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Abstract

Antimicrobial peptides (AMPs) are host-encoded antibiotics that combat invading microbes. These short immune effectors are conserved in plants, animals, and fungi. Early work showed that AMPs killed bacteria in generalist fashions in vitro: i.e. AMPs that killed *Escherichia coli* also killed many Gram-negative bacteria when tested. At the genome level, AMP gene families rapidly expand or contract, which suggested single genes were unlikely to be important. At the signalling level, AMPs are induced as a suite of peptides by conserved NF-κB immune pathways across organisms (e.g. Toll, Imd). Together these observations led to the assumption that individual genes contributed only small effects, and instead the cumulative cocktail of AMPs was key to a successful defence response. This idea was never robustly tested in vivo owing to technical limitations. In 2015, two studies stumbled onto remarkable effects of fruit fly immune effectors. In one case a polymorphism in a single AMP gene (*Diptericin A* or *DptA*) greatly affected the fly defence against a specific bacterium (*Providencia rettgeri*). In another case, deleting just the *Bomanin* gene family caused immune susceptibility mimicking loss of Toll signalling generally. The prevailing model of generalist AMP action was ill equipped to explain these findings.

In my PhD, I have systematically deleted the AMP genes of fruit flies to clarify AMP defences in vivo. This confirmed AMPs can act in generalist or redundant fashions in some cases. However some AMP-pathogen interactions are remarkably specific. Deletion of just the *Drosocin* gene explains much of the susceptibility of NF-κB/Imd immune deficient flies to *Enterobacter cloacae* infection. Meanwhile deletion of just the two *Diptericins* recapitulates the susceptibility of Imd mutants to *P. rettgeri*.

Contrary to previous assumptions, our findings suggested AMPs are not simple generalist peptides. There are many more short peptide immune genes waiting to be characterized that may be relevant to specific infections. I next investigated three lesser-characterized genes: *Baramicin A, DptB, and Drosocin*. The *Baramicin A* gene encodes multiple products, including one peptide (IM22) that was first annotated in my study. I found *BaraA* is key to the fly defence against pathogenic fungi. Next, I dissected the roles of individual *Diptericin* genes. Surprisingly, *DptB* alone is required for survival after infection by a lab isolate of *Acetobacter*. Finally, I identified *Drosocin* as the source gene for IM7, a mystery peptide first detected in 1998. A polymorphism in IM7 previously obscured its identification. This polymorphism affects fly defence against *Providencia burhodogranariea*, where one immune-poor allele effectively rivals deletion of IM7 entirely against this microbe.

These AMP-microbe interactions reveal that survival after infection can be mediated at the level of single AMP genes or even common alleles of those genes. Contrary to previous assumptions, it appears the AMP response is composed of silver bullets, wooden stakes, and other specialized defence tools required to fight specific enemies. In this light, the diverse AMPs induced upon infection may be an evolutionary solution to optimize survival: general microbe patterns induce immune signalling, there is a need for timely production of antimicrobial peptides, and so a battery of antimicrobial peptides are produced immediately even though only a few are likely to be relevant for any given pathogen.

Keywords

Drosophila – antimicrobial peptides – host defence peptides - AMPs - HDPs – Toll – Imd – Host-pathogen interactions

Résumé

Les peptides antimicrobiens (AMP) sont des antibiotiques peptidiques qui combattent les microbes infectieux. Ces effecteurs sont observés chez les plantes, les animaux et les champignons. Des études *in vitro* suggèrent que les AMPs éliminent les microbes de manière généraliste. Au niveau de la signalisation, les AMP sont induits par les voies immunitaires NF-kB, très conservées (ex: Toll, Imd). Ces observations ont conduit à l'hypothèse que chaque gène codant pour un AMP ne contribuait que modestement à la réponse immunitaire à titre individuel. Au lieu de cela, un cocktail associant plusieurs AMPs serait la clé d'une réponse immunitaire efficace. Cette idée n'avait jamais été testée in vivo en raison de limitations techniques. En 2015, deux études réalisées chez la drosophile ont infléchi notre point de vue sur les AMPs. Tout d'abord, il fut observé qu'un polymorphisme de *Diptericin A* affectait spécifiquement la défense de la mouche contre la bactérie *Providencia rettgeri*. Ensuite, une étude a montré que la délétion des gènes codants pour une seule famille d'effecteurs, les Bomanins, provoquait une susceptibilité à l'infection aussi marquée que la perte totale de la voie de signalisation immunitaire Toll. Ces nouvelles données étaient en désaccord avec le modèle dominant, qui suggérait un mode d'action généraliste des AMPs.

Ici, j'ai systématiquement délété les gènes codant pour des AMPs chez la drosophile pour étudier le rôle de chacun dans la défense immunitaire *in vivo*. Ces études ont confirmé que les AMPs pouvaient agir de manière additive ou redondante, en accord avec l'idée de cocktail. Cependant, certaines interactions AMP-pathogène se sont avérées remarquablement spécifiques. Ainsi, la *Drosocin* contribue en grande partie à la résistance apportée par la voie Imd à *Enterobacter cloacae*. A l'inverse, la simple délétion des deux *Diptericins* cause une susceptibilité totale à *P. rettgeri*. Cela démontre que, contrairement aux hypothèses initiales, les AMPs ne sont pas de simples peptides généralistes.

De nombreux effecteurs immunitaires de la drosophile n'ont pas été caractérisés. Dans la deuxième partie de ma thèse, j'ai analysé le rôle de trois gènes codant des AMPs: *Baramicin A*, *Drosocin*, et *Diptericin B*. J'ai montré que *Baramicin A* est un peptide régulé par la voie Toll, importante pour la défense contre le champignon entomopathogène *Beauveria bassiana*. Ensuite, j'ai montré que le gène *Drosocin* code un autre peptide, IM7, qui avait été détecté pour la première fois en 1998, mais jamais caractérisé au niveau génétique. Enfin, j'ai disséqué la contribution de chacun des deux gènes *Diptericins* à la défense de l'hôte. Étonnamment, le gène *Diptericin B* est requis pour la survie après infection par un isolat d'*Acetobacter* tandis que *Diptericin A* confère une résistance à *P. rettgeri,* illustrant une fois encore l'extrême spécificité de certaines interactions AMP-bactérie.

En conclusion, mon travail montre que les peptides antimicrobiens ne sont pas des effecteurs généralistes, mais peuvent avoir un spectre d'action extrêmement spécifique. Ainsi, l'existence d'un grand nombre de peptides antimicrobiens chez la drosophile lui permettrait de se défendre contre de nombreux pathogènes via la production d'effecteurs très spécialisés. Ces travaux sur les peptides antimicrobiens de drosophile ont une pertinence plus générale compte tenu de la conservation des mécanismes immunitaires innées, notamment chez les mammifères.

Mots-clés

Drosophila – AMPs - HDPs – Toll – Imd – interactions des hôtes et pathogen

Contents

List of Figures

Chapter 1 Introduction

1.1 The insect immune response

Insects typically live in a microbe-rich environment that requires a potent immune system to prevent infection by opportunistic pathogens. In contrast to vertebrates, insects possess only innate immune defence mechanisms. Insect immune defence begins at behavioural and epithelial barriers that prevent would-be pathogens from invading the body cavity. After this, the insect immune response relies on detection of pathogen associated molecular patterns (PAMPs) that are recognized by host pattern recognition receptors (PRRs). Common molecular patterns include bacterial peptidoglycan, fungal ß-glucan, and other microbial cell wall components (Lemaitre and Hoffmann, 2007). These interactions are mediated by host cells and tissues in a global response upon systemic infection. Pioneering work on insect immunity was first carried out in moths and flies (Carton, 2019; Faye and Lindberg, 2016). Since then, the immune responses of many other insects have been studied, each with their own relevance to particular research questions. For instance, beetles, moths, and bees are larger insects that are capable of donating large volumes of hemolymph (insect blood) from individuals, greatly facilitating protein purifications and macromolecule assays. These models are also commonly species of economic interest, either as pests or species of agricultural importance (Rolff and Schmid-Hempel, 2016). Two major lineages of flies (Diptera) have been extensively studied for their immune response: i) mosquitoes and ii) *Drosophila*. Mosquito immunity is of significant interest to human disease transmission, however rearing mosquitos is somewhat arduous due to their ecologically diverse life stages. The fruit fly *Drosophila melanogaster* is not agriculturally important, nor is it a vector of human disease. However fruit flies possess invaluable characteristics that have made them a de facto model of not just insect immunity, but innate immunity more generally. *Drosophila* are **i)** easily reared with short generation times and many offspring, allowing very rapid progress **ii)** boast an unparalleled genetic and molecular toolkit to dissect the biology underlying host-pathogen interactions (Roote and Prokop, 2013), and **iii)** share highly conserved immune pathways of other animals. For these reasons, research in *Drosophila* has contributed greatly to the study of innate immunity (Lemaitre et al., 1996, 1995; Volchenkov et al., 2012), and fruit flies continue to be powerful re-

search tools to understand interactions between host and pathogen. Flies possess a potent innate immune system composed of many modules (Fig. 1.1), which I will describe below.

1.2 Intrinsic antiviral immunity and programmed cell death

Fruit flies are infected by many viruses including *Drosophila* C virus (Pisuviricota, ssRNA+), *Drosophila* sigmavirus (Negarnaviricota, ssRNA-), *Drosophila* Kallithea virus (Naldaviricetes, dsDNA) and more (Habayeb et al., 2006; Hill and Unckless, 2020; Jousset et al., 1977; Longdon et al., 2012; Palmer et al., 2018). Genome-wide analyses have revealed the antiviral defence against certain infections can rely on highly specific polymorphisms that protect flies against viruses such as *ref(2)P* or *pastrel* genes, suggesting coevolutionary arms races are major drivers of host-virus interactions (Cao et al., 2017; Carré-Mlouka et al., 2007; Martins et al., 2014). Viral infection triggers a unique subset of responses in *Drosophila*, including the activation of the arthropod RNA interference pathway (Cottrell and Doering, 2003). RNA interference is triggered by detection of double stranded RNA, a PAMP restricted to viral genomes and present as a viral reproduction intermediate. The RNA-induced silencing complex (RISC) binds double stranded RNA whereupon the protein Dicer2 cleaves these double stranded RNAs into smaller pieces that act as template for further recognition and suppression of homologous sequence. Recent investigations have also demonstrated an intact *Drosophila* cGAS/STING pathway required for antiviral defence (Cai et al., 2020; Goto et al., 2018; Holleufer et al., 2021; Slavik et al., 2021). Autophagy is also involved in resistance against certain viruses (Nakamoto et al., 2012; Shelly et al., 20hanson09). Viral infection also triggers a need for policing of infected cells, possibly requiring programmed cell death and clearance of cell debris. The JNK signalling pathway regulates cell death during development and immunity (Igaki, 2009), and molecules required for the activation of the JNK pathway are also involved in the *Drosophila* Imd pathway (discussed below). Indeed the TAK1 kinase phosphorylates downstream components in both Imd and JNK signalling (Ertürk-Hasdemir et al., 2009; Park et al., 2004; Valanne et al., 2007). This JNK-mediated programmed cell death clears virus-infected bodies.

1.3 The *Drosophila* cellular immune response

The *Drosophila* systemic immune response can be broadly divided into two major categories: the cellular and humoral responses. The cellular response is mediated by circulating *Drosophila* blood cells (hemocytes) that act as sentinels of the body. These hemocytes are crucial in orchestrating wound-healing, as well as detection and suppression of invading

parasites and pathogens (Gold and Brückner, 2015; Meister and Lagueux, 2003). *Drosophila melanogaster* has three major kinds of hemocytes (Lemaitre and Hoffmann, 2007). First, plasmatocytes are analogues of mammalian macrophages, responsible for phagocytosis of invading pathogens and secretion of immune effectors and cytokines that communicate the signal of infection to the rest of the body. Second, *Drosophila* crystal cells ("oenocytoids" in other insects (Banerjee et al., 2019)) are specialized hemocytes containing bundles of prophenoloxidase enzyme. Upon injury or infection, crystal cells burst to release ready-made prophenoloxidase into the hemolymph. These prophenoloxidases mediate one of the most impressive arthropod immune reactions, the melanization response. A few specific populations of plasmatocyte-like blood cells and all crystal cells generate prophenoloxidases (Tattikota et al., 2020), but it is the crystal cells that are uniquely specialized as reserves of inactive enzyme that can be rapidly released into the hemoylmph to combat infection and promote wound clotting. Finally, *Drosophila* larval blood cells differentiate into plate-like lamellocytes upon infestation by macroparasites such as parasitic wasps (Rizki and Rizki, 1992). These lamellocytes are specialists at encapsulating invading parasites, and express a third prophenoloxidase protein (PPO3) that is constitutively active to both melanize the capsule and kill the invading parasite (Dudzic et al., 2015).

1.4 The *Drosophila* humoral immune response

Systemic infection triggers a coordinated immune response largely regulated by the insect fat body (analogue of the mammalian liver) and also the circulating hemocytes (Lemaitre and Hoffmann, 2007). Other tissues (e.g. trachea, malpiguian tubules, gut) can also respond to systemic infection, but these responses are typically more localized and contribute less to systemic immunity (Ferrandon et al., 1998; Tzou et al., 2000). Upon infection, the fat body synthesizes and secretes a massive array of peptides and proteins into the hemolymph (Liu et al., 2006; Uttenweiler-Joseph et al., 1998; Verleyen et al., 2006). These molecules perform key roles to prevent pathogen growth and adapt the host for the changing metabolic needs of the infection (Lemaitre and Hoffmann, 2007). These various mechanisms contributing to the humoral response to infection are described below.

1.4.1 Metabolic regulation by insulin signalling

The insulin signalling pathway controls sugar metabolism and is transcriptionally regulated by the Forkhead transcription factor FOXO and the ribosomal kinase S6K (Lizcano et al., 2003), and translationally regulated by the translation suppressor Thor/4eBP (Jünger et al.,

2003). Both injury and the presence of free amino acids in the hemolymph trigger changes to the activity of the *Drosophila* insulin pathway in opposite fashions. Injury triggers the tyrosine kinase Stitcher, which triggers epidermal wound healing through the transcription factor Grainy head (Wang et al., 2009), and also depresses the level of the insulin signalling FoxO transcription factor through upregulation of Akt signalling (O'Farrell et al., 2013). The presence of free amino acids in the hemolymph, which occurs upon injury and protein degradation associated with immediate immune responses, suppresses the translation initiation protein *4eBP/Thor* through *TORC1* (Kramer et al., 2008; Tettweiler, 2005), tightly regulating *Drosophila* insulin signalling during infection. This shift adjusts the nutritional quality of the hemolymph compartment in an effort to prepare the fly for the protein synthesis demand of the innate immune response and to sequester resources away from opportunistic invading pathogens (Musselman et al., 2018).

1.4.2 Iron sequestration

Another major shift in the hemolymph compartment concerns the presence of free iron. Iron can act as a key growth factor for pathogen proliferation. As a consequence, during infection animals sequester iron, which has driven the evolution of human iron sequestering transferrin proteins to evade binding of bacterial transferrin receptors (Barber and Elde, 2014). In *Drosophila*, it was recently shown that transferrin 1 is a key regulator of free iron in the hemolymph after infection, shuttling free iron to the fat body for storage (Iatsenko et al., 2020). Loss of transferrin 1 leads to increased susceptibility to certain bacteria and fungi, emphasizing the importance of this nutritional immune response.

1.4.3 Infection-induced responses to stress

Systemic infection imposes stress on the organism. The circadian rythym of infected flies is disrupted causing inconsistent sleep (Toda et al., 2019), the nutritional quality in the hemolymph compartment changes drastically, and a massive demand for protein synthesis leads to oxidative stress and metabolic waste products (Clark et al., 2013). Indeed, damaging lipid peroxidation occurs after infection, and so the fly flushes free lipids from the hemolymph by route of the malpighian tubules (analogue of mammalian kidneys). This response is coordinated by the p38 stress response pathway, critically regulating the gene Materazzi, which is required for this lipid purge (Li et al., 2020). The JAK-STAT signalling pathway also coordinates a variety of stress response proteins upon infection that help the organism tolerate ongoing infection, mediated by the fat body and circulating hemocytes (Sanchez Bosch et al.,

2019; Zeidler et al., 2000). One well-known family of stress-induced peptides are the *Drosophila* Turandots, which have acted as readouts of JAK-STAT signalling for decades but whose function remains enigmatic (Ekengren and Hultmark, 2001). Production of the JAK-STAT ligands by hemocytes remotely stimulates stem cells and affects muscle metabolism contributing to a systemic wound repair response (Agaisse and Perrimon, 2004; Chakrabarti et al., 2016; Woodcock et al., 2015).

1.4.4 Toll and Imd NF-κB Signalling

Two of the most prominent signalling pathways involved in Drosophila innate immunity are the Toll and Imd NF-κB pathways. They were initially identified for their role in regulating the transcription of antimicrobial peptide genes (Lemaitre et al., 1996, 1995). Microarrays later revealed that these pathways regulate hundred of genes in addition to the AMPs (De Gregorio et al., 2002). Thus, these two pathways coordinate a grand diversity of antimicrobial responses, and affect almost all other innate immune pathways either directly or indirectly. Consequently, mutations affecting these pathways result in severe immune deficiencies against microbial infection (Lemaitre et al., 1996, 1995). Toll signalling can regulate other immune responses directly via production of serine proteases and inhibitors that act on the melanization response (Binggeli et al., 2014; Dudzic et al., 2019; Levashina et al., 1999). Imd signalling intermediates cross-talk with JNK pathway components (e.g. TAB2/TAK1 in (Fernando et al., 2014)), or act upstream of cGAS/STING signalling in the antiviral response (Cai et al., 2020). Indirectly, the effector products of Toll and Imd signalling (such as AMPs) suppress microbial growth and so prevent the theft of host sugars, proteins, and lipids by opportunistic microbes, protecting the metabolic competence of the fly in the face of infection (Lemaitre and Hoffmann, 2007). A dedicated discussion of the effector peptides of *Drosophila* NF-κB signalling can be found later in section 1.5.3.

1.4.5 The *Drosophila* Toll pathway

The *Drosophila* Toll signalling pathway begins extracellularly through the sequential activation of serine proteases by microbial proteases and microbial molecular patterns. This process is extremely complex and our current understanding is incomplete. Serine protease gene families are complicated and different insects encode varying numbers of related genes with possibly redundant or complementary functions. Nevertheless the extracellular serine protease architecture of *Drosophila* Toll is shared by other insects including mosquitoes, beetles, and moths (Cerenius et al., 2008; Dudzic et al., 2019; Kanost and Jiang, 2015). In fruit flies, this

sequential activation begins through the recognition of Lysine-type peptidoglycan of Gram-positive bacteria by the peptidoglycan recognition protein PGRP-SA and Gram-negative binding protein GNBP1 (an unfortunate misnomer), or fungal beta glucans by GNBP3 that activate the apical serine protease ModSP (Buchon et al., 2009; Dudzic et al., 2019; Pili-Floury et al., 2004; Vaz et al., 2019). ModSP then cleaves the serine protease Grass, which acts upstream of the sister serine proteases Hayan and Persephone (Dudzic et al., 2019). At this stage, Toll signalling can also be activated by microbial proteases acting directly on a "bait region" present in the Hayan and Persephone precursor proteins (Dudzic et al., 2019; El Chamy et al., 2008; Gottar et al., 2006; Issa et al., 2018). Also at this stage, the Toll extracellular cascade branches to activate Sp7 that can cleave prophenoloxidase leading to the melanization response (An et al., 2013), or to SPE that cleaves the Toll ligand spätzle (Dudzic et al., 2019). Spätzle then binds to dimers of the transmembrane Toll receptor protein. The intracellular part of *Drosophila* Toll signalling is homologous to TLR signalling and TNFR signalling of mammals. Toll signalling is also essential for establishing the dorsal-ventral axis, and has many other functions in development (Imler and Hoffmann, 2001; Lemaitre and Hoffmann, 2007; Lindsay and Wasserman, 2014). Intracellular Toll signalling propagates through the sequential recruitment and activation of death domain-containing proteins MyD88, Tube, and Pelle, which frees the NF-κB transcription factors Dif and Dorsal from their negative repressor Cactus (Lemaitre and Hoffmann, 2007). These transcription factors dimerize either as homodimers or heterodimers to induce downstream target genes that encode proteins like serine proteases and their inhibitors, as well as immune effector proteins and peptides (Busse et al., 2007; Tanji et al., 2010). One of the best known targets of *Drosophila* Toll signalling is the antimicrobial peptide gene *Drosomycin*, which is induced up to ~100x following infection relative to its basal state (Ferrandon et al., 1998; Hanson and Lemaitre, 2020; Lemaitre et al., 1997).

1.4.6 The *Drosophila* Imd pathway

The *Drosophila* immune deficiency (Imd) pathway has many similarities to TLR and TNFR alpha signalling (Myllymäki et al., 2014). *Drosophila* Imd signalling is initiated through the recognition of microbial DAP-type peptidoglycan found in most Gram-negative bacteria and also the cell wall of some Gram-positive bacteria (e.g. *Bacillus subtilis, Listeria innocua*) by the secreted peptidoglycan recognition protein PGRP-SD and transmembrane peptidoglycan receptor PGRP-LC (Choe et al., 2005; Gottar et al., 2002; Iatsenko et al., 2016; Kaneko et al., 2004; Leulier et al., 2003). PGRP-LC encodes three isoforms that recognize either monomeric (TCT) or polymeric peptidoglycan (Kaneko et al., 2004; Neyen et al., 2012). The intracellular

domain of PGRP-LC recruits the Imd protein through their cRHIM domains (Kleino and Silverman, 2019). Alternatively, intracellular bacterial tracheal cytotoxin (TCT) can be sensed by PGRP-LE, notably in hemocytes and the midgut, to induce Imd signalling (Bosco-Drayon et al., 2012; Kaneko et al., 2006; Kurata, 2014; Lim et al., 2006). Activation of the Imd pathway initiates a complex cascade leading to i) the cleavage of the ankyrin repeat domain from the NF-κB transcription factor Relish, freeing the transactivating domain, and ii) the phosphorylation of Relish. After both processes, Relish dimers then translocate to the nucleus to initiate transcription of Imd target genes (Myllymäki et al., 2014). The activation of Relish occurs through two distinct arms, either involving the death domain containing protein FADD and caspase Dredd, or the TAB2/Tak1 complex and the complex of immune kinase kinases including IKKγ ("Kenny" in flies) and IKKβ/Ird5. A common readout of Imd signalling is the antimicrobial peptide *Diptericin A* (Lemaitre and Hoffmann, 2007), which is expressed at a low basal level yet achieves incredible induction hundreds to thousands of times its basal state (Lemaitre et al., 1997).

Initial work in *Drosophila* immunity identified several antimicrobial peptides and their genes. In the years that followed, the attention of scientists quickly turned towards the immune pathways that regulate them. This work, involving the careful dissection of Toll and Imd NF-κB signalling, was made possible in large part due to antimicrobial peptide genes like *Drosomycin* and *Diptericin* that serve as unmistakeable beacons of pathway activation (Faye and Lindberg, 2016; Lemaitre and Hoffmann, 2007). The work put in to initially characterize these immune effectors typically receives less attention than the profound conservation of NF-κB signalling in animals. However those insights were only made possible through the characterization of immune effectors as critical tools for immune signalling research. In the next section, I hope to do this work justice and expand on the principle function of such immune effectors.

1.5 Antimicrobial peptides and other immune effectors

Antimicrobial peptides or "AMPs" (also called Host Defence Peptides or HPDs) are short, typically cationic, and amphipathic molecules that directly act on invading microorganisms in the micromolar range. They display varied mechanisms of action, including pore formation or other forms of membrane destabilization, or inhibit intracellular targets such as DNA chaperone proteins or microbial ribosomes that ultimately lead to cell death (Hancock et al., 2016; Imler and Bulet, 2005; Lazzaro et al., 2020; Mookherjee et al., 2020). Antibacterials derived from microbes are famous for their revolutionary contributions to medicine such as

penicillin or bacteriocins, ushering in the antibiotic era (Gratia, 2000; Hutchings et al., 2019). But it would not be until decades later that immune research would come to appreciate that animals also encoded genes effectively functioning as antibiotics.

1.5.1 Antimicrobial peptides : directly microbicidal agents

The story of animal AMP discovery begins with the characterization of the insect antimicrobial peptide response. This research provided the foundation for a paradigm shift in innate immunity (Faye and Lindberg, 2016). Work by the group of Hans Boman and colleagues at Stockholm university in Sweden isolated the first antibacterial peptides of an animal in the silk moth *Hyalophora cecropia*. This molecule, termed "Cecropin", displayed a potent in vitro activity against a diversity of gram-negative bacteria. The characterization of Cecropin provided a mechanism to explain how the insect hemolymph rapidly transformed into a bactericidal environment (Steiner et al., 1981). Boman's group later described a second antimicrobial peptide family from Cecropia moths, the Attacins (Hultmark et al., 1983). This pioneering work was followed by the identification of an antibacterial molecule secreted from rabbit granulocytes that came to be known as Defensin (work done by the group of Robert Lehrer) (Selsted et al., 1984), and shortly after antibacterial Defensins secreted by human neutrophils were also discovered (also called HNPs for "human neutrophil peptides" (Ganz et al., 1985)). These early studies revealed a layer of immunity neglected by the thinking of the era, which typically focused only on adaptive immune mechanisms with memory and high specificity (Faye and Lindberg, 2016). The following decade saw the characterization of a great many AMP families from humans and many diverse insect and vertebrate models (Casteels et al., 1990; Casteels-Josson et al., 1993; Dimarcq et al., 1988; Lambert et al., 1989; Moore et al., 1993; Selsted et al., 1992; Zanetti et al., 1995). Since then, AMPs have been extensively studied.

1.5.2 Early applications of antimicrobial peptide study

An immediate use for AMPs was to act as readouts of infection, as these molecules are induced to an incredible extent reaching hundreds or even thousands of times their basal expression level (Engström et al., 1993; Ferrandon et al., 1998; Lemaitre et al., 1997, 1996, 1995; Reichhart et al., 1992; Uttenweiler-Joseph et al., 1998). For instance, the discovery of Toll-like receptors as key regulators of innate immunity was made possible thanks to the use of the fruit fly AMP *Drosomycin* as a readout of *Drosophila* Toll pathway activation (Lemaitre et al., 1996). A second major motivation has been to understand their antimicrobial mechanisms for the

design of novel antibiotics (Mylonakis et al., 2016). Perhaps the most famous case study of this motivation is the frog AMP Magainin, first identified concurrently in 1987 by the groups of Michael Zasloff and David Williams (Giovannini et al., 1987; Zasloff, 1987). Magainins act by forming toroidal pores in bacterial membranes, permeabilizing the membrane and disrupting membrane integrity (Ludtke et al., 1996). While commercial Magainin development never succeeded in becoming a novel therapeutic, it was found to be safe and effective as a topical antibiotic. The reason given by the United States Food and Drug administration in 1999 for rejecting Magainin for commercial use was simply because it performed equal to current standard of care, but did not represent an improvement (Moore, 2003). Magainin thus acts as a proof of principle, despite the wisdom of the era rejecting the introduction of more diverse antibiotics into the market. Modern concerns over the development of antibiotic resistance have renewed interest in learning from the potent innate defence mechanisms of animals, utilizing AMPs directly or as inspiration for the development of more shelf-stable antibiotics (Lazzaro et al., 2020; Mylonakis et al., 2016). While various antibiotics of bacterial and fungal origin have been approved for clinical use, to date no antibiotic inspired by an animal AMP has entered the market. Insects boast a promising diversity of antimicrobial peptides for clinical development, particularly given their lack of adaptive immune mechanisms. The implication being that the insect AMP response forms a critical defence, dictating life or death upon infection (Hanson and Lemaitre, 2020). A few AMP families are conserved across insects, indicating their utility in host defence goes back hundreds of millions of years (Gerdol et al., 2020; Hanson et al., 2019b; Rolff and Schmid-Hempel, 2016). The fact that the core of these defence peptides has remained relevant over such geological time scales makes them promising candidates for the development of antibiotics that prevent evolution of resistance (Lazzaro et al., 2020; Yu et al., 2016). In this thesis, I will discuss these ancient immune effectors through the lens of the fruit fly, *Drosophila melanogaster*.

1.5.3 The *Drosophila* antimicrobial peptide response

In the decades following the discovery of Cecropin, several major AMP families of insects were described. In *Drosophila*, seven "classical" AMP families were known by 1995. These AMP families were: Cecropin, Attacin, Defensin, Diptericin, Drosocin, Drosomycin, and Metchnikowin, whose transcriptional regulation is summarized in Fig. 1.2. Notably, insect Cecropins (Steiner et al., 1981) and Attacins (Hultmark et al., 1983) are conserved throughout hemi- and holometabolous insects (dating back ~350 million years (Misof et al., 2014)) and Defensins (Lambert et al., 1989) are conserved across arthropods and crustaceans, stemming from a

common ancestral molecule (Froy and Gurevitz, 2003; Gerdol et al., 2020). Insect Cecropins are small (<40 residues) peptides whose secondary structure is composed of two α helices upon incorporation into the lipid bilayer. Cecropins aggregate into either barrel-stave or toroidal pores composed of both Cecropin and lipid molecules, with diameters of ~40 angstroms (4nm) (Christensen et al., 1988; Durell et al., 1992; Efimova et al., 2014). In vitro, Cecropins primarily act against a broad panel of Gram-negative bacteria, though disparate studies have also suggested an antifungal role (Carboni et al., 2021; Ekengren and Hultmark, 1999). The *Drosophila* genome encodes 4 functional *Cecropin* genes clustered in the genome together with the testes-specific antibacterial peptide gene *Andropin*. The *Attacins* (*Att*) are larger genes encoding proteins up to 241AA (e.g. *AttC*). However *Attacins* commonly encode furin-cleaved polypeptides, united by a glycine-rich C-terminal domain (Hedengren et al., 2000; Hultmark et al., 1983). The mature Attacin structures are thus smaller than their precursor proteins. The propeptides of Attacins in *Drosophila* bear some similarity to Proline-rich AMPs of other insects known to bind irreversibly to bacterial DnaK or the bacterial ribosome translational tunnel (e.g. Abaecin, Pyrrhocoricin) (Kragol et al., 2001; Peng et al., 2018; Rahnamaeian et al., 2016). Indeed, the *Drosophila* N- terminal peptide of Attacin C synergizes with pore-forming Cecropins (Rabel et al., 2004). Two bumblebee peptides with sequence similarity to *Drosophila* Attacin C Proline-rich peptide or Attacin domain pore-forming products similarly synergize to kill *Escherichia coli* bacteria (Rahnamaeian et al., 2016). Four *Attacin* genes are named as such in the *Drosophila* genome, although another *Attacin* gene (named *edin*) is known (Vanha-Aho et al., 2012). Additionally, *AttD* is strongly expressed in hemocyte and lacks a signal peptide, