). We have preferred to use $NimC1^{1}$; $eater^{1}$
257 to study the contribution of hemocytes rather than other approaches with important indirect effects such
258 as the pre-injection of latex beads to saturate phagocytes (Elrod-Erickson et al., 2000), or expression of
259 a pro-apoptotic gene in plasmatocytes (Charroux and Royet, 2009; Defaye et al., 2009). All the
260 mutations in this study were partly isogenized for seven or more generations into the DrosDel *iso* w^{1118}
261 genetic background (Ferreira et al., 2014; Ryder et al., 2004). We then generated by recombination six
262 out of the seven combinations of flies simultaneously lacking two immune modules with the exception
263 of *ΔTOLL*, *ΔPhag* as $NimC1^{1}$; $eater^{1}$, spz^{rm7} flies were not viable (see fly strains in **Table 1**). Of note,
264 in our hands recombinant Rel^{E20}, spz^{rm7} flies were not viable in the DrosDel isogenic background forcing
265 us to use non-iso flies to assess the host defense of *ΔIMD*, *ΔTOLL* flies. Over the course of our study,
266 isogenic $NimC1^{1}$;$eater^{1}$ flies also displayed viability issues, and specific experiments used non-isogenic
267 flies to improve confidence in phenotypes; these experiments are noted in figure captions. We used two
268 approaches to simultaneously delete the Toll and Melanization modules: **i)** $PPO1^{\Delta}$, $PPO2^{\Delta}$; spz^{rm7} flies
269 (referred to as *ΔTOLL*, ΔMel^{PPS}) and **ii)** flies carrying a small viable deletion $Hayan\text{-}psh^{Def}$ (referred to
270 as *ΔTOLL*, ΔMel^{HP}) that removes two clustered serine protease genes, *Hayan,* and *persephone,* which
271 blocks the TOLL-PO SP cascade that regulates both humoral-Toll and melanization (Dudzic et al., 2019;
272 Westlake et al., 2024). This allowed us to delete the Toll and melanization pathways using only one
273 deletion event on the *X* chromosome. Excitingly, we succeeded in generating a fly line deficient for all

four immune modules with the genotype: *Hayan-psh^{Def}; NimC1^{1}; eater^{1}, Rel^{E20}* hereafter named *ΔITPM (ΔIMD, ΔTOLL, ΔPhag, ΔMel).* These flies were homozygous viable with very poor viability, and homozygous fertile, despite major deficiencies in these four important systemic immune mechanisms. These flies allow us to monitor survival kinetics and pathogen growth in the near-total absence of an immune system. Finally, we added two iso fly lines in our analysis that delete key host defense peptides downstream of the Toll and Imd pathways: **i)** *Bom$^{\Delta 55C}$*, a short deletion removing ten Bomanins at position 55C, which causes a major susceptibility to infection by Gram-positive bacteria and fungi (Clemmons et al., 2015), and **ii)** Δ*AMP14*, a compound fly line of eight mutations removing 14 AMP genes (4 Cecropins, 4 Attacins, 2 Diptericins, Drosocin, Defensin, Drosomycin, and Metchnikowin), which causes a major susceptibility to infection by Gram-negative bacteria (Carboni et al., 2022; Hanson et al., 2019). This set of immune-deficient fly lines in a defined genetic background provides a unique tool kit to address the function of immune modules individually or collectively.

Imd, Toll, Phagocytosis, and Melanization modules function largely independently

The Imd, Toll, Phagocytosis, and Melanization modules are interconnected (Westlake et al., 2024). For instance, the Toll and Imd pathways both regulate a subset of genes involved in melanization (De Gregorio et al., 2002; Ligoxygakis et al., 2002). Moreover, one module can indirectly affect another. For instance, a lack of melanization could increase the bacterial load after infection, resulting in higher activation of the Toll and Imd pathways (Binggeli et al., 2014). In the first step, we analyzed the activation of each of the four modules in single- and multiple-module deficient flies using classical readouts of these modules. This also allowed monitoring of the possible effects of deleting one module on the activity of another. We analyzed the immune response in single module deficient flies by monitoring **i)** the expression of the antibacterial peptide gene *Diptericin* (*DptA* - a target of the Imd pathway), and **ii)** the antifungal peptide gene *Drosomycin* (*Drs* - a target of the Toll pathway) upon systemic infection with a mixture of heat-killed *Escherichia coli* and *Micrococcus luteus*, **iii)** ex vivo phagocytosis of *E. coli* and S*taphylococcus aureus* coated bio-particles incubated with larval hemocytes, and **iv)** melanization at the injury site of adult flies. This analysis first confirmed that Δ*IMD*, Δ*TOLL*, Δ*Phag,* and Δ*Mel* flies are deficient for Imd, Toll, phagocytosis, and melanization respectively (**Figure 1A, B, C, D**). We note that the expression level of *DptA* was roughly similar to the wild-type in Δ*TOLL* and Δ*Mel* flies, however, *ΔPhag* flies show reduced *DptA* induction at 6h compared to WT (~50%) (**Figure 1A**). This still represents an immense induction of *DptA* after infection, but may suggest a lag or lesser overall Imd response in *ΔPhag* flies. *DptA* expression in *ΔPhag* was, however, comparable to wild-type at 12h and 24h. On the other hand, the *Drs* induction at 6h of *ΔMel* flies was significantly higher than WT (~1.6x), as found previously using live *M. luteus* infection (Binggeli et al., 2014) (**Figure 1B**). The phagocytic assay confirmed that the Δ*Phag* mutant was significantly impaired in its ability to phagocytose bacterial bio-particles. Furthermore, Δ*IMD,* and Δ*Mel* mutant larvae had similar levels of phagocytic index to the wild-type (**Figure 1C**), while Δ*TOLL* had a higher phagocytic index, if anything. As expected, there was no apparent melanization at the site of the injury in Δ*Mel* flies while melanization blackening was readily observed in the wild-type and in other single-module mutants (**Figure 1D**). Collectively, this analysis indicates that each of the four immune modules can be selectively blocked in the isogenic DrosDel background without interfering with the competence of the others. We sometimes noticed a modest difference of module activity in the absence of another (higher Toll activity in Δ*Mel,* lower IMD activity in Δ*Phag* and higher phagocytic activity in Δ*TOLL*), findings that require further exploration to be fully established.

We then repeated our analysis using our set of module compound mutants. Not surprisingly, all compound flies display the expected immune deficiencies validating the lines we constructed. This analysis confirms that *ΔITPM* flies, although viable, are indeed fully immune deficient for the four host defense modules. We did not detect any striking synergistic or antagonistic effects in which deleting

322 two modules has a vastly different impact compared to the additive effect of deleting the two modules
323 individually. Notably, a small level of *Drs* expression is retained in Δ*TOLL* mutant flies, but was fully
324 abolished in Δ*IMD,* Δ*TOLL* flies **(Figure 1A vs 1E)**, confirming the *Drs* gene receives a small input
325 from the Imd pathway (Leulier et al., 2000). The Δ*TOLL,* ΔMel^{PPS} and, Δ*TOLL,* ΔMel^{HP} flies display
326 *Drs* induction at 6h that is resolved to unchallenged WT levels by 12h, consistent with this induction
327 coming from an early input from the IMD pathway in these backgrounds. Taken together, all the
328 compound mutant flies behave largely as expected given the sum effects of their individual mutations,
329 confirming that these modules largely function independently.
330
331 **Both the Melanization and TOLL modules contribute to wounding and lifespan**
332 Before assessing the resistance to infection, we monitored fly survival at 25°C and 29°C to
333 determine if these mutations impact viability in the absence of challenge. At 25°C, no single mutants
334 died significantly more than the wild-type in the absence of challenge within the ten-day window
335 (**Figure 2A**). However, looking at compound mutants, even in the absence of challenge, each compound
336 mutant other than Δ*IMD, ΔTOLL* suffered 25% cumulative mortality by 10 days post-eclosion, while
337 Δ*TOLL, ΔMel*^{PPS} and Δ*ITPM* flies suffered even greater cumulative mortality. Similar trends were
338 observed at 29°C with slightly elevated mortality (**Figure 2supp1**). The observation that *ΔTOLL,*
339 *ΔMel*^{PPS} (*PPO1*^{1}*,PPO2*^{1}*, spz*^{rm7}) flies have shorter lifespan compared to *ΔTOLL*, *ΔMel*^{HP} (*Hayan-psh*^{Def})
340 could be explained by residual Toll and/or PO activity in *Hayan-psh*^{Def} or additional indirect effects in
341 *ΔTOLL, ΔMel*^{PPS} flies. As such, the combined effect of Toll and Melanization deficiencies in
342 unchallenged and clean injury survival is best assessed by considering signals from both genotypes.
343 *ΔITPM* flies (*Hayan-psh*^{Def}*; NimC1*^{1}*; Eater*^{1}*, Rel*^{E20}) have the lowest unchallenged survival rate of
344 genotypes included in this study. Taken together, the Imd, Toll, Melanization, and Phagocytosis
345 modules contribute little to lifespan maintenance individually, but are crucial to maintain lifespan
346 collectively (**Figure 2B**).
347 Systemic infections were performed by septic injury (pricked with a contaminated needle). We
348 therefore monitored the survival of single and double mutant flies after a clean injury to disentangle the
349 effects of injury from those of infection. We found a cumulative mortality of ~50% for Δ*Mel* flies by
350 day 10 (**Figure 2C**), while other single module mutants displayed relatively little mortality over the 10-
351 day window; *ΔIMD* had notable late-onset mortality in a minority of experimental replicates, which may
352 be due to stochastic dysbiosis effects during aging (Hanson and Lemaitre, 2023; Marra et al., 2021).
353 Double mutant *ΔIMD, ΔTOLL* and Δ*IMD, ΔPhag* flies retained a high survival rate. Consistent with an
354 importance of melanization in the injury response (Binggeli et al., 2014), all the compound mutants
355 affected in melanization were susceptible to clean injury, with similar mortality seen in *ΔPhag, ΔMel*,
356 *ΔIMD, ΔMel*, *ΔTOLL, ΔMel*^{PPS}, *ΔTOLL, ΔMel*^{HP}, and *ΔITPM* flies (**Figure 2D**). Thus, while these
357 genotypes differ in their base lifespan, their viability upon injury is remarkably similar. These results
358 emphasize an interconnectedness of the melanization response with each other immune module for
359 maintaining health after injury.
360 Similar results were obtained for both unchallenged and clean injury lifespans at 29°C. Of note,
361 *ΔTOLL*, *ΔMel*^{PPS} suffered significantly greater mortality in unchallenged conditions than other
362 genotypes, even including *ΔITPM* flies (**Figure 2supp1**). Other compound mutant genotypes displayed
363 a continuum of mortality in unchallenged conditions between *ΔITPM* (~50% cumulative mortality) and
364 *ΔTOLL, ΔMel*^{HP} (~15% cumulative mortality). Upon clean injury, results were broadly consistent with
365 25°C trends, with a more uniform mortality across all genotypes.
366 Collectively, our study highlights that all these immune modules contribute somewhat to the
367 survival of flies upon clean injury, with a major role of the melanization response in maintaining lifespan
368 and survival to injury. In the next steps of our study, we compared survival upon infection to both

369 unchallenged and clean injured flies to distinguish the impact of microbial infection from the injury
370 itself.
371
372 **Multiple mechanisms contribute to resistance to microbial infection**

373 To compare the contribution of each module to host defense, we performed infection
374 experiments of wild-type and single- and multiple-module mutant flies following septic
375 infection with five viruses, eight Gram-positive bacteria, eight Gram-negative bacteria, septic
376 injury for two fungal species, and natural infection (i.e. spore deposition on the cuticle) for two
377 fungal species. Temperature, dose, and days monitored are shown in **Figure 3**, and see **Table**
378 **S1** for other key parameters. Survival curves are shown in **Supplementary file 2** for each
379 pathogen and results of these survivals are summarized in **Figure 3** (and **Supplementary file**
380 **3**) as mean lifespans given 7 or 10 days as the maximum lifespan per treatment, with number
381 of tested flies indicated on the right and darker blue intensity as indicative of lower lifespan.

382 We performed infection experiments with the following viruses and microbial species: DCV,
383 FHV, DXV, IIV-6, SINV, *Aspergillus fumigatus*, *Beauveria bassiana*, *Candida albicans*,
384 *Mycobacterium marinum*, *Corynebacterium diphtheriae, Micrococcus luteus, Streptococcus*
385 *pneumoniae, Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes, Bacillus*
386 *subtilis, Vibrio parahemolyticus, Providencia rettgeri, Providencia burhodogranariea,*
387 *Pectobacterium carotavorum Ecc15, Klebsiella pneumoniae, Enterobacter cloacae,*
388 *Escherichia coli, and Salmonella enterica ser. typhimurium.* Collectively, this represents
389 infections with 24 pathogens across 14 genotypes and two infection routes (336 genotype-by-
390 pathogen interactions). When combined with comparisons to clean injury and unchallenged
391 controls to validate if a lifespan difference is meaningful, and to wild-type or *ΔITPM* treatments,
392 this inflates to 1000+ interactions. We therefore focused our survival analysis on major effects.
393 We report the core summary statistics and overt trends, and comment only where difference in
394 mean lifespan between reference genotypes or control treatments was a minimum of 1 day.

