UNIVERSITY OF CALIFORNIA,
IRVINE

Mechanisms of innate immune memory in the fruit fly *Drosophila melanogaster*

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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RESEARCH EXPERIENCE

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PUBLICATIONS

"Drosophila immune priming to Enterococcus faecalis relies on immune tolerance rather than resistance"
K. Cabrera, D. Hoard, O. Gibson, D. Martinez, Z. Wunderlich (in revision) bioRxiv: https://www.biorxiv.org/content/10.1101/2022.07.20.500468v1

"Intraspecific Transcriptome Variation and Sex-Biased Expression in Anopheles arabiensis"
V. Jayaswal, C. Ndo, H. Ma, B.D. Clifton, M. Pombi, K. Cabrera, A. Cohuet, K. Mouline, A. Diabaté, R. Dabiré, D. Ayala, J.M. Ranz (2021) Genome Biology and Evolution 13(9): evab199.

RESEARCH PRESENTATIONS

"Mechanisms of Enterococcus faecalis-mediated immune priming in Drosophila melanogaster"
Biological Design Center Seminar – (2022) oral presentation

"Transcriptional Mechanisms Controlling Immune Priming in Drosophila melanogaster"
GSA Dros22 Conference – (2022) poster presentation

"Transcriptional Mechanisms Controlling Immune Priming in Drosophila melanogaster"
Boston Area Drosophila Meeting – (2021) oral presentation

"Transcriptional Mechanisms Controlling Immune Priming in Drosophila melanogaster"
GSA Dros22 Conference – (2022) poster presentation

"Transcriptional Mechanisms Controlling Immune Priming in Drosophila melanogaster"
Asilomar Chromatin and Epigenetics Conference – (2019) oral presentation

"Characterization of the Drosophila Innate Immune Epigenome Using ATAC-seq"
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- Aided large group discussion and led weekly small group discussions
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ABSTRACT OF THE DISSERTATION

Mechanisms of innate immune memory in the fruit fly *Drosophila melanogaster*

By

Kevin Cabrera

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2023

Professor Zeba Wunderlich, Chair

Insects, like any other organisms, are in constant contact with pathogenic biotic elements in their environment. Though they only possess an innate immune response (which is typically considered a non-specific and short-lived response to infection), immune memory from previous infections has been observed in insects. Innate immune priming increases an organism's survival of a second infection after an initial, non-lethal infection. We used *Drosophila melanogaster* and an insect-derived strain of *Enterococcus faecalis* to study transcriptional, physiological, and temporal control of priming. In contrast to work with other pathogens, the enhanced survival in primed animals does not correlate with decreased bacterial load, and further analysis shows that primed organisms tolerate, rather than resist infection. Using RNA-seq of immune tissues, we found many genes were up-regulated in only primed flies. In contrast, few genes continuously express throughout the experiment or more efficiently re-activate upon reinfection. Priming experiments in immune deficient mutants revealed Imd signaling is largely dispensable for responding to a single infection but needed to fully prime. The fly's innate immune response is plastic — differing in immune strategy, transcriptional program, and pathway use depending on

infection history. We explore this plasticity by varying infecting pathogens, re-infection times, and host genotype. Ability to prime against *Enterococcus faecalis* re-infection was dependent on host genotype and the rest time between exposures. Although there was no homologous priming with Serratia marcescens, heterologous priming between *E. faecalis* and *S. marcescens* was observed. Immune priming is not a static phenotype – it is highly dependent on assay and environmental conditions.

1: INTRODUCTION

1.1: INSECT INNATE IMMUNITY

Insects, like any other organisms, are in constant contact with potentially pathogenic biotic elements in their environment. These take the shape of bacteria, viruses, fungi, or parasitoid predators that pose a significant risk to an insect's survival. Given the near ubiquitous presence of insects across all biomes and their robust fitness across environments, it should come as no surprise that insects have developed successful strategies for fending off infection. Despite the lack of a canonical adaptive immune response (which evolved much later at 500 million years ago with gnathostomes) (Flajnik & Kasahara 2010), insects have successfully countered infection using only an innate immune system.

An innate immune response has been typically described as a non-specific, first line of defense. It is a deeply ancestral system of pathogen defense with high functional and genetic homology throughout the entire animal lineage (Kimbrell & Beutler 2001). For the purposes of this thesis, the fruit fly *Drosophila melanogaster* will be used as the model for centering discussions of innate immune response. This is an appropriate choice given not only the genetic tractability afforded from working in this system, but also given the extensive body of knowledge about its ability to respond to infection. It would be remiss to not mention the field of research that has studied the *Drosophila* innate immune response, culminating in the 2011 Nobel Prize in Physiology or Medicine, and which has been expanding ever since.

1.1.1: Molecular mechanisms of insect innate immune response

The *Drosophila* immune response is divided into two canonical signaling pathways: the Gram-positive and fungal-responsive Toll pathway, and the Gram-negative Immune-Deficient (IMD) pathway (Buchon, et al. 2014) (Figure 1.1). The Toll pathway is functionally homologous to the mammalian Toll-like pathway, and the IMD pathway functionally homologous to the TNF-α pathway. Both result in downstream activation of NF-κB factors that translocate to the nucleus of the cell to initiate transcription. Induction of both pathways leads to activation of cellular immunity as well as humoral immunity through expression of antimicrobial peptides (AMPs) as well as other immune effectors.

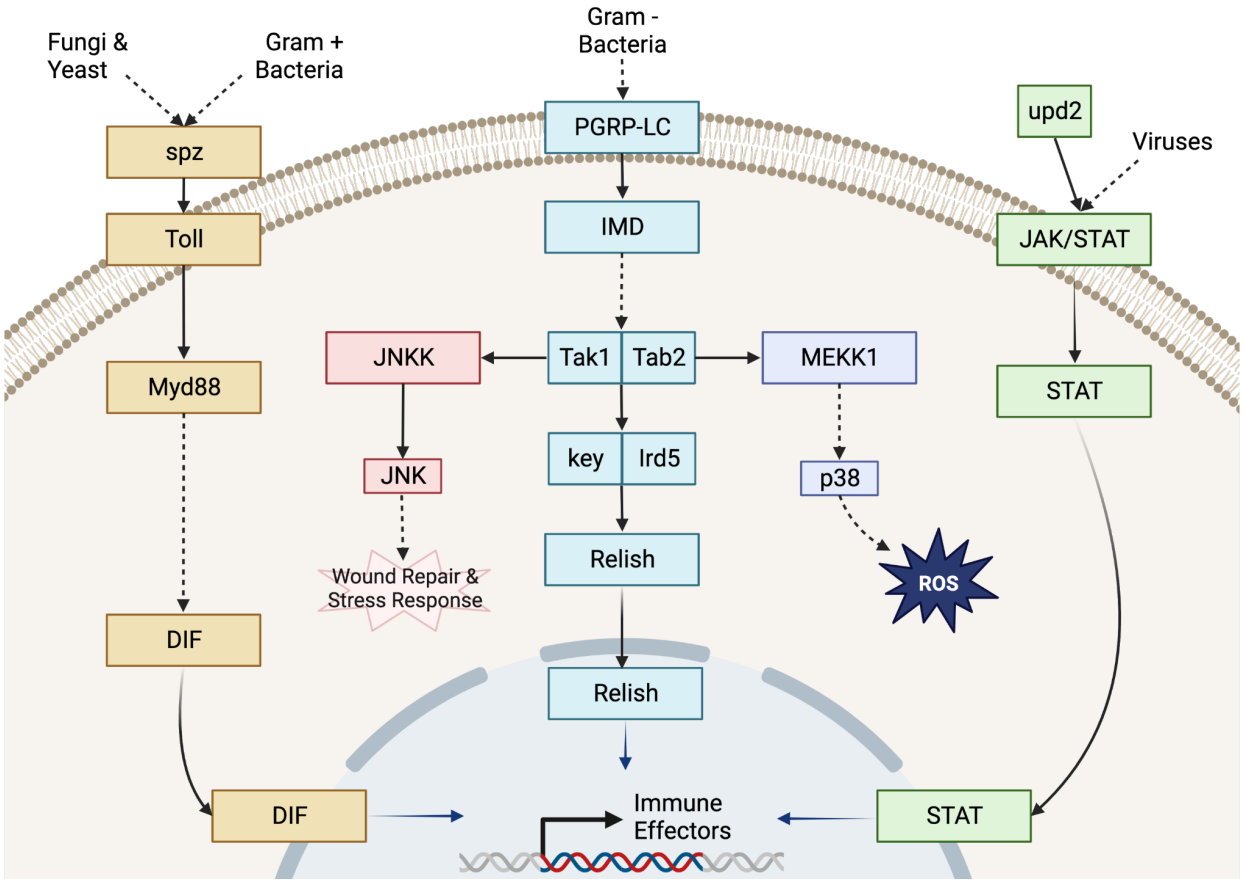

Figure 1.1: Molecular pathways involved in immune response in *D. melanogaster*

The Toll pathway is predominantly activated in response to infection with Gram-positive bacteria, filamentous fungi, lys-type peptidoglycan, or viruses. Extracellular recognition factors scavenge for pathogen virulence markers and activate protease cascades that result in the cleavage of the Toll receptor ligand Spätzle (Spz) by the Spätzle processing enzyme (SPE). Binding of Spz to the transmembrane Toll receptor causes formation of a cytoplasmic signaling complex of three death-domain containing proteins: Myd88, Tube, and Pelle. From this complex, Pelle subsequently phosphorylates the IkB factor Cactus. Under non-signaling conditions, Cactus is bound to the paralogs Dorsal or Dorsal-related immunity factor (DIF). Toll signaling shares the dual purpose of also organizing developmental dorso-ventral patterning. It differentially employs Dorsal during embryonic development versus DIF during immune response. Upon phosphorylation however, Cactus is degraded and Dorsal/DIF is able to freely translocate into the nucleus and initiate transcription of several Toll effector proteins (Valanne, et al. 2011). Several Toll-dependent AMPs have been identified through experiments that assayed their expression upon Toll pathway deletions. These inferred Toll-regulated AMPs include Drosomycin (*Drs*), Defensin (*Def*), Daisho 1&2 (*Dso1/2*), and Baramicin (*BaraA*) (Hanson, et al. 2021; Cohen, et al. 2020; Imler & Bulet, 2005; Lee, et al. 2001). Singular loss of these AMPs has been found to mildly affect survival against Gram-positive bacteria/fungi, whereas combined loss drastically affects survival suggesting they function in a synergistic fashion (Hanson, et al. 2019). Another major Toll effector family, the Bomanins, has been shown to be involved in resistance against fungi and Gram-positive bacteria (Clemmons, et al. 2015).

IMD response is activated by binding of DAP-type peptidoglycan from Gram-negative bacteria to peptidoglycan-recognition proteins (PGRPs) anchored to the cell surface. This induces formation of an intracellular protein complex of Imd, Fadd, and Dredd. This complex

cleaves Imd itself which then activates the Tab2/Tak1 complex. This complex phosphorylates the IKK complex that subsequently phosphorylates the NF-κB homologue Relish. Relish is then cleaved and translocates into the nucleus to activate immune effector gene expression through its TF activity (Myllymäki, et al. 2014). As with Toll signaling, several AMPs are induced by IMD signaling including Diptericins (*DptA/B*), Drosocin (*Dro*), Attacins (*AttA/B/C/D*), and Cecropins (*CecA1/2, CecB, CecC*) (Hanson, et al. 2019; Imler & Bulet, 2005). Metchnikowin can also be expressed by IMD as well as Toll, and responds to both Gram-positive and Gram-negative bacteria (Levashina, et al. 1998).

While useful in categorizing general responses to infection, this binary description falls short in portraying not only the myriad of other pathways that feed into and are affected by immune response, but also the high degree of molecular cross-talk between even these two pathways (Yu, et al. 2022). Viral infection, septic injury, heat-shock, and dehydration activate the JAK/STAT pathway via the pro-inflammatory cytokine Upd3. JAK-STAT activation in the fat body leads to expression of several pro-inflammatory cytokines and other stress-response proteins. IMD activation can lead to activation of the c-Jun N-terminal Kinases (JNK) stress-response pathway via bifurcation at the Tak1/Tab2 complex. JNK signaling is required for response to various stressors that induce cell damage (ex. reactive oxygen species, heat, irradiation, bacterial antigens, etc.) and repairing the tissues they disrupt (Tafesh-Edwards & Eleftherianos 2020). Ectopic and physiological activation of Toll signaling induces ROS-mediated apoptosis via the JNK pathway (Li, et al. 2020). Sterile wound repair alone activates Toll, ERK, and JNK signaling to mediate epithelial barrier repair downstream of hydrogen peroxide and protease production from the melanization response (Capilla, et al. 2017). In an example of Toll/IMD cross-reactivity, a Toll extracellular recognition factor, PGRP-SD, is able

to bind Gram-negative DAP-type peptidoglycans and induce Toll signaling (Leone, et al. 2008). While AMPs are predominantly expressed by either IMD or Toll, expression of a subset of AMPs can be induced by the other pathway to a lesser extent (ex. *Mtk*, *Dro*, *AttA*, *CecA1*). In short, immune activation orchestrates a large-scale molecular response that is not limited to the canonical immune signaling of the Toll and IMD pathways.

1.1.2: Strategies for infection response in insects

While the molecular pathways for infection response have been intensely studied, there has been considerable interest in the field into how these pathways are activated or repressed in response to an infection. When faced with a pathogen, an organism has two choices of how to confront it. The first option is to actively resist the pathogen with the intent of eliminating it. This involves mounting a coordinated attack against the pathogen through extensive transcription and coordination of immune effectors. Alternatively, an organism can tolerate the infection below some lethal threshold. This is accomplished by producing some low level of immune effectors that maintains the pathogen load at a level that does not outright kill the organism, but does not fully clear the infection (Ayres & Schneider 2012). Tolerance is used as a way to mitigate the sequelae of immune hyper-activity that could kill the organism in the process of attempting to eliminate a pathogen.

A study by Chambers, et al. 2019 found that flies infected with *Providencia rettgeri*, *S. marcescens*, and *E. faecalis* never cleared the infection. In fact, they showed a strong tolerance phenotype in that the flies kept producing AMPs, and even had non-specific protection against re-infection. Dampening of JAK-STAT activation early in viral immune response was also found to prevent immunopathology while maintaining a manageable viral load that did not increase

lethality (Merkling, et al. 2015). Chronic infections also change the ability to actively tolerate or resist an infection. A study by Wuktich, et al. 2023 showed that chronic infections with *E. faecalis* and the Gram-negative *Serratia marcescens* caused an increase in both tolerance and resistance of a secondary *P. rettgeri* infection.

It has become more appreciated that there is a metabolic trade off to resisting an infection. The extensive transcriptional burden, intricate signaling coordination, and mobilization of cellular action carries with it a high metabolic price. As such, survival depends not only on the ability to mitigate the harmful effects of a pathogen, but also on correctly managing the energetic reserves needed to combat foreign invaders (Lazzaro & Tate 2022). Multiple studies have shown that insects modulate their metabolic activity to compliment an immune response. Infection with *Streptococcus pneumoniae* in *D. melanogaster* causes hemocytes to activate aerobic glycolysis and rapidly deplete glucose stores (Krejčová, et al. 2019). Temporally dense assaying of transcription after LPS stimulation in *D. melanogaster* showed an initially acute up-regulation of immune response genes coupled with down-regulation of metabolic-responsive genes (Schlamp, et al. 2021). Though metabolic gene expression returned to pre-injection baselines 12-24 hours after stimulation, immune genes continued up-regulated. This antagonistic expression pattern early on suggests a large trade-off at the beginning of an infection. It has also been shown that Toll signaling induction during an immune response shifts anabolic lipid metabolism towards phospholipid synthesis and endoplasmic reticulum expansion for AMP production (Martinez, et al. 2020).

1.1.3: The fat body and hemocytes are the central immune organs in *D. melanogaster*

Although immune response results from the integration of many molecular actions throughout the body, there are two main organs that coordinate immunity in the fly: the fat body and the hemocytes. The fat body is an adipocyte-heavy organ functionally homologous to the mammalian liver that extends along the dorsal side of the fly and predominantly located in the abdomen, but extending as far as the head. Adult fat body tissue is functionally and morphologically distinct from the larval fat body, which it completely replaces by four days after eclosion. It functions as the control center for metabolism and humoral immune response as the main site of AMP production within the adult fly (Hoffmann & Reichhart 2002). Practically, fat bodies are isolated via dissection by removal of the digestive tract and gonads from the abdomen, and collecting the abdominal filet that contains fat body cells attached to the inner wall of the exoskeleton (Krupp & Levine 2010; Ramirez-Corona, et al. 2021).

Hemocytes are the circulating and secretory blood cell equivalent in invertebrates. These cells make up the bulk component of the hemolymph: the central component of the invertebrate open circulatory system that circulates through the animal interior and directly interfaces with tissues. Antimicrobial activity by hemocytes constitutes the cellular immune response. While their hemocyte subtypes and nomenclature vary between insect species, the three subtypes in *D. melanogaster* are the plasmatocytes, lamellocytes, and crystal cells. About 95% of adult hemocytes are made up of the phagocytic plasmatocytes. Like mammalian macrophages, plasmatocytes are functionally programmed into distinct subtypes during embryonic and larval development, but lose most of the subtype distinction into adulthood (Coates, et al. 2021) They are responsible for removal of cell debris, pathogen elimination upon infection, and cell signaling. Predation by parasitoid wasps induces a cellular response by differentiating

plasmatocytes into lamellocytes to encapsulate and kill the deposited wasp egg in developing larvae (Zettervall, et al. 2004). Crystal cells are responsible for the melanization response wherein pathogens are isolated in a gel-like matrix and eliminated by several antimicrobial peptides. Lamellocytes and crystal cells are not typically found in adult *D. melanogaster*.

1.2: IMMUNE PRIMING REEVALUATES THE NON-SPECIFICITY OF AN INNATE IMMUNE RESPONSE

Innate immunity has historically been considered a non-specific, first line of defense. However, it has now accepted that despite lacking canonical adaptive machinery, innate immune systems are able to retain some level of immune memory. In so-called 'immune priming', the cells of the innate immune system are able to recall a past immune response and modulate their activity when presented with a secondary challenge (Figure 1.2). The functional consequence of priming is usually defined as an increased chance of survival in primed organisms after secondary infection as opposed to organisms that did not have a prior immune challenge.

Many studies have assessed whether immune priming is specific (i.e. does priming with a certain stimulus only confer protection to a specific secondary pathogen), or whether protection is conferred in most cases where there has been previous exposure to a pathogen or stressor. The details for defining immune memory are not firmly established across phyla or even across species. This ambiguity is part of the motivation behind delineating terms like 'immune priming' for insects, and 'trained immunity' in mammals (discussed in detail below). Insect immune priming and its classification as true immune memory is a conversation that is still in progress. Broadly speaking, insect immune priming is unspecific (Moret & Siva-Jothy 2003; Chambers, et al. 2019; Ben-Ami, et al. 2020). However, key examples of specificity have emerged that

demonstrate that priming will only occur in certain pathogen/stimuli combinations, host genotypes, and temporal re-infection schema (Cooper & Eleftherianos 2017; Medina-Gomez, et al. 2018; Ferro, et al. 2019; Hidalgo & Armitage 2022). It is currently hypothesized that the specificity of immune memory in insects is related to the level of risk or repeated exposure a pathogen presents to that particular host species (Dhinaut, et al. 2018).

The central focus of Chapter 2 will be understanding the transcriptional mechanisms that control immune priming with the Gram-positive bacteria *Enterococcus faecalis*. The details of what sort of primary immune challenge elicits a primed response, the pathogen specificity of priming, and the temporal dynamics of priming are all points of study and will also be partially explored in this thesis in Chapter 3.

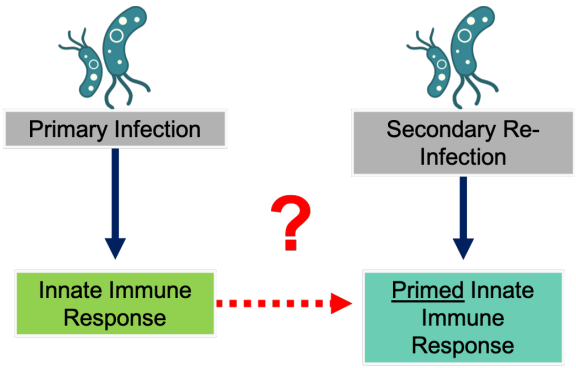

Figure 1.2: Schema of a primed immune response

1.2.1: Arthropod immune priming

Evidence of innate immune memory has been found in diverse organisms, including both plants and animals (Cooper & Ton 2022). This near ubiquitous presence of innate immune memory (especially as compared to the relatively recent emergence of adaptive immunity in jawed fishes) suggests that it is an evolutionary advantageous trait necessary for the fitness of a broad range of organisms. While the focus of this thesis will be on characterizing innate immune

priming in *D. melanogaster*, it is worth discussing the trait in closely related species within the phylum arthropoda as well as in more evolutionarily distant classes that have adaptive immunity in addition to innate immune memory, such as mammalia. In this way, we can put the response in flies within evolutionary context as well as compare and contrast their version of immune priming to that of other animals.

There are several parameters on which immune priming can be characterized in arthropods. As mentioned before, the main way to define immune priming is through comparing survival after re-infection in animals previously exposed to a stimulus versus not. Trans-generational inheritance of enhanced survival has also been used to characterize immune priming in arthropods, although it will not be explored in this thesis (Tetreau, et al. 2019). Tracking pathogen load across infection time complements survival data by indicating whether enhanced survival results from more efficient clearance of the pathogen or not. Tracking the numbers of phagocytic cells and their relative phagocytic ability also helps delineate whether increased survival rates are due to an expanding pool of hemocytes, or due to reprogramming of the already available pool of phagocytes without expansion. Assaying of transcriptomic response to singular and repeated infection also highlights the molecular basis that underlies the large-scale physiological effects of immune priming.

Three functional hypotheses have been proposed to explain the transcriptional basis of immune priming in arthropods (Melillo, et al. 2018; Pradeu & Du Pasquier 2018; Coustau, et al. 2016). The first hypothesis of immune loitering posits that immune effectors are expressed during the initial immune response, but do not get degraded before the secondary immune challenge (Figure 1.3A). In this way, effectors loiter into the secondary challenge and can continue contributing to an immune response without the lag time of having to initiate

production of said effectors. The second hypothesis of a potentiated recall response shows that effectors do get cleared before the secondary challenge, but are expressed in a potentiated manner upon secondary challenge (Figure 1.3B). When re-expressed, these effectors can either more quickly initiate expression than when expressed in the primary immune response, and/or they can express at higher levels upon secondary challenge. Finally, it could be that immune priming elicits a qualitatively different immune response when compared to the primary immune response (Figure 1.3C). This would mean expression of a completely different set of effectors upon re-infection that qualitatively change the nature of the immune response. While these three explanations are presented as separate hypotheses, it is most likely that these three mechanisms are all contributing, to varying extents, to what is observed as immune priming.

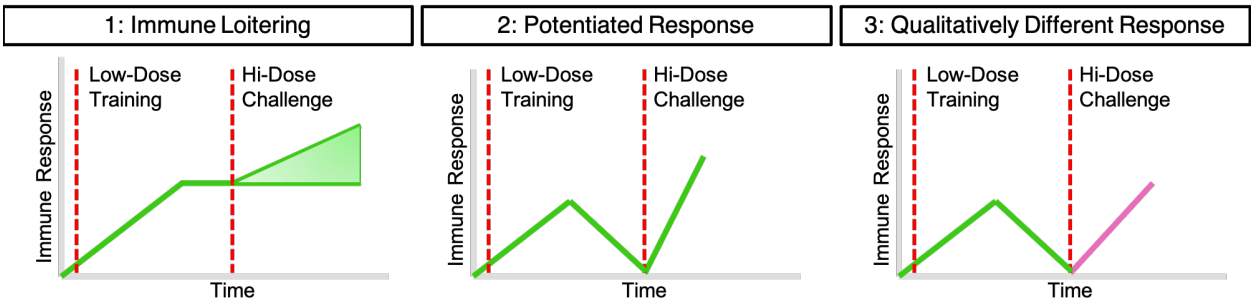

Figure 1.3: Graphical representation of the three hypotheses explaining arthropod immune priming.

One of the common trends in insect immune priming is the increase in numbers of circulating hemocytes during priming. Adult *Anopheles gambiae* mosquitoes increase and maintain the number of granulocytes upon *Plasmodium* infection (Rodrigues, et al. 2010). *Galleria mellonella* (greater wax moth) larvae exposed to low doses of the fungus *Aspergillus fumigatus* caused increased numbers of circulating hemocytes (Fallon, et al. 2011). Infection of a

queen honeybee *Apis mellifera* with the bacterium *Paenibacillus larvae* caused prohemocyte-hemocyte differentiation in its larvae, showing the extension of this trend into trans-generational immune priming (Hernandez Lopez, et al. 2014). In contrast, *D. melanogaster* increases phagocytic activity of plasmatocytes in a primed response, while not necessarily increasing the overall number of circulating hemocytes (Pham, et al. 2007). While immune response is fairly conserved across insects, it does always come with the caveat that there is species-to-species differences. The role of phagocytes as it relates to priming with other pathogens as well as how their transcriptional activity changes in response to priming is yet to be explored.

1.2.2: *Drosophila melanogaster* as a model for understanding immune priming

There have been a bevy of studies assaying immune priming in *D. melanogaster*. However, differences in experimental techniques have made consolidating findings into a singular mechanism somewhat challenging. This leads to experiments that test infection/reinfection with the same pathogen versus infection/reinfection with two different pathogens; on top of experiments that prime using non-virulent immune stimuli all across varying re-infection time spans. The commonality between all these studies then is that previous exposure to a stimuli (whether it be a *de facto* live pathogen, a heat-killed pathogen, or a non-virulent immune stimulus) resulted in increased survival probability against a secondary infection and were thus identified as instances of immune priming.

The first major study to focus on immune priming in *D. melanogaster* assayed double-infections with the Gram-positive bacteria *Streptococcus pneumoniae*. It found that priming was dependent on Toll signaling and phagocytosis, but Toll activation alone was not enough to cause priming. They also found that priming with *S. pneumoniae* and the fungus *Beauveria bassiana*

elicited self-protection, but priming with *Listeria monocytogenes*, *Mycobacterium marinum*, *Salmonella typhimurium* did not elicit self- or cross-protection (Pham, et al. 2007).

Another study found that sterile wounding causes expression of reactive oxygen species (ROS) genes, expression of Toll AMPs, and elevated H_2O_2 levels. Sterile wounding and re-injecting two days later with *E. faecalis* increases survival compared to no wounding control (Chakrabarti, et. al 2020). Induction of IMD response with LPS caused expression of Imd-regulated genes for up to five days after exposure (Schlamp, et al. 2021). A study focusing on immune tolerance found that *P. rettgeri* and *S. marcescens* priming confers significant protection when re-infecting with all three bacteria. *E. faecalis* priming gave significant protection against *S. marcescens* and *P. rettgeri* re-infection, but not significant protection against an *E. faecalis* re-infection (Chambers, et al. 2019). Current research also suggests that while trans-generational priming is observed in other arthropod species, it is not observed specifically in *D. melanogaster* (Radhika & Lazzaro, 2023). This highlights the necessity to consider the differences in experimental approach when considering results across multiple priming experiments in the same species.

1.2.3: Comparing insect immune priming, and mammalian immune training

There has been a lot of interest recently in immune memory of mammalian innate immune cells (namely macrophages, monocytes, and natural killer cells). While the morphology and functions of mammalian innate immune cells differs from that of insects, the large degree of functional homology between the two systems means that it is worth comparing immune memory between classes.

Firstly, there are several key differences in the nomenclature used in both fields. The phenomenon of increasing the rate and intensity of an immune response against a secondary pathogen after primary response is termed 'trained immunity' (Netea, et al. 2016). This is practically defined through increased expression of inflammatory cytokines mediated through epigenetic and metabolic reprogramming. Functionally, this is most similar to the potentiated response hypothesis proposed for explaining arthropod immune priming (Figure 1.3). While this definition of trained immunity does not explicitly cover the possibility of a qualitatively different response after re-infection, a comparison can be drawn between the two.

The effect of heterologous protection conferred from vaccination with live pathogens has been recently attributed to trained immunity. Bacillus Calmette–Guérin (BCG) vaccination against tuberculosis was found to provide non-specific protection against other bacterial and viral infections, predicted overall better respiratory tract disease prognosis, and reduced mortality rates in children via trained immunity (Blok, et al. 2015; Arts, et al. 2018). Short-term, global chromatin remodeling shifting transcription towards a mature immune inflammatory effector response has also been reported in both monocytes and natural killer cells collected from COVID-19 patients (You, et al. 2021). However, while monocytes continued to downregulate interferon signaling & maintain some DNA methylation changes in convalescing COVID-19 patients, it did not differentially affect cytokine production (accompanied by a higher homeostatic release of IL-1Ra and IFN-γ) in subsequent bacterial, fungal, and viral infection (Liu, Kilic, & Li, et al. 2022). This highlights that while there was an initial immune training phenotype and epigenetic remodeling associated with Sars-Cov2 infection, the effect was not long-lived.

Phenotypes of trained immunity typically last 3 months to a year, although live vaccine-mediated training has been shown to last upwards of five years (Netea, et al. 2020). However, there has been some evidence for trans-generational BCG-mediated priming in mammals (Berendsen, et al. 2020), as has been shown in insects (Tetreau, et al. 2019). While there are mechanistic and physiological similarities between mammalian trained immunity and insect immune priming, it is still not known whether both mechanisms result from convergent or divergent evolutionary histories.

'Immune priming', as defined in mammalian immunity circles, describes the re-activation of immune response without the return to basal immune response post primary infection. This is most closely related to what has been called 'immune loitering' in arthropods as they both output as additive/synergistic effects in re-infection. A similar tolerance phenotype has been described in mammals as well. In LPS tolerance, repeated exposure of high doses of lipopolysaccharide (LPS) or other Toll receptor ligands leads to a reduced inflammatory response and immune paralysis in macrophages after a secondary stimulus (Divangahi, et al. 2020). Additionally, stimuli-induced differentiation of immune cells has been delineated from 'trained immunity'. This is similar to the increased pro-hemocyte differentiation characteristic of most insect immune priming (*D. melanogaster* excluded; discussed in Section 1.2.1). While similar molecular mechanisms may underlie these different responses in both mammals and insects (i.e. they may all exhibit some level of epigenetic and metabolic reprogramming), what differentiates them is the targets and extents to which they are altered.

1.2.4: Epigenetics and immune priming

One of the central questions in the field is how priming-induced transcription is encoded. Research in mammalian systems has shown that innate immune memory is epigenetically encoded through deposition of activating histone marks throughout the initial infection that then primes for gene expression during a subsequent infection (van der Heijden, et al. 2018). In macrophages, NF-kB that does not oscillate between going in and out of the nucleus most efficiently opens up chromatin so it can displace nucleosomes and cause H3K4me1 deposition at latent immune enhancers (Cheng & Ohta, et al. 2021). Human monocytes have been shown to drive global epigenetic re-organization associated with a secondary infection through shifts in metabolic activity caused by the initial "training" infection (Fanucchi, et al. 2021). In these instances, metabolites that are connected to histone modification are differentially allocated in trained individuals. Innate immune memory-based metabolic reprogramming was also observed in *Caenorhabditis elegans* which sparked the conversation on whether it was an evolutionary ancestral trait common to any related organisms with an innate immune system (Penkov, et al. 2019).

As in mammalian monocytes, epigenetic reprogramming has been implied to play a role in maintenance of a primed immune response in insects. In the mosquito *A. gambiae*, enhanced immune response was associated with changes in histone acetylation mediated by the histone acetyltransferase AgTip60 (Gomes, at al. 2021). Introduction of *Escherichia coli* and *Serratia entomophila* into tobacco hornworms (*Manduca sexta*) caused global changes in DNA methylation and histone acetylation. Bacterial-mediated epigenetic changes were maintained trans-generationally in an example of TGIP (Gegner, et al. 2019). It has also been posited that part of a pathogen's attack strategy involves epigenetic suppression of host immune response.

Wasp egg deposition in the moth *Galleria mellonella* caused transient loss of DNA methylation, down-regulation of histone acetylation/deacetylation gene expression, changes in histone acetylation, and changes in microRNA expression (Özbek, et al. 2020). Specific comparisons between mammalian and invertebrate epigenetic regulation of innate immune memory will delineate whether there is an evolutionarily common mechanism between the two groups, or whether it is a case of convergently evolved traits.

1.3: RATIONALE

In light of the current research, several experiments have been conducted to survey mechanistic aspects of immune priming in *D. melanogaster*. We begin by describing the ability to mount a primed immune response using *E. faecalis* re-infection as a model. In this fashion, we assay the survival phenotype, bacterial load dynamics, and transcriptional response characteristic of an *E. faecalis*-based primed immune response (Chapter 2). We then build upon this foundation by investigating how altering host genotype, temporal dynamics of infection, and pathogen identity affects the ability to mount a primed immune response (Chapter 3). In an effort to create more sophisticated tools to assay immune response as a whole on the fly, we also focus on molecular tools that were developed in the lab to selectively label immune tissues in vivo for dissection-free isolation.

2: *DROSOPHILA* IMMUNE PRIMING TO *ENTEROCOCCUS FAECALIS* RELIES ON IMMUNE TOLERANCE RATHER THAN RESISTANCE

The contents of this chapter are currently under review for publication in the journal PLoS Pathogens and is available on BioRxiv:
(https://www.biorxiv.org/content/10.1101/2022.07.20.500468v1)

2.1: ABSTRACT

Innate immune priming increases an organism's survival of a second infection after an initial, non-lethal infection. We used *Drosophila melanogaster* and an insect-derived strain of *Enterococcus faecalis* to study transcriptional control of priming. In contrast to work with other pathogens, the enhanced survival in primed animals does not correlate with decreased bacterial load, and further analysis shows that primed organisms tolerate, rather than resist infection. Using RNA-seq of immune tissues, we found many genes were upregulated in only primed flies. In contrast, few genes continuously express throughout the experiment or more efficiently re-activate upon reinfection. Priming experiments in immune deficient mutants revealed Imd signaling is largely dispensable for responding to a single infection but needed to fully prime; while Myd88-dependent Toll signaling is required to respond to a single infection, but dispensable for priming. The fly's innate immune response is plastic — differing in immune strategy, transcriptional program, and pathway use depending on infection history.

2.2: INTRODUCTION

The fruit fly *Drosophila melanogaster* inhabits environments rich in bacteria, fungi, and viruses. The fly has to mitigate these pathogens to survive. To this end, it has evolved a tightly controlled innate immune response. It has long been appreciated that the fly immune pathways can distinguish between Gram-positive bacteria and fungi versus Gram-negative bacteria (Buchon, et al. 2014). Recent findings have elaborated on these models by showing specificity within Gram-classifications, cross-talk between the two individual pathways, and a coordination between tissues (Kleino, et al. 2014; Lin, et al. 2020; Hanson, et al. 2019).

Among these refined characteristics is the potential for immune memory in the innate immune system. While flies lack the canonical antibody-mediated immune memory of the adaptive immune response, an initial non-lethal infection can sometimes promote survival of a subsequent infection. This phenomenon, termed immune priming, has been observed in evolutionarily distant organisms such as plants (Cooper & Ton 2022), multiple arthropod species (Milutinović, et al. 2016), and mammals (Netea, et al. 2016; Divangahi, et al. 2020). The fact that this mechanism is present in animals that have an adaptive response hints at its importance in organismal fitness.

Despite immune priming's effect on survival, the underlying mechanism controlling it in flies is not completely understood. Three hypotheses have been proposed to explain the physiological effects of priming (Cooper & Eleftherianos 2017; Coutasu, Kurtz, Moret 2016). The first is that there is a qualitatively different response, e.g. a difference in the identity of the effectors produced or cellular processes, between primed versus non-primed insects, leading to a more effective response. A second hypothesis is that insects will initiate an immune response during priming, but will re-initiate the same immune function in a potentiated manner upon

reinfection. This is most similar to the phenomenon of what has been observed in mammalian trained immunity (Divangahi, et al 2020). Lastly, immune effectors created during the initial immune response may be persistently expressed, eliminating the lag time in initiating effector production. Since flies can harbor low-level chronic infections instead of completely clearing them (Duneau, et al. 2017; Chambers, et al. 2019), these chronic infections may contribute to immune priming by providing a consistent mild stimulus. Priming may be driven by a combination of these three mechanisms. Delineating the relative contributions of each may not only reveal the drivers of infection survival, but may also suggest epigenetic mechanisms of gene regulation and tradeoffs between the immune response and other biological processes.

Drosophila is a good model for dissecting the mechanisms driving immune priming due to its genetic tractability, extensively characterized innate immune pathways, and its homology to mammalian innate immune pathways. There has been extensive characterization of the fly's transcriptional response to a variety of bacteria (Troha, et al. 2018; Schlamp, et al. 2021; De Gregorio, et al. 2002) and the progression of bacterial load during infection with different bacteria or in different host genotypes (Duneau, et al. 2017). Studies of priming have revealed the key role of phagocytosis. Blocking phagocytosis in adults decreases priming with the Gram-positive bacterium *Streptococcus pneumoniae* (Pham, et al. 2007). Blocking developmental phagocytosis of apoptotic debris also makes larvae more susceptible to bacterial infection (Weavers, et al. 2016). In addition, the production of reactive oxygen species as a result of wounding contributes to immune priming with the Gram-positive bacterium *Enterococcus faecalis* (Chakrabarti & Visweswariah 2020). These findings lay the foundation for testing the mechanistic hypotheses that underlie immune priming.

In this study, we present a multifaceted approach to understand immune priming in the fly using an *E. faecalis* reinfection model. *E. faecalis*, a Gram-positive, naturally occurring pathogen of the fly, has been previously used to induce an immune response with dose-dependent lethality. We characterize not only the physiological response to priming by way of survival and bacterial load to immune priming, but also the transcriptional response that underlies the physiology. By assaying transcription separately in both the hemocytes and fat body, we explore the organ-specific program that mounts a more effective primed immune response.

2.3: RESULTS

2.3.1: *E. faecalis* priming increases survival after re-infection

To determine whether we could elicit a priming response in flies, we needed to find appropriate priming and lethal doses. For these experiments, 4-day old male Oregon-R (OrR) flies were infected with a strain of the Gram-positive bacteria *Enterococcus faecalis* originally isolated from wild-caught *D. melanogaster* (Figure 2.1A) (Lazzaro, et al. 2006). Survival was scored as the hazard ratio (HR) of the bacterial-infected flies against a PBS-injected control; a HR > 1 indicates worse survival of the experimental sample compared to the control. The HR also gives a quantitative summary of the survival curve – higher the HR, the more quickly the animals died. Initial infection with *E. faecalis* showed dose-dependent survival (Figure 1B; *Efae* Low Dose vs. PBS HR = 1.4 [95% CI 0.96-2.1], *Efae* High Dose vs PBS HR = 5.7 [3.9-8.3]). Flies infected with a dose of ~30,000 CFU/fly (*Efae* High Dose) gradually died off, with more than fifty percent of flies dying by day 2, making it a practical choice for representing a lethal dose. Flies injected with a lower dose of ~3,000 CFU/fly (*Efae* Low Dose) had survival

comparable to those injected with PBS, with a HR not significantly different from 1, indicating that death was largely due to the injection process itself, rather than from bacterial challenge.

To model re-infection, flies were initially injected either with a low bacterial dose (i.e. *Efae*-primed flies) or a negative control of PBS (i.e. Mock-primed flies) (Figure 2.1A). After resting for seven days, flies were re-injected with a high dose of *E. faecalis* and assayed. Seven days was chosen as the priming interval because we found that flies had gained enhanced re-infection survival from priming (Supplementary Figure 2.1A), reached a stable chronic bacterial load (Figure 2.2A), and survived in high enough numbers to practically collect for re-infection. The median survival time after re-injection was significantly increased from Mock-primed flies (1 day) to *Efae*-primed flies (4 days) (Figure 2.1C). We define priming as an increase in survival in *Efae*-primed flies compared to Mock-primed flies. Quantitatively, we assessed priming by comparing *Efae*-primed to Mock-primed survival using the HR; priming is indicated by a HR that is significantly less than 1 (*Efae*-Primed vs. Mock-Primed HR = 0.29 [0.20-0.41]). Though there was a decrease in survival from double sterile wounding compared to a single sterile wound with PBS (Supplementary Figure 2.1B), *Efae*-primed flies had survival comparable to this double-PBS injected baseline. We can again use the HR to define this "full" priming – when the *Efae*-primed flies survive as well as the double-PBS control, this results in a HR that is not different from 1 (*Efae*-Primed vs. PBS/PBS HR = 0.75 [0.53-1.1]). In fact, *Efae*-primed flies not only survived as well as the PBS/PBS control, but also showed improved survival when compared to single, High Dose-infected flies (Supplementary Figure 2.1C).

To see what bacterial signals are required for priming, we attempted to prime flies with heat-killed *E. faecalis*, which retains its signaling-responsive components but lacks any additional virulence factors (Itoh, et al. 2012; Adams, et al. 2010). This experiment resulted in a

more moderate increase in survival rate compared to live bacteria priming (Figure 2.1D, HK-*Efae*-Primed vs. Mock-Primed HR = 0.52 [0.37-0.74]). As can be seen by comparing the HK-*Efae*-Primed survival curve to the PBS/PBS survival curve, these animals do not achieve "full" priming as is the case with live bacteria. This implies some level of priming is conferred simply through bacterial sensing, but that the effect is not as robust as when the fly is exposed to the live microbe. This may either be because the live microbe produces other virulence factors or damage that is needed for priming or because the heat-killed microbe's products are cleared too quickly to create an equally strong priming response.

To compare *E. faecalis* priming to the priming described for *Streptococcus pneumoniae*, which was dependent on phagocytosis (Pham, et al. 2007), we disrupted phagocytosis in two ways. We first performed the double injections in an *eater* mutant background (Bretscher, et al. 2015). The hemocytes in these flies are unable to carry out bacterial phagocytosis and have cell adhesion defects in the larva but can still mount a full Toll and Imd immune response (Kocks, et al. 2005). By comparing the *Efae*-primed to Mock-primed flies, we can observe a modest amount of immune priming, with a median survival time of 3 days and 1 day, respectively (Figure 2.1E). However, the *Efae*-primed flies have a shorter median survival time than the PBS/PBS controls. This trend is mirrored by the HR comparing *eater Efae*-primed flies to the PBS/PBS control, which is greater than 1 (HR = 3.8 [2.3-6.4], Supplementary Table 2.1), indicating that the mutants are unable to achieve full priming. An orthogonal method of assessing the role of phagocytosis in priming – blocking phagocytosis with beads during the initial *E. faecalis* infection in OrR flies as was done previously (Pham, et al. 2007) – caused a complete loss of priming ability (Figure 2.1F). Differences in the intensity of priming loss between assays may be

due to genetic background effects. Together, this indicates that phagocytosis is needed to fully prime.

2.3.2: Priming increases tolerance to *E. faecalis*

To measure the infection dynamics underlying both the un-primed and primed response to *E. faecalis*, we tracked bacterial load throughout the course of the infection. Infected flies were collected at 24 hour intervals after injection, homogenized, and plated in a serial dilution. As a baseline, we followed bacterial load in flies solely injected with either a high (~30,000 CFU/fly) or low dose (~3,000 CFU/fly) of *E. faecalis* (Figure 2.2A). By day 2 after injection, the bacterial loads in flies infected with a high dose were generally above 100,000 CFU/fly. This indicates that without priming, the bacterial load in flies infected with a lethal dose increases to a high plateau. In contrast, by day 1 the distribution of bacterial loads in flies initially infected with a low dose was bimodal, consistent with what has been previously reported (Duneau, et al. 2017). This suggests a subset of flies were more effectively resisting the infection and attempting to clear it, while another subset tolerated a relatively high bacterial load. The data from the low dose flies indicate two things. First, even a low dose of *E. faecalis* is not completely eliminated from the animals. Second, upon reinfection, there are likely two distinct populations of flies, harboring either a relatively high or low bacterial burden, which could alter their capability to survive a subsequent infection.

We then tested the relationship between bacterial burden and the enhanced survival seen in primed flies. Flies that are primed could increase their survival by either more efficiently clearing the infection or more effectively tolerating a chronic bacterial burden. When looking at bacterial load in double-injected flies, there was no significant difference between Mock-primed

and *Efae*-primed cohorts across the time course (Kruskal-Wallis rank sum test: $p = 0.26$) (Figure 2.2B). Despite their significant differences in survival (Figure 2.1C), this does not correlate with a difference in the bacterial load between the two conditions, indicating that the improved survival of *Efae*-primed flies relative to the Mock-primed flies is likely due to tolerance, not resistance. To further confirm that bacterial tolerance is driving the survival of *Efae*-primed flies, we also measured the bacterial load upon death (BLUD; Duneau, et al. 2017) for double-injected flies. The higher an animal's BLUD, the higher its tolerance for a particular microbe. We found that *Efae*-primed flies harbored a significantly higher bacterial burden at the time of death (Figure 2.2C). This experiment further supports the idea that primed flies are able to tolerate a higher bacterial load than Mock-primed flies before they succumb to an infection.

2.3.3: Fat bodies show priming-specific transcription

To correlate increased survival in primed flies with transcriptional response, we measured gene expression in the fat body using RNA-seq. The fly fat body is a liver-like tissue responsible for driving an extensive transcriptional program in response to bacterial infections (DiAngelo, et al. 2009; Dionne 2014). As in previous experiments, flies were injected either singly or twice, with samples collected 24 hours after each injection (Figure 2.3A; Supplementary Table 2.2). To identify genes differentially expressed in response to each injection, we performed differential gene expression analysis against a non-injected, age-matched control. In this way, we generated four lists of up-regulated genes to compare – those upregulated in the animals with a single low dose infection, a single high dose infection, a mock-priming protocol, or a *Efae*-priming protocol. Genes that were differentially up-regulated only, for example, in *Efae*-primed flies were identified as "priming-specific". As a comparison to prior work, we analyzed the

expression profiles of a previously published list of "core" immune genes in our samples and found a subset was induced upon infection in our samples (Supplementary Figure 2.2A) (Troha, et al. 2018).

The comparison of fat body transcription across conditions showed a high amount of *Efae* primed-specific and Mock-primed specific upregulation (149 genes & 408 genes, respectively, using an FDR cutoff of 0.05) (Figure 2.3B & C, full list for all conditions and overlap in Supplementary Table 2.3). Only a small fraction of these genes has been previously annotated with immune functions (19 *Efae*-Primed genes, ~13%; 15 Mock-Primed genes, ~4%) (Ramirez-Corona, et al. 2021; Troha, et al. 2018), although gene ontology (GO) analysis indicated immune response as one of the highest enriched terms (Supplementary Figure 2.2B, top). Mock-primed specific GO term enrichment indicated response to stimuli, but also included genes involved specifically in response to mechanical stimuli and post-transcriptional gene regulation (Supplementary Figure 2.2B bottom & Supplementary Table 2.3).

To delineate pathways whose component genes were upregulated in *Efae*-primed fat body versus Mock-primed fat body transcriptomes, we applied gene set enrichment analysis (GSEA) on the full transcriptome for both conditions. GSEA is an approach that looks for the coordinated up- or down-regulation of a set of genes involved in a common pathway or function. Since it uses all the transcriptome data, as opposed to differentially expressed genes identified by a fixed threshold, it can reveal differentially expressed pathways between samples that GO analysis may not detect (Subramanian, et al. 2005). *Efae*-primed samples were enriched for pathways involved in protein and lipid metabolism and metabolite transport, while Mock-primed fat bodies were enriched for pathways involved in the cell cycle (Supplementary Figure 2.3; full analysis in Supplementary Table 2.4). This suggests there is metabolic reprogramming

associated with priming and altered regulation of cell division in Mock-primed fat bodies. Despite the high degree of unique transcriptional activity in Mock-primed fat bodies, Mock-primed flies die more quickly than either *Efae*-primed or high dose-infected flies. This suggests that this transcriptional reaction is not necessarily advantageous for infection survival. Taken together, fat bodies showed a strong transcriptional response to infection, with a high degree of Mock-primed and *Efae*-primed-specific transcription.

We also noted that all conditions shared a set of 40 commonly up-regulated genes, which we call "core genes." Seventeen of these core genes are known or suspected AMPs, including several *Bomanins* (*Boms*), *Daisho 1 & 2*, and the AMPs *Metchnikowin*, *Drosomycin*, *Diptericin B*, and *Baramicin A* (Supplementary Figure 2.2B) (Cohen, et al. 2020; Hanson, et al. 2019; Hanson, et al. 2021; Lindsay, et al. 2018). Previous experimental work has shown that survival of *E. faecalis* infection is strongly dependent on the *Bom* gene family (Clemmons, et al. 2015). Flies lacking 10 out of the 12 *Boms* succumb to a single *E. faecalis* infection as quickly as flies that lack Toll signaling. Bacterial load data indicates that flies lacking either these 10 *Boms* resist an individual *E. faecalis* infection more weakly than wild type flies. Conversely, flies with deletions of several AMPs (4 *Attacins*, 2 *Diptericins*, *Drosocin*, *Drosomycin*, *Metchnikowin*, and *Defensin*) or *Baramicin A* show only modest decreases in survival of *E. faecalis* infections (Hanson, et al. 2019; Hanson, et al. 2021).

Given their differing effects on *E. faecalis* infection survival, we decided to analyze the expression patterns of the core *Boms* separately from the other core known or suspected AMPs. We displayed the distribution of expression levels of each gene group using transcripts per million (TPMs). When comparing expression of the core *Boms*, we found no significant difference in expression between the Mock-primed and *Efae*-primed flies (Wilcoxon rank sum

test: p = 0.075) (Figure 2.3D, right). Likewise, a comparison of expression levels for the core AMP or AMP-like genes yielded no significant difference between the Mock-primed and *Efae*-primed flies (Wilcoxon rank sum test: p = 0.64) (Figure 2.3D, left). This indicates that increased survival of *Efae*-primed flies is not due to the primed fat bodies producing more transcripts associated with bacterial resistance. This observation is consistent with the lack of increased bacterial clearance for *Efae*-primed relative to Mock-primed flies in Figure 2.2B and further supports the notion that priming promotes survival through bacterial tolerance.

2.3.4: Loss of Imd negatively impacts the fly's ability to prime against *E. faecalis*

We also observed priming-specific down-regulation of *imd* (Figure 2.3E), which led us to consider the role of Imd signaling in the priming response. While Imd signaling is canonically associated with response to Gram-negative bacterial infections, it is also connected to regulation of the MAPK-mediated reactive oxygen species production and wound response, as well as a generalized stress response (Ragab, et al. 2011; Myllmäki, et al. 2014). We first hypothesized that the downregulation of *imd* in *Efae*-primed flies might lead to lower expression levels of Imd-responsive AMPs, perhaps as a way to avoid transcribing genes that do not contribute to the animal's survival of the Gram-positive *E. faecalis* infections. However, the Imd-responsive AMPs were not down-regulated in a priming-specific manner (Supplementary Figure 2.2C & D).

To further explore the role Imd signaling plays in a primed immune response, we tested survival of an *imd* mutant (Pham, et al. 2007) to single and double injections (Figure 2.3F & G, Supplementary Figure 2.2E & F). As has been previously shown, the *imd* mutant showed a dose dependent response to *E. faecalis* infection with levels of lethality similar to a non-immunocompromised OrR control (Figure 2.3F & Supplementary Table 2.1; OrR HRs [Low

Dose = 1.4 (0.97-2.1) , High Dose = 5.7 (3.9-8.3)] & *imd* HRs [Low Dose = 1.3 (1.0-1.8), High Dose = 3.3 (2.4-4.6)]). However, when subjecting the flies to dual injections, we observed a significant, though not total, loss of priming ability in these *imd*-mutant flies (Figure 2.3G). *Efae*-primed flies still survive a second injection more effectively than Mock-primed flies (*Efae*-Primed vs. Mock-Primed HR = 0.39 [0.27-0.58]), but less successfully than control flies twice injected with sterile PBS (*Efae*-Primed vs. PBS/PBS HR = 4.14 [2.7-6.3]). We further probed the role of the Imd pathway in immune priming and found mutants in three additional pathway components, *kenny, Tab2,* and *Relish*, also show diminished immune priming (Supplementary Figure 2.4). Together, this demonstrates that while the loss of the *imd* does not impact the survival of the flies with a single bacterial infection, it does negatively impact survival in animals that have been infected more than once.

2.3.5: The hemocytes of primed animals up-regulate metabolic and translational pathways

Using the same approach as in fat bodies, we determined priming-specific transcription in adult hemocytes (Supplementary Figure 2.5A, full list of up-regulated and down-regulated genes in Supplementary Table 2.5). Hemocytes have several roles in the immune response, including bacterial phagocytosis, pathogen sensing, and signaling. Compared to fat bodies (Figure 2.3B), hemocytes showed a low amount of priming-specific up-regulation, with only 17 genes specifically up-regulated in the *Efae*-primed condition (Figure 2.4A, Supplementary Figure 2.5B). Most of these genes are poorly characterized or functionally unrelated (Supplementary Table 2.5). There were also 458 genes specifically up-regulated in animals with a single *Efae* High dose infection, indicating that the hemocyte transcriptional response to *E. faecalis* infection depends on the dose, previous injection state, and age of the animal. A GO term analysis reveals

that many of these high dose specific genes are involved in immune response, as expected, and regulation of metabolic processes (Supplementary Figure 2.5C). This analysis indicates that, in contrast to the fat body, hemocytes only upregulate a small number of genes specifically in the primed condition.

Similar to the fat body analysis, we identified hemocyte "core" genes as the up-regulated genes in all four conditions – animals with a single low dose infection, a single high dose infection, a mock-priming protocol, or a *Efae*-priming protocol. Of the 17 hemocyte core genes, 11 of them (~64%) overlapped with the 40 core genes found in fat bodies (Supplementary Figure 2.5D & Supplementary Table 2.5). Among these were several Bomanins, *Drosomycin*, *SPH93*, *IBIN*, and *Metchnikowin-like*, implying a role for these genes in response to *E. faecalis* infection in both hemocytes and fat body. As with our fat body data, we again separately analyzed the levels of expression of the AMPs versus Bomanin effectors for hemocytes. When comparing expression levels of the core *Boms*, we found no significant difference in expression between the Mock-primed and *Efae*-primed flies (Wilcoxon test: $p = 0.32$) (Figure 2.4B, right). Likewise, a comparison of the expression levels for the core AMP genes yielded no significant difference between the Mock-primed and *Efae*-primed flies (Wilcoxon test: $p = 0.45$) (Figure 2.4B, left). This indicates that, similar to the comparison between *Efae*-primed and Mock-primed fat bodies, transcripts associated with bacterial resistance are not specifically up-regulated in primed hemocytes.

Given the diverse functions of hemocytes in immune response, we decided to use GSEA to again systematically delineate priming-enriched pathways (Figure 2.4C, full GSEA analysis in Supplementary Table 2.6). Figure 2.4C shows individual gene sets enriched in either *Efae*-primed or Mock-primed hemocytes as nodes whose size represents the proportion of genes

within a set that were found to be enriched. Edges connect nodes that share overlapping genes between gene sets, and their thickness represents how many genes are shared. This analysis of hemocyte transcription in *Efae*-primed samples versus Mock-primed samples indicated a wider picture of metabolic reprogramming (Clusters 2, 6, 8, 10, 11, and 13) and altered protein production (Clusters 4, 5, 6, and 7) in the primed samples. There was also enrichment for genes involved in antigen-presenting and neutrophil degranulation functions in mammalian orthologs, which contained several lysosomal and metabolic genes associated with bacterial immune response, such as the GILT family of genes.

2.3.6: Several Toll effectors continuously express into re-infection, but Myd88-mediated Toll signaling is not needed for immune priming

We further leveraged our transcriptomic data to identify genes that continuously express from the first infection into reinfection (Figure 2.5A). We defined continuously expressing genes as those that were up-regulated both 1 day and 6 days after a low dose infection (*Efae* Low-d1 & *Efae* Low-d7) and 1 day after the subsequent high dose infection (*Efae*-Primed-d8). Fat bodies had 14 genes that were identified as continuously expressing (Figure 2.5B), while hemocytes only had two (Figure 2.5C). For fat bodies, 13 of the 14 (~93%) continuously expressing genes overlapped with the identified core *E. faecalis* response genes (Figures 2.3B & C; annotated in Supplementary Table 2.3). Most of these genes are either known or suspected AMPs, and the list also includes a recently characterized lncRNA (lncRNA:CR33942) that can enhance the Toll immune response (Zhou, et al. 2022). The fat body continuously expressing genes are largely Toll-regulated.

To further investigate the role Toll signaling is playing in creating a primed response to *E. faecalis*, we assayed infection response in flies with a *Myd88* mutation that eliminates intracellular Toll signaling (Figure 2.5D) (Charatsi, et al. 2003). In the single injection conditions, we continued to see a dose-dependent effect on survival (Low Dose vs PBS HR = 4.3 [2.8-6.6]; High Dose vs PBS HR = 13 [8.8-22]), with expected increased lethality when compared to our immune-competent control (Supplementary Figure 2.6A) (Clemmons, et al. 2015; Hanson, et al. 2019). When assaying for survival against double-injected conditions, we found that *Myd88* mutants were still able to effectively prime against *E. faecalis* re-infection (Figure 2.5E; *Efae*-Primed vs Mock-Primed HR = 0.32 [0.20-0.53]). Despite lacking canonical Toll-mediated immune signaling, these mutants were able to respond to double-injections and mount a primed immune response, with equivalent survival between the *Efae*-primed flies and the control flies injected twice with PBS (Supplementary Figure 2.6B; *Myd88*: *Efae*-Primed vs PBS/PBS HR = 0.56 [0.34-0.92]).

We additionally parsed the effects of eliminating extracellular Toll signaling versus intracellular signaling by assaying *spz* mutants. Like *Myd88* mutants, we found that ablating *spz* maintained the dose-dependent response to *E. faecalis* single infections (Figure 2.5F; Efae Low Dose vs PBS HR = 2.9 [1.9-4.5], Efae Hi Dose vs PBS HR = 6.1 [4.1-9.1]). However, the *spz* mutants lacked the ability to prime against *E. faecalis* (Figure 5G; *Efae*-Primed vs Mock-Primed HR = 1.2 [0.83-1.7]). This indicates that immune priming against the Gram-positive *E. faecalis* does not strictly require Myd88-mediated Toll signaling but does require extracellular Spz activity.

2.3.7: Potentiated recall gene expression plays a minor role in *E. faecalis* immune priming

In addition to priming-specific and continuously expressing genes, we were also identified as "recall response genes" (Melillo et al. 2018). These genes were defined as genes that are up-regulated in response to an initial low dose infection, turned off 6 days later, and up-regulated more strongly in response to a subsequent infection (Figure 2.6A). In fat bodies, we identified 7 recall genes (Figure 2.6B), and we did not identify any recall genes in hemocytes. Of these few fat body recall genes, we found two Polycomb interacting elements (*jing* & *cg*) and a component of the Mediator complex (*MED23*), suggesting a potential role for transcriptional regulation. However, we did not find a strong role for recall transcription in our experiments.

2.4: DISCUSSION

In this study we have shown the transcriptional underpinnings of a primed immune response against *Enterococcus faecalis* infection in *Drosophila melanogaster*. We demonstrated that a low dose of *E. faecalis* can prime the flies to better survive a high dose infection at least 7 days later, and the increase in survival is not linked to more effective clearance of the bacteria, but to increased tolerance of *E. faecalis* in primed animals. When comparing *Efae*-primed and Mock-primed animals, we found that the transcriptional profiles of antimicrobial peptides and *Bomanins* do not differ between the two conditions in either the fat body nor the hemocytes, indicating that their differential expression is not driving survival in primed animals. However, there are ample transcriptional differences between the conditions, and GSEA analysis points to differences in cell cycle regulation and metabolic response. When testing priming ability in *imd*, *Myd88*, and *spz* mutants, we found that these mutants have unexpected survival phenotypes in the double injection conditions – *imd* mutants prime less effectively than wild type flies, *Myd88*

mutants show no apparent loss of priming ability, and *spz* mutants completely lose the ability to prime.

Overall, we have seen evidence for tolerance, phagocytosis, and transcriptional reprogramming as a driver of priming against *E. faecalis* infection. Flies primed against *E. faecalis* re-infection did not actively clear bacteria more efficiently than Mock-primed controls (Figure 2.2B) and did harbor a higher bacterial load upon death (Figure 2.2C), both hallmarks of infection tolerance. We also found that phagocytosis was needed to fully prime, as supported by the decrease in priming ability in both our *eater*-deficient flies (Figure 2.1E) and bead-blocking experiments (Figure 2.1F). Given that primed flies seem to survive infection by tolerating, rather than clearing bacteria, this suggests a role for phagocytes in priming other than their canonical responsibility of eliminating pathogens. One possibility is that phagocytes are working to sense an infection and relay that signal to other tissues through functional reprogramming (Nehme, et al. 2011; Gold & Brückner 2014). This is supported by the large transcriptional shift in metabolic pathways seen in hemocytes, and specifically, the up-regulation of lysozyme-related pathways, including the "MHC Class II Antigen Presentation" and "Neutrophil Degranulation gene sets (Figure 2.4C). Explicit proof of phagocyte reprogramming as a potential mechanism of priming merits further investigation. Transcriptionally, there are three primary mechanisms suggested that may underlie immune priming – (1) primed animals may drive a qualitatively different expression program than mock primed flies, differentially regulated distinct genes, (2) primed flies may continually express key immune genes between a priming and subsequent infection, or (3) primed flies may re-active an immune response more quickly than unprimed flies. Our transcriptional data shows that most priming differences in both fat bodies and hemocytes can be attributed to gene expression that is unique to priming (Figure 2.3B & 2.4A). We saw continuous

expression of a small number of Toll effectors in fat bodies (Figure 2.5B), and very little evidence of potentiated gene expression (Figure 2.6B).

There are previous studies of immune priming in flies, which taken together with this work paint a more complete picture of the phenomenon. One of the early descriptions of immune priming in *D. melanogaster* found a phagocytosis-dependent, AMP-independent priming response against *Streptococcus pneumoniae* (Pham, et al. 2007). Our study uses a different Gram-positive microbe, but a similar re-infection timescale. Similar to that study, we find that phagocytosis is needed to mount a primed immune response, as was demonstrated by the impaired priming in the *eater* mutant and bead-blocked flies. We also corroborated that survival is not correlated with AMP production. However, Pham et al. found that primed flies resist *S. pneumoniae* more effectively than naive flies, while our *Efae*-primed flies appeared to rely on immune tolerance to enhance survival. It is possible that this difference is due to the increased virulence of the pathogen, *S. pneumoniae,* which can kill a wild type fly with a relatively low dose of 3,000 CFU, relative to *E. faecalis*. The difference could also be due to the specificity of the host's primed response to different pathogens. More recent work also studied priming mechanisms in flies infected with *M. luteus* and *S. typhimurium* and found evidence of resistance and tolerance mechanisms, respectively (Fuse, et al. 2022). *M. luteus* primed flies show potentiated gene expression upon re-infection. Prakash and co-workers have probed priming using *P. rettgeri* and found that a host of factors, including sex and infection route can also shape immune priming (Prakash, et al., 2023). In sum, these findings suggest that there may be multiple, bacteria-specific priming mechanisms.

Another study found that sterile wounding 2 days, but not 7 days, prior to infection with *E. faecalis* conferred some level of ROS-mediated protection (Chakrabarti, et al. 2020). This

study's assay most closely matches our Mock-primed re-infections, and we also did not see enhanced survival when the wounding occurred 7 days prior to the infection. This indicates that the protection conferred from sterile wounding is effective in the short-term (i.e., 2 days), but not in the long-term (i.e. 7 days). However, both this study and our observations support the idea that hemocytes activate new functions in response to prior stimuli exposure (as was found in Weaver, et al. 2016, as well). Finally, a study looking at the effects of chronic bacterial infection did not find immune priming with *E. faecalis* when using the same re-injection time points (Chambers, et al. 2019). However, in that study flies were injected with two low-doses (~3,000 CFU/fly) and injected first in the abdomen and second in the thorax. This suggests a dose-dependent and/or injection site-dependent effect on priming ability.

One of the most surprising findings of this study is the priming responses found in the *imd, Myd88*, and *spz* mutant flies. As others have previously reported, our work demonstrates that the disruption of *imd* does not affect the fly's survival against a single low dose infection of *E. faecalis*, while the elimination of Toll signaling greatly reduces the fly's survival of the same infection. This is consistent with the well-described sensing of Gram-positive bacteria via Toll signaling and Gram-negative bacteria via Imd signaling (Buchon, et al. 2014). However, we find that *imd* mutants lose some, though not all, of their priming capacity, *Myd88* mutants have similar survival between flies injected twice with PBS or *Efae*-primed flies, and *spz* mutants completely lose their ability to prime. The requirement of *imd* for survival was surprising for two reasons: first because Imd signaling has not been implicated in the survival of Gram-positive bacteria (or priming, in the case of *S. pneumoniae* in Pham, et al. 2007), and second, because we saw down regulation of the *imd* gene in the fat body primed transcriptome. This suggests while downregulation of *imd* may be useful in priming, complete eradication of the pathway in the

animal removes some priming ability. This could be due to the role the Imd pathway plays in modulating other key immune response pathways such as JAK/STAT, JNK, and MAPK signaling (Kleino & Silverman 2014).

We were also surprised to see the variable role of Toll signaling for priming. Toll signaling plays a key role in surviving Gram-positive infections, and virtually all the persistently expressed genes we found here are known Toll targets (Figure 2.5B). While both Toll pathway mutants, *Myd88* and *spz*, showed markedly worse survival in response to a single low *E. faecalis* dose, they showed opposite effects in their ability to prime. For the *Myd88* mutants, one possible explanation of this observation is that *Myd88* mutants show markedly lower survival of the initial low dose *E. faecalis* infection. This implies that, when we select survivors to re-infect 7 days later, this may be representative of a specific subset of flies with an advantage that allows them to survive the initial infection despite the lack of a Myd88-mediated Toll response. This contrasted with the complete loss of priming in *spz* mutants. The precise mechanism driving this difference between the Toll mutants remains unclear – the response of the *Myd88* and *spz* mutants to a single injection, whether of PBS, or a high or low dose of *E. faecalis* are remarkably similar. Further, their response to the PBS/PBS or Mock-priming dual injections are also virtually indistinguishable. This implies some extracellular activity involved in upstream Toll signaling is necessary to mount a primed immune response, but that Myd88 intracellular activity is not and suggests further probing into the underlying mechanism is warranted.

While our data did not indicate a difference in bacterial clearance between *Efae*-primed and Mock-primed flies (Figure 2.2B), we acknowledge the possibility that the number of bacteria remaining in the animal from the initial infection may affect priming responses. As has been previously noted (Duneau, et al. 2017), we found variability in the bacterial burden during the

initial low dose infection, consistent with some flies more effectively resisting infection than others (Figure 2.2A). Chronic infections tend to lead to low-level activation of the immune response throughout the animal's lifetime, causing expression of immune effectors that can loiter into re-infection and may contribute to enhanced survival (Chambers, et al. 2019). It is not yet clear what effect the intensity of a chronic infection would have on priming ability, but it should be considered in the future. It is possible that a more severe chronic infection could either put the animal in a heightened state of "readiness" for a new infection or exhaust its resources.

Our data implies a major role for metabolic reprogramming in mediating a primed immune response against *E. faecalis*. Given the high energetic cost of mounting an immune response, it is logical to imagine immune priming as a more efficient re-allocation of metabolic resources to fine tune an immune defense strategy in a short-lived animal (as discussed in Lazzaro & Tate 2022; Schlamp, et al. 2021). Interestingly, evidence of metabolic shifts was not just relegated to the fat body (Supplementary Figure 2.3), which acts as the site of integration for metabolic and hormonal control, but was found to be the case with hemocytes, as well (Figure 2.4C). Similarly, in mammalian trained immunity where metabolic reprogramming drives epigenetic changes in innate immune cell chromatin (Fanucchi, et al. 2021). Further characterization of *Drosophila* immune priming could explore the extent of differential metabolite usage when mounting a primed immune response and whether the transcriptional differences observed are encoded through epigenetic reprogramming of histone mark deposition, akin to what is observed in mammalian systems. Our study lays the groundwork for understanding the interplay between a physiological primed immune response and the transcriptional regulatory logic defining it.

2.5: METHODS

2.5.1: Fly Strains and Husbandry

Experiments, unless otherwise indicated, were performed using 4-day old Oregon-R male flies. *Eater* mutants are described in Bretscher et al. (2015) and were obtained from the Bloomington Stock Center (RRID:BDSC_68388). These flies knocked out the *eater* gene through homologous recombination that replaced 745bp of the TSS, exons 1 and 2, and part of exon 3 with a 7.9 kb cassette carrying a $w^{[+]}$ gene. The imd^{1091} line, the *w*; key^{1}, *cn*, *bw*; $gIKK\gamma^{WT}$ line, $Tab2^{AOII3}$ line, and the Rel^{E20} line were provided by Neal Silverman. The imd^{1091} mutants were generated by creating a 26bp deletion at amino acid 179 that creates a frameshift mutation at the beginning of the death domain in *imd* (Pham 2007). $Myd88^{[kra-1]}$ flies were provided by Steve Wasserman and Lianne Cohen. This line was created by excising 2257bp of the *Myd88* gene spanning the majority of the first exon and inserting a P-element (Charatsi 2003). Stable lines were balanced against a CyO balancer with homozygous mutant males being selected for injections. *Spätzle* mutants were obtained from the Bloomington Stock Center ($spz^{2}ca^{1}$/TM1, RRID:BDSC_3115). Stable lines were balanced against a TM1 balancer with homozygous mutant males being selected for injections. Flies were housed at 25˚C with standard humidity and 12 hr-light/12 hr-dark light cycling.

2.5.2: Injections

All bacterial infections were done using a strain of *Enterococcus faecalis* originally isolated from wild-caught *Drosophila melanogaster* (Lazzaro 2006). Single colony inoculums of *E. faecalis* were grown overnight in 2mL BHI shaking at 37˚C. 100uL of overnight *E. faecalis* inoculum was then added to 2mL fresh BHI and grown shaking at 37˚C for 2.5 hours before

injections to ensure it would be in the log-phase of growth. Bacteria was then pelleted at 5,000 rcf for 5 minutes, washed with PBS, re-suspended in 200uL PBS, and measured for its OD600 on a Nanodrop. Flies were injected with either PBS, *E. faecalis* at OD 0.05 for low dose experiments (~3,000 CFU/fly), or *E. faecalis* at OD 0.5 for high dose experiments (~30,000 CFU/fly). Due to the high heat resistance of *E. faecalis*, heat-killed inoculums were produced by autoclaving 10mL cultures that were in log-phase growth. Successful heat-killing was determined by streaking 50uL on a BHI plate and checking it had no growth. Adult flies were injected abdominally using one of two high-speed pneumatic microinjectors (Tritech Research Cat. # MINJ-FLY or Narishige IM 300) with a droplet volume of ~50nL for both PBS and bacterial injections. Injections into a drop of oil on a Lovin's field finder were used to calibrate the droplet volume. Injections were performed in the early afternoons to control for circadian effects on immune response. Flies were not left on the CO_2 pad for more than 10 minutes at a time. Injected flies were housed in vials containing a maximum of 23 flies at 25˚C with standard humidity and 12 hr-light/12 hr-dark light cycling.

2.5.3: Survival Assays

To track survival, flies were observed every 24 hours at the time they were injected. Media was changed every three days with flies being exposed to CO_2 for no more than two minutes between vial transfers. Survival is plotted as Kaplan-Meier curves using the R 'survival' and 'survminer' packages. Cox proportional hazards and median survival times were used to compare survival experiments. Comparisons on survival between two conditions is presented as a hazard ratio (HR) that scores survival rate of a test group against survival in a referent group. A

HR is reported alongside its 95% confidence interval as well as a Wald test p-value reporting whether the HR significantly deviates from 1.

2.5.4: Bead Blocked Infection

To ablate phagocytosis during the initial low dose infection, flies were first abdominally injected with 50nL Cml latex beads (Thermo Scientific Cat. # C37480), allowed to rest for 4 hours, and then injected with ~3,000 *E. faecalis*. Primed survival was then assayed for after injection with ~30,000 CFU of *E. faecalis* 7 days after the initial bacterial infection (as was previously described in Pham, et al. 2007).

2.5.5: Dilution Plating

Single flies were suspended in 250uL PBS and homogenized using an electric pestle. The homogenate was then serially diluted five-fold and plated on BHI plates and left to grow in aerobic conditions for two days at 25˚C. Using this method there was little to no background growth of the natural fly microbiome. Images were then taken of each plate using an iPhone XR and analyzed using ImageJ with custom Python scripts to calculate colony forming units (CFU) per fly. Plotting was done using the R package ggplot2 (Wickham 2016).

2.5.6: Hemocyte Isolation

For each biological replicate, 20 flies were placed in a Zymo-Spin P1 column with the filter and silica removed along with a tube's-worth of Zymo ZR Bashing Beads. Samples were centrifuged at 10,000 rcf at 4˚C for one minute directly into a 1.5mL microcentrifuge tube

containing 350uL TriZol (Life Technologies) (schematic in Supplementary Figure 5A). Samples were then snap frozen and stored at -80˚C for future RNA extraction.

2.5.7: Fat Body Isolation

Each biological replicate consisted of 3 extracted fat bodies. Flies were anesthetized with CO_2 and pinned with a dissection needle at the thorax, ventral side up, to a dissection pad. The head, wings, and legs were then removed using forceps. Using a dissection needle, the abdomen was carefully opened longitudinally, and the viscera removed using forceps. The remaining abdominal filet with attached fat body cells was then removed from the thorax and transferred to a 1.5mL microcentrifuge tube on ice containing 350uL TriZol. Samples were then snap frozen and stored at -80˚C for future RNA extraction. Dissection of fat bodies includes some level of testes and sperm contamination, which was monitored by tracking expression of sperm-related genes in RNA-seq libraries and throwing out any libraries that have relatively high expression of said genes (Supplementary Figure 2.7).

2.5.8: RNA-seq Library Preparation

RNA from either fat bodies or hemocytes was extracted using a Zymo Direct-zol RNA Extraction kit and eluted in 20uL water. Libraries were prepared using a modified version of the Illumina Smart-seq 2 protocol as previously described **(**Ramirez-Corona 2021**).** Libraries were sequenced on an Illumina Next-seq platform using a NextSeq 500/550 504 High Output v2.5 kit to obtain 43bp paired-end libraries.

2.5.9: Differential Gene Expression Analysis

Sequenced libraries were quality checked using FastQC and aligned to *Drosophila* reference genome dm6 using Bowtie 2 (Langmead & Salzberg 2012). Counts were generated using the subread function featureCounts. Counts were then loaded into EdgeR (Robinson 2010), libraries were TMM normalized, and genes with CPM < 1 were filtered out. Full code used in downstream analysis can be found at https://github.com/WunderlichLab/ImmunePriming-RNAseq.

2.5.10: Priming-Specific Transcription Analysis

To identify priming-specific up-regulation, we first identified genes that were significantly up-regulated ($log_2FC>1$ & FDR<0.05) in each condition that assayed for immune response 24 hours after infection (i.e. *Efae* Hi Dose-d1, *Efae* Low Dose-d1, Mock-Primed-d8, and *Efae*-Primed-d8) (the effect of modulating significance and log_2FC cut-offs can be seen in Supplementary Figure 2.8). These gene lists were then compared to each other for overlap. Genes that were only up-regulated in *Efae*-Primed-d8 samples, but in no other condition were labeled as "priming-specific". Average expression of AMPs and *Bomanins* plotted as a box-and-whisker plot of $log_{10}(TPM+1)$ to show variance. Significant differences between conditions were calculated using a Wilcoxon rank sum test.

2.5.11: Continuous Expression Analysis

To determine genes that were continuously being expressed throughout initial immune priming into re-infection, we focused on the transcription in samples assayed at *Efae* Low-d1, *Efae* Low-d7, and *Efae*-Primed-d8. We first selected genes that were expressed at the above time

points relative to a non-stimulated, age-matched control ($log_2FC > 0$). We then filtered that shortlist on the following conditions: genes had to significantly up-regulated at *Efae* Low-d1 compared to its age-matched control ($log_2FC > 0$ & $FDR < 0.05$), genes had to significantly up-regulate at *Efae*-Primed-d8 compared to its age-matched control ($log_2FC > 0$ & $FDR < 0.05$), and genes had to either stay at similarly expressed levels or increase in expression between *Efae* Low-d7 and *Efae*-Primed-d8 compared to their age-matched controls ($log_2FC \geq 0$).

2.5.12: Potentiated Recall Response Analysis

We termed genes as being "recalled" if they were initially transcribed during priming (*Efae* Lo-d1 log_2FC over age-matched control > 0.5), ceased being expressed by the end of priming (*Efae* Lo-d7 log_2FC over age-matched control ≤ 0), and were then re-expressed upon re-infection (*Efae*-Primed-d8 log_2FC over age-matched control > 0.5 & FDR < 0.1). Our significance threshold had to be somewhat relaxed for expression after re-infection to detect any recalled gene expression at all. To delineate genes that were truly re-activating transcription in a potentiated manner (i.e., at a higher level upon re-infection as compared to when they were initially expressed during priming), we also filtered on the conditional that log_2FC over age-matched controls had to be higher in *Efae*-Primed-d8 versus *Efae* Low-d1. Finally, to identify genes that were recalled only in our primed samples, we further filtered on the condition that genes had to have a $log_2FC \leq 0$ over age-matched controls for Mock-primed-d8 samples.

2.5.13: GO Term Enrichment

All GO Term Enrichment was done using Metascape's online tool (Zhou, et al. 2019) and plotted using custom ggplot2 scripts.

2.5.14: Gene Set Enrichment Analysis

Gene set enrichment analysis was run using the GSEA software v. 4.2.3 (Subramanian, et al. 2005). *Drosophila*-specific gene matrices for both KEGG and Reactome-based GSEA aliases were taken from Cheng 2021. TMM-normalized TPMs were extracted from EdgeR analysis and used as input for two-condition comparisons using GSEA software. Due to the low number of replicates (< 7 replicates per condition), analysis was run using a gene set permutation. Full tabular results are found in Supplementary Tables 2.4 & 2.6. An enrichment map visualizing the network of enriched gene sets was created using Cytoscape (Node Cutoff = 0.1 FDR; Edge Cutoff = 0.5) and clusters describing the mapping manually curated (Merico, et al. 2010).

2.6: ACKNOWLEDGEMENTS

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2.7: AUTHOR CONTRIBUTIONS

Z.W. and K.C. are responsible for conceptualization and formal analysis throughout. K.C. was responsible for data curation, software development, visualization, and investigation for experiments throughout the manuscript. D.S.H. contributed to investigation and software

development of dilution plating experiments. O.G. assisted in investigation of dilution plating experiments. D.M. was responsible for investigation of heat-killed *E. faecalis* survival experiments. K.C. and Z.W. wrote the original draft and edited the manuscript. Z.W. supervised the project.

2.8: COMPETING INTERESTS

The authors do not declare any competing interests.

2.9: FIGURES

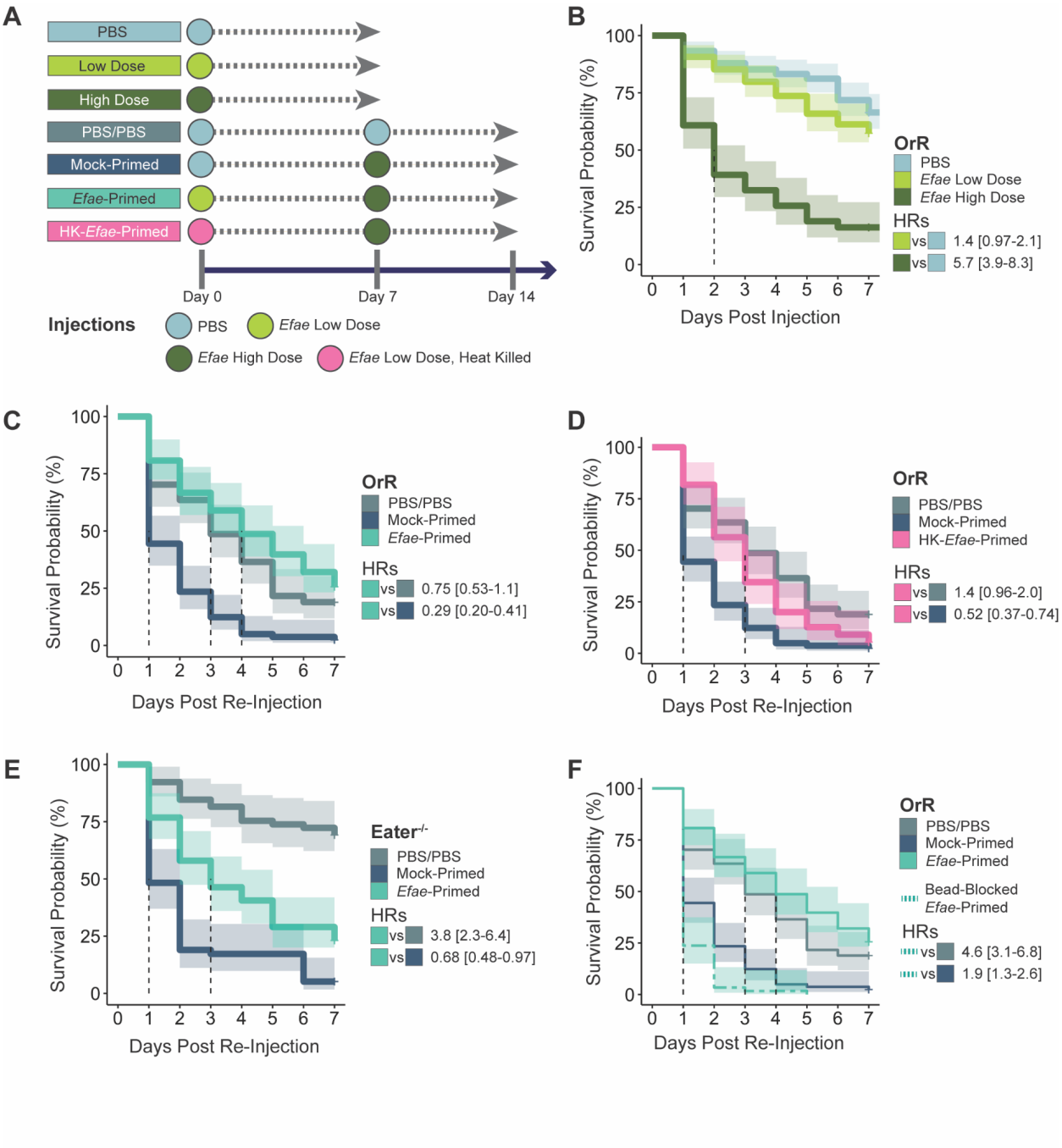

A
PBS
Low Dose
High Dose
PBS/PBS
Mock-Primed
Efae-Primed
HK-Efae-Primed
Day 0
Day 7
Day 14
Injections
PBS
Efae Low Dose
Efae High Dose
Efae Low Dose, Heat Killed
B
Survival Probability (%)
Days Post Injection
OrR
PBS
Efae Low Dose
Efae High Dose
HRs
vs 1.4 [0.97-2.1]
vs 5.7 [3.9-8.3]
C
Survival Probability (%)
Days Post Re-Injection
OrR
PBS/PBS
Mock-Primed
Efae-Primed
HRs
vs 0.75 [0.53-1.1]
vs 0.29 [0.20-0.41]
D
Survival Probability (%)
Days Post Re-Injection
OrR
PBS/PBS
Mock-Primed
HK-Efae-Primed
HRs
vs 1.4 [0.96-2.0]
vs 0.52 [0.37-0.74]
E
Survival Probability (%)
Days Post Re-Injection
Eater-/-
PBS/PBS
Mock-Primed
Efae-Primed
HRs
vs 3.8 [2.3-6.4]
vs 0.68 [0.48-0.97]
F
Survival Probability (%)
Days Post Re-Injection
OrR
PBS/PBS
Mock-Primed
Efae-Primed
Bead-Blocked
Efae-Primed
HRs
vs 4.6 [3.1-6.8]
vs 1.9 [1.3-2.6]

Figure 2.1*: E. faecalis* can induce immune priming in *D. melanogaster*
A). Schematic of single and double-injection experiments. **B).** Survival of OrR flies injected with PBS (n = 149), *Efae* Low Dose (~3,000 CFU/fly, n = 129), and *Efae* High Dose (~30,000 CFU/fly, n = 74). Dotted line indicates median survival time. Shaded area indicates 95% confidence interval. Low Dose vs PBS: HR = 1.4, p = 0.072; High Dose vs PBS: HR = 5.7, p = 2.0E-16; pairwise comparisons are calculated using a Cox proportional hazard model with hazard ratios and Wald statistic values reported for experimental conditions versus their PBS negative control. Full survival statistics can be found in Supplementary Table 1. **C).** Survival of primed OrR flies versus double-injected, non-primed controls (PBS/PBS: n = 74, Mock-Primed: n = 81, *Efae*-Primed: n=78). *Efae*-Primed vs PBS/PBS: HR = 0.75, p = 1.3E-01; Mock-Primed vs. PBS/PBS: HR = 2.6, p = 9.5E-08. **D).** Survival of OrR flies primed with heat-killed *E. faecalis* (HK-*Efae*-Primed: n = 55) versus flies primed with live *E. faecalis*: HR = 1.4, p = 8.0E-02; data for *Efae*-Primed & Mock-Primed same as C. **E).** Survival of primed, phagocytosis-deficient *eater*-mutant flies versus double-injected, non-primed controls (PBS/PBS: n = 65, Mock-Primed: n = 58, *Efae*-Primed: n = 69). *Efae*-Primed vs PBS/PBS: HR = 3.8, p = 4.2E-07; Mock-Primed vs. PBS/PBS: HR = 5.6, p = 1.9E-11. **F).** There is a significant loss in priming ability when phagocytes are bead-blocked in the initial low-dose *E. faecalis* infection (*Efae*-Primed vs Mock-Primed HR = 0.29, p = 2.7E-12; BB-*Efae*-Primed vs Mock-Primed HR = 1.9, p = 4.4E-04). All data except for BB-*Efae*-Primed are the same as **C**, replotted for comparison.

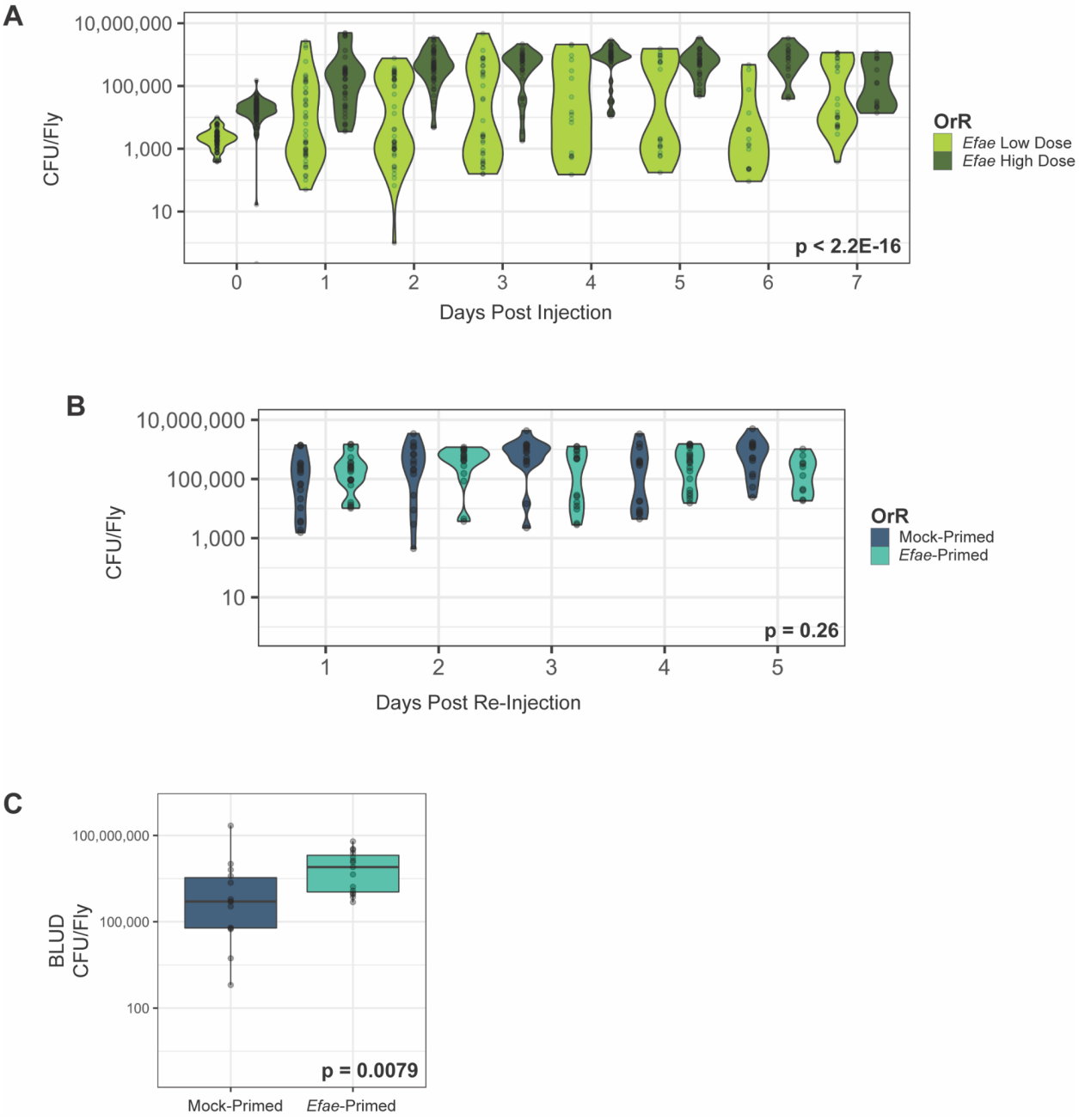

A
10,000,000
100,000
1,000
10
CFU/Fly
p < 2.2E-16
0
1
2
3
4
5
6
7
Days Post Injection
OrR
Efae Low Dose
Efae High Dose
B
10,000,000
100,000
1,000
10
CFU/Fly
p = 0.26
1
2
3
4
5
Days Post Re-Injection
OrR
Mock-Primed
Efae-Primed
C
100,000,000
100,000
100
BLUD CFU/Fly
p = 0.0079
Mock-Primed
Efae-Primed

Figure 2.2: Bacterial clearance is not correlated with primed survival against *E. faecalis* re-infection

A). Bacterial load of single-injected flies. Flies were abdominally injected with either *E. faecalis* Low Dose (~3,000 CFU/fly) or *E. faecalis* High Dose (~30,000 CFU/fly), and a subset was dilution plated every 24 hours. There is a significant difference in bacterial load over time between initially low-dose and high-dose infected flies (Kruskal-Wallis rank sum test: df = 7, X^2 = 106.38, p < 2.2E-16). **B).** Bacterial load of double-injected flies. Mock-Primed and *Efae*-Primed flies do not differ in their bacterial load over time (Kruskal-Wallis rank sum test: df = 4, X^2 = 7.2423, p = 0.12). Data displays up to day 5 because of the strong survivor bias inherent to selecting flies that are still alive after that point (reference survival at day 5 and after in **Fig 2.1C**). **C).** Bacterial load upon death (BLUD) of double-injected flies (Wilcoxon rank sum test: W = 45, p = 0.0079).

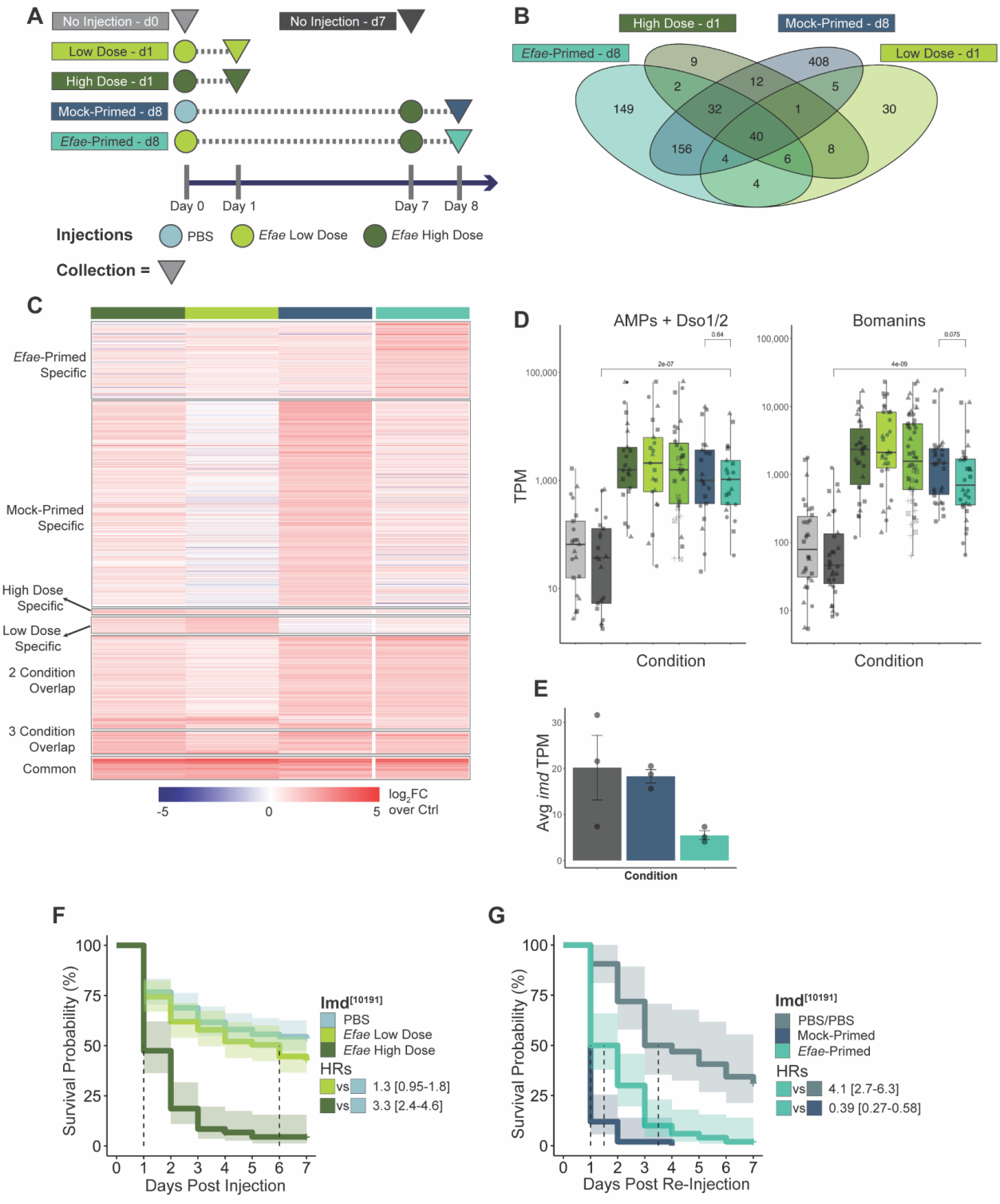

A
No Injection - d0
No Injection - d7
Low Dose - d1
High Dose - d1
Mock-Primed - d8
Efae-Primed - d8
Day 0
Day 1
Day 7
Day 8
Injections
PBS
Efae Low Dose
Efae High Dose
Collection =
B
High Dose - d1
Mock-Primed - d8
Efae-Primed - d8
Low Dose - d1
9
408
2
12
5
149
32
1
30
40
156
4
6
8
4
C
Efae-Primed Specific
Mock-Primed Specific
High Dose Specific
Low Dose Specific
2 Condition Overlap
3 Condition Overlap
Common
log2FC over Ctrl
-5
0
5
D
AMPs + Dso1/2
Bomanins
TPM
Condition
E
Avg imd TPM
Condition
F
Survival Probability (%)
Days Post Injection
Imd[10191]
PBS
Efae Low Dose
Efae High Dose
HRs
vs 1.3 [0.95-1.8]
vs 3.3 [2.4-4.6]
G
Survival Probability (%)
Days Post Re-Injection
Imd[10191]
PBS/PBS
Mock-Primed
Efae-Primed
HRs
vs 4.1 [2.7-6.3]
vs 0.39 [0.27-0.58]

Figure 2.3: Fat bodies have a high degree of priming-specific transcriptional up-regulation **A).** Sample collection for RNA-seq experiments. Conditions are the same as Figure 1A, with the addition of age-matched, non-injected controls at Day 0 and Day 7. Circles represent injections and triangles represent time of collection. **B).** Venn-diagram of significantly up-regulated genes (log fold change (log_2FC) >1 & false discovery rate (FDR) <0.05) for conditions in A compared to age-matched controls. **C).** Heat map of significantly up-regulated genes as corresponding to B (scale: log_2FC over age-matched controls) **D).** Expression in $log_{10}(TPM+1)$ of ubiquitously up-regulated AMPs [left] and *Bomanins* [right]. Biological replicates are designated by the shape of individual points. While there is a significant difference in AMP and *Bom* expression in *Efae*-Primed fat bodies compared to their age-matched, non-injected controls (Wilcoxon test; AMPs: p = 2.0E-07, *Boms*: p = 4.0E-09), there is not a significant difference in ubiquitous AMP and *Bom* expression between Mock-Primed and *Efae*-Primed fat bodies (Wilcoxon test; AMPs: p = 0.64, *Boms*: p = 0.075). **E).** Average TPMs for the gene *imd* in double-injected fat body samples. **F).** Survival of *imd*-mutant flies injected with PBS (n = 167), *Efae* Low Dose (n = 121), and *Efae* High Dose (n = 86). Dotted line indicates median survival time. Shaded area indicates 95% confidence interval. Low Dose vs PBS: HR = 1.3 [0.95-1.8]; High Dose vs PBS: HR = 3.3 [2.4-4.6]; pairwise comparisons are calculated using a Cox proportional hazard model with hazard ratios and Wald statistic values reported for experimental conditions versus their PBS negative control. **G).** Survival of primed *imd*-mutant flies versus double-injected, non-primed controls (PBS/PBS: n = 61, Mock-Primed: n = 60, *Efae*-Primed: n=71). *Efae*-Primed vs PBS/PBS: HR = 4.1 [2.7-6.3]; Mock-Primed vs. PBS/PBS: HR = 10.7 [6.5-17].

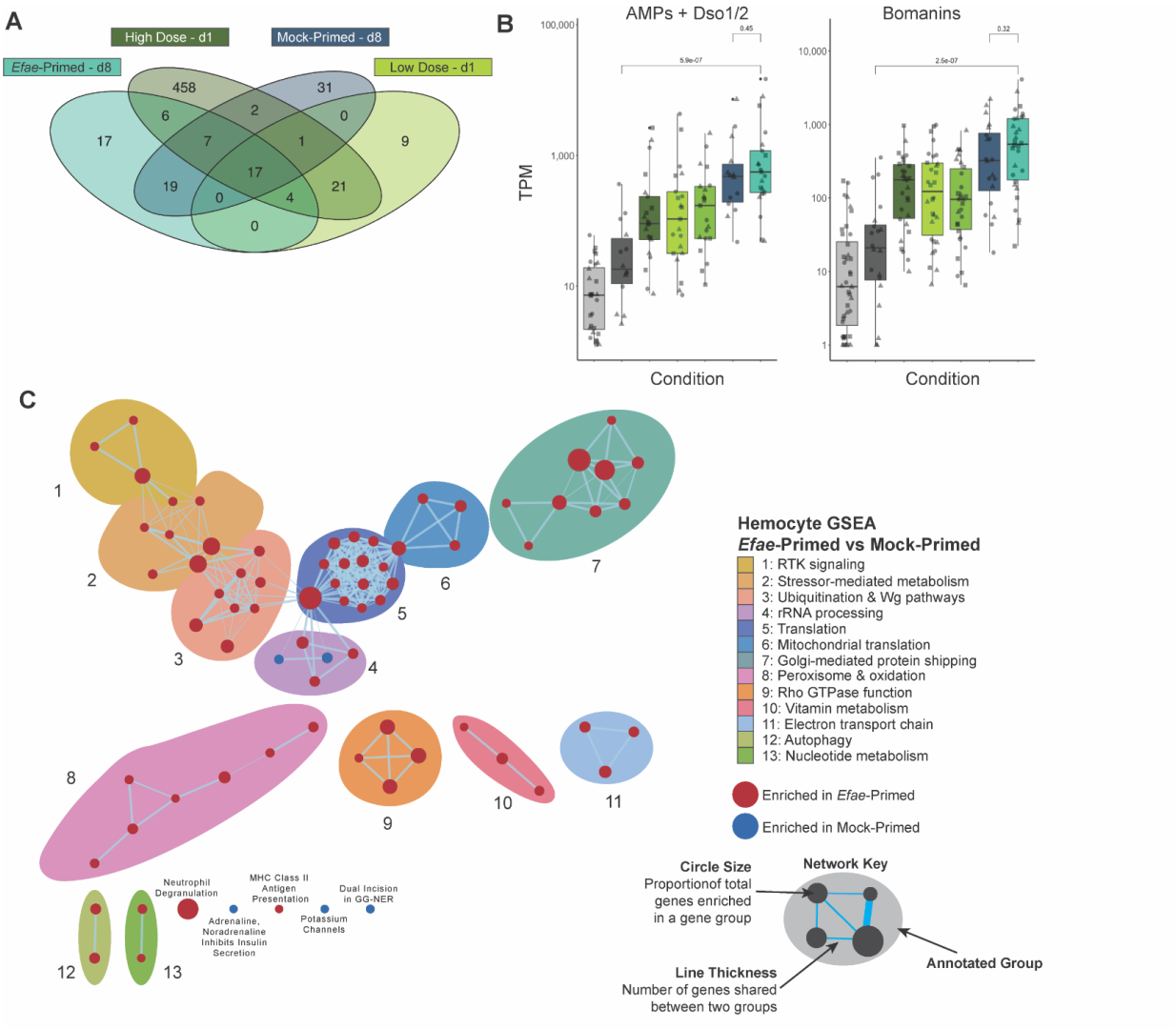

A
High Dose - d1
Mock-Primed - d8
Efae-Primed - d8
Low Dose - d1
458
31
6
2
0
17
7
1
9
17
19
0
4
21
0
B
AMPs + Dso1/2
Bomanins
TPM
100,000
1,000
10
10,000
1,000
100
10
1
5.9e-07
0.45
2.5e-07
0.32
Condition
Condition
C
1
2
3
4
5
6
7
8
9
10
11
12
13
Neutrophil Degranulation
Adrenaline, Noradrenaline Inhibits Insulin Secretion
MHC Class II Antigen Presentation
Potassium Channels
Dual Incision in GG-NER
Hemocyte GSEA
Efae-Primed vs Mock-Primed
1: RTK signaling
2: Stressor-mediated metabolism
3: Ubiquitination & Wg pathways
4: rRNA processing
5: Translation
6: Mitochondrial translation
7: Golgi-mediated protein shipping
8: Peroxisome & oxidation
9: Rho GTPase function
10: Vitamin metabolism
11: Electron transport chain
12: Autophagy
13: Nucleotide metabolism
Enriched in Efae-Primed
Enriched in Mock-Primed
Circle Size
Proportionof total genes enriched in a gene group
Network Key
Annotated Group
Line Thickness
Number of genes shared between two groups

Figure 2.4: Hemocytes do not significantly increase effector expression when primed, but differentially activate metabolic pathways

A). Venn diagram of significantly up-regulated ($log_2FC > 1$ & FDR <0.05) genes for hemocytes collected in the same conditions as **Fig 2.3A**. **B).** Expression in log_{10} (TPM+1) of ubiquitously up-regulated AMPs [left] and *Bomanins* [right]. Condition colors match conditions in Figure 3A. Biological replicates are designated by the shape of individual points. While there is a significant difference in AMP and *Bom* expression in *Efae*-Primed hemocytes compared to their age-matched, non-injected controls (Wilcoxon test; AMPs: p = 5.9E-07, *Boms*: p = 2.5E-07), there is not a significant difference in ubiquitous AMP and *Bom* expression between Mock-Primed and *Efae*-Primed fat bodies (Wilcoxon test; AMPs: p = 0.45, *Boms*: p = 0.32).

C). Network representation of Gene Set Enrichment Analysis (GSEA) for *Efae*-Primed versus Mock-Primed hemocytes. This visualization represents relationships between statistically significant terms (FDR < 0.05), manually curated with clusters that summarize the relationships between terms. Each circle represents an enriched gene set, circle size represents the relative proportion of genes within a set that were enriched, and the line thickness represents the number of genes that are enriched between any two gene sets. Full results are found in Supplementary Table 6.

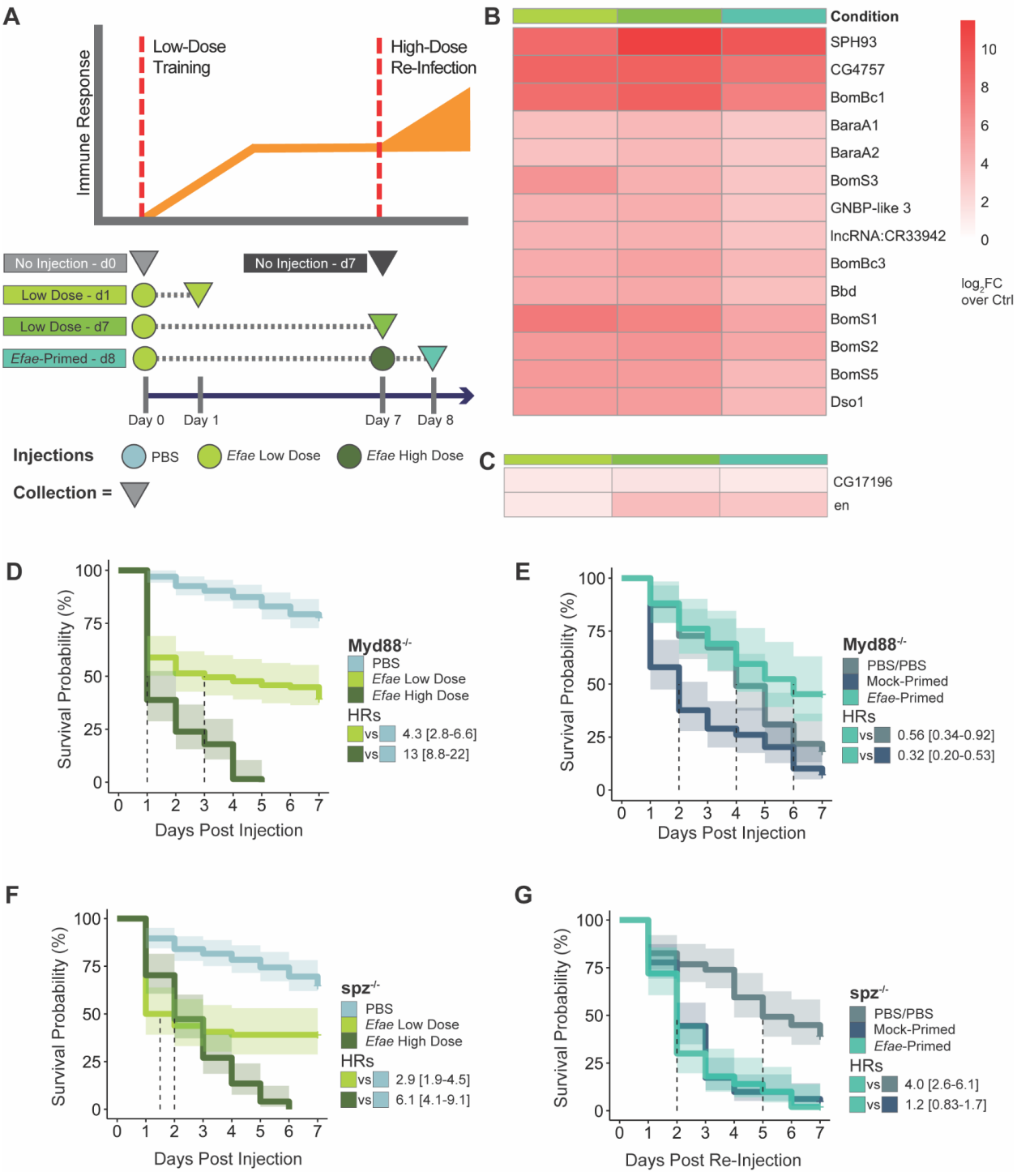

A
Immune Response
Low-Dose Training
High-Dose Re-Infection
No Injection - d0
No Injection - d7
Low Dose - d1
Low Dose - d7
Efae-Primed - d8
Day 0
Day 1
Day 7
Day 8
Injections
PBS
Efae Low Dose
Efae High Dose
Collection =
B
Condition
SPH93
CG4757
BomBc1
BaraA1
BaraA2
BomS3
GNBP-like 3
lncRNA:CR33942
BomBc3
Bbd
BomS1
BomS2
BomS5
Dso1
log2FC over Ctrl
C
CG17196
en
D
Survival Probability (%)
Days Post Injection
Myd88-/-
PBS
Efae Low Dose
Efae High Dose
HRs
4.3 [2.8-6.6]
13 [8.8-22]
E
Myd88-/-
PBS/PBS
Mock-Primed
Efae-Primed
HRs
0.56 [0.34-0.92]
0.32 [0.20-0.53]
F
spz-/-
PBS
Efae Low Dose
Efae High Dose
HRs
2.9 [1.9-4.5]
6.1 [4.1-9.1]
G
spz-/-
PBS/PBS
Mock-Primed
Efae-Primed
HRs
4.0 [2.6-6.1]
1.2 [0.83-1.7]
Days Post Re-Injection

Figure 2.5: Toll effector genes continuously express throughout *E. faecalis* immune priming A). Schematic of continuous gene expression from priming into re-infection. Experimental conditions are the same as **Figure 2.1A**, with the addition of age-matched, non-injected controls at Day 0 and Day 7 as well as an additional time point at Day 7 for collection of samples late in priming. Circles represent injections and triangles represent time of collection **B).** Continuously expressing genes in fat bodies (scale: log_2FC over age-matched controls). **C).** Continuously expressing genes in adult hemocytes (scale: log_2FC over age-matched controls). **D).** Survival of single-injected *Myd88* mutant flies versus PBS control (PBS: n = 135, *Efae* Low Dose: n = 107, *Efae* High Dose: n=67). Low Dose vs PBS: HR = 4.3 [2.8-6.6]; High Dose vs PBS: HR = 13 [8.8-22]. **E).** Survival of primed *Myd88* mutant flies versus double-injected, non-primed controls (PBS/PBS: n = 60, Mock-Primed: n = 69, *Efae*-Primed: n=60). *Efae*-Primed vs PBS/PBS: HR = 0.56 [0.34-0.92]; Mock-Primed vs. PBS/PBS: HR = 1.8 [1.2-2.7]. **F).** Survival of single-injected *spz* mutant flies versus PBS control (PBS: n = 64, *Efae* Low Dose: n = 65, *Efae* High Dose: n=74). Low Dose vs PBS: HR = 2.9 [1.9-4.5]; High Dose vs PBS: HR = 6.1 [4.1-9.1]. **G).** Survival of primed *spz* mutant flies versus double-injected, non-primed controls (PBS/PBS: n = 69, Mock-Primed: n = 81, *Efae*-Primed: n=50). *Efae*-Primed vs PBS/PBS: HR = 4.0 [2.6-6.1]; Mock-Primed vs. PBS/PBS: HR = 3.4 [2.3-5.1].

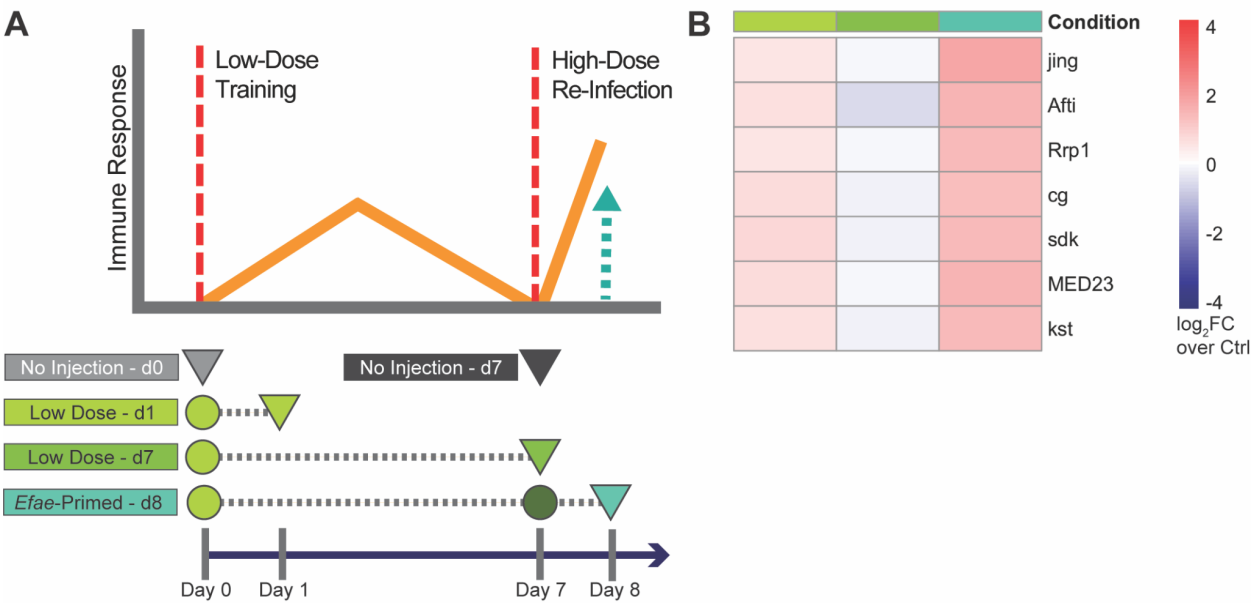
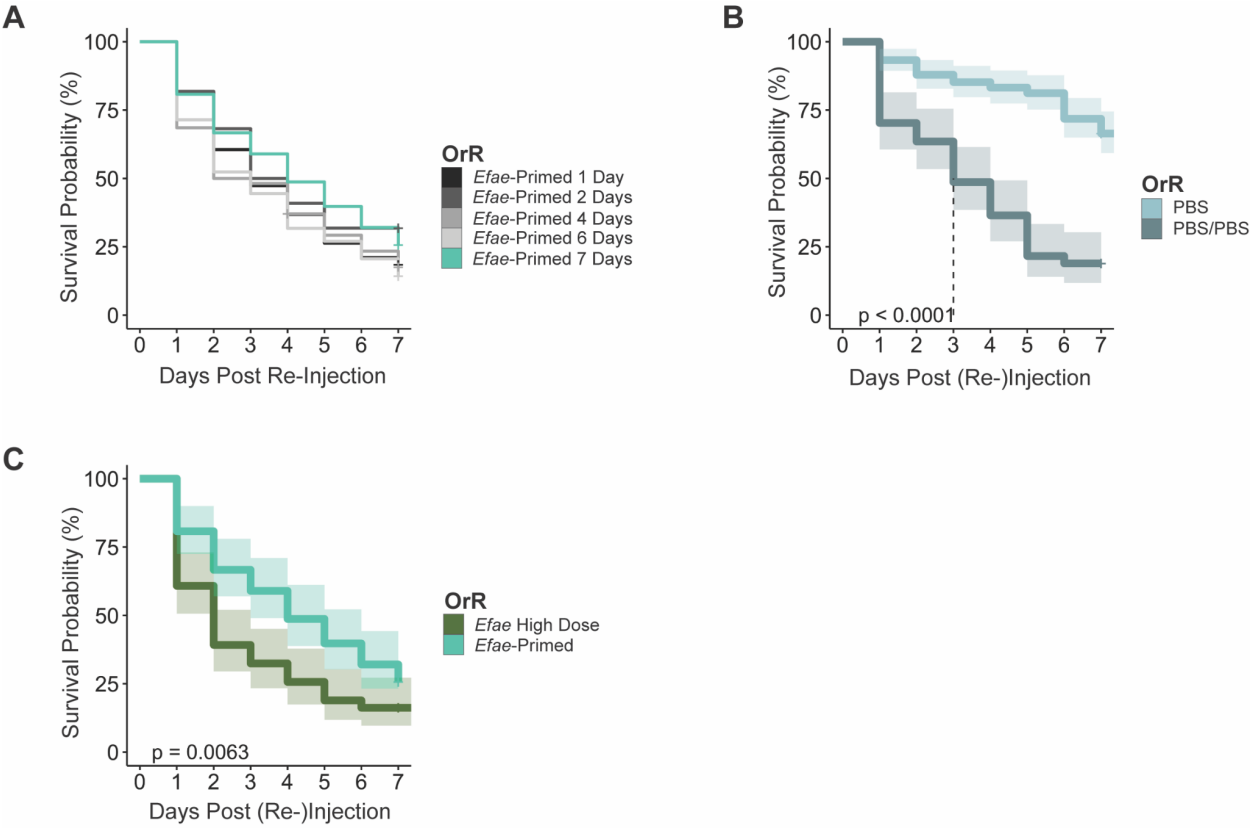


Figure 2.6: Few potentiated genes are recalled in *E. faecalis* immune priming
A). Schematic of immune recall response. **B).** Potentiated recall genes in fat bodies (scale: log_2FC over age-matched controls).

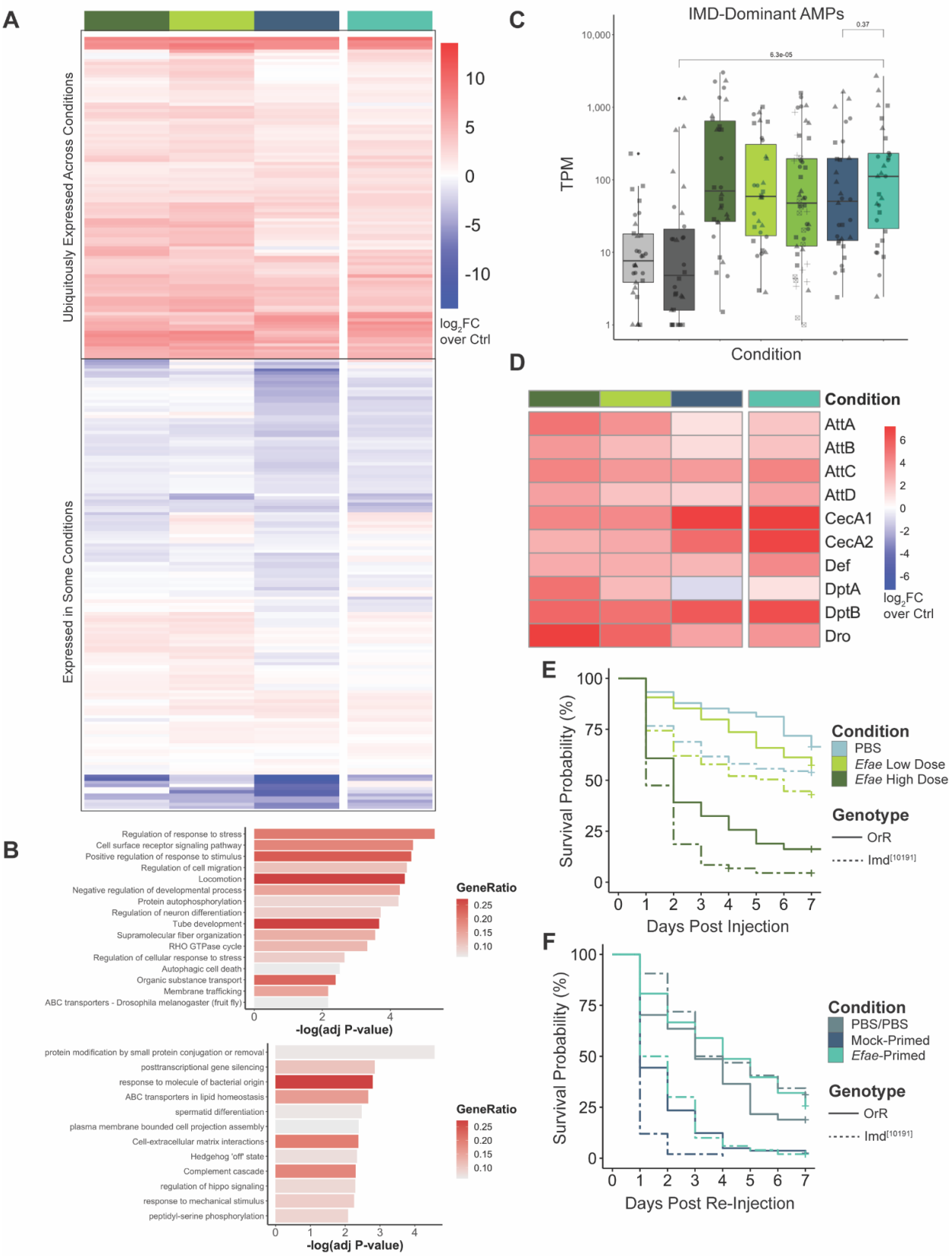
2.10: SUPPLEMENTARY MATERIAL

2.10.1: Supplementary Figures

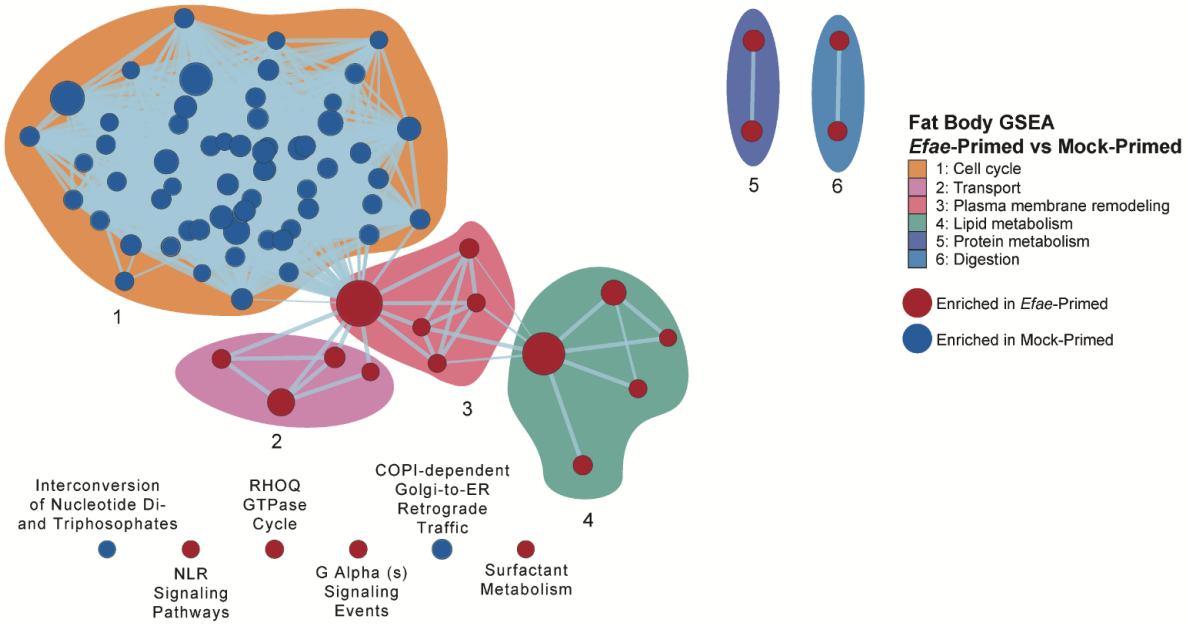


Supplementary Figure 2.1: Dynamics of *E. faecalis* priming and double-injection survival in OrR flies

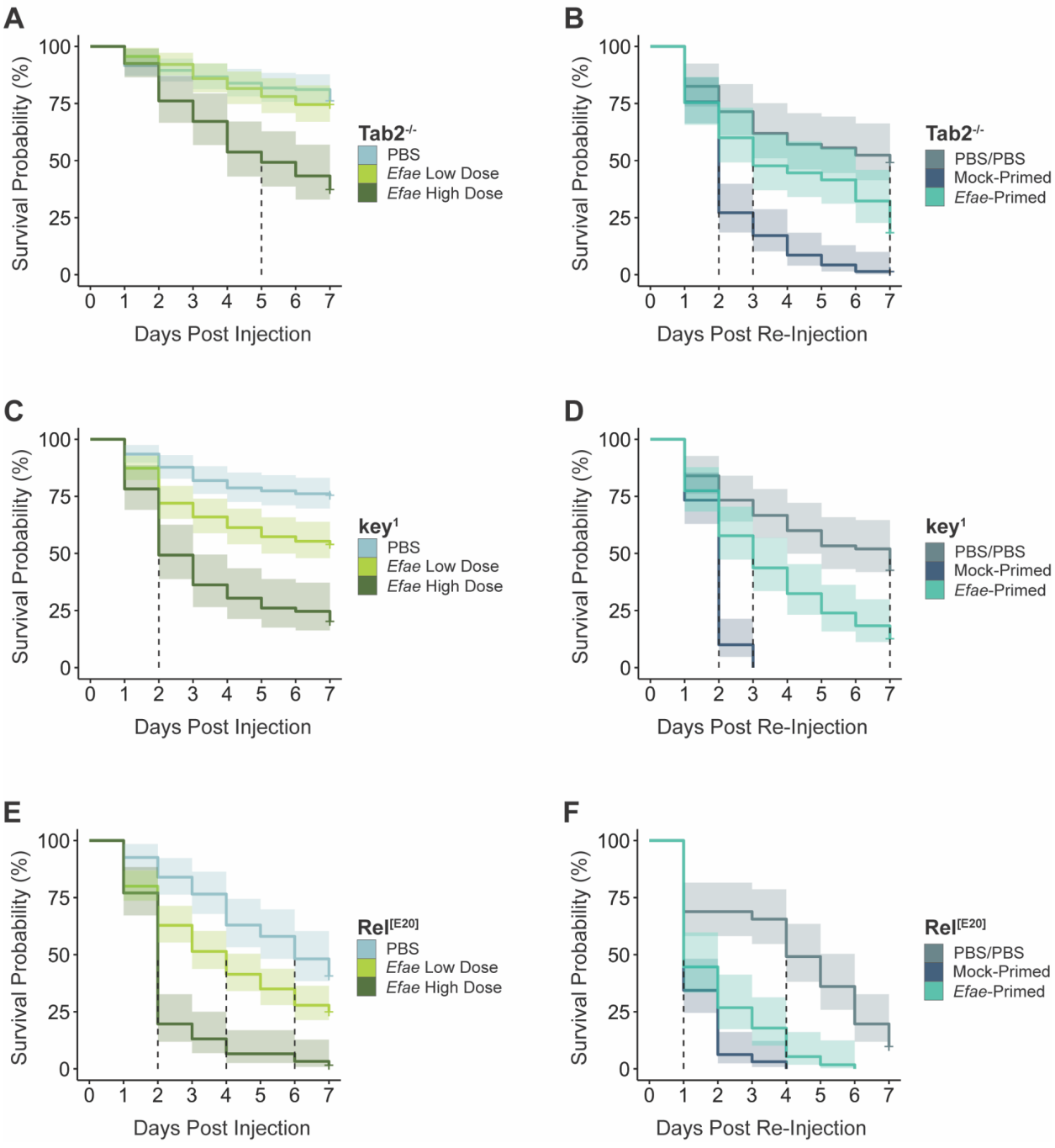
A). Survival is similar in flies allowed to prime with a low-dose of *E. faecalis* (~3,000 CFU/fly) for varying amounts of time before re-infection with a high dose of *E. faecalis* (~30,000 CFU/fly) (n: 1 Day = 38, 2 Days = 22, 4 Days = 54, 6 Days = 63, 7 Days = 78). **B).** There is a significant difference in survival (log-rank sum test, $p<0.0001$) in OrR flies injected once with PBS (PBS, n = 149) or twice with PBS with seven days of rest between repeated injections (PBS/PBS, n = 74). Dotted lines indicate median survival time; shaded regions indicate 95% confidence intervals. **C).** There is a significant difference in survival (log-rank sum test, $p=0.0063$) in OrR flies injected once with a high dose of *E. faecalis* (*Efae* High, ~30,000 CFU/fly, n = 74) versus primed with a low dose of *E. faecalis* for seven days and then re-infected with a high dose of *E. faecalis* (*Efae*-Primed, n = 78). Data are the same as **Figure 2.1B-C**, replotted for comparison.


A
Ubiquitously Expressed Across Conditions
Expressed in Some Conditions
10
5
0
-5
-10
log2FC over Ctrl
B
Regulation of response to stress
Cell surface receptor signaling pathway
Positive regulation of response to stimulus
Regulation of cell migration
Locomotion
Negative regulation of developmental process
Protein autophosphorylation
Regulation of neuron differentiation
Tube development
Supramolecular fiber organization
RHO GTPase cycle
Regulation of cellular response to stress
Autophagic cell death
Organic substance transport
Membrane trafficking
ABC transporters - Drosophila melanogaster (fruit fly)
0
2
4
-log(adj P-value)
GeneRatio
0.25
0.20
0.15
0.10
protein modification by small protein conjugation or removal
posttranscriptional gene silencing
response to molecule of bacterial origin
ABC transporters in lipid homeostasis
spermatid differentiation
plasma membrane bounded cell projection assembly
Cell-extracellular matrix interactions
Hedgehog 'off' state
Complement cascade
regulation of hippo signaling
response to mechanical stimulus
peptidyl-serine phosphorylation
0
1
2
3
4
-log(adj P-value)
GeneRatio
0.25
0.20
0.15
0.10
C
IMD-Dominant AMPs
0.37
6.3e-05
10,000
1,000
100
10
1
TPM
Condition
D
Condition
AttA
AttB
AttC
AttD
CecA1
CecA2
Def
DptA
DptB
Dro
6
4
2
0
-2
-4
-6
log2FC over Ctrl
E
100
75
50
25
0
Survival Probability (%)
0
1
2
3
4
5
6
7
Days Post Injection
Condition
PBS
Efae Low Dose
Efae High Dose
Genotype
OrR
Imd[10191]
F
100
75
50
25
0
Survival Probability (%)
0
1
2
3
4
5
6
7
Days Post Re-Injection
Condition
PBS/PBS
Mock-Primed
Efae-Primed
Genotype
OrR
Imd[10191]

Supplementary Figure 2.2: Additional analysis for fat body RNA-seq
A). Heatmap of log_2FC over non-injected controls of whole-body, core immune response genes from Troha, et al. 2018. Only a subset of the core genes were ubiquitously expressed across all conditions assayed for in this study. The differences are likely due to distinctions in time point and tissue. **B).** GO term enrichment from fat body *Efae*-Primed-specific [top] and Mock-Primed-specific [bottom], up-regulated genes. **C).** Expression in log_{10}(TPM+1) of IMD-dominant AMPs. Biological replicates are designated by the shape of individual points. While there is a significant difference in IMD AMP expression in *Efae*-Primed fat bodies compared to their age-matched, non-injected controls (Wilcoxon test; p = 6.3E-05), there is not a significant difference in expression between Mock-Primed and *Efae*-Primed fat bodies (Wilcoxon test; p =0.37). **D).** Heatmap of log_2FC over non-injected controls of IMD-dominant AMPs across collected fat body samples. **E).** Single-injection survival comparison between OrR and *imd*-mutant flies. **F).** Double-injection survival comparison between OrR and *imd*-mutant flies. Data from D & E are the same as in **Figure 2.3G&H**, replotted for comparison.

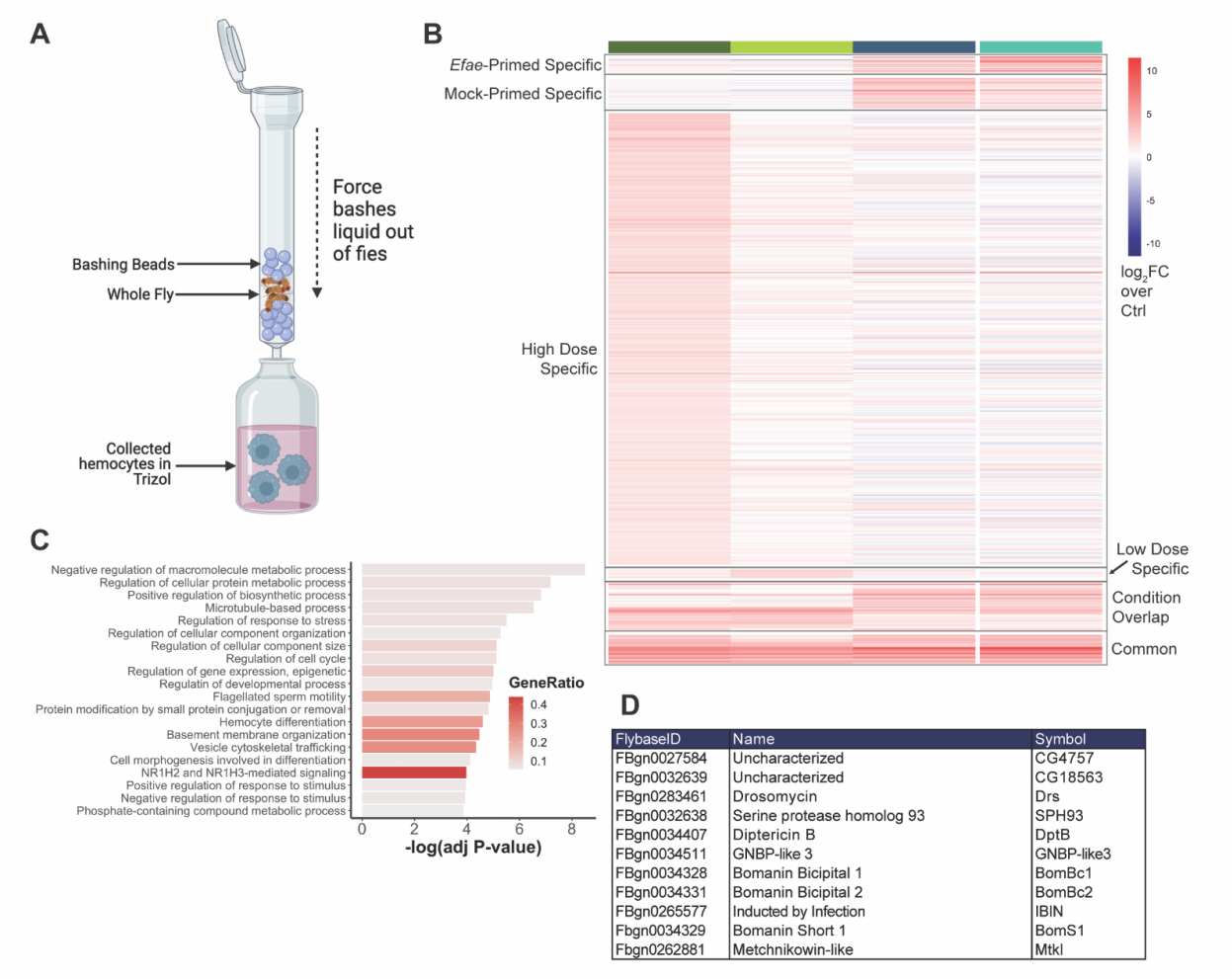


Supplementary Figure 2.3: GSEA for *Efae*-primed versus Mock-primed fat bodies
This visualization represents relationships between statistically significant terms (FDR < 0.05), manually curated with clusters that summarize the relationships between terms. Full results are found in Supplementary Table 2.3.


A
Survival Probability (%)
100
75
50
25
0
0 1 2 3 4 5 6 7
Days Post Injection
Tab2-/-
PBS
Efae Low Dose
Efae High Dose
B
Survival Probability (%)
Days Post Re-Injection
Tab2-/-
PBS/PBS
Mock-Primed
Efae-Primed
C
Survival Probability (%)
Days Post Injection
key1
PBS
Efae Low Dose
Efae High Dose
D
Survival Probability (%)
Days Post Re-Injection
key1
PBS/PBS
Mock-Primed
Efae-Primed
E
Survival Probability (%)
Days Post Injection
Rel[E20]
PBS
Efae Low Dose
Efae High Dose
F
Survival Probability (%)
Days Post Re-Injection
Rel[E20]
PBS/PBS
Mock-Primed
Efae-Primed

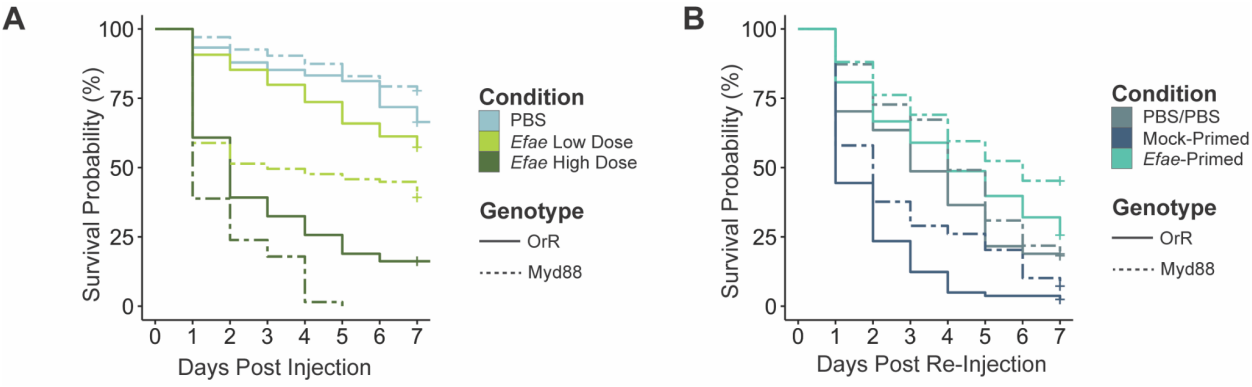
Supplementary Figure 2.4: Single- and double-injection survival for additional IMD & Toll pathway mutants

A). Survival of single-injected *Tab2* mutant flies versus PBS control (PBS: n = 143, *Efae* Low Dose: n = 114, *Efae* High Dose: n = 67). Dotted line indicates median survival time. Shaded area indicates 95% confidence interval. Low Dose vs PBS: HR = 1.1, p = 0.76; High Dose vs PBS: HR = 3.4, p = 1.1E-07; pairwise comparisons are calculated using a Cox proportional hazard model with hazard ratios and Wald statistic values reported for experimental conditions versus their PBS negative control. **B).** Survival of primed *Tab2* mutant flies versus double-injected, non-primed controls (PBS/PBS: n = 63, Mock-Primed: n = 70, *Efae*-Primed: n=65). *Efae*-Primed vs PBS/PBS: HR = 2.0, p = 0.0015; Mock-Primed vs. PBS/PBS: HR = 4.6, p = 1.7E-11. **C).** Survival of *key* mutant flies injected with PBS (n = 155), *Efae* Low Dose (~3,000 CFU/fly, n = 148), and *Efae* High Dose (~30,000 CFU/fly, n = 69). Low Dose vs PBS: HR = 2.2, p = 9.5E-05; High Dose vs PBS: HR = 5.3, p = 5.4E-15. **D).** Survival of primed *key* mutant flies versus double-injected, non-primed controls (PBS/PBS: n = 75, Mock-Primed: n = 60, *Efae*-Primed: n=71). *Efae*-Primed vs PBS/PBS: HR = 2.3, p = 3.5E-05; Mock-Primed vs. PBS/PBS: HR = 7.1, p = 2.0E-16. **E).** Survival of single-injected *Rel* mutant flies versus PBS control (PBS: n = 140, *Efae* Low Dose: n = 63, *Efae* High Dose: n=60). Low Dose vs PBS: HR = 0.57, p = 0.0015; High Dose vs PBS: HR = 2.8, p = 1.2E-09. **F).** Survival of primed *Rel* mutant flies versus double-injected, non-primed controls (PBS/PBS: n = 55, Mock-Primed: n = 64, *Efae*-Primed: n = 56). *Efae*-Primed vs PBS/PBS: HR = 3.7, p = 2.4E-09; Mock-Primed vs. PBS/PBS: HR = 5.7, p = 7.3E-14. Like *imd* mutants, *Rel*, *key*, and *Tab2* mutants lost the ability to fully prime against *E. faecalis* infections. The relative severity of the loss does depend on the mutant, with *Relish* mutants showing the weakest priming ability, followed by *key*, and then *Tab2* (which shows only a minor priming defect).

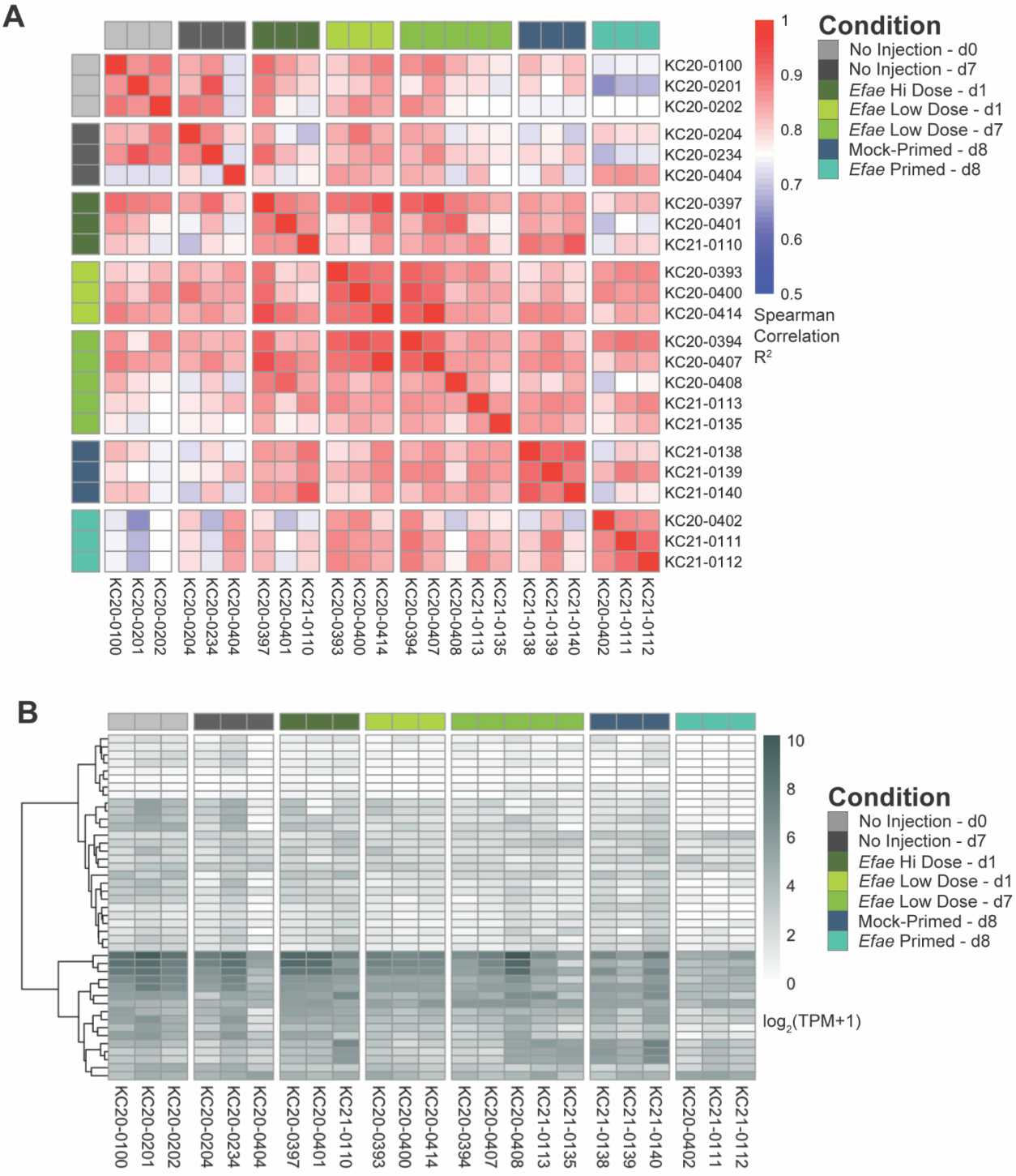


FlybaseID	Name	Symbol
FBgn0027584	Uncharacterized	CG4757
FBgn0032639	Uncharacterized	CG18563
FBgn0283461	Drosomycin	Drs
FBgn0032638	Serine protease homolog 93	SPH93
FBgn0034407	Diptericin B	DptB
FBgn0034511	GNBP-like 3	GNBP-like3
FBgn0034328	Bomanin Bicipital 1	BomBc1
FBgn0034331	Bomanin Bicipital 2	BomBc2
FBgn0265577	Inducted by Infection	IBIN
Fbgn0034329	Bomanin Short 1	BomS1
Fbgn0262881	Metchnikowin-like	Mtkl

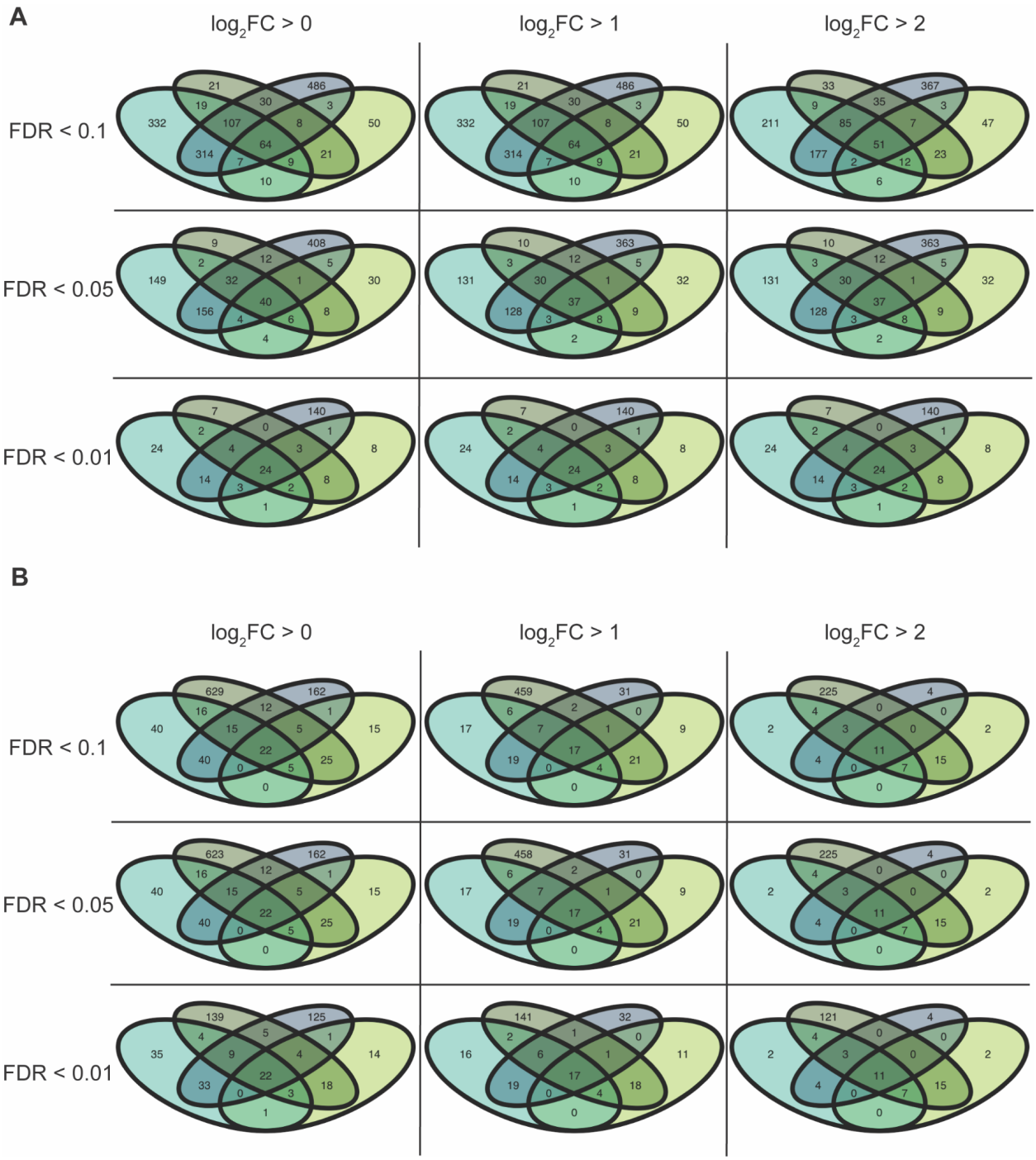
Supplementary Figure 2.5: Additional data for hemocyte RNA-seq
A). Schematic diagram of hemocyte RNA extraction. **B).** Significantly up-regulated genes as corresponding to conditions in Fig 4A (scale: log_2FC over age-matched controls). **C).** GO term enrichment from hemocyte *Efae* Hi Dose-specific, up-regulated genes. **D).** Overlap of up-regulated core genes (4-condition overlap in Venn diagram) between hemocytes and fat bodies.



Supplementary Figure 2.6: Additional data for continuous expression RNA-seq
A). Single-injection survival comparison between OrR and *Myd88*-mutant flies.
B). Double-injection survival comparison between OrR and *Myd88*-mutant flies. Data are the same as in **Figure 2.5 D&E**, replotted for comparison.



Supplementary Figure 2.7: Quality control of fat body RNA-seq libraries
A). Spearman correlation heatmap of fat body RNA-seq libraries. Values are R^2 spearman correlation values. **B).** Expression of sperm motility genes in fat body RNA-seq libraries. Values are $\log_2(TPM+1)$.



Supplementary Figure 2.8: Modulation of significance and fold-change cutoffs in differential analysis

A). Overlap analysis between 24-hour RNA-seq in fat bodies when changing fold-change cut-offs along the y-axis ($log_2FC>0$, $log_2FC>1$, $log_2FC>2$) and significance cutoffs along the x-axis (FDR<0.1, FDR<0.05, FDR<0.01). **B).** Same analysis as **A**, with hemocytes.

2.10.2: Supplementary Data

(Supplementary Tables 2.2-6 can be found in the online print version of this article)

Genotype	Condition	Referent	HR	Lower	Upper	Pval	Prime Against Mock?	Full Priming?
eater	*Efae Low*	*PBS*	2.127	1.431	3.162	1.92E-04		
eater	*Efae High*	*PBS*	32.965	20.552	52.875	2.00E-16		
eater	*Efae Mock*	*PBS/PBS*	5.589	3.381	9.24	1.94E-11		
eater	*Efae Primed*	*PBS/PBS*	3.803	2.267	6.38	4.16E-07		NO
eater	*Efae Primed*	*Efae Mock*	0.6807	0.4758	0.9737	3.52E-02	YES	
Imd [10191]	*Efae Low*	*PBS*	1.313	0.949	1.818	1.00E-01		
Imd [10191]	*Efae High*	*PBS*	3.328	2.415	4.587	2.03E-13		
Imd [10191]	*Efae Mock*	*PBS/PBS*	10.657	6.543	17.359	2.00E-16		
Imd [10191]	*Efae Primed*	*PBS/PBS*	4.141	2.739	6.261	1.63E-11		NO
Imd [10191]	*Efae Primed*	*Efae Mock*	0.3942	0.2666	0.5831	3.15E-06	YES	
Myd88	*Efae Low*	*PBS*	4.284	2.776	6.61	4.88E-11		
Myd88	*Efae High*	*PBS*	13.861	8.752	21.95	2.00E-16		
Myd88	*Efae Mock*	*PBS/PBS*	1.8181	1.239	2.6678	2.25E-03		
Myd88	*Efae Primed*	*PBS/PBS*	0.5554	0.3354	0.9199	2.23E-02		YES
Myd88	*Efae Primed*	*Efae Mock*	0.3241	0.1998	0.5257	5.00E-06	YES	
OrR	*Efae Low*	*PBS*	1.422	0.9691	2.087	0.0719		
OrR	*Efae High*	*PBS*	5.704	3.9228	8.293	2.00E-16		
OrR	*Efae Mock*	*PBS/PBS*	2.573	1.8184	3.641	9.51E-08		

OrR	*Efae Primed*	*PBS/PBS*	0.7541	0.5251	1.083	1.26E-01		YES
OrR	*HK-Efae Primed*	*PBS/PBS*	1.3958	0.9611	2.027	7.99E-02		NO
OrR	*BB-Efae Low*	*PBS*	1.929	1.2257	3.035	4.52E-03		
OrR	*BB-Efae Primed*	*PBS/PBS*	4.6096	3.1245	6.801	1.34E-14		NO
OrR	*Efae Primed*	*Efae Mock*	0.2855	0.201	0.4057	2.66E-12	YES	
OrR	*HK-Efae Primed*	*Efae Mock*	0.5205	0.3658	0.7406	2.84E-04	YES	
OrR	*BB-Efae Primed*	*Efae Mock*	1.8547	1.3143	2.6173	4.40E-04	NO	
spz	*Efae Low*	*PBS*	2.896	1.88	4.462	1.42E-06		
spz	*Efae High*	*PBS*	6.115	4.109	9.101	2.00E-16		
spz	*Efae Mock*	*PBS/PBS*	3.434	2.321	5.083	6.87E-10		
spz	*Efae Primed*	*PBS/PBS*	3.984	2.598	6.109	2.36E-10		NO
spz	*Efae Primed*	*Efae Mock*	1.188	0.8294	1.702	0.347	NO	
key	*Efae Low*	*PBS*	2.202	1.481	3.272	9.47E-05		
key	*Efae High*	*PBS*	5.277	3.477	8.007	5.39E-15		
key	*Efae Mock*	*PBS/PBS*	7.12	4.501	11.26	2.00E-16		
key	*Efae Primed*	*PBS/PBS*	2.299	1.549	3.41	3.54E-05		NO
key	*Efae Primed*	*Efae Mock*	0.2777	0.1798	0.4289	7.64E-09	YES	
Tab2	*Efae Low*	*PBS*	1.079	0.6573	1.771	0.764		
Tab2	*Efae High*	*PBS*	3.425	2.1746	5.394	1.09E-07		
Tab2	*Efae Mock*	*PBS/PBS*	4.618	2.958	7.212	1.70E-11		
Tab2	*Efae Primed*	*PBS/PBS*	2.044	1.315	3.175	0.00148		NO
Tab2	*Efae Primed*	*Efae Mock*	0.3951	0.27671	0.5844	3.33E-06	YES	
Rel [E20]	*Efae Low*	*PBS*	0.5746	0.4081	0.8091	0.00151		

Rel [E20]	*Efae High*	*PBS*	2.7967	2.0073	3.8965	1.22E-09		
Rel [E20]	*Efae Mock*	*PBS/PBS*	5.744	3.634	9.079	7.28E-14		
Rel [E20]	*Efae Primed*	*PBS/PBS*	3.686	2.401	5.657	2.42E-09		NO
Rel [E20]	*Efae Primed*	*Efae Mock*	0.606	0.4144	0.8862	0.00979	YES	

Supplementary Table 2.1: Summary statistics for all survival curves calculated using Kaplan-Meier visualizations and Cox proportional hazard modeling.

Supplementary Table 2.2: Sequencing information for fat body and hemocyte RNA-seq

Supplementary Table 2.3: Lists of up-regulated genes specific to each fat body condition assayed in **Figure 2.3**, common between all fat body conditions, and specifically down-regulated in *Efae*-Primed-d8 fat bodies.

Supplementary Table 2.4: Gene set enrichment analysis for *Efae*-Primed vs Mock-Primed fat bodies. Clustering and terms are shown in **Supplementary Figure 2.3**. This represents the tabular output directly from the GSEA software v. 4.2.3 (Subramanian, et al. 2005).

Supplementary Table 2.5: Lists of up-regulated genes specific to each hemocyte condition assayed in **Figure 2.4**, common between all hemocyte conditions, specifically down-regulated in *Efae*-Primed-d8 fat bodies and overlap between common *Efae*-response genes in fat bodies and hemocytes.

Supplementary Table 2.6: Gene set enrichment analysis for *Efae*-Primed vs Mock-Primed hemocytes. Clustering and terms are shown in **Figure 2.4C**. This represents the tabular output directly from the GSEA software v. 4.2.3 (Subramanian, et al. 2005).

3: DYNAMICS OF IMMUNE RESPONSE AND PRIMING WITH DIFFERING BACTERIAL SPECIES AND CONTROL GENOTYPES

3.1: ABSTRACT

The innate immune system is able to retain some amount of immunological memory from a primary infection. This change can then be used to more successfully respond to a secondary infection in a phenomenon known as immune priming. In this study, we have characterized the priming ability in *Drosophila melanogaster* while varying infecting pathogens, re-infection times, and host genotype. Ability to prime against *Enterococcus faecalis* re-infection was dependent on host genotype and the rest time between exposures. Although there was no homologous priming with *Serratia marcescens*, heterologous priming between *E. faecalis* and *S. marcescens* was observed. Finally, within our experiments we find that survival against single-dose infections is not predictive of survival against *E. faecalis* re-infection. Immune priming is not a static phenotype – it is highly dependent on assay and environmental conditions.

3.2: INTRODUCTION

One of the major challenges in describing immune priming has been the variability between published studies. Between any two publications there are documented differences in host genotypes, bacterial species used, method of pathogen introduction, time between primary and secondary infection, and many more (Arch, et al. 2022). One of the first choices that needs to be considered is the identity of initial and secondary infections. Experiments can either employ homologous priming (in which the primary and secondary infection is of the same

species), or heterologous priming (in which the primary and secondary infections are different) (Contreras-Garduño, et al. 2016). Given the large variation in study design and overall outcomes, there is no clear evidence as to whether heterologous or homologous priming confers optimal immune priming. This has brought forth questions on the specificity of a primed immune response and whether specificity is specific to only a subset of pathogens (Sheehan, et al. 2020).

It has also been previously appreciated that genetic differences among control lines of *D. melanogaster* contribute to their ability to combat infection (Lazzaro, et al. 2006). Despite the differences between control genotypes to respond to different pathogens, there is not a high degree of evolutionary adaptation in *Drosophila* immune genes at the population-level. A study comparing immune gene evolution between *Drosophila melanogaster* and *Drosophila simulans* populations found that there was low core immune pathway gene sequence variability between populations adapted to distinct microbial environments – most of the variability was between Drosophila species(Early, et al. 2017). However, it is clear that environmental factors (both biotic and abiotic) have marked effects on immune response (Lazzaro, et al. 2008; Lazzaro & Little 2009). Population-level genotypic variability that affects immune outcomes is lying outside of the core of immune pathways. Immune-affecting genetic variation has been mapped to effector/bacterial sensing genes (Early, et al. 2017), X-linked JAK-STAT and JNK genes (Hill-Burns & Clark 2009), and mitochondrial chromosomes (Salminen & Vale 2020).

Natural variation between control genotypes can be leveraged to study the differential effects of polymorphic traits on immune response. One such resource available to the fly community is the *Drosophila* Synthetic Population Resource (DSPR) which collects various fully-sequenced, inbred control genotypes with significant genetic dissimilarity (King, et al. 2012). Population-level genetic variation has been used to identify cis- and trans-acting factors

that are responsible for regulating both Gram-positive and Gram-negative immune responses (Ramirez-Corona, et al. 2021). Large population-scale studies have also been used to characterize the effect of genetic variation in heterologous priming with *Micrococcus luteus* and *Staphylococcus aureus* (Tang, et al. 2022). This study found that variation in ~80 genes (some of them related to metabolic activity) was responsible for describing the differences in survival seen in their priming set up.

To investigate the effects host genotype, bacterial species, and temporal dynamics have on priming, we have repeated and modified our *E. faecalis*-priming experiments (detailed in Chapter 2) with different control genotypes and priming times. We also introduce priming experiments with the Gram-negative entomopathogen *Serratia marcescens* and observe a lack of survival protection when priming. We also introduce a genetically engineered control strain with GFP-labeled tissue that not only recapitulates our immune priming phenotypes, but can be used for functional characterization of immune response in the future. Finally, we leverage our large amount of priming data to show that survival against a single-dose infection is not predictive of survival to a secondary infection.

3.3: RESULTS

3.3.1: Control genotypes vary in their ability to prime against *E. faecalis*

After having bench-marked the ability to prime against *E. faecalis* using OrR as our control genotype, we asked whether the ability to enhance re-infection survival was specific to OrR or observed across control genotypes. We began by measuring priming ability in genotypes previously assayed for survival against the same wild-caught strain of *E. faecalis* (Lazzaro, et al. 2006; Ramirez-Corona, et al. 2021; Wukitch, et al. 2023) (Figure 3.1). As in our previous *E.*

faecalis assays (detailed in **2.5.2**), we first injected flies with either PBS, a low-dose of *E. faecalis* (~3,000 CFU/fly), or a high-dose of *E. faecalis* (~30,000 CFU/fly). CantonS experiments (Figure 3.1G & H) are preliminary as our lab stock was eliminated before experiments were completed. As explained in **2.3.1**, survival is summarized using hazard ratios (HRs) referent against PBS negative controls.

With the exception of B6 (Figure 3.1A), we observed a dose-dependent response to single *E. faecalis* infection (Figure 3.1C, E, G). In general, we have found B6 to be robust against most pathogenic infections (see Figure 3.4 as well), which is consistent with previously published survival data for the genotype (Ramirez-Corona, et al. 2021). Iso1 and A4 had *Efae* low dose and *Efae* high dose survival levels comparable to our original OrR (Low Dose vs PBS HRs: OrR = 1.4 [95% CI 0.96-2.1], A4 = 1.6 [1.1-2.2], Iso1 = 1.2 [0.84-1.7]; (High Dose vs PBS HRs: OrR = 5.7 [3.9-8.3], A4 = 3.9 [2.7-5.7], Iso1 = 2.6 [1.8-3.8]). CantonS flies were somewhat more susceptible to low-dose infections compared to OrR (HR = 2.2 [1.5-3.3]). Overall, this demonstrated that survival against single *E. faecalis* infections in previously reported control genotypes (with the exception of B6) was comparable to our internal OrR standard.

While these genotypes had been previously assayed for response to single-dose *E. faecalis* infections, they had not been assayed for double infections using our priming schema. In contrast to our OrR standard, flies of these control genotypes were not able to mount a primed immune response against *E. faecalis*. In accordance with its lower lethality to single-dose *E. faecalis*, B6 flies had only moderately lower survival in both Mock-Primed and *Efae*-Primed cohorts versus PBS/PBS cohorts (HRs: Mock-Primed vs PBS/PBS = 1.8 [1.2-2.7]; *Efae*-Primed vs PBS/PBS = 1.5 [0.94-2.3]) (Figure 3.1B). Continuing our definition of priming ability as an *Efae*-Primed vs Mock-Primed HR significantly less than 1, we saw no priming in B6, A4, Iso1,

or CantonS (HRs: B6 = 0.83 [0.54-1.3], A4 = 0.91 [0.64-1.3], Iso1 = 0.92 [0.64-1.3], CantonS = 1.1 [0.58-1.9]) (Figure 3.1B, D, F, H). These data extend the conclusion that genotype has an effect on immune response by showing that host genotype can alter the ability to prime after re-infection, even if there is no overt difference in ability to respond to a single infection.

To extend our observations beyond previously reported-on genotypes, we assayed priming in several other founder lines from the DSPR (Figure 3.2). We decided to include more genotypes in an effort to understand whether *E. faecalis* priming according to our injection and timing methods was specific to OrR or whether it could be recapitulated in any other control genotypes. Again we found that the newly tested genotypes (A5, A7, B2, and B7) all showed dose-dependent survival against *E. faecalis* (Figure 3.2A, C, E, G). However, three of these new genotypes (A5, A7, B2) were able to successfully prime against *E. faecalis* re-infection (Efae-Primed vs. Mock-Primed HRs: A5 = 0.76 [0.54-1.1], A7 = 0.23 [0.14-0.36], B2 = 0.50 [0.34-0.74], B7 = 1.3 [0.88-1.84]) (Figure 3.2B, D, F, H). Unlike OrR however, none of the new genotypes were able to match *Efae*-primed survival to the PBS/PBS baseline (*Efae*-Primed vs PBS/PBS HRs: A5 = 4.2 [2.6-6.9], A7 = 1.3 [0.85-2.0], B2 = 1.5 [1.1-2.2], B7 = 1.4 [0.98-2.0]). Overall, this shows that *E. faecalis* priming is not OrR-specific, but the OrR still showed the greatest *E. faecalis* priming potential out of any of the genotypes tested.

3.3.2: The degree of protection after re-injection is dependent on the amount of time between the first and second injection

Timing between primary and secondary injections has been one of the major differences between prior priming experiments. To test how timing would affect the ability to prime, we modulated the amount of time between primary and secondary injections for both Efae-Primed

and Mock-Primed flies. We previously modulated the time for *Efae*-Primed flies (Supplementary Figure 2.1A). All of the assayed time intervals were able to successfully prime according to our *Efae*-Primed vs Mock-Primed HR < 1 metric (1 Day = 0.38 [0.25-0.58], 2 Days = 0.29 [0.16-0.50], 4 Days = 0.41 [0.28-0.59], 6 Days = 0.44 [0.31-0.62], 7 Days = 0.29 [0.20-0.41]). However, only 2 Day-primed and 7 Day-primed flies were able to prime at equivalently optimal levels, with survival the closest to the PBS/PBS control (Efae-Primed vs PBS/PBS HR: 1 Day = 0.96 [0.62-1.5], 2 Days = 0.73 [0.41-1.3], 4 Days = 1.0 [0.69-1.5], 6 Days = 1.1 [0.76-1.6], 7 Days = 0.75 [0.53-1.1]). These results support the idea that there are optimal priming intervals between initial and secondary pathogen exposure.

While not observed in our seven day Mock-priming experiments, it has been previously reported that previous wounding alone can confer some protection against infection from *E. faecalis* (Chakrabarti, et al. 2020) – given that the time between the two events is short enough. To test the dynamics of this, we modulated the amount of time between initial PBS injection and secondary *E. faecalis* high dose infection and tested its effects on survival (Figure 3.3).

To quantify survival enhancement, we analyzed hazard ratios for each Mock-Primed experiment relative to *Efae*-Primed survival. Since optimal survival protection is baselined by our *Efae*-Primed flies, the closer Mock-Primed vs *Efae*-Primed HRs get to 1, the closer they are to optimal protection capacity; the greater the HRs, the worse those flies are surviving compared to *Efae*-Primed flies. We found that flies allowed to rest 4 days between PBS and *E. faecalis* injections had the greatest survival enhancement while 7 day Mock-Primed flies died the most overall (Mock-Primed vs *Efae*-Primed HRs: 1 Day = 2.0 [1.3-3.2], 3 Days = 2.8 [1.6-5.0], 4 Days = 1.3 [0.80-2.1], 5 Days = 1.7 [1.2-2.4], 6 Days = 1.7 [1.2-2.5], 7 Days = 3.3 [2.4-4.7]). As

with our *Efae*-priming time experiments, we found that there was an optimal time interval for conferring optimal survival enhancement in Mock-primed flies.

3.3.2: Initial infection with *S. marcescens* does not provide protection against subsequent *S. marcescens* infection

To explore how ubiquitous priming ability is across bacterial species, we decided to repeat our priming experiments with the Gram-negative bacteria *Seratia marcescens*. *S. marcecens* and *E. faecalis* belong to different Gram classifications and do not share many surface proteins. Comparable to *E. faecalis*, we observed dose-dependent survival for single-injections in OrR (Figure 3.3A) and in A4 flies (Figure 3.3E), but not for B6 (Figure 3.3C). However, we did not observe an increase in survival for *Smar*-Primed flies in any of the three tested genotypes (Figure 3.3B, D, F). Instead, we find that Mock-Primed B6 (Figure 3.3D) and A4 (Figure 3.3F) flies have increased survival chances. Taken together, this indicates that *S. marcescens* re-infections are different from *E. faecalis* infections. While initial exposure to a low-dose of *S. marcescens* does not confer protection against re-infection in any of the tested genotypes, initial wounding alone does confer some level of enhanced survival upon re-infection in select control genotypes. While it has been previously reported that wounding does confer some level of protection in *E. faecalis* infections given that the time between wounding and infection is short enough (Chakrabarti, et al. 2020), such protection has not been previously documented for *S. marcescens*.

3.3.3: Heterologous priming with *E. faecalis* and *S. marcescens* sub-optimally enhances survival upon re-infection

After characterizing priming in homologous re-infection with both *E. faecalis* and *S. marcescens,* we then tested heterologous priming between the two bacterial species. Though limited in just testing two of many possible bacterial combinations, this begins probing into how nonspecific the ability to prime against reinfection is. For these experiments, we benchmark heterologous priming against both Mock-Primed and PBS/PBS survival as previously described.

In both experiments, we found that heterologous priming caused some enhanced survival compared to Mock-Primed flies (Figure 3.4). However, heterologously primed flies were not able to reach survival comparable to PBS/PBS controls, as had been previously observed in homologous *Efae*-priming experiments (Figure 2.1C). This indicates that heterologous priming with both *E. faecalis* and *S. marcescens* offers some enhanced survival, but at a sub-optimal level in the case of *E. faecalis* secondary infection.

3.3.4: Novel immune-labeled flies recapitulate immune response to *E. faecalis*

One of the main difficulties in extending functional descriptions of immune response in *D. melanogaster* is the lack of methods of easily assaying immune tissue. Consolidating findings from immune priming and attaching mechanisms to them would be ideally done in a host genotype that facilitates future functional characterization. Traditional methods of immune tissue isolation require a high degree of training and manual dexterity, and can cause stress-related gene expression induced from the isolation procedure itself (Krupp & Levine, 2010). New methods use Gal4-driven expression of a UAS-GFP^{KASH} construct to selectively label tissues within the fly (Jaregui-Lozano, et al. 2021). These fly lines anchor GFP domains to the cytosolic

face of the nuclear lamina tissues specific to the Gal4 construct. Selective labeling of *D. melanogaster* immune tissues using this system would create ideal lines for future characterization of immune response using functional genomic techniques (Figure 3.6A).

To make stably immune-labeled fly lines we crossed a UAS-GFP^{KASH} line to immune tissue-driven Gal4 lines (Hml>Gal4 for hemocytes, Cg>Gal4 for adult fat body, and Lsp2>Gal4 for larval fat body). Full cross details are outlined in the methods. Stably GFP-expressing flies fluoresced under blue light (Figure 3.6B) and had GFP-labeled nuclei when nuclear lysates were imaged under a fluorescent microscope (Figure 3.6C). We also assayed these flies for survival against *E. faecalis* and found that they were able to mount an immune response to the bacteria in ways that mimicked other control genotypes.

While it was clear that single-dose *E. faecalis* infections were pathogenic to these lines, Hml>GFP^{KASH} (aka NH2) and Cg>GFP^{KASH} (aka NF1) did not show a dose-dependent response (Figure 3.6D & F). However, Lsp2>GFP^{KASH} (aka NL1) did show dose-dependent single-dose survival (Figure 3.6H). We have also noted internally, that NH2 has relatively low fecundity and is a bit "sicklier" similar to CantonS, while NL1 and NF1 are relatively robust lines like OrR. When testing their ability to prime, we found that NH2 was able to "fully" prime like OrR (i.e. match survival of *Efae*-Primed cohorts to PBS/PBS controls) (Figure 3.6E). NF1 flies were able to prime significantly better than Mock-Primed cohorts (Figure 3.6G), but NL1 flies were unable to prime against *E. faecalis* re-infection (Figure 3.6I). These findings further solidify the idea that background genotype is playing a role in dictating their immune response and establish a baseline for using these lines in future experiments.

3.3.5: Survival against a single *E. faecalis* infection is not predictive of response to a secondary *E. faecalis* infection

We leveraged the large amounts of survival data collected from multiple genotypes to test the ability to predict survival to double-infections as a function of survival to single infections. Given the variation in survival to *Efae*-Low Dose and *Efae*-High Dose infections, we tested the correlation between single- and double-dose infection hazard ratios (Figure 3.7). This approach delineates whether higher or lower lethality to a low-dose infection would correlate with the ability to prime. If so, this may hint at an evolutionary logic that would dictate why some instances of initial infection confer protection and others do not.

When testing for correlation of Mock-Primed referent *Efae*-Primed hazard ratios (i.e. if these HRs < 1 then these flies were able to prime), we find that there is no significant correlation to single-dose infections (Figure 3.7A&B). The lack of correlation between single-dose infections and double-dose infections held whether mutant genotypes were included in the calculation (Figure 3.7A) or whether correlations were calculated using only control genotypes (Figure 3.7B).

We also found that single-dose HRs did not correlate with *Efae*-Primed HRs calculated with PBS/PBS survival as a referent (i.e. if these HRs = 1, then these flies were able to survive reinfection just as well as the PBS/PBS negative control) (Figure 3.7C&D). As in the Mock-Primed referent group, there was no correlation regardless of whether mutant genotypes were included in the calculations (Figure 3.7C), or not (Figure 3.7D). Across all comparisons, *Efae* High Dose and *Efae* Low Dose hazard ratios were positively correlated. This is concordant with the general observation that survival to single infections across all genotypes was dose-dependent. We also found that Mock-Primed HRs were negatively correlated with *Efae*-Primed

HRs calculated with Mock-Primed survival as a referent (Figure 3.7A&B). However, Mock-Primed and *Efae*-Primed HRs were positively correlated when *Efae*-Primed HRs are calculated usingPBS/PBS survival as the referent (Figure 3.7C) [albeit, the correlation is lost when only control genotypes are used (Figure 3.7D)]. This indicates that increased lethality in Mock-Primed flies is significantly correlated with increased ability to prime in the tested priming schema.

3.4: DISCUSSION

Immune priming is a complex phenotype liable to effects from host genotype, bacterial identity, training times, and exposure to stressors. In this manuscript we have tested how varying these parameters changed the ability to respond to a secondary stimulus and how poorly response to the initial stimulus predicts response to a secondary insult.

Taking a lateral approach to immune priming by keeping the infection schema constant and varying the different genotypes used highlights a caveat of any priming experiment – the results are contextual to the host they were done in. Despite these control genotypes having full canonical immune molecular pathways, something in them does not allow them to prime. Building from the findings from Chapter 2, the genetic variation between genotypes that prime and do not prime could be in genes that are largely priming-specific or that modulate tolerance. Such analyses require eQTL-like calculations obtained from repeating survival experiments with many more genotypes. However, a previous experiment testing M. luteus/S. aureus priming in hundreds of genotypes found that variation in ~80 genes, including Ald-h, were responsible for explaining differences in priming ability (Tang, et al. 2022). A subset of these genes, including Ald-h, were identified as ‘priming-specific’ in our study and as such lay the groundwork for comparative analysis.

Re-infection with the Gram-negative *S. marcescens* did not match the survival improvement logic we observed with the Gram-positive *E. faecalis*. However, it is not appropriate to attribute this difference in priming ability solely to Gram classification as many other studies have shown successful priming with other Gram-negative pathogens (Contreras-Garduño, et al. 2016). Priming with *S. marcescens* may still be possible with different timings, injection concentrations, or host genotypes. Future studies should assess how bacterial species and host genotypes determine the amount of time between primary and secondary insult needed to optimize survival enhancement.

3.5: METHODS

3.5.1: Fly Strains and Husbandry

All experiments were carried out with 4-day old males. Flies were housed at 25˚C with standard humidity and 12 hr-light/12 hr-dark light cycling. All DSPR founder lines were obtained directly from the DSPR at University of California, Irvine.

Nuclear GFP-expressing flies were generated by crossing immune tissue-specific Gal4 lines (Cg>GAL4 & Lsp2>GAL4 for fat bodies and Hml>GAL4 for hemocytes) to UAS-GFP^{KASH} lines. The full mating scheme is detailed below.

Cross 1: Initially cross the immune-driving Gal4 lines to the UAS-GFP^{KASH} line.

$$w^{1118}; Cg > GAL4\,; + \quad \times \quad w^{*}; +; P\{UAS - EGFP.KASH - Msp300\}attP2$$

Cross 2: Select Gal4 & UAS-GFP^{KASH} heterozygotes (which fluoresce under the blue light microscope) and mate them to a double-balancer line.

$$\frac{w^{1118}}{w^{*}}; \frac{Cg > GAL4}{+}; \frac{UAS - GFP^{KASH}}{+} \quad \times \quad w^{*}; \frac{Kr[IF - 1]}{CyO}; \frac{D[1]}{TM3, Ser[1]}$$

Cross 3: Select males and virgin females that are heterozygous for Gal4 & UAS-GFP^{KASH} (which fluoresce under the blue light microscope) AND are heterozygous for the double-balancer (curly and serrated wings). Self-cross to remove the balancer

$$\frac{w^{1118}}{w^{*}};\frac{Cg>GAL4}{CyO};\frac{UAS-GFP^{KASH}}{TM3,Ser[1]} \; --SelfCross\; --$$

$$> \frac{w^{1118}}{w^{*}};\frac{Cg>GAL4}{Cg>GAL4};\frac{UAS-GFP^{KASH}}{UAS-GFP^{KASH}}$$

3.5.2: Injections and Survival Tracking

Injections and subsequent survival tracking was done as detailed in **2.5.2** and **2.5.3**, with modifications where appropriate. Single colony inoculums of *S. marcescens* were grown overnight in 2mL LB shaking at 37˚C. 50uL of overnight *S. marcescens* inoculum was then added to 2mL fresh LB and grown shaking at 37˚C for 1.5 hours before injections to ensure it would be in the log-phase of growth. Bacteria was then pelleted at 5,000 rcf for 5 minutes, washed with PBS, re-suspended in 200uL PBS, and measured for its OD600 on a Nanodrop. Flies were injected with either PBS, *S. marcescens* at OD 0.05 for low dose experiments (~3,000 CFU/fly), or *S. marcescens* at OD 0.5 for high dose experiments (~30,000 CFU/fly). To insure correct bacterial doses were injected into each fly cohort, a subset of at least 5 flies would be collected throughout the injection session and dilution plated on LB plates.

3.5.3: Nuclei Isolation and visualization

Nuclei were isolated as previously described in Jauregui-Lozano, et al. 2021. Briefly, 50 4-7 day old male flies were collected in 1.5mL Eppendorf tubes and initially dissociated through five cycles of flash freezing with liquid nitrogen and vortexing. Flies were transferred to a sound

homogenizer containing 1mL homogenization buffer (40 mM HEPES, pH 7.5, 120 mM KCl, and 0.4% v/v NP-40). Flies were homogenized using 7 strokes with “loose pestle” followed by 7 strokes with “tight” pestle. Homogenized lysate was then filtered using 40 μm cell strainers (Corning, Tewksbury MA, USA Catalog# 352340), and NP-40 was diluted to 0.1% final concentration by adding three volumes of Dilution buffer (40 mM HEPES, pH 7.5 and 120 mM KCl). Homogenate was then flash frozen and stored at -80˚C for future use. For nuclei imaging, 10uL homogenate was stained with Hoechst dye and visualized using a confocal microscope.

3.5.4: Correlation Analysis

Spearman correlations were calculated between hazard ratios using custom R codes. Correlations and p-values were then plotted using the R package ‘corrplot’.

3.6: ACKNOWLEDGEMENTS

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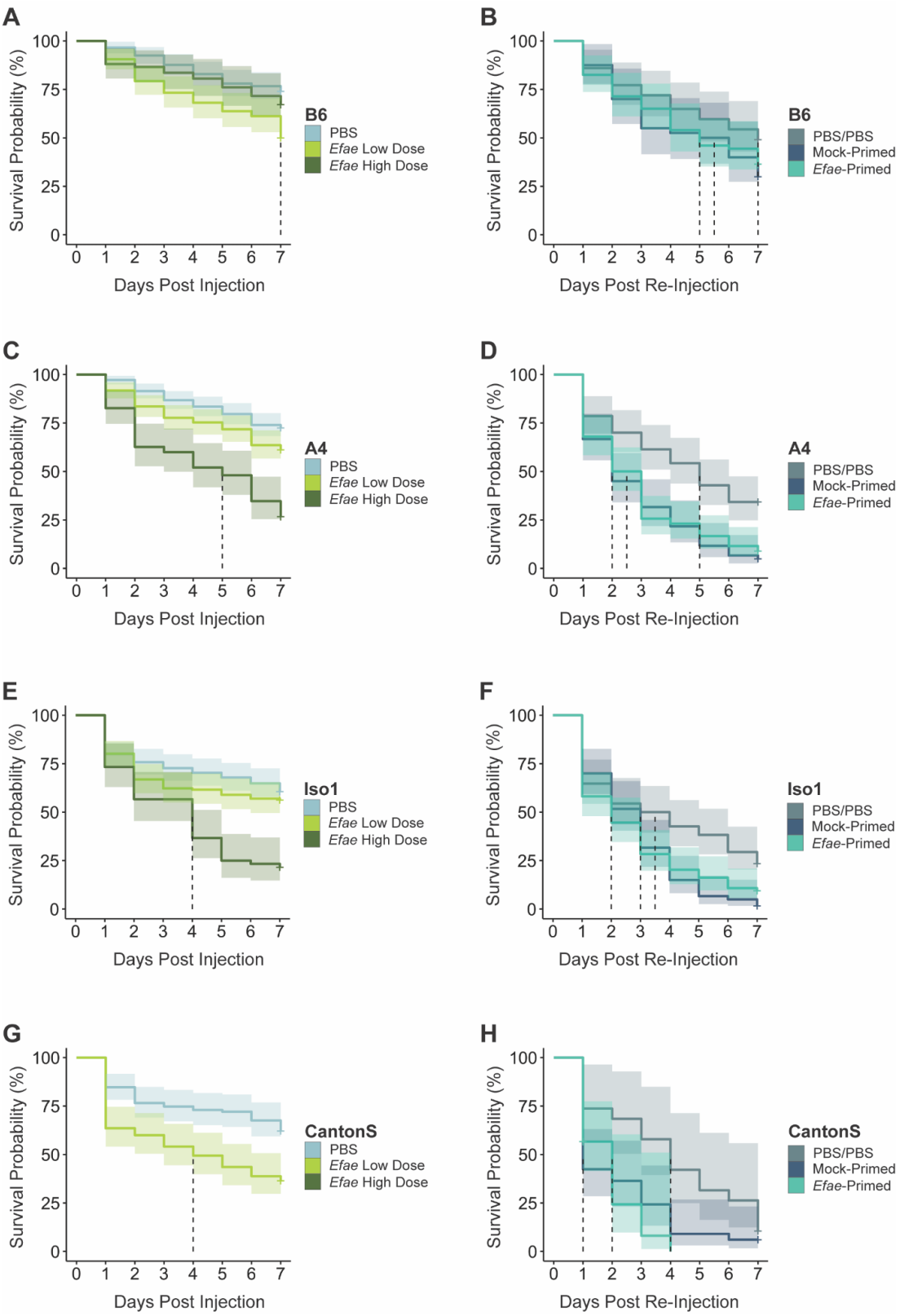
3.7: AUTHOR CONTRIBUTIONS

Z.W. and K.C. are responsible for conceptualization and formal analysis throughout. K.C. was responsible for data curation, software development, visualization, and investigation for experiments throughout the manuscript. O.G. was responsible for the temperature priming experiments and assisted in tracking survival. K.C. and Z.W. wrote the original draft and edited the manuscript. Z.W. supervised the project.

3.8: COMPETING INTERESTS

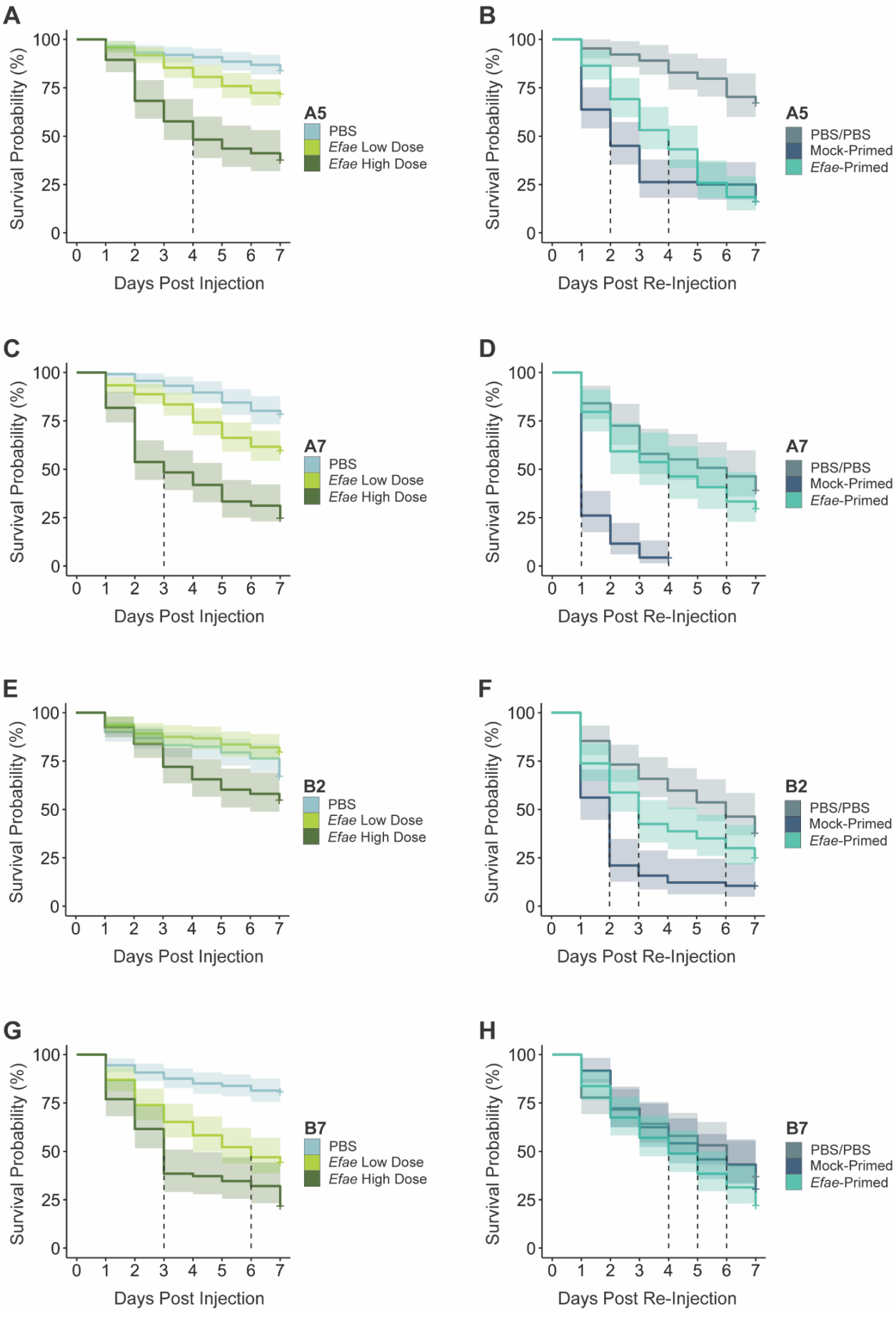
The authors do not declare any competing interests.

3.9: FIGURES


A
Survival Probability (%)
100
75
50
25
0
0 1 2 3 4 5 6 7
Days Post Injection
B6
PBS
Efae Low Dose
Efae High Dose
B
Survival Probability (%)
Days Post Re-Injection
B6
PBS/PBS
Mock-Primed
Efae-Primed
C
Survival Probability (%)
Days Post Injection
A4
PBS
Efae Low Dose
Efae High Dose
D
Survival Probability (%)
Days Post Re-Injection
A4
PBS/PBS
Mock-Primed
Efae-Primed
E
Survival Probability (%)
Days Post Injection
Iso1
PBS
Efae Low Dose
Efae High Dose
F
Survival Probability (%)
Days Post Re-Injection
Iso1
PBS/PBS
Mock-Primed
Efae-Primed
G
Survival Probability (%)
Days Post Injection
CantonS
PBS
Efae Low Dose
Efae High Dose
H
Survival Probability (%)
Days Post Re-Injection
CantonS
PBS/PBS
Mock-Primed
Efae-Primed

<u>Figure 3.1</u>: Priming in control genotypes previously assayed for *E. faecalis* immune response

A). Survival of single-injected B6 flies versus PBS control (PBS: n = 146, *Efae* Low Dose: n = 116, *Efae* High Dose: n = 86). Dotted line indicates median survival time. Shaded area indicates 95% confidence interval. Low Dose vs PBS: HR = 2.3, p = 9.9e-05; High Dose vs PBS: HR = 1.3, p = 0.27; pairwise comparisons are calculated using a Cox proportional hazard model with hazard ratios and Wald statistic values reported for experimental conditions versus their PBS negative control. **B).** Survival of primed B6 flies versus double-injected, non-primed controls (PBS/PBS: n = 78, Mock-Primed: n = 70, *Efae*-Primed: n=63). *Efae*-Primed vs PBS/PBS: HR = 1.5, p = 0.092; Mock-Primed vs. PBS/PBS: HR = 1.8, p = 0.0084. **C).** Survival of A4 flies injected with PBS (n = 211), *Efae* Low Dose (n = 170), and *Efae* High Dose (n = 75). Low Dose vs PBS: HR = 1.6, p = 0.015; High Dose vs PBS: HR = 3.9, p = 4.7e-13. **D).** Survival of primed A4 flies versus double-injected, non-primed controls (PBS/PBS: n = 70, Mock-Primed: n = 60, *Efae*-Primed: n=78). *Efae*-Primed vs PBS/PBS: HR = 2.2, p = 2.4e-05; Mock-Primed vs. PBS/PBS: HR = 2.4, p = 1.0e-05. **E).** Survival of Iso1 flies injected with PBS (n = 165), *Efae* Low Dose (n = 151), and *Efae* High Dose (n = 60). Low Dose vs PBS: HR = 1.2, p = 0.34; High Dose vs PBS: HR = 2.6, p = 8.3e-07. **F).** Survival of primed Iso1 flies versus double-injected, non-primed controls (PBS/PBS: n = 68, Mock-Primed: n = 60, *Efae*-Primed: n=74). *Efae*-Primed vs PBS/PBS: HR = 1.7, p = 0.0048; Mock-Primed vs. PBS/PBS: HR = 1.8, p = 0.0017. Experiments for CantonS flies are preliminary. **G).** Survival of CantonS flies injected with PBS (n = 111), *Efae* Low Dose (n = 85). Low Dose vs PBS: HR = 2.2, p = 1.6e-04. **H).** Survival of primed CantonS flies versus double-injected, non-primed controls (PBS/PBS: n = 19, Mock-Primed: n = 33, *Efae*-Primed: n=30). *Efae*-Primed vs PBS/PBS: HR = 2.3, p = 0.022; Mock-Primed vs. PBS/PBS: HR = 2.1, p = 0.019.


A
Survival Probability (%)
Days Post Injection
A5
PBS
Efae Low Dose
Efae High Dose
B
Survival Probability (%)
Days Post Re-Injection
A5
PBS/PBS
Mock-Primed
Efae-Primed
C
Survival Probability (%)
Days Post Injection
A7
PBS
Efae Low Dose
Efae High Dose
D
Survival Probability (%)
Days Post Re-Injection
A7
PBS/PBS
Mock-Primed
Efae-Primed
E
Survival Probability (%)
Days Post Injection
B2
PBS
Efae Low Dose
Efae High Dose
F
Survival Probability (%)
Days Post Re-Injection
B2
PBS/PBS
Mock-Primed
Efae-Primed
G
Survival Probability (%)
Days Post Injection
B7
PBS
Efae Low Dose
Efae High Dose
H
Survival Probability (%)
Days Post Re-Injection
B7
PBS/PBS
Mock-Primed
Efae-Primed

<u>Figure 3.2</u>: *E. faecalis* priming in DSPR control genotypes

A). Survival of single-injected A5 flies versus PBS control (PBS: n = 174, *Efae* Low Dose: n = 170, *Efae* High Dose: n = 85). Dotted line indicates median survival time. Shaded area indicates 95% confidence interval. Low Dose vs PBS: HR = 1.9, p = 0.0076; High Dose vs PBS: HR = 5.7, p = 1.1e-13; pairwise comparisons are calculated using a Cox proportional hazard model with hazard ratios and Wald statistic values reported for experimental conditions versus their PBS negative control. **B).** Survival of primed A5 flies versus double-injected, non-primed controls (PBS/PBS: n = 64, Mock-Primed: n = 80, *Efae*-Primed: n=81). *Efae*-Primed vs PBS/PBS: HR = 4.2, p = 1.1e-08; Mock-Primed vs. PBS/PBS: HR = 5.5, p = 1.1e-11. **C).** Survival of A7 flies injected with PBS (n = 116), *Efae* Low Dose (n = 151), and *Efae* High Dose (n = 93). Low Dose vs PBS: HR = 2.2, p = 0.0012; High Dose vs PBS: HR = 6.2, p = 6.5e-15. **D).** Survival of primed A7 flies versus double-injected, non-primed controls (PBS/PBS: n = 69, Mock-Primed: n = 69, *Efae*-Primed: n=54). *Efae*-Primed vs PBS/PBS: HR = 1.3, p = 0.22; Mock-Primed vs. PBS/PBS: HR = 5.9, p = 6.6e-16. **E).** Survival of B2 flies injected with PBS (n = 131), *Efae* Low Dose (n = 128), and *Efae* High Dose (n = 93). Low Dose vs PBS: HR = 0.59, p = 0.033; High Dose vs PBS: HR = 1.5, p = 0.048. **F).** Survival of primed B2 flies versus double-injected, non-primed controls (PBS/PBS: n = 82, Mock-Primed: n = 57, *Efae*-Primed: n=80). *Efae*-Primed vs PBS/PBS: HR = 1.5, p = 0.025; Mock-Primed vs. PBS/PBS: HR = 3.1, p = 2.0e-08. **G).** Survival of B7 flies injected with PBS (n = 161), *Efae* Low Dose (n = 115), and *Efae* High Dose (n = 78). Low Dose vs PBS: HR = 3.7, p = 2.1e-09; High Dose vs PBS: HR = 6.7, p = <2.0e-16. **H).** Survival of primed B7 flies versus double-injected, non-primed controls (PBS/PBS: n = 81, Mock-Primed: n = 72, *Efae*-Primed: n=86). *Efae*-Primed vs PBS/PBS: HR = 1.4, p = 0.067; Mock-Primed vs. PBS/PBS: HR = 1.1, p = 0.61.

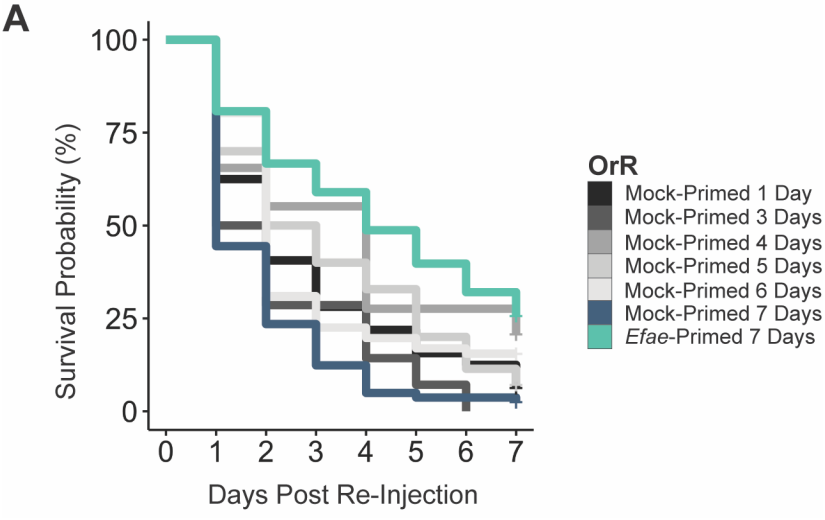


Figure 3.3: Temporal dynamics of *E. faecalis* Mock-priming

A). Survival changes for varying amounts of time between initial injection with PBS and infection with a high dose of *E. faecalis* (~30,000 CFU/fly) (n: 1 Day = 32, 3 Days = 31, 4 Days = 30, 5 Days = 70, 6 Days = 71); HRs: Mock-Primed 1 Day = 1.5 (p = 0.053), Mock-Primed 3 Days = 2.1 (p = 0.012), Mock-Primed 4 Days = 0.99 (p = 0.98), Mock-Primed 5 Days = 1.3 (p = 0.15), Mock-Primed 6 Days = 1.3 (p = 0.12),. 7 Day Mock-Primed and 7 Day *Efae*-Primed survival is the same as in Figure 2.1C, replotted for comparison.

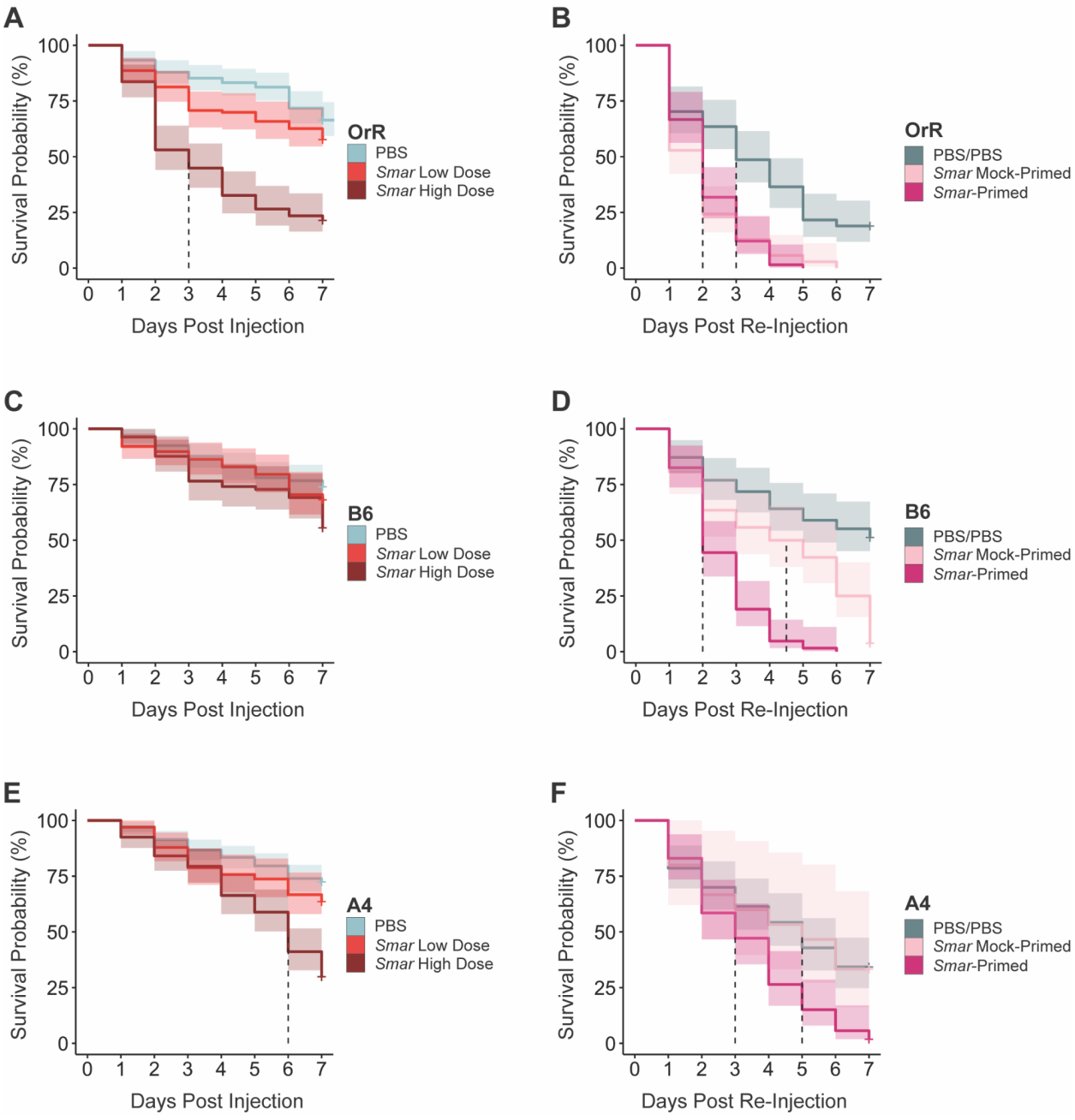

A
Survival Probability (%)
100
75
50
25
0
0 1 2 3 4 5 6 7
Days Post Injection
OrR
PBS
Smar Low Dose
Smar High Dose
B
Survival Probability (%)
Days Post Re-Injection
OrR
PBS/PBS
Smar Mock-Primed
Smar-Primed
C
Survival Probability (%)
Days Post Injection
B6
PBS
Smar Low Dose
Smar High Dose
D
Survival Probability (%)
Days Post Re-Injection
B6
PBS/PBS
Smar Mock-Primed
Smar-Primed
E
Survival Probability (%)
Days Post Injection
A4
PBS
Smar Low Dose
Smar High Dose
F
Survival Probability (%)
Days Post Re-Injection
A4
PBS/PBS
Smar Mock-Primed
Smar-Primed

Figure 3.4: *S. marcescens* single & double infections in control genotypes
A). Survival of single-injected OrR flies versus PBS control (PBS: n = 149, *Smar* Low Dose, ~3,000 CFU/Fly: n = 123, *Smar* High Dose, ~30,000 CFU/Fly: n = 98). Dotted line indicates median survival time. Shaded area indicates 95% confidence interval. Low Dose vs PBS: HR = 1.4, p = 0.08; High Dose vs PBS: HR = 4.0, p = 4.3e-14; pairwise comparisons are calculated using a Cox proportional hazard model with hazard ratios and Wald statistic values reported for experimental conditions versus their PBS negative control. **B).** Survival of primed OrR flies versus double-injected, non-primed controls (PBS/PBS: n = 74, *Smar* Mock-Primed: n = 70, *Smar*-Primed: n=66). *Smar*-Primed vs PBS/PBS: HR = 2.7, p = 2.9e-07; *Smar* Mock-Primed vs. PBS/PBS: HR = 2.8, p = 1.7e-08. **C).** Survival of B6 flies injected with PBS (n = 146), *Smar* Low Dose (n = 88), and *Smar* High Dose (n = 81). Low Dose vs PBS: HR = 1.3, p = 0.35; High Dose vs PBS: HR = 1.9, p = 0.0075. **D).** Survival of primed B6 flies versus double-injected, non-primed controls (PBS/PBS: n = 146, *Smar* Mock-Primed: n = 52, *Smar*-Primed: n=63). *Smar*-Primed vs PBS/PBS: HR = 6.9, p = 2.5e-16; *Smar* Mock-Primed vs. PBS/PBS: HR = 3.0, p = 7.0e-07. **E).** Survival of A4 flies injected with PBS (n = 211), *Smar* Low Dose (n = 99), and *Smar* High Dose (n = 107). Low Dose vs PBS: HR = 1.4, p = 0.099; High Dose vs PBS: HR = 3.3, p = 1.7e-11. **F).** Survival of primed A4 flies versus double-injected, non-primed controls (PBS/PBS: n = 70, *Smar* Mock-Primed: n = 45, *Smar*-Primed: n=53). *Smar*-Primed vs PBS/PBS: HR = 2.2, p = 1.2e-04; *Smar* Mock-Primed vs. PBS/PBS: HR = 1.0, p = 0.97.

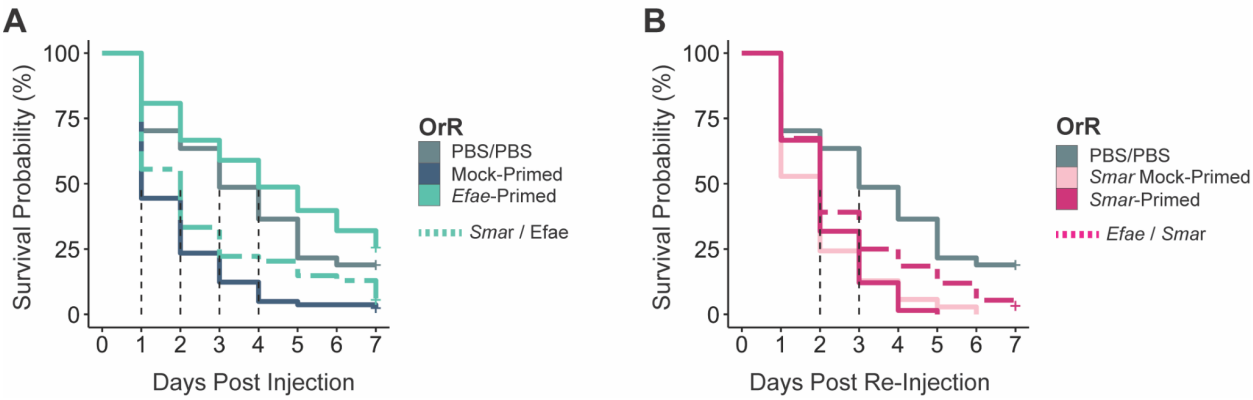


Figure 3.5: Heterologous priming with *E. faecalis* and *S. marcescens*

A). Survival of homologous and heterologous *E.faecalis* primed OrR flies versus double-injected, non-primed controls (PBS/PBS: n = 74, *Efae* Mock-Primed: n = 81, *Efae*-Primed: n=78, *Smar*/*Efae*-Primed: n=54). *Smar*/*Efae*-Primed vs PBS/PBS: HR = 1.7, p = 0.0052. Homologous priming survival is the same as in **Figure 2.1C**, plotted for comparison. **B).** Survival of homologous and heterologous *S. marcescens* primed OrR flies versus double-injected, non-primed controls (PBS/PBS: n = 74, *Smar* Mock-Primed: n = 70, *Smar*-Primed: n=66, Efae/Smar-Primed: n = 92). *Efae*/*Smar*-Primed vs. PBS/PBS: HR = 1.7, p = 0.0015. Homologous priming survival is the same as in **Figure 3.3B**, plotted for comparison.

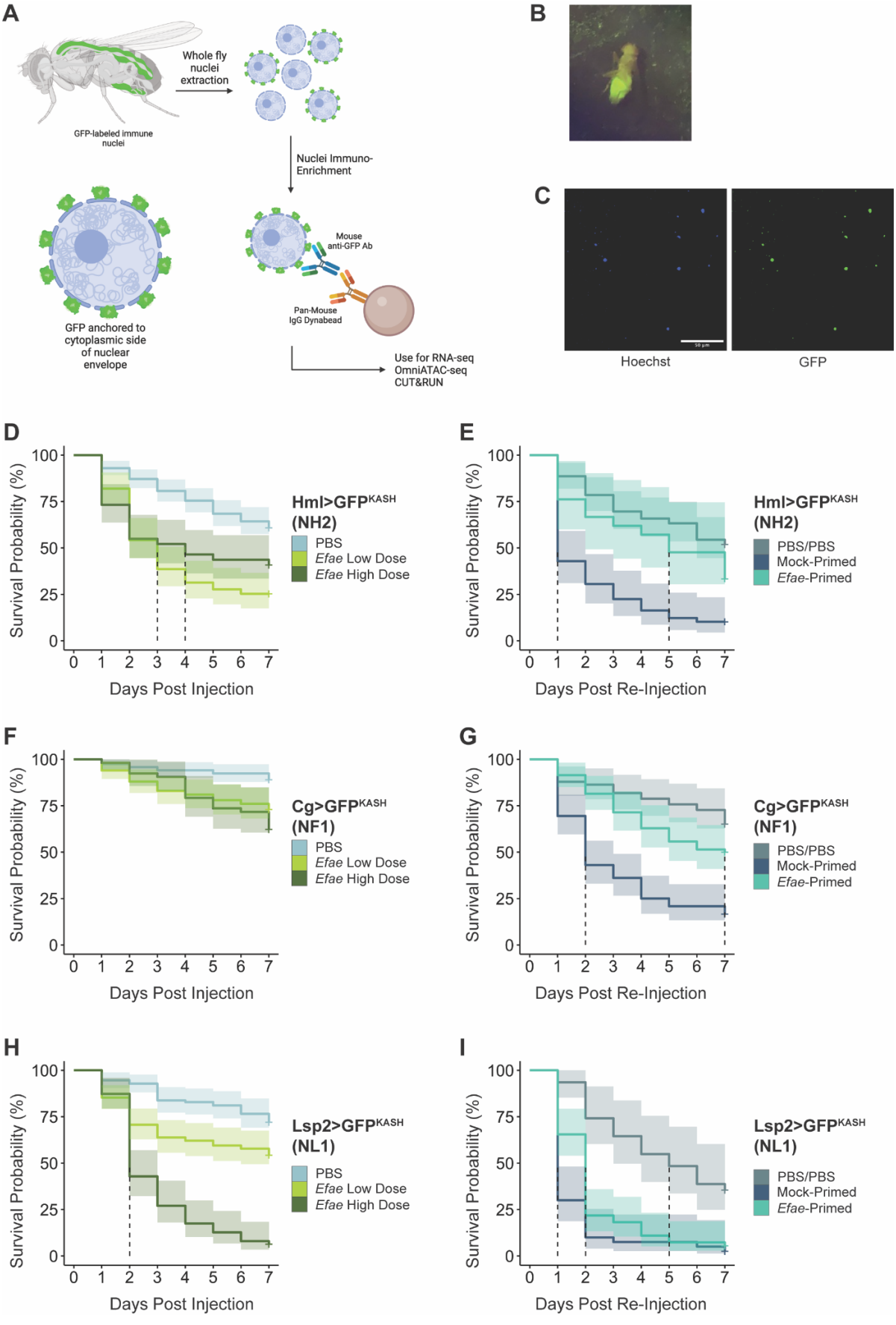

A
Whole fly nuclei extraction
GFP-labeled immune nuclei
Nuclei Immuno-Enrichment
Mouse anti-GFP Ab
Pan-Mouse IgG Dynabead
GFP anchored to cytoplasmic side of nuclear envelope
Use for RNA-seq OmniATAC-seq CUT&RUN
B
C
Hoechst
GFP
D
Survival Probability (%)
Days Post Injection
Hml>GFPKASH (NH2)
PBS
Efae Low Dose
Efae High Dose
E
Days Post Re-Injection
Hml>GFPKASH (NH2)
PBS/PBS
Mock-Primed
Efae-Primed
F
Cg>GFPKASH (NF1)
G
H
Lsp2>GFPKASH (NL1)
I

Figure 3.6: Immune-labeled GFP lines for assaying immune response.
A). Schematic of immune-labeled GFP^{KASH} flies and isolation of GFP^{+} nuclei via nuclear immuno-enrichment. **B).** Image of a 5-day old male Hml>GFP^{KASH} fly under blue fluorescent light, showing GFP^{+} fluorescence . **C).** Fluorescence imaging of a Hml>GFP^{KASH} nucleus isolate. [Left] Hoechst staining, [Right] **D).** Survival of single-injected Hml>GFP^{KASH} (aka NH2) flies versus PBS control (PBS: n = 171, *Efae* Low Dose: n = 83, *Efae* High Dose: n = 71). Dotted line indicates median survival time. Shaded area indicates 95% confidence interval. Low Dose vs PBS: HR = 3.0, p = 8.9e-10; High Dose vs PBS: HR = 2.1, p = 1.4e-04; pairwise comparisons are calculated using a Cox proportional hazard model with hazard ratios and Wald statistic values reported for experimental conditions versus their PBS negative control. **E).** Survival of primed Hml>GFP^{KASH} (aka NH2) flies versus double-injected, non-primed controls (PBS/PBS: n = 79, Mock-Primed: n = 49, *Efae*-Primed: n=41). *Efae*-Primed vs PBS/PBS: HR = 1.6, p = 0.14; Mock-Primed vs. PBS/PBS: HR = 4.1, p = 5.0e-10. **F).** Survival of Cg>GFP^{KASH} (aka NF1) flies injected with PBS (n = 119), *Efae* Low Dose (n = 100), and *Efae* High Dose (n = 53). Low Dose vs PBS: HR = 2.8, p = 0.0025; High Dose vs PBS: HR = 3.9, p = 1.4e-04. **G).** Survival of primed Cg>GFP^{KASH} (aka NF1) flies versus double-injected, non-primed controls (PBS/PBS: n = 66, Mock-Primed: n = 72, *Efae*-Primed: n=70). *Efae*-Primed vs PBS/PBS: HR = 1.6, p = 0.077; Mock-Primed vs. PBS/PBS: HR = 4.5, p = 1.5e-09. **H).** Survival of Lsp2>GFP^{KASH} (aka NL1) flies injected with PBS (n = 111), *Efae* Low Dose (n = 116), and *Efae* High Dose (n = 63). Low Dose vs PBS: HR = 2.0, p = 0.0017; High Dose vs PBS: HR = 7.1, p = <2.0e-16. **I).** Survival of primed Lsp2>GFP^{KASH} (aka NL1) flies versus double-injected, non-primed controls (PBS/PBS: n = 31, Mock-Primed: n = 40, *Efae*-Primed: n=55). *Efae*-Primed vs PBS/PBS: HR = 3.1, p = 2.7e-05; Mock-Primed vs. PBS/PBS: HR = 5.2, p = 6.3e-09.

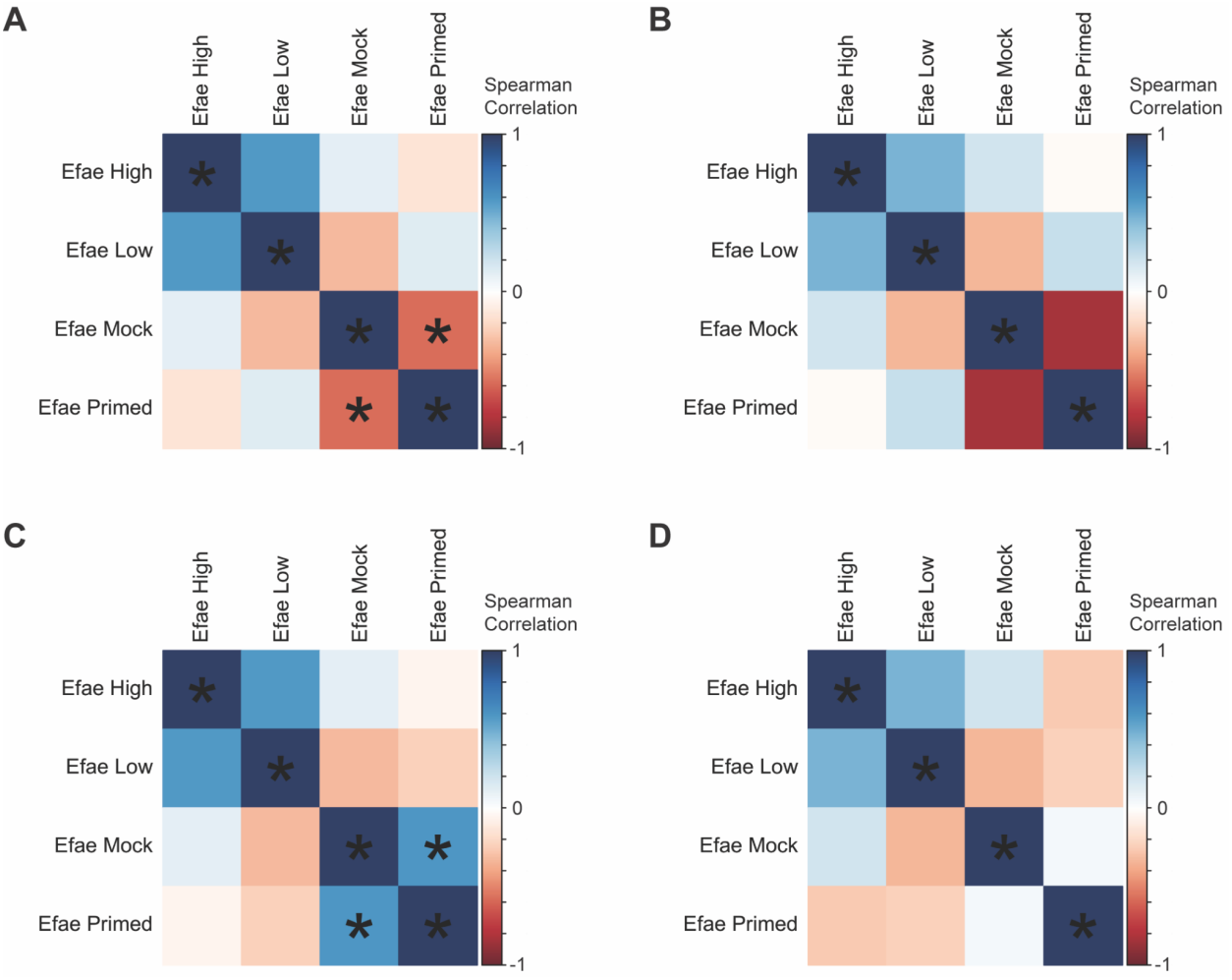


Figure 3.7: Correlation of hazard ratios between different single & double infections
A). Spearman correlation of survival hazard ratios when comparing all tested genotypes from Chapter 2, and using HRs calculated with *Efae* Mock-Primed as the referent group for *Efae*-Primed flies. Correlations marked with '*' indicate statistically significant correlations (p-value < 0.05). **B).** Same as **A**, but using only non-mutant genotypes. **C).** Spearman correlation of survival hazard ratios when comparing all tested genotypes from Chapter 2, and using HRs calculated with PBS/PBS as the referent group for *Efae*-Primed flies. **D).** Same as **C**, but using only non-mutant genotypes.

4: DISCUSSION

The classical view of immune response is divided into non-specific innate immunity and antibody-mediated adaptive immunity. However, it is now appreciated that immune response functions through the coordinated actions of a myriad of biological pathways and that immune memory is not solely relegated to the actions of the adaptive immune response. Even in more evolutionarily simple organisms like fruit flies, response to a pathogen is an orchestrated coordination of complex actions that, when mismanaged, spells death for the organism. Mechanistic insight into how such a complex phenotype is controlled demystifies the stochasticity that is often associated with immune response.

In the above work we have chipped away at the larger aspects of innate immunity by characterizing primed immune responses in the fruit fly *D. melanogaster*. We began by characterizing the physiological and transcriptional changes that happen upon re-infection when previously exposed to *E. faecalis* (Chapter 2). In these experiments we found that *E. faecalis*-primed flies generally tolerated the infection more efficiently and mounted a large-scale transcriptional response specific to being in a primed state. We then modulated our priming experiment set ups by changing the temporal dynamics, host genotypes, and bacterial species (Chapter 3). In this way we found that priming is a flexible phenotype contingent on all thew above variables. Our studies have highlighted how complex of a phenomenon innate immune priming is and how much more work needs to be done to fully grasp the molecular control of such a tightly orchestrated feat.

The work presented above has laid the foundation for assaying *E.faecalis*-mediated and *S. marcescens*-mediated immune priming in *D. melanogaster*. However, this is only a jumping off point for more nuanced and thorough questions that can be asked about the mechanisms of

immune priming. One such question builds on the observed split in bacterial loads at the end of the seven day training with low dose *E. faecalis* (Figure 2.2A, *Efae* Low Dose Day 7). Using bioluminescent strains of bacteria currently in development in our lab, it would be worth testing the bacterial load of trained flies prior to re-infection and measuring whether the chronic bacterial load in those flies has an effect on the ability to prime against re-infection. In these experiments, flies would be initially infected with the bioluminescent *E. faecalis* and assayed for bacterial load and survival across time on an individual rather than population-wide scale. While survival to the initial infection proved to be a poor predictor of priming ability (Figure 3.7), such a conclusion was drawn on a population-scale and may benefit from an individual scale that can be achieved using a bioluminescent bacteria approach.

Another line of inquiry branches from the transcriptomic characterization of *E. faecalis* priming. We observed a large amount of metabolism-related gene expression that was unique to *Efae*-primed flies (Figure 2.4 for hemocytes & Supplementary Figure 2.3 for fat bodies). Assaying for changes in metabolite usage throughout initial training and then into the primed response would validate the gene expression results and draw a more complete picture of the role tolerance is playing in the phenotype. Such an experiment would also provide a point of comparison to the metabolic characterization of the trained immunity phenotype described in mammalian monocytes.

To delve deeper into the mechanisms controlling priming-specific changes in transcription, it would make sense to assay the epigenetic changes that accompany immune response. Given that epigenetic modification during initial infection is important for driving immune memory in mammals and certain arthropod innate immune response, it is logical to assay it in *D. melanogaster*. This would involve tracking what genes are primed for expression

when infected with a low-dose of *E. faecalis* through characteristic open chromatin and histone mark signatures.

Lastly, it would be worth building upon the work done to create immune nuclear GFP-tagged fly lines. Experiments attempting to pull-down GFP-tagged nuclei using either bead-based immuno-enrichment or FACS sorting would characterize the viability of using such nuclei for downstream functional genomics. Once optimized, those protocols can be used to develop a transcriptome for the different fly lines which should be compared to the already published transcriptomes of dissected tissues (Chapter 2). Given the difficulty in attaining viable nuclei from dissected tissue, these lines should be an invaluable tool in characterizing the epigenetic immune response as well as in visualizing the immune tissues in a myriad of assays.

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