395 Our study confirms that the Imd pathway plays a major role against all tested Gram-negative
396 bacteria, as Δ*IMD* flies succumb more rapidly than wild-type flies to all of them. This pathway
397 also contributes, albeit to a lesser extent, to survival against the Gram-positive bacteria *B.*
398 *subtilis* and *L. monocytogenes,* which bear DAP-type peptidoglycan at their membrane that
399 triggers Imd activation (Kaneko et al., 2004; Leulier et al., 2003). We were interested to see
400 the impact of our Δ*IMD* flies in host defense to viruses as the specific mutation we used (Rel^{E20})
401 deletes a transcription factor common to both the canonical Imd pathway and the recently-
402 appreciated cGLR-Sting-Relish pathway (Goto et al., 2018). We found that Δ*IMD* mutant flies
403 were somewhat susceptible to the four viruses DCV, FHV, DXV, and IIV6, but not SINV,
404 agreeing with previous studies showing a reduction in lifespan of 1-2 days for *Rel* mutant flies
405 after viral infection (Costa et al., 2009; Goto et al., 2018; Sansone et al., 2015).

406 This study also confirms a prominent role of the Toll pathway in survival upon infection by all
407 virulent Gram-positive bacteria and fungi upon septic injury. Less virulent microbes and
408 infectious routes (such as *A. fumigatus* by natural infection) did not result in increased mortality
409 in individual *ΔTOLL* mutant flies. A contribution of the Toll pathway to survival was also

410 observed for some Gram-negative bacteria, including a minor contribution to survival upon *Pr.*
411 *rettgeri* infection, and a major contribution after *V. parahemolyticus* infection. In addition, we
412 found a susceptibility of ΔTOLL flies to FHV, DXV, and IIV6, suggesting Toll-regulated
413 effectors mediate defence against viral infection.

414 Use of *ΔPhag* mutants reveals a role of the cellular response in survival against the fungus *B.*
415 *bassiana*. *ΔPhag* flies further show a minor susceptibility to *S. aureus,* and a subset of Gram-
416 negative bacteria (*Pr. rettgeri*, *Ecc15,* and *E. coli*). Susceptibilities to Gram-negative bacteria
417 could result from the observed lag in Imd pathway activation (Figure 1A); indeed, previous
418 studies have shown a full *DptA* response is pivotal for defence against *Pr. rettgeri* (Hanson et
419 al., 2023, 2019; Unckless et al., 2016), and lifespans are greatly impacted by subtle differences
420 when using pathogens with intermediate levels of virulence (Duneau et al., 2017a). A role for
421 Imd in explaining *ΔPhag* susceptibilities does not detract from the importance of the cellular
422 response, but instead offers a mechanistic route to explore for these phenotypes.

423 Surprisingly, our study reveals a relatively consistent role of melanization against all the tested
424 viruses, notably so for DXV, IIV-6, and SINV. Δ*Mel* flies also display a minor increased
425 susceptibility to *B. bassiana* natural infection and septic injury. As previously described
426 (Dudzic et al., 2019), *ΔMel* flies are highly susceptible to the Gram-positive bacterium *S.*
427 *aureus*, rivaling the susceptibility of *ΔITPM* flies. *ΔMel* flies further show a strong
428 susceptibility to *Pr. rettgeri* rivalling *ΔIMD* flies, and also a minor susceptibility to infection
429 by other Gram-negative bacteria (*Pr. burhodogranariea, E. coli*).

430 Collectively, the use of single module mutants confirms the major role of the Imd pathway
431 against Gram-negative bacteria and Toll against virulent Gram-positive bacteria and fungi.
432 Interestingly, melanization was consistently important to survive virus infection, and plays a
433 prominent and important role in defense against specific bacterial species. While it was almost
434 never the most critical module, phagocytosis and the cellular response contributes to survival
435 against various germs. Use of single module deficient flies alongside *ΔITPM* flies reveals that
436 survival to some of the microbes tested can rely almost entirely on one pathway (Imd – *Ecc15,*
437 *K. pneumoniae, E. cloacae, Pr. burhodogranariea*; Toll – *E. faecalis*), or can receive notable
438 contributions from two pathways (Toll and IMD – *L. monocytogenes, B. subtilis, V.*
439 *parahemolyticus*; Toll and Phagocytosis – *A. fumigatus* and *B. bassiana* natural infection*;* Imd
440 and Melanization - DCV). Strikingly, many pathways contribute to host defense to *S. aureus*
441 and *Pr. rettgeri*.

442 **Contribution of AMPs and Bomanins to Toll and Imd activities**

443 Recent studies have described a prime importance of antimicrobial peptides (AMPs) and host
444 defense peptides (HDPs) in responding to infection (reviewed in Hanson (2024)). These
445 peptides are the principal effectors of the Toll and Imd pathways, yet mutations for AMPs and
446 HDPs typically have not been tested alongside mutations for both the Imd and the Toll pathways
447 across pathogens, as studies have typically included just the one relevant pathway mutation as
448 a positive control. Thus, the importance of AMPs and HDPs relative to the pathways that

449 regulate them has been only partially investigated. We used *ΔAMP14* and *ΔBom* flies to assess
450 the extent by which AMPs and Bomanins contribute to Toll and Imd activities (**Figure 3**). Here
451 we found that AMPs are critical for survival to infection against all Gram-negative bacteria
452 tested, often rivaling the susceptibility of *ΔIMD* flies. Notably, *ΔAMP14* flies were also
453 somewhat susceptible to the yeast *C. albicans,* and the Gram-positive bacteria *B. subtilis* and
454 *M. luteus*, reinforcing minor susceptibilities seen previously (Carboni et al., 2022; Hanson et
455 al., 2019). Interestingly, we found that *ΔAMP14* survivals to DCV, FHV, DXV, and IIV6,
456 largely paralleled those of *ΔIMD* flies (Figure 3, Supplemental File 1), suggesting Imd-
457 mediated survival phenotypes rely greatly on the presence of the seven classical AMP families.

458 On the other hand, *ΔBom* flies were susceptible to septic injury by *B. bassiana* and also
459 somewhat to *C. albicans* consistent with previous studies (**Supplementary file 2**), as well as
460 all virulent Gram-positive bacteria, consistent with their important role downstream of the Toll
461 pathway (Clemmons et al., 2015; Lindsay et al., 2018; Xu et al., 2023). In addition, *ΔBom* flies
462 paralleled the *ΔTOLL* susceptibility to DCV, FHV, and DXV, but were not susceptible to IIV6
463 like *ΔTOLL*.

464 Collectively, our study confirms the key role of antimicrobial peptides downstream of the Imd
465 pathway in the defense against Gram-negative bacteria, and a primary role of Bomanins
466 downstream of Toll in defense against fungi and Gram-positive bacteria, with little input to
467 survival against Gram-negative bacteria. We additionally find these secreted peptides can
468 contribute to survival after certain viral infections.

469 **Immune modules mostly contribute additively to host defense**

470 The results described above revealed the key contribution of single modules and immune
471 effectors to host defense. However, they did not assess the possible additive, synergistic, or
472 antagonistic contribution of these four modules. Moreover, they did not monitor to which extent
473 these modules were important in the absence of other modules. To address this, we compared
474 the survival of single-module deficient flies to double module-deficient flies lacking two
475 modules, and *ΔITPM* flies lacking all four modules (**Figure 3**).

476 We first observed that *ΔITPM* flies, which lack the four modules, were always as or more
477 susceptible than single and double module deficient flies. In multiple cases, single or double
478 mutants already rivalled the susceptibility of *ΔITPM* flies in microbe-specific ways. In
479 particular, the susceptibility of *ΔIMD*-flies to most Gram-negative bacteria rivalled *ΔITPM,* and
480 for some germs *ΔTOLL* (*L. monocytogenes*, *E. faecalis*, *B. bassiana* injury) or *ΔMel* (*S. aureus*)
481 alone were comparable to *ΔITPM*. In other cases, however, combined loss of two pathways was
482 necessary to rival *ΔITPM* susceptibilities. For instance, *ΔIMD, ΔTOLL* deletion causes
483 complete *ΔITPM*-like susceptibility to *B. subtilis*, *M. luteus*, and *E. coli*, and near-complete
484 susceptibility to *Sa. typhimurium*. *ΔIMD, ΔTOLL* further causes increased susceptibility to most
485 viruses compared to their individual mutant strains. In these cases, there is clear synergy or
486 additivity of the contributions of the two pathways, as either pathway alone results in only a
487 minor or no susceptibility. We also observed intriguing differences between our two versions

488 of *ΔTOLL, ΔMel* flies, as *ΔTOLL, ΔMel*^{PPS} tended to succumb to infection to a greater extent
489 than *ΔTOLL, ΔMel*^{HP}, consistent with the differences observed upon clean injury (**Figure 2**).
490 *ΔTOLL*, *ΔMel*^{PPS} displayed a minor increase in susceptibility compared to *ΔTOLL*, *ΔMel*^{HP} to
491 *A. fumigatus* natural infection, and more prominent susceptibilities to the yeast *C. albicans*, the
492 Gram-positive bacteria *M. marinum*, *M. luteus* and also to Gram-negative bacteria *K.*
493 *pneumoniae*, *E. coli*, *Pr. rettgeri* and *Pr. burhodogranariea*.

494 Collectively, use of double module deficient flies uncovers the role of immune modules to host
495 defense that were masked by other modules. While *ΔIMD* and *ΔTOLL* deficiencies sometimes
496 displayed a synergistic effect (i.e. little mortality of single mutants, but *ΔITPM*-like mortality
497 of double mutants), for combinations of other pathways the results were more often minor with
498 a cumulative effect on susceptibility, suggesting most modules contribute to defence
499 independently. The use of two variations of *ΔTOLL*, *ΔMel*, with *ΔTOLL*, *ΔMel*^{PPS} having a
500 stronger impact than *ΔTOLL*, *ΔMel*^{HP}, also highlights how the level of pathway disruption can
501 affect infection outcomes, particularly for certain fungal or bacterial pathogens like *Providencia*
502 species that may cleave host proteases (suggested by Duneau et al., 2017b; Issa et al., 2018).

503 **Timing of Toll, Imd, Phagocytosis, and Melanization contribution to host defense**

504 Host defense programs can rely on resistance mechanisms that directly target or limit growth
505 of pathogens (Howick and Lazzaro, 2017; Lafont et al., 2021; Medzhitov et al., 2012). When
506 effectors reach a critical concentration threshold that promotes resistance, pathogen growth is
507 inhibited; for instance, the time to pathogen control of *Pr. rettgeri* is ~7 hours, relying heavily
508 on the expression and production of DptA (Duneau et al., 2017a; Hanson et al., 2023).
509 Phagocytosis and the melanization response are often described as providing near-immediate
510 immune protection (Haine et al., 2008), with reactions ex vivo progressing within minutes.
511 Meanwhile activation of the Imd and Toll pathways takes hours to reach peak concentrations
512 of host defense peptides (Uttenweiler-Joseph et al., 1998). Thus, we were curious if different
513 mutations would display differences in their time of action to control microbial growth. We
514 therefore measured the microbial growth rate for *Pr. rettgeri, S. aureus,* and *C. albicans* at
515 different time points post-infection in wild-type and single module mutant flies as these
516 pathogens were combatted by several immune modules in our survival data.

517 Agreeing with contributions to survival, flies singly deficient for IMD, Phagocytosis, or
518 Melanization modules display a higher bacterial load than wild-type when infected with *Pr.*
519 *rettgeri* **(Figure 4A)**, which was apparent after 12 hours post-infection. Our observation that
520 *ΔPhag* showed impaired IMD signalling at 6h (**Figure 1A**) might explain the contribution of
521 phagocytosis to *Pr. rettgeri* microbial growth control with the same timing as *ΔIMD* flies. We
522 observed that *ΔIMD* flies, *ΔMel* flies, and *ΔITPM* flies that each succumb completely to *Pr.*
523 *rettgeri* infection, also show high and consistent microbe loads at 24h indicative of sepsis-
524 induced death. On the other hand, wild-type, *ΔTOLL*, and *ΔPhag* flies show a greater
525 stochasticity in bacterial load at 24h, consistent with a fraction of individuals surviving the
526 infection by controlling the pathogen. This indicates deletion of the Toll pathway and
527 phagocytosis does not fully ablate essential resistance mechanisms for combatting *Pr. rettgeri*.

528 In the case of *S. aureus* **(Figure 4B)**, we observed some individuals with higher bacterial loads
529 in Δ*Phag*, and Δ*IMD* flies at 2h and 6h, which was not seen in wild-type, Δ*TOLL,* or Δ*Mel* flies.
530 This suggests the Imd pathway can suppress early *S. aureus* growth, and possibly also
531 phagocytosis, although this Δ*Phag* could ultimately stem from lesser Imd induction.
532 Interestingly, flies lacking melanization displayed the highest susceptibility, but did not depart
533 from wild-type or other modules in pathogen load until 12h post-infection. While the
534 melanization reaction is a rapid response, these results suggest that the killing activity of the
535 melanization response acts with slower kinetics. In *ΔITPM* flies, we further observed earlier *S.*
536 *aureus* growth at 2h and 6h like *ΔIMD* and *ΔPhag* flies. However, *S. aureus* loads at 12-24h
537 were more comparable in *ΔITPM* flies to *ΔMel* flies. These trends agree both with survival data
538 kinetics, and an independent action of each of these pathways in their resistance effects against
539 *S. aureus*. This suggests that *ΔITPM* flies succumbs even more quickly than individual modules
540 due to loss of multiple resistance mechanisms with different kinetics of activation that
541 independently contribute to defense.

542 For *C. albicans* **(Figure 4C)**, mortality begins 2-3 days post-infection for Δ*TOLL* mutants, but
543 takes place at later time points for Δ*IMD,* Δ*Phag,* and *ΔMel* flies. In microbial growth kinetics,
544 Δ*TOLL* already shows high *C. albicans* loads at 24h consistent with a previous study (Hanson
545 et al., 2019) and observations done with *Candida glabrata* (Quintin et al., 2013). Interestingly,
546 a few Δ*IMD* flies also showed elevated growth of *C. albicans* at early time points. This suggests
547 Imd-regulated effectors could help to suppress initial *C. albicans* growth, while Toll-regulated
548 genes (e.g. Bomanins, Drs) contribute to this pathogen suppression to a greater extent at later
549 time points. On the other hand, Δ*Phag,* and Δ*Mel* flies did not show any increased *C. albicans*
550 loads within the 24h time window despite onset of mortality at later time points. Finally, Δ*ITPM*
551 flies displayed more consistent and rapid growth of *C. albicans* than *ΔTOLL* or *ΔIMD* alone,
552 particularly visible at 12-24h post-infection, consistent with independent contributions of both
553 Toll and Imd in resistance to this yeast.

554 All survival experiments to this point were done with males. We therefore assessed key survival
555 trends for these infections in females to learn whether the dynamics we observed were
556 consistent across sexes **(Figure 4supp1)**. For all three pathogens (*Pr. rettgeri*, *S. aureus*, *C.*
557 *albicans)* the rank order of susceptibility was broadly similar between males and females, with
558 higher rates of mortality in females overall. Thus, we found no marked sex-by-genotype
559 interaction. Interestingly, the greater susceptibility of females in our hands is true even for
560 Δ*ITPM* flies, although there were only a few surviving flies on which we can base these
561 conclusions. However, these data may suggest the sexual dimorphism in defense against
562 infection that we see against these pathogens is due to factors independent of the immune
563 modules we disrupted.

564 Collectively, we observed that microbial growth parallels the susceptibility of modules
565 indicating that they work in resistance. Surprisingly we did not observe an early role in pathogen
566 killing for melanization despite melanization regulating a rapid blackening response in ex vivo
567 assays (Nakhleh et al., 2017). We further recover stochasticity in microbe loads across
568 genotypes as expected when only a subset of individuals suppress the pathogen and survive the

infection (Duneau et al., 2017a). The more rapid and more consistent microbial growth kinetics of *ΔITPM* flies, particularly visible by 18h, further demonstrate that these pathways contribute to resistance collectively.

Contributions of individual modules to disease tolerance

An increasingly used metric to delineate roles of resistance and tolerance is the Pathogen Load Upon Death (PLUD) (Duneau et al., 2017a; Duneau et al., 2025). The PLUD is determined by the virulence of the pathogen to set an upper limit, but also the tolerance of the host in surviving to a given pathogen burden before succumbing to infection. We monitored PLUDs for the same pathogens: *Pr. rettgeri, S. aureus,* and *C. albicans*. The PLUD of both *Pr. rettgeri* and *S. aureus* upon death was overall similar in wild-type and single-module mutant flies **(Figure 5A-B)**. Notably, there was a greater stochasticity in *Pr. rettgeri* infections trending towards lower PLUD in Δ*ITPM* flies, suggesting a stochastic reduction in disease tolerance of *ΔITPM* flies to this bacterium, although this was not significant compared to wild-type (P>.05). We saw no statistical differences in PLUD across genotypes for *S. aureus*.

For *C. albicans*, the distribution of PLUD values for *ΔPhag* flies was markedly different from wild-type, also seen to some extent for *ΔMel*. Interestingly, flies with these genotypes suppress *C. albicans* growth well **(Figure 4C)**. Yet here we recovered many individuals from both of these module-deficient lines that died with a far lower PLUD **(Figure 5C)**. Taken together, this suggests that the humoral immune pathways, particularly Toll, contribute to resistance against *C. albicans*. However, the cellular response and melanization reaction instead regulate tolerance to *C. albicans* infection. The use of Δ*ITPM* flies further emphasizes that when both resistance (Toll, Imd) and tolerance (Phagocytosis, Melanization) mechanisms are deficient, the loss of resistance plays a primary importance, and can mask the loss of tolerance from being observed.

Collectively, monitoring of PLUD suggests that some modules, specifically phagocytosis and melanization, contribute to tolerance of certain infections. Importantly, this tolerance effect was only seen in genotypes that can resist infection. These findings demonstrate the collective contribution to resistance and/or tolerance of the four immune modules studied here.

Discussion

In this article, we have generated a set of single and compound immune module deficient fly lines in a controlled genetic background. Using various assays, we confirmed the validity of our lines, revealing that each module can be activated independently. We did, however, observe a higher Toll activation in Δ*Mel* flies upon systemic infection with dead bacteria. Future studies may reveal if this higher Toll activity in Δ*Mel* flies involves a regulatory pathway, or perhaps reflects higher persistence of microbial elicitors (e.g. peptidoglycan) that activate the Toll pathway. We also observed a lesser Imd activation in Δ*Phag* flies at 6 hours post-infection, which could reflect a role for hemocytes to stimulate the Imd systemic response. In this study, we used $NimC1^{1}$; $eater^{1}$ double mutants to assess the cellular response. These flies have defects in phagocytosis, but also adhesion and sessility (Melcarne et al., 2019). $NimC1^{1}$; $eater^{1}$ larvae

608 also have increased hemocyte number at the larval stage, but adults rapidly lose Hml positive
609 cells (Melcarne, 2020). Despite these limitations, we believe that the mutations we have used
610 here are among the best available to assess the role of these four modules to host defense.
611 Surprisingly, we could produce a fly line lacking the four main defense mechanisms of the
612 systemic immune response, indicating that none of these modules is essential for survival.
613 Theoretically, Δ*ITPM* flies are almost completely immune deficient but they can still clot
614 wounds, activate the JNK and JAK-STAT pathways that mediate the wound healing response,
615 and retain constitutive immune defense molecules that could also provide a certain degree of
616 protection. Consistent with several studies (Capilla et al., 2017; Carvalho et al., 2014; Rämet et
617 al., 2002), we show that Toll and melanization synergistically contribute to wound healing in
618 adults. However, the observation that Δ*Phag,* Δ*Mel* or Δ*IMD,* Δ*Mel* double mutant flies are also
619 more susceptible to clean injury than Δ*Mel* flies indicates that the Imd pathway and
620 phagocytosis also contribute to wound healing. Thus, although immune deficient fly lines for
621 the four modules are viable, the use of compound mutation and Δ*ITPM* flies reveals a clear role
622 of the immune system in response to wounding and lifespan maintenance.

623 Use of single and double module-deficient flies provides key insights on the mechanisms used
624 by *Drosophila* to combat infection. We could confirm previous studies revealing the role of
625 Imd against Gram-negative bacteria, Toll against Gram positive bacteria and virulent fungi, and
626 an importance of melanization and phagocytosis against specific pathogens (Charroux and
627 Royet, 2009; Defaye et al., 2009; Garg and Wu, 2014; Nehme et al., 2011). Surprisingly,
628 melanization was a consistently important module in survival to virus infection. It seems
629 unlikely that prophenoloxidase activity taking place in the hemolymph could combat viral
630 agents that are intracellular. We instead speculate this contribution of melanization to surviving
631 virus infection could be due to its role in wound healing, or perhaps autotoxic contributions of
632 melanization reaction intermediates that fail to convert in phenoloxidase-deficient flies. We
633 additionally recovered a role of Imd signalling (i.e. *Relish*) in antiviral defense, which was
634 expected given recent characterizations of cGLR-Sting-Relish antiviral immunity (Ai et al.,
635 2024; Cai et al., 2022; Goto et al., 2018). However, susceptibilities of Rel^{E20} flies were
636 paralleled by Δ*AMP14* in all cases, suggesting AMPs contribute to this susceptibility. While
637 Sting regulates a number of genes likely important for antiviral defense (Goto et al., 2018), the
638 susceptibility we observe here could be a direct action of AMPs on enveloped viruses as
639 described in some studies (Feng et al., 2020; Huang et al., 2013; Yasin et al., 2004), or an
640 indirect effect, such as a failure to regulate gut microbes after the damage induced by viral
641 replication (Marra et al., 2021). We additionally used dual modes of infection for the fungus *B.*
642 *bassiana,* finding an importance of melanization and Toll signalling in both infection modes.
643 However, our study reveals that Bomanin effectors explain most of the Toll contribution upon
644 septic injury but not natural infection, for *B. bassiana*. This observation is in line with different
645 importance of effectors or modules according to the route of infection (Martins et al., 2013).

646 Our double module mutant analysis reveals that most modules contribute additively to host
647 defense. This is consistent with these modules functioning independently. However, we noticed
648 instances of synergy between two pathways, notably including Toll and Imd. Synergy between
649 Toll and Imd can be explained by the fact that many immune-inducible regulated genes receive

650 input from both the Imd and Toll pathways, including genes like *Metchnikowin*, *Drosomycin*
651 and *Transferrin1* (De Gregorio et al., 2002). Finally, we also observed rarer cases of synergistic
652 susceptibility in flies deficient for Toll and Melanization or Imd and Melanization.

653 Our study confirms that the Toll and Imd humoral modules provide a broad role against certain
654 classes of pathogens, Imd for Gram-negative bacteria and DAP-type containing Gram positive
655 bacteria, and Toll for Fungi and Gram-positive bacteria. Use of AMP and Bomanin mutants
656 revealed that this can be largely explained by the effectors they control. In contrast, the
657 contribution of phagocytosis and melanization appears to be critical to a more specific and
658 diverse set of pathogens. We speculate that phagocytes or melanization are critical to handle
659 bacteria that resist host defense peptides (Hanson et al., 2019), or can hide from them (Touré et
660 al., 2023). The melanization reaction is a source of ROS that is potent against pathogens such
661 as *S. aureus* that have been shown to be sensitive to ROS, and resistant to Toll and Imd defenses
662 (Dudzic et al., 2019; Ford and King, 2021; Needham et al., 2004; Ramond et al., 2021). These
663 two modules play important roles in immune related processes such as encapsulation
664 (melanization), the uptake of bacteria escaping from the gut, or tissue homeostasis
665 (phagocytosis) (Braun et al., 1998; C. Melcarne et al., 2019; Nehme et al., 2007) that were not
666 assessed here. Collectively, our study validates, with minor discrepancies, many studies that
667 have assessed the contribution of these modules individually (ex. (Apidianakis et al., 2005;
668 Binggeli et al., 2014; Lamiable et al., 2016; Lemaitre et al., 1996, 1995; Nehme et al., 2011).
669 Our mutant lines can now be used to analyze the contribution of these immune modules in
670 resistance to other pathogens, notably wasps, nematodes, microsporidia and protozoans or in
671 other contexts such as mating and local infection.

672 While some immune modules play a predominant role against some pathogens, other pathogens
673 are handled by multiple modules. We hypothesize that pathogens that have intermediate levels
674 of virulence, killing only a fraction of wild-type flies, may better reveal the role of multiple
675 modules. Indeed, the stochasticity in survival analysis partly stems from the arms-race
676 occurring between the pathogen and host immunity, as shown for *Pr. rettgeri* (Duneau et al.,
677 2017a). In this condition, any small change in the immune system may tip the outcome of the
678 arm-race toward lethality or survival. In this line, it is notable that multiple modules were
679 important to survive *Pr. rettgeri* infection. Previous studies have revealed a major role of the
680 Imd pathway-regulated AMP Diptericin A against this bacterium (Hanson et al., 2023, 2019;
681 Unckless et al., 2016). However, Duneau et al. showed that survival patterns to *Pr. rettgeri*
682 bifurcate into two outcomes based on time taken to fully activate systemic defenses (Duneau
683 et al., 2017a), and showed a role for a Toll-PO SP cascade regulating serine protease in defense
684 against this microbe (Duneau et al., 2017b). We may speculate that melanization, although not
685 as uniquely critical as Diptericin, might tip the balance of this arms race towards host lethality,
686 resulting in comparable survival phenotypes. The observation that *ΔPhag* flies expressed less
687 *DptA* (**Figure 1**) can also explain their susceptibility. Future studies will clarify how these
688 multiple modules can contribute to host survival according to additive or achilles dynamics –
689 the concept that microbes have generic or specific weaknesses that host effectors target
690 (Hanson, 2024). It will also be important to consider the distinct roles of resistance, tolerance,

691 and resilience in host defense (Howick and Lazzaro, 2017; Wukitch et al., 2023; Duneau et al.,
692 2025).

693 We observed a good correlation between survival analysis and pathogen growth in single
694 module flies for *Pr. rettgeri*, *S. aureus,* and *C. albicans*. This indicates that these immune
695 modules mostly contribute to resistance mechanisms that target pathogen growth. Our study
696 did not reveal key early contributions of phagocytosis or melanization to control pathogen
697 growth, despite their quasi-immediate activation; higher *S. aureus* growth at 2 hours in *ΔPhag*
698 flies was paralleled by *ΔImd* flies, and we observed lesser Imd activation in *ΔPhag* flies.
699 Melanization, while being critical to survive *S. aureus* infection, impacts bacterial growth
700 beginning only at the 12 hour time point. This indicates that the Melanization microbicidal
701 activity in vivo takes place slower than the blackening reaction seen from bled hemolymph.
702 Interestingly, Δ*Phag* and also Δ*Mel* flies could suppress *C. albicans* yeast growth, but
703 ultimately some individuals succumb to infection with lower PLUD levels. We confirmed
704 Δ*Phag* PLUD results using both an isogenic and a second wild-type genetic background,
705 suggesting this lower PLUD is genuine. Susceptibility to fungal infection independent of fungal
706 proliferation has also been reported using an *A. fumigatus* septic infection model, and relies on
707 the protection offered by Bomanins from pathogen-secreted toxins (Xu et al., 2023). It is
708 tempting to speculate, based on the modules involved, that the loss of tolerance to *C. albicans*
709 we observe has to do with wound repair or accumulating damage, such as what has been
710 reported in beetles that suffer renal failure (Khan et al., 2017; Li et al., 2020), or flies with
711 autotoxic trachea degradation upon stress pathway disruption (Rommelaere et al., 2024). Thus,
712 this tolerance effect could rely on pathogen-mediated or autotoxic damage, which may be
713 elucidated in a future study.

714 Here we have provided a single and double mutant analysis of *Drosophila* immune module
715 functions. Our study provides several insights on what modules are most important to survive
716 infection by defined pathogens. However, we also highlight the collective contribution of
717 modules to defence even when one module is of an outsized importance. We extend our
718 comprehension of innate immune responses by revealing higher complexity, implicating
719 multiple host defense modules in survival to various germs, including some with more cryptic
720 contributions. As illustrated by our previous characterizations of AMP function (Carboni et al.,
721 2022; Hanson et al., 2019), the melanization response (Dudzic et al., 2015), and stress-induced
722 Turandot proteins (Rommelaere et al., 2024), a combinatorial mutation approach to deciphering
723 immune functions can be extended even to the broad level of immune modules. Of note, we
724 were unable to systematically sample all genotype-by-pathogen interactions equally. We have
725 therefore been highly conservative in our reporting of major effects. There are likely many
726 important interactions not discussed in our study. Future investigations may highlight important
727 biology that is apparent in our data, but which we may not have mentioned here. To this end,
728 we have deposited our isogenic immunity fly stocks in the Vienna Drosophila Resource Centre
729 to facilitate their use. Beyond immunity, our tools can also be of use to study various questions
730 at the cutting edge of aging, memory, neurodegeneration, cancer, and more, where immune
731 genes have been implicated repeatedly. We hope that this set of lines will be useful to the
732 community to better characterize the *Drosophila* host defense.

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1058 # Figures

1059 **Table 1: List of Mutants used in this study.** "-" indicates the deleted immune module.

			Deficient pathway			
Drosophila lines	**Reference**	**Name**	**IMD**	**Toll**	**Phagocytosis**	**Melanization**
iso ; iso ; Rel^{E20}	Hedengren et al., 1999	*ΔIMD*	-	+	+	+
iso ; iso ; spz^{rm7}	Lemaitre et al., 1996	*ΔTOLL*	+	-	+	+
iso ; NimC1^{1} *; Eater*^{1}	Melcarne et al., 2019	*ΔPhag*	+	+	-	+
iso ; PPO1Δ, PPO2Δ; iso	Bingelli et al., 2014	*ΔMel*	+	+	+	-
+ ; + ; Rel^{E20}*, spz*^{rm7}	this paper	*ΔIMD, ΔTOLL*	-	-	+	+
iso ; PPO1Δ, PPO2Δ ; NimC1^{1}*,Eater*^{1}	this paper	*ΔPhag, ΔMel*	+	+	-	-
iso ; NimC1^{1} *; Eater*^{1}*, Rel*^{E20}	this paper	*ΔIMD, ΔPhag*	-	+	-	+
iso ; PPO1Δ PPO2Δ ; Rel^{E20}	this paper	*ΔIMD, ΔMel*	-	+	+	-
iso ; PPO1Δ PPO2Δ ; spz^{rm7}	this paper	*ΔTOLL, ΔMel*^{PPS}	+	-	+	-
iso Hay-psh^{Def} *; iso ; iso*	Dudzic et al., 2019	*ΔTOLL, ΔMel*^{HP}	+	-	+	-
iso Hay-psh^{Def} *; NimC1*^{1} *; Eater*^{1} *Rel*^{E20}	this paper	*ΔITPM*	-	-	-	-

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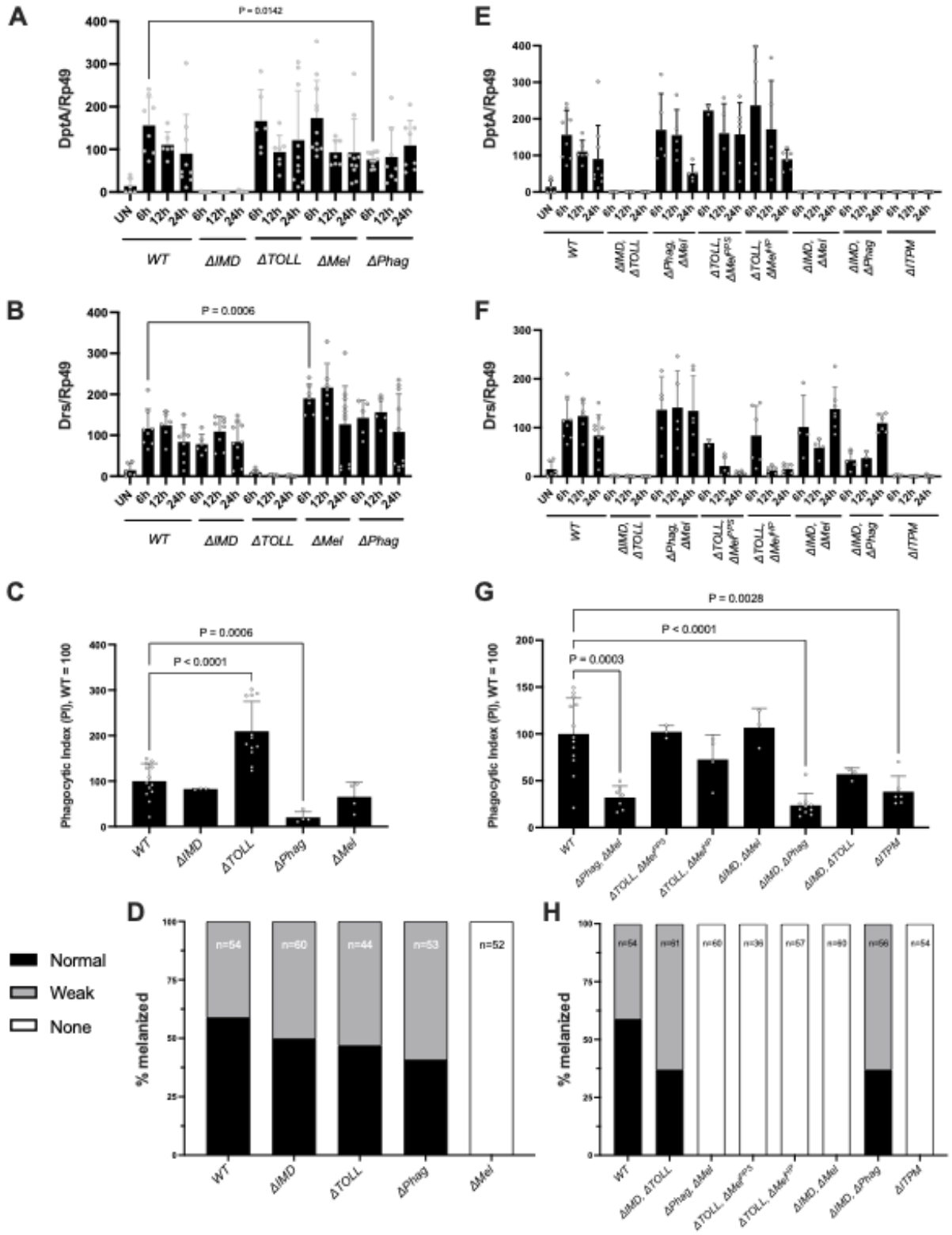

Figure 1: Each immune module functions largely independently from other immune modules. **A)** Activation of the Imd pathway, as revealed by *DptA* expression following infection with a mixture of heat-killed *E. coli* and *M. luteus,* is broadly wild-type in single module mutants other than Imd. In the case of *ΔPhag* flies, we observed a lesser induction at 6h compared to wild-type. **B)** Activation of the Toll pathway, as revealed by the expression of *Drs* is broadly wild-type in single module mutants other than *ΔTOLL* flies. In the case of *ΔMel*,

1069 we observed a slightly greater Toll pathway activity. **C)** The ability of plasmatocytes to
1070 phagocytose bacterial particles is not negatively affected in single module mutants except
1071 *ΔPhag* (also see Figure 1supp1) **D)** Cuticle blackening after clean injury is not impaired except
1072 in *ΔMel* flies. **E)** Activation of the Imd pathway remains strongly inducible in compound
1073 mutants except when deficient for the Imd pathway. **F)** Activation of the Toll pathway remains
1074 strongly inducible in compound mutants except when flies were deficient of the Toll pathway.
1075 Note that *Drs* receives a minor input from Imd signaling (Leulier et al., 2000), explaining minor
1076 induction of *Drs* in *ΔToll* flies at early time points. **G)** The ability of plasmatocytes to
1077 phagocytose bacterial particles is not significantly affected in compound mutants except those
1078 including *ΔPhag*. **H)** Cuticle blackening after clean injury is not impaired in compound mutants
1079 except in *ΔMel* flies.

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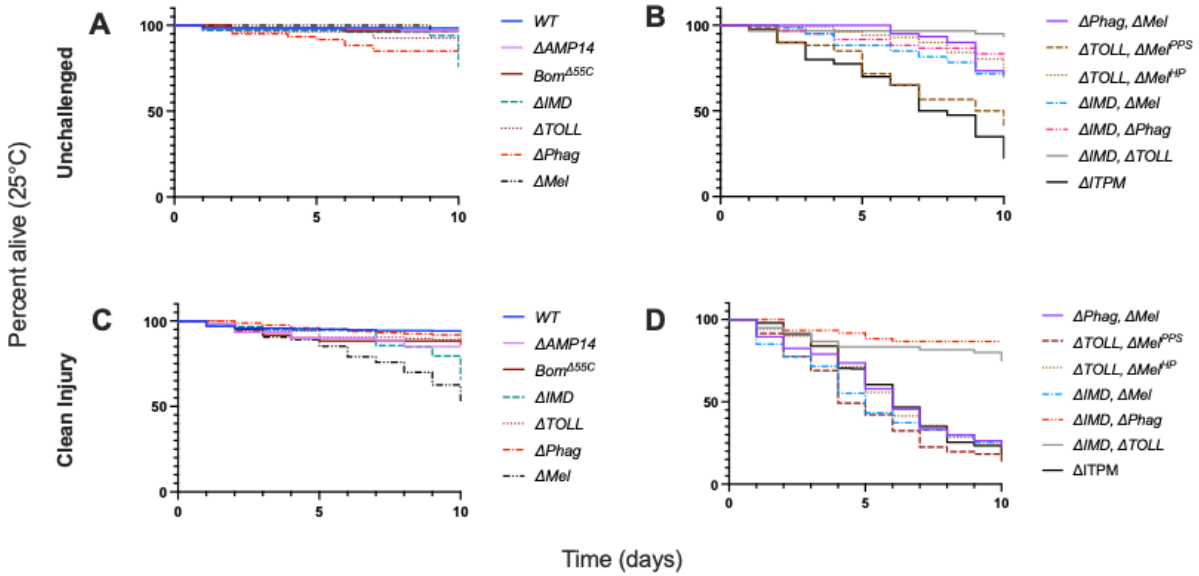


Figure 2: Lifespans of mutants used in this study in unchallenged flies (A-B) and upon clean injury (C-D) conditions at 25°C. See Figure 2supp1 for 29°C comparisons.

Type	Species	Temp	Dose	Days	N exp	WT		ΔAMP14		ΔBom		ΔIMD		ΔTOLL		ΔPhag		ΔMel		ΔPhag, ΔMel		ΔTOLL, ΔMel^PPS		ΔTOLL, ΔMel^HP		ΔIMD, ΔMel		ΔIMD, ΔPhag		ΔIMD, ΔTOLL		ΔITPM	
Controls	Unchallenged	25°C	n/a	7	13	6.9	177	6.9	60	6.9	60	6.8	150	6.8	80	6.6	60	7.0	110	7.0	60	6.0	60	6.8	239	6.6	60	6.6	60	6.8	60	5.8	60
	Clean Injury	25°C	n/a	7	36	6.7	709	6.6	60	6.5	60	6.7	540	6.6	280	6.8	249	6.4	526	5.3	57	4.6	71	5.4	70	4.7	67	6.5	60	6.2	60	5.5	445
	Clean Injury	29°C	n/a	7	11	6.6	211	6.5	60	5.9	60	6.3	100	6.1	75	6.6	60	6.4	119	4.9	56	3.0	47	4.8	56	3.7	57	6.0	64	5.6	60	3.9	64
	Clean Injury	25°C	n/a	10	36	9.6	709	9.1	60	9.2	60	9.2	540	9.3	280	9.6	249	8.5	526	6.2	57	5.2	71	6.3	70	5.6	67	9.1	60	8.7	60	6.3	445
	Unchallenged	29°C	n/a	10	11	9.9	139	9.3	60	9.5	60	8.9	60	7.1	34	9.3	40	9.2	60	8.7	60	5.9	64	9.3	40	8.5	60	9.1	60	9.4	49	7.7	61
Viruses	Drosophila C virus	25°C	2000 TCID50	10	5	8.3	100	6.7	89	7.7	88	6.8	73	7.8	83	8.1	78	6.3	86	6.3	88	5.5	58	5.0	92	5.1	80	6.7	84	3.8	53	4.1	78
	Flock House virus	25°C	250000 TCID50	10	5	8.5	122	7.1	73	7.2	84	7.3	79	7.3	77	7.3	81	6.9	82	6.2	82	5.1	56	6.0	85	4.9	61	6.8	73	5.2	59	5.1	84
	Drosophila X virus	25°C	431000 TCID50	10	2	9.0	40	6.9	41	8.2	20	7.1	40	7.9	34	7.8	41	5.4	38	7.4	46	4.9	37	5.1	38	6.0	15	6.2	40	5.6	19	4.6	43
	Invertebrate iridescent-6 virus	25°C	31700 TCID50	10	3	9.4	59	7.5	50	9.4	64	8.1	58	8.4	54	8.8	60	5.6	59	6.0	61	5.8	59	5.8	59	6.1	48	8.3	54	5.9	47	4.4	69
	Sindbis virus	25°C	50000 TCID50	10	3	9.4	58	8.4	61	8.6	61	8.9	60	8.8	60	8.3	60	5.4	65	5.9	66	4.9	51	4.8	59	4.5	60	7.0	50	4.5	45	4.3	61
Fungi	Aspergillus fumigatus (natural)	29°C	rolled on plate	10	5	10.0	80	9.2	61	9.9	20	8.8	56	7.2	81	7.6	41	9.1	60	8.2	60	6.8	62	8.6	60	7.7	60	8.7	66	6.9	60	4.7	62
	Beauvaria bassiana (natural)	29°C	30mg	10	5	9.6	120	8.2	61	9.8	60	9.4	60	7.3	70	7.8	43	8.7	78	7.7	60	5.0	64	6.0	36	6.9	66	6.4	40	5.0	57	4.4	62
	Beauvaria bassiana	29°C	OD=5	7	6	6.4	81	6.0	74	3.6	58	5.7	58	3.6	66	5.2	39	5.3	62	4.4	57	2.6	58	3.4	63	4.4	21	4.7	51	3.1	56	3.3	65
	Candida albicans	29°C	OD=200	7	8	6.7	160	5.6	45	6.0	102	5.9	54	5.0	99	6.4	65	5.9	60	6.4	102	1.7	20	3.1	156	5.7	36	5.9	69	3.2	27	2.4	62
Gram positive bacteria	Mycobacterium marinum	25°C	OD=1	7	6	6.1	132	6.0	50	6.5	51	6.5	111	5.9	108	5.2	77	5.8	89	5.7	108	4.1	62	5.8	64	5.1	51	5.1	64	6.3	56	3.9	117
	Corynebacterium diphtheriae	25°C	OD=100	7	3	6.0	73	6.2	84	6.5	87	5.6	79	5.1	90	6.6	87	4.6	82	5.8	69	3.2	47	3.3	103	4.9	72	5.9	68	4.5	61	3.0	75
	Micrococcus luteus	29°C	OD=200	7	8	6.7	121	5.7	60	6.8	60	6.2	120	6.4	148	6.5	71	6.5	81	5.6	81	4.6	68	6.5	80	4.8	77	6.3	64	2.1	102	2.1	91
	Streptococcus pneumoniae	25°C	OD=0.3	7	3	6.8	41	6.1	60	6.4	60	6.7	61	6.6	70	6.3	60	6.2	60	6.4	60	5.7	60	5.0	61	5.1	60	6.0	60	5.5	60	4.5	50
	Enterococcus faecalis	25°C	OD=5	7	10	5.0	130	4.5	100	2.1	62	4.2	122	1.8	126	5.2	79	5.1	122	3.9	78	1.6	63	1.8	102	4.2	62	4.0	79	1.4	61	1.3	151
	Staphylococcus aureus	25°C	OD=0.5	7	8	5.1	119	4.8	58	5.7	21	4.2	119	3.4	73	3.6	79	2.4	73	1.9	78	2.0	60	2.0	112	2.1	78	4.1	59	3.4	40	1.9	67
	Listeria monocytogenes	25°C	OD=0.5	7	5	4.8	81	4.7	78	2.2	60	2.3	62	1.6	112	4.6	71	5.3	78	3.9	78	2.1	70	2.2	73	3.5	88	3.3	60	2.0	62	1.6	62
	Bacillus subtilis	25°C	OD=5	7	7	5.7	130	3.3	49	4.1	20	4.0	123	3.8	119	6.0	55	5.4	110	4.5	98	2.8	69	2.7	84	4.5	76	5.4	20	2.1	41	2.0	81
Gram negative bacteria	Vibrio parahemolyticus	25°C	OD=10	7	2	3.1	58	1.1	61	2.9	62	1.1	62	1.3	70	2.8	64	2.3	70	3.2	57	1.8	68	1.9	41	1.0	59	1.1	63	1.0	59	1.0	64
	Providencia rettgeri	25°C	OD=0.1	7	7	5.5	146	1.1	57	5.1	58	2.0	78	4.4	70	3.6	100	1.8	95	1.5	61	1.5	75	5.3	40	1.3	75	2.1	62	1.0	21	1.4	123
	Providencia burhodogranaria	25°C	OD=10	7	7	5.1	161	1.7	78	4.6	40	1.6	58	4.6	88	4.9	63	4.0	124	4.2	84	2.9	61	4.9	60	1.3	78	1.0	61	1.4	40	1.3	88
	Pectobacterium carotovorum	29°C	OD=10	7	8	6.4	151	1.5	57	5.8	40	1.8	111	5.8	108	4.4	120	5.8	100	5.2	102	4.7	60	5.2	60	1.8	72	1.8	8	1.4	62	1.4	106
	Klebsiella pneumoniae	25°C	OD=1	7	4	6.7	80	1.4	68	6.7	88	1.4	87	6.8	80	6.9	80	5.8	60	6.3	60	3.9	80	6.9	60	1.3	39	2.0	56	1.0	33	1.3	64
	Enterobacter cloacae	25°C	OD=10	7	8	6.6	149	1.3	83	6.7	80	1.0	100	6.6	90	6.7	60	6.4	80	6.1	80	4.9	81	5.8	39	1.3	73	1.0	27	1.6	81	1.2	83
	Escherichia coli	25°C	OD=200	7	8	6.6	141	5.0	79	6.9	40	3.8	80	6.2	70	5.4	20	5.5	43	5.5	85	3.9	63	5.3	63	3.8	60	4.6	60	1.7	70	1.6	60
	Salmonella enterica ser. typhimurium	25°C	OD=200	7	4	6.6	80	4.8	40	6.7	100	5.3	80	6.3	40	6.4	40	6.3	40	6.6	63	5.5	40	4.8	64	4.7	67	3.9	19	2.7	56	1.8	69

Figure 3: Heatmap of lifespans of immune module-deficient flies upon infection by various pathogens. Darker blue indicates lower survival, while white indicates maximum survival. Experiments used either 7 or 10 days as a maximum lifespan/time course, and the heatmap is adjusted accordingly per row to have white fill for the maximum possible lifespan of that row. Heatmap colours: blue (low survival), white (high survival). Survival curves are presented in **Supplementary file 2**, and a summary of susceptibilities is provided in Figure 3supp1. Small numbers beside mean lifespans indicate total sample size per genotype per treatment.

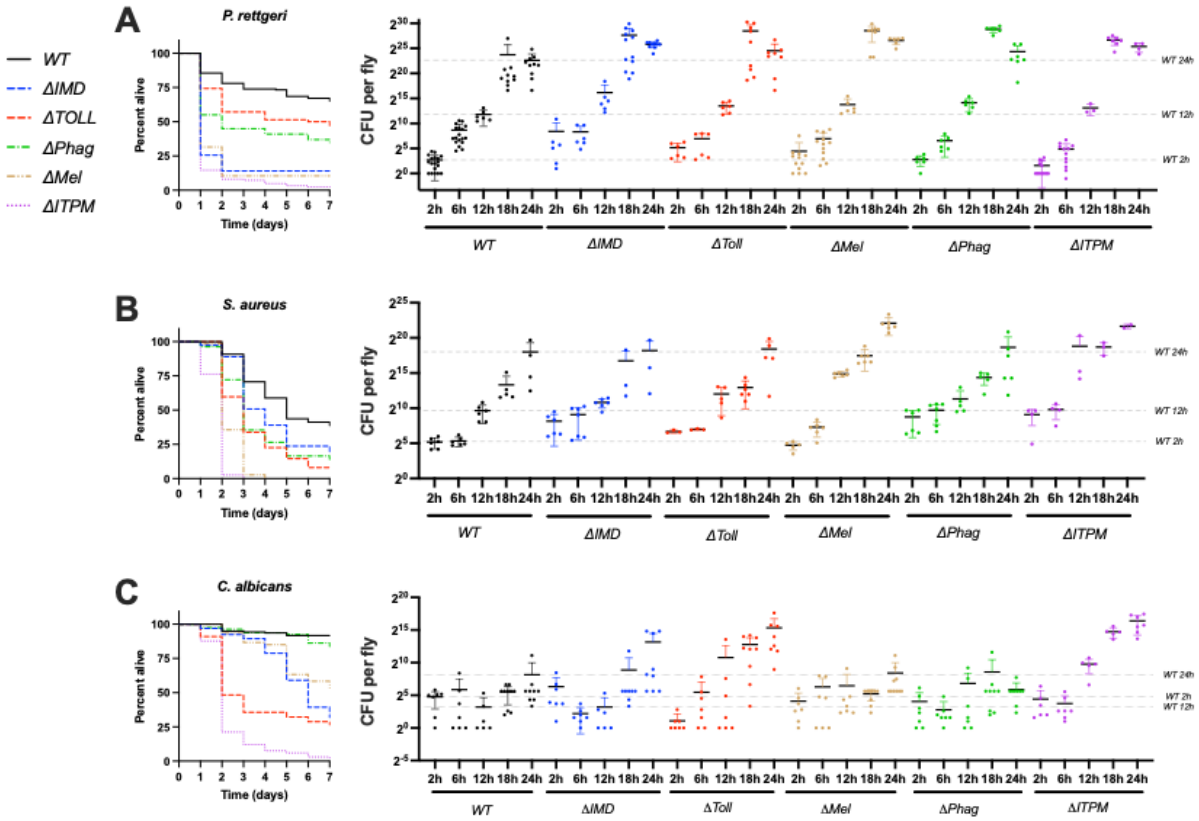
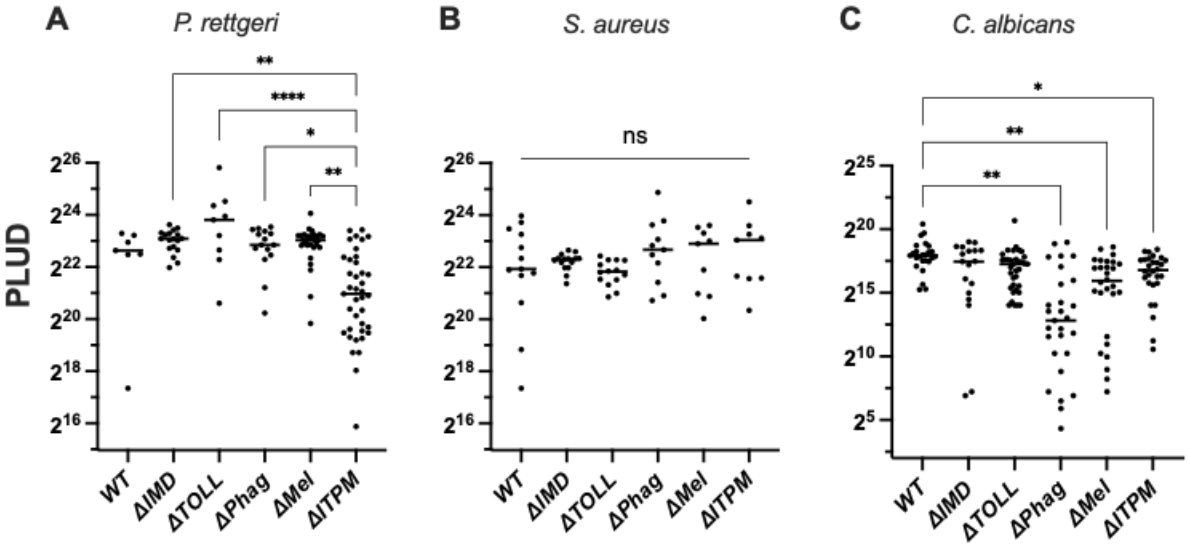


Figure 4: Growth kinetics of *Pr. rettgeri* (A), *S. aureus* (B), and *C. albicans* (C) in wild-type, single module mutant and *ΔITPM* flies. Survival curves underlying data in Figure 3 are shown for comparison. Each data point reflects a pooled sample of 5 flies. Error bars reflect 1 standard deviation from the mean.



1099

1100 **Figure 5: Measurement of PLUD upon infection with *Pr. rettgeri*, *S. aureus*, and *C.***
1101 ***albicans*. A)** The PLUD of *Pr. rettgeri* in individual module mutant flies is not significantly
1102 different from wild-type. The PLUD of *ΔITPM* flies was not significantly different, although
1103 censoring of a single low-PLUD outlier in the wild-type would result in a significant difference
1104 between wild-type and *ΔITPM* flies ($p < .01$). **B)** The PLUD of *S. aureus*-infected flies is not
1105 different across any genotype. **C)** The PLUD of *C. albicans*-infected flies was significantly
1106 lower in *ΔPhag* flies with a notably lower mean PLUD. This difference was robust to use of
1107 different *ΔPhag* genetic backgrounds (merged data shown here). *ΔMel* flies and *ΔITPM* flies
1108 also had significantly lower PLUD. Error bars reflect 1 standard deviation from the mean.

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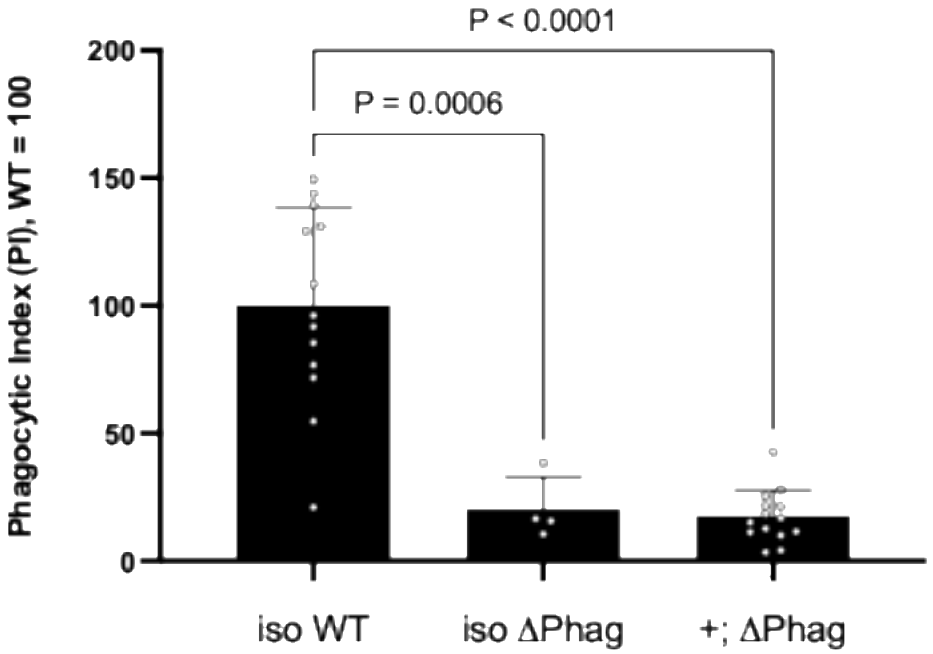


Figure 1supp1: We used *ΔPhag* mutants in the genetic background of Melcarne et al. (2019) *(+; ΔPhag)* in some experiments, and confirm here that these mutants are equally deficient in phagocytic capacity to *ΔPhag* flies from the iso DrosDel genetic background (*ΔPhag)*.

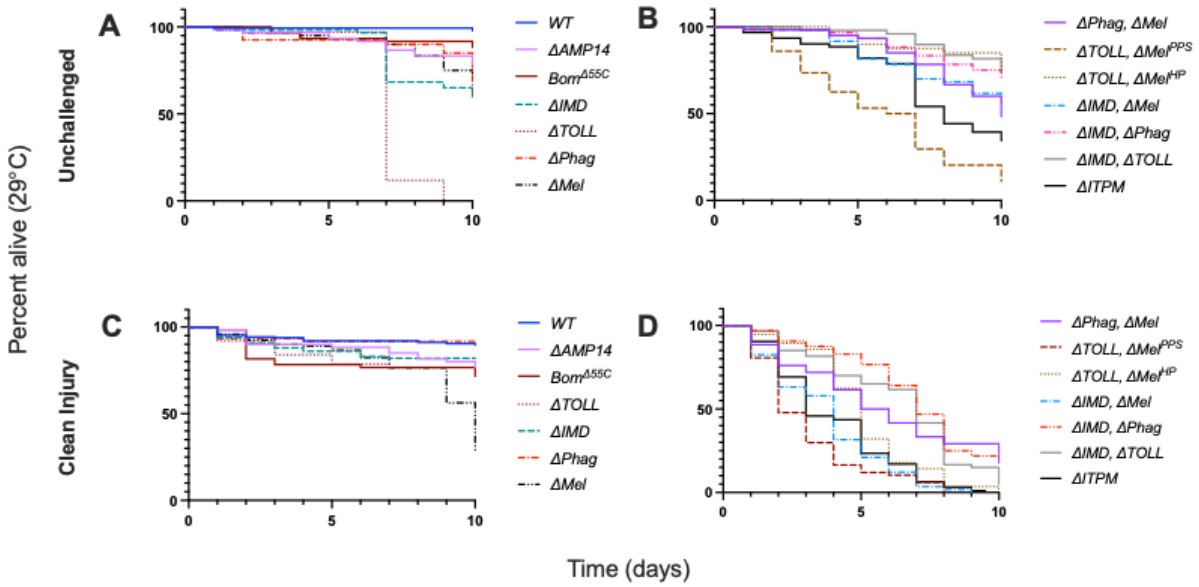


Figure 2supp1: Lifespans of mutants used in this study in unchallenged flies and upon clean injury conditions at 29°C.

Figure 3supp1: Summary table of susceptibilities per the three conditions outlined in section "Systemic infections and survival." A ✓ indicates the pathway contributes to defence against a given pathogen. In the event that a single module mutant is not more susceptible to the infection, but contributes to increased mortality in a double mutant line, a ✓ is given and the co-deleted relevant pathways highlighted by their first letter. Black boxes indicate no difference to wild-type or to clean injury.

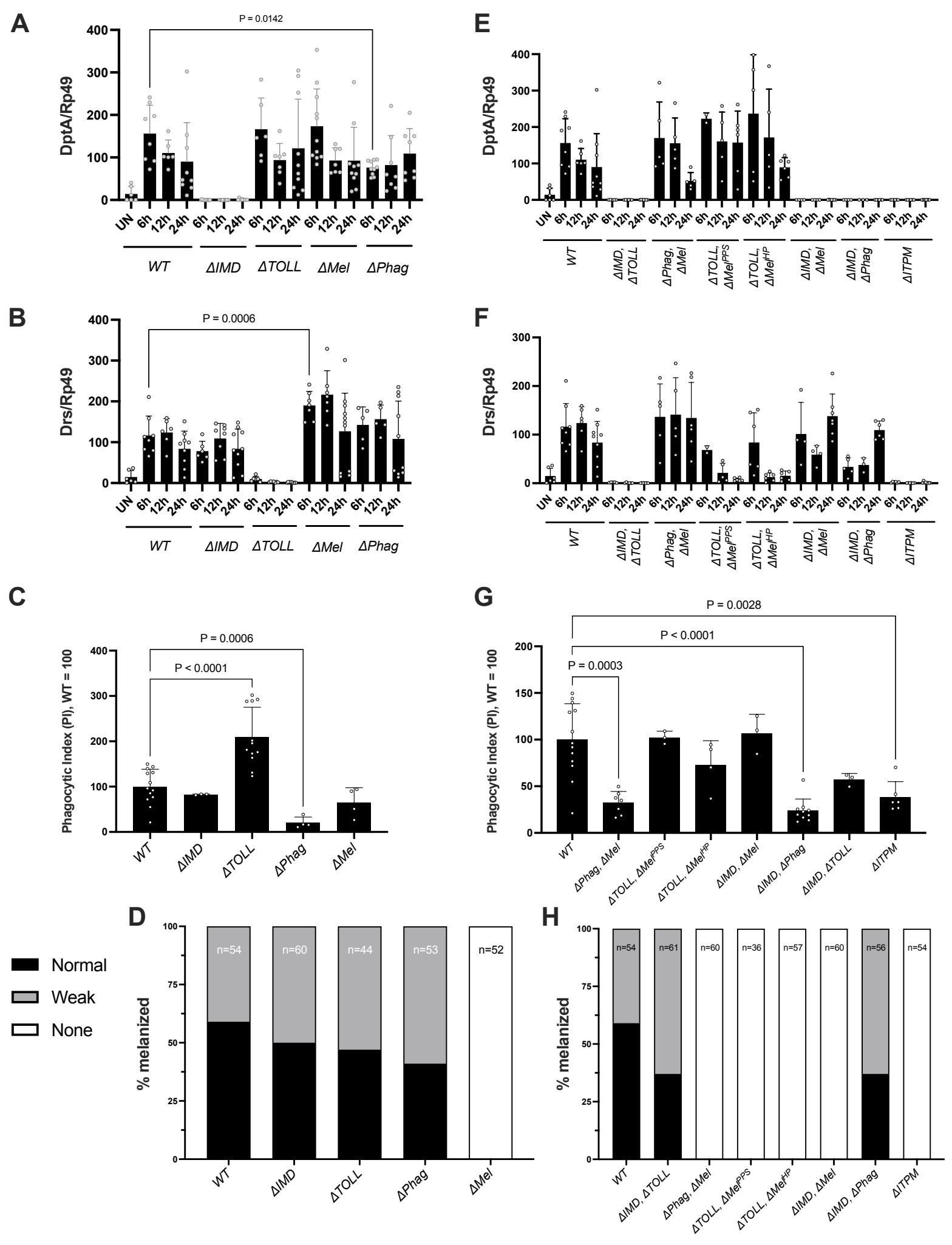
1123 **Figure 4supp1:** Survival to infection of females against *P. rettgeri* **(A)**, *S. aureus* **(B)**, and *C.*
1124 *albicans* **(C)**. Survival trends largely parallel the rank order of males (**Figure 4**).

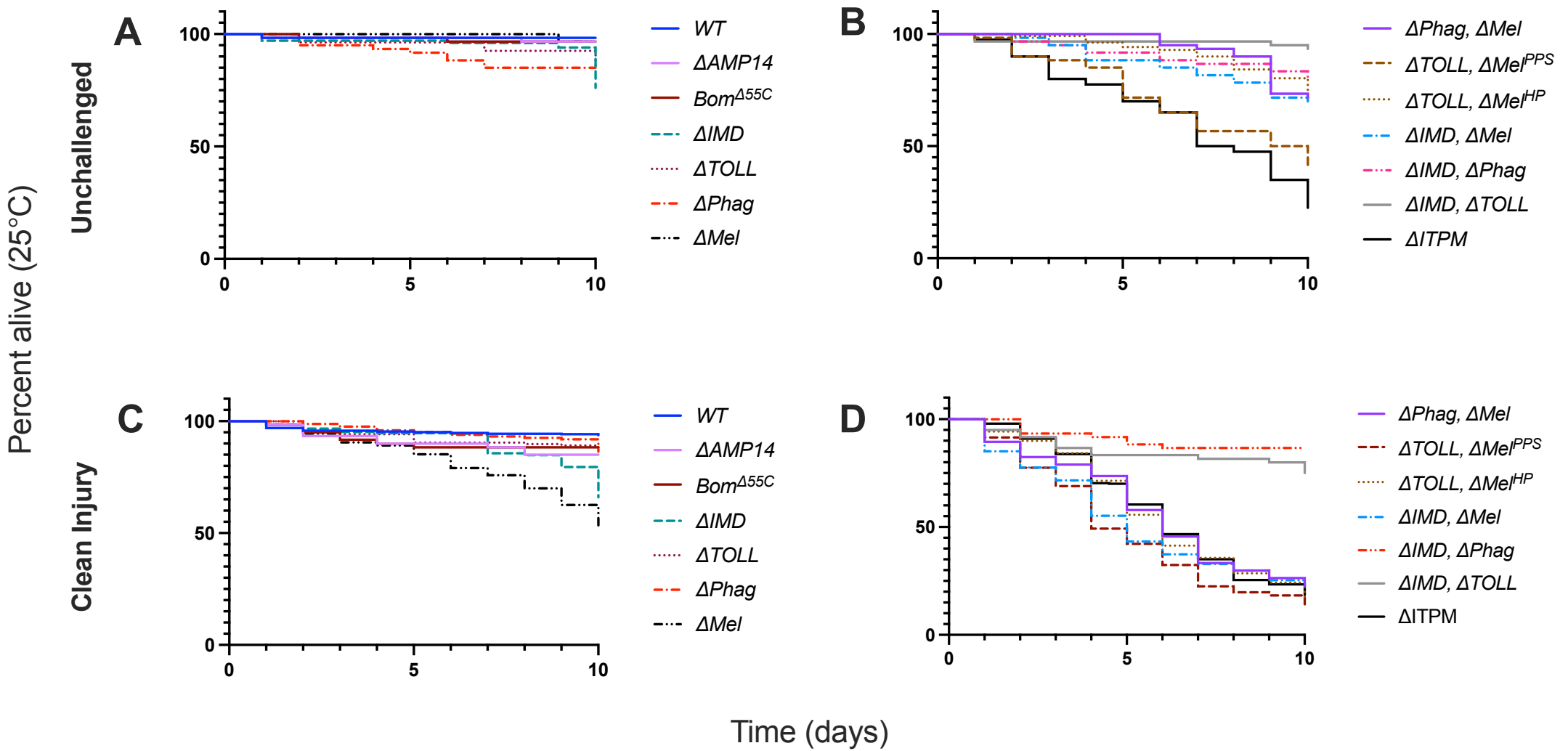
1125 **Table S1:** Microbe characteristics and growth conditions.

1126 **Supplementary file 1:** Supplemental discussion of fly genetics.

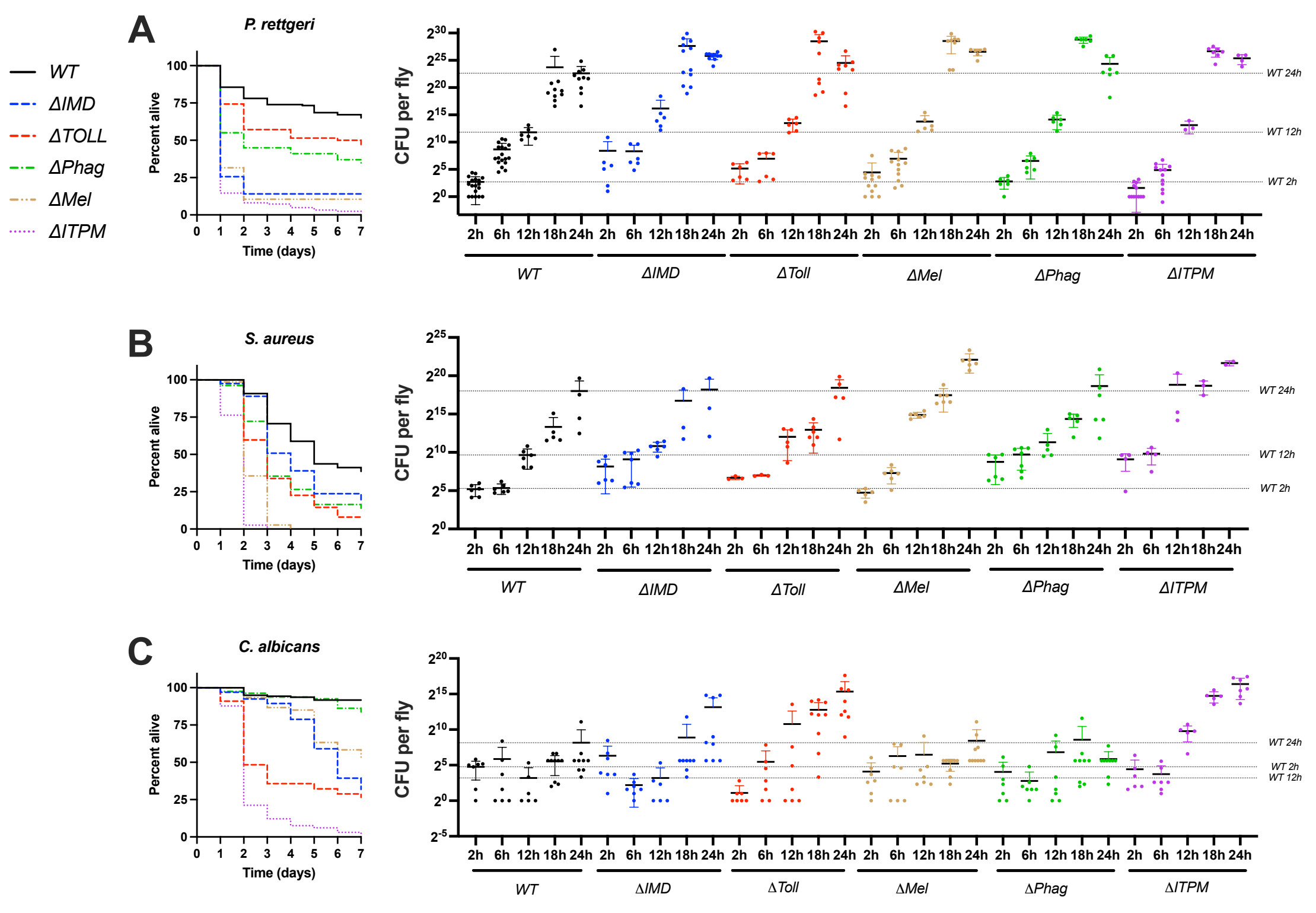
1127 **Supplementary file 2:** Survival curves for all data summarized in Figure 3.

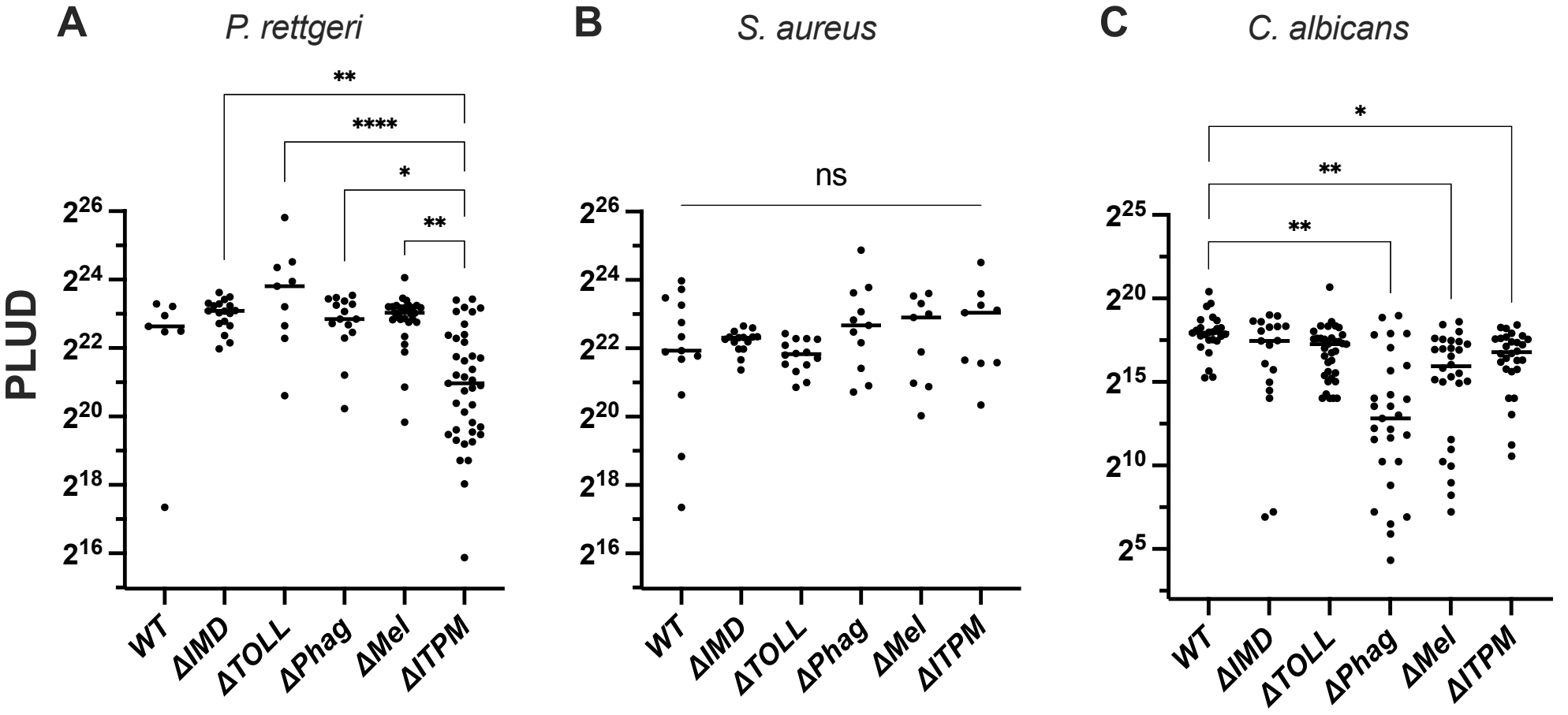
1128 **Supplementary file 3:** Supplemental discussion of fly genetics and considerations for the use
1129 of genotypes described in this study.


A
P = 0.0142
DptA/Rp49
400
300
200
100
0
UN
6h
12h
24h
WT
ΔIMD
ΔTOLL
ΔMel
ΔPhag
B
P = 0.0006
Drs/Rp49
C
P = 0.0006
P < 0.0001
Phagocytic Index (PI), WT = 100
ΔIMD
ΔTOLL
ΔPhag
ΔMel
D
% melanized
n=54
n=60
n=44
n=53
n=52
Normal
Weak
None
E
F
ΔIMD, ΔTOLL
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔITPM
G
P = 0.0028
P < 0.0001
P = 0.0003
H
n=54
n=61
n=60
n=36
n=57
n=60
n=56
n=54

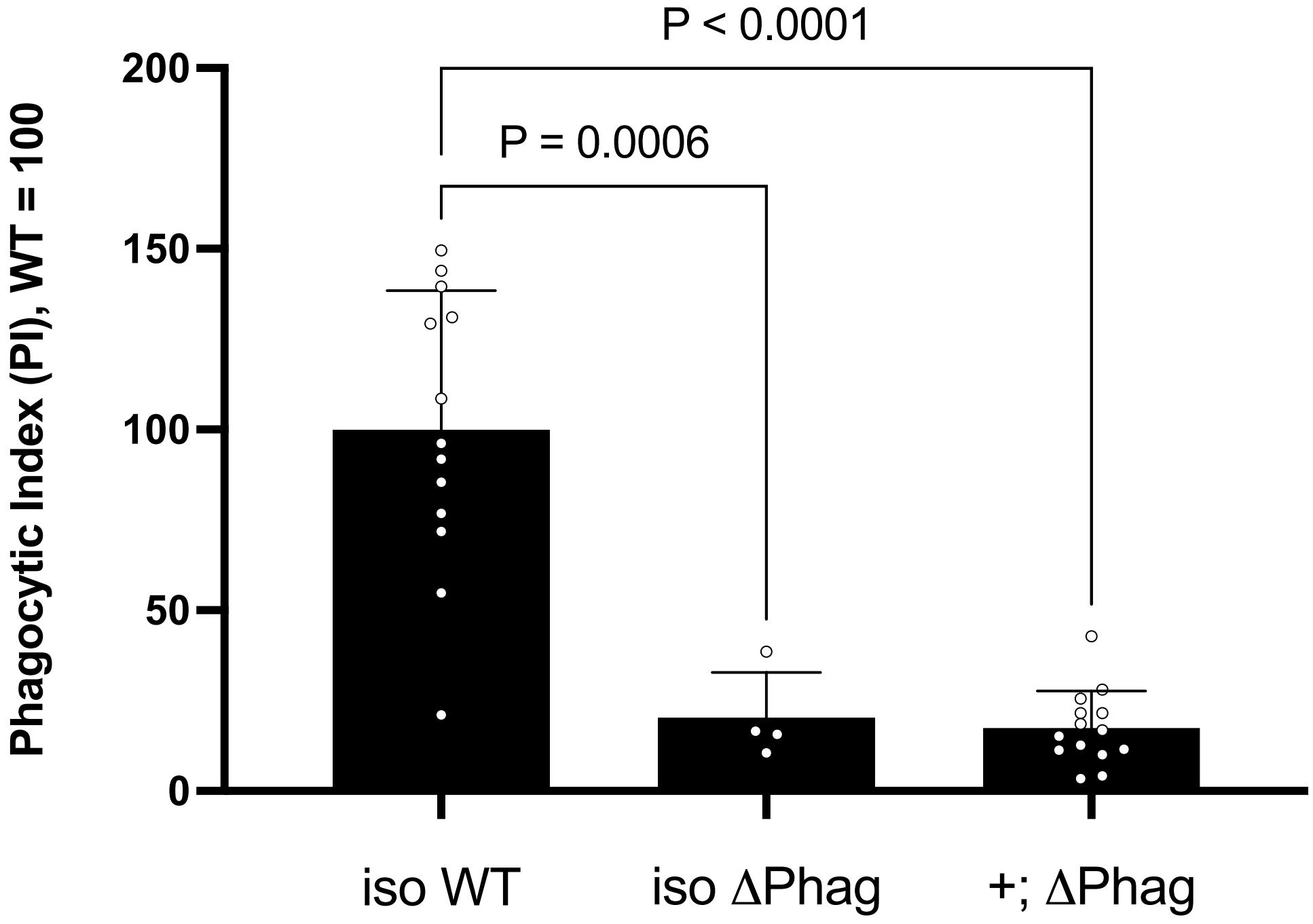

A
B
C
D
Unchallenged
Clean Injury
Percent alive (25°C)
Time (days)
100
50
0
0
5
10
WT
ΔAMP14
Bom^Δ55C
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM

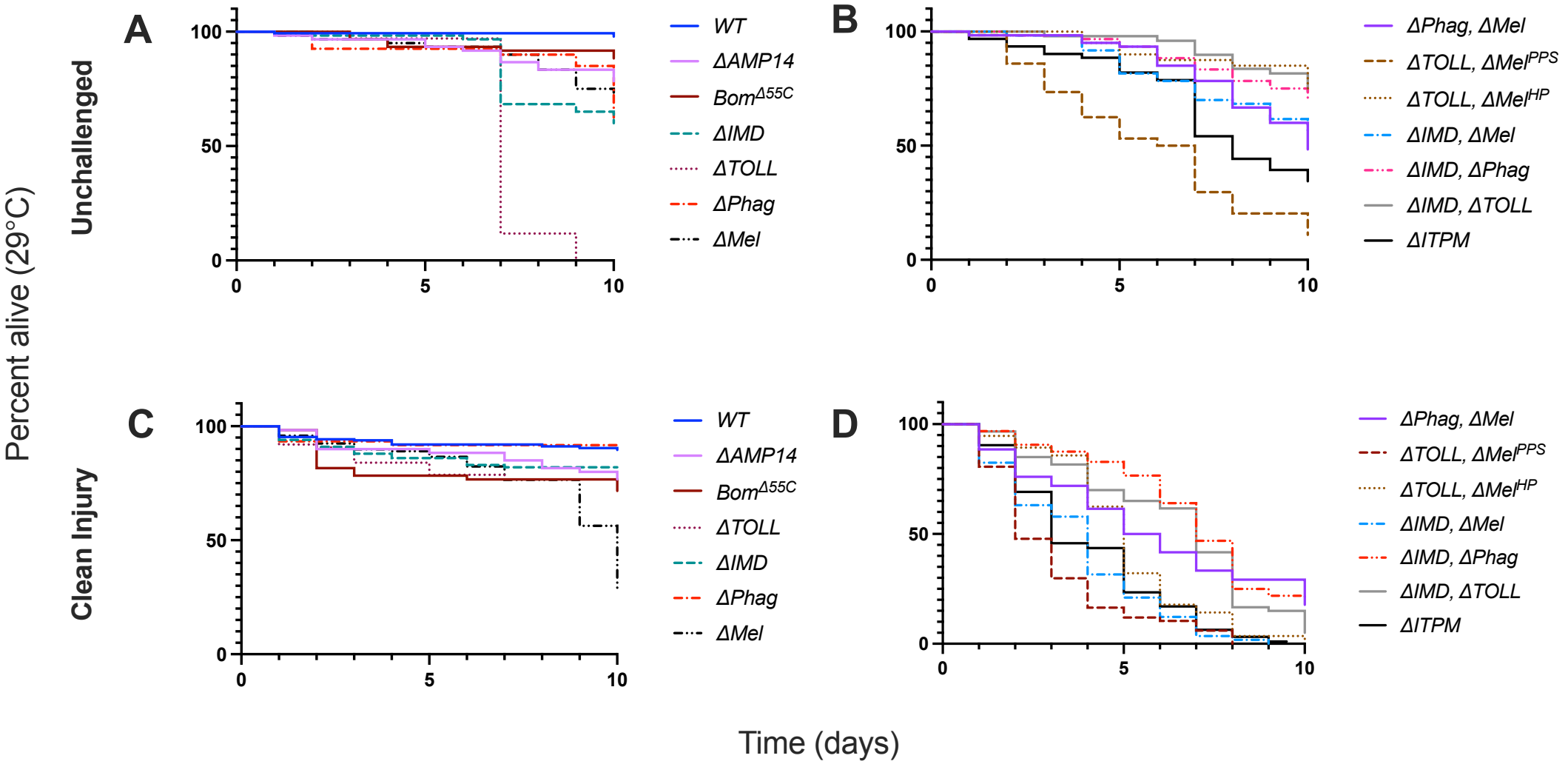
Type	Species	Temp	Dose	Days	N exp	*WT*	*ΔAMP14*	*ΔBom*	*ΔIMD*	*ΔTOLL*	*ΔPhag*	*ΔMel*	*ΔPhag, ΔMel*	*ΔTOLL, ΔMel^{RR5}*	*ΔTOLL, ΔMel^{up}*	*ΔIMD, ΔMel*	*ΔIMD, ΔPhag*	*ΔIMD, ΔTOLL*	*ΔTPM*
Controls	*Unchallenged*	25°C	nil	7	13	6.9	177	6.9	6.8	6.8	6.8	6.8	7.0	6.6	6.6	6.8	6.8	6.8	5.8
	Clean Injury	25°C	nil	7	36	6.7	208	6.6	6.5	6.7	6.6	6.8	6.8	6.4	5.8	5.3	6.6	5.4	5.5
	Clean Injury	25°C	nil	10	21	6.5	159	6.5	6.4	6.5	6.5	6.8	6.4	6.4	5.8	4.9	6.5	5.0	5.6
	Clean Injury	25°C	nil	10	36	9.6	109	9.1	9.2	9.2	9.4	9.3	9.2	8.5	5.8	6.2	9.1	5.7	6.3
	Unchallenged	29°C	nil	10	11	9.9	139	9.3	8.9	8.9	7.1	9.4	9.3	9.2	8.9	8.7	8.9	9.4	7.7
Viruses	*Drosophila C virus*	25°C	2000 TCID50	10	5	8.3	100	8.7	8.9	7.7	6.8	7.2	7.8	7.3	6.3	6.3	8.8	5.5	4.1
	Flock House virus	25°C	250000 TCID50	10	5	8.5	100	7.1	7.5	7.2	7.3	7.3	7.3	6.9	6.7	6.2	6.9	5.1	5.6
	Drosophila X virus	25°C	431000 TCID50	10	2	9.0	49	6.9	8.1	8.2	7.1	4.8	7.9	7.4	5.8	7.4	6.9	6.2	4.8
	Invertebrate iridescent-6 virus	25°C	31700 TCID50	10	3	9.4	59	7.5	9.4	8.4	8.1	5.8	8.4	8.8	5.6	6.0	8.1	5.8	5.9
	Simbiu virus	25°C	50000 TCID50	10	3	9.4	58	8.4	8.1	8.6	8.7	8.9	8.8	8.9	8.3	6.5	5.4	6.7	5.9
Fungi	*Aspergillus fumigatus* (natural)	25°C	rolled on plate	10	5	10.0	80	9.2	9.1	9.9	9.2	8.8	9.2	7.3	7.1	8.7	9.1	6.0	6.2
	Beauveria bassiana (natural)	25°C	filming	10	5	9.6	120	8.2	8.7	8.8	8.6	8.7	8.7	7.3	6.9	7.7	8.7	5.0	5.4
	Beauveria bassiana	25°C	OD=5	7	6	6.4	51	6.0	7.4	3.6	6.9	5.7	3.6	6.8	5.2	4.3	5.2	4.4	4.7
	Candida albicans	25°C	OD=200	7	8	6.7	160	5.6	6.6	6.0	5.9	5.9	6.0	6.4	6.5	5.9	6.4	5.0	5.7
Gram positive bacteria	*Micrococcus luteus*	25°C	OD=1	7	6	6.1	110	6.0	6.5	6.5	6.7	5.9	6.5	6.7	5.8	5.7	6.0	4.1	4.0
	Streptococcus pneumoniae	25°C	OD=3	7	3	6.8	61	6.1	6.4	6.5	6.7	6.1	6.6	6.7	6.0	5.7	6.0	5.1	5.2
	Enterococcus faecalis	25°C	OD=1	7	10	6.0	100	4.5	5.8	5.8	5.8	5.8	5.8	5.8	5.1	5.1	5.1	4.2	4.2
	Staphylococcus aureus	25°C	OD=0.5	7	8	5.1	114	4.8	5.6	5.7	4.2	5.6	3.4	5.3	3.6	3.9	2.4	3.9	1.9
	Listeria monocytogenes	25°C	OD=0.5	7	5	4.8	91	4.7	5.9	2.2	6.3	2.3	5.2	1.6	1.2	4.6	5.7	1.3	1.2
	Bacillus subtilis	25°C	OD=5	7	7	5.7	139	3.5	4.1	3.9	4.0	3.3	3.8	3.1	1.0	3.9	3.8	1.0	1.0
Gram negative bacteria	*Vibrio parahaemolyticus*	25°C	OD=10	7	2	3.1	54	1.1	1.1	2.9	2.3	1.1	1.1	2.8	2.4	2.3	1.0	3.2	1.0
	Providencia rettgeri	25°C	OD=0.1	7	7	5.5	146	5.1	5.1	5.1	5.0	5.1	4.4	4.9	3.6	3.6	1.8	3.5	1.5
	Providencia burhodogranaria	25°C	OD=10	7	7	5.1	181	1.7	1.6	4.6	1.6	3.4	4.6	1.9	4.3	4.0	1.0	4.2	1.0
	Pectobacterium carotovorum	25°C	OD=10	7	8	6.4	154	1.5	5.7	5.8	4.9	1.8	1.7	5.8	1.8	4.4	1.2	4.7	1.0
	Klebsiella pneumoniae	25°C	OD=0.1	7	8	5.7	114	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.3
	Enterobacter cloacae	25°C	OD=10	7	8	6.6	169	1.3	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
	Escherichia coli	25°C	OD=200	7	8	6.6	141	5.0	7.6	6.9	6.9	6.9	6.9	6.9	6.9	6.9	6.9	6.9	6.9
	Salmonella enterica ser. typhimurium	25°C	OD=200	7	8	6.5	160	4.8	4.7	5.8	5.0	5.0	5.0	4.7	4.7	4.7	4.7	4.7	4.7

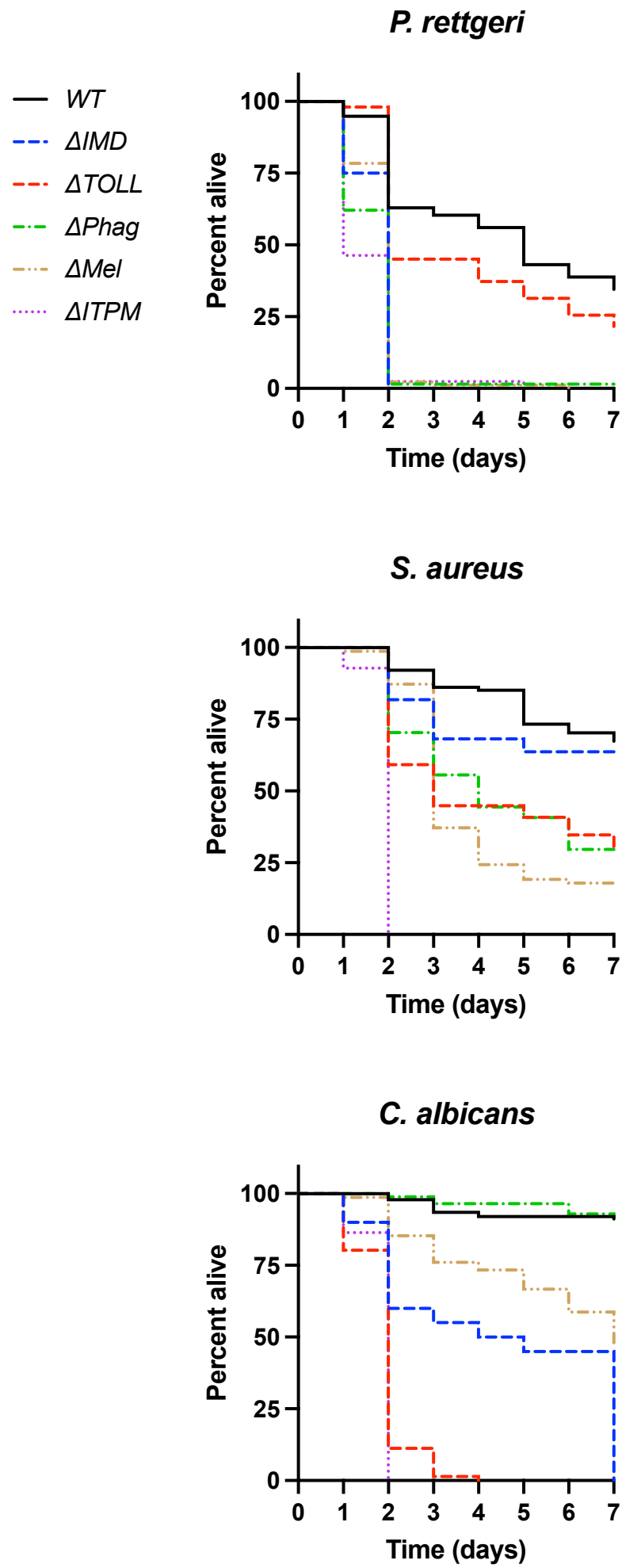

A
P. rettgeri
WT
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔITPM
Percent alive
Time (days)
CFU per fly
WT 24h
WT 12h
WT 2h
2h 6h 12h 18h 24h
WT
ΔIMD
ΔToll
ΔMel
ΔPhag
ΔITPM
B
S. aureus
C
C. albicans


A
P. rettgeri
B
S. aureus
C
C. albicans
PLUD
**
****
*
**
ns
*
**
**
2^{26}
2^{24}
2^{22}
2^{20}
2^{18}
2^{16}
2^{25}
2^{20}
2^{15}
2^{10}
2^{5}
WT
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔITPM

			Deficient pathway			
Drosophila lines	**Reference**	**Name**	**IMD**	**Toll**	**Phagocytosis**	**Melanization**
iso ; iso ; Rel^{E20}	Hedengren et al., 1999	Δ*IMD*	-	+	+	+
iso ; iso ; spz^{rm7}	Lemaitre et al., 1996	Δ*TOLL*	+	-	+	+
iso ; NimC1^{1} *; Eater*^{1}	Melcarne et al., 2019	Δ*Phag*	+	+	-	+
iso ; PPO1Δ, PPO2Δ; iso	Bingelli et al., 2014	Δ*Mel*	+	+	+	-
+ ; + ; *Rel*^{E20}, *spz*^{rm7}	this paper	Δ*IMD*, Δ*TOLL*	-	-	+	+
iso ; PPO1Δ, PPO2Δ ; NimC1^{1}*,Eater*^{1}	this paper	Δ*Phag*, Δ*Mel*	+	+	-	-
iso ; NimC1^{1} *; Eater*^{1}*, Rel*^{E20}	this paper	Δ*IMD*, Δ*Phag*	-	+	-	+
iso ; PPO1Δ PPO2Δ ; Rel^{E20}	this paper	Δ*IMD*, Δ*Mel*	-	+	+	-
iso ; PPO1Δ PPO2Δ ; spz^{rm7}	this paper	Δ*TOLL*, Δ*Mel*^{PPS}	+	-	+	-
iso Hay-psh^{Def} *; iso ; iso*	Dudzic et al., 2019	Δ*TOLL*, Δ*Mel*^{HP}	+	-	+	-
iso Hay-psh^{Def} *; NimC1*^{1} *; Eater*^{1} *Rel*^{E20}	this paper	Δ*ITPM*	-	-	-	-


P < 0.0001
P = 0.0006
Phagocytic Index (PI), WT = 100
200
150
100
50
0
iso WT
iso ΔPhag
+; ΔPhag


A
B
C
D
Unchallenged
Clean Injury
Percent alive (29°C)
Time (days)
100
50
0
0
5
10
WT
ΔAMP14
Bom^Δ55C
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔPhag, ΔMel
ΔTOLL, ΔMel^PPS
ΔTOLL, ΔMel^HP
ΔIMD, ΔMel
ΔIMD, ΔPhag
ΔIMD, ΔTOLL
ΔITPM


P. rettgeri
WT
ΔIMD
ΔTOLL
ΔPhag
ΔMel
ΔITPM
Percent alive
100
75
50
25
0
0 1 2 3 4 5 6 7
Time (days)
S. aureus
Percent alive
100
75
50
25
0
0 1 2 3 4 5 6 7
Time (days)
C. albicans
Percent alive
100
75
50
25
0
0 1 2 3 4 5 6 7
Time (days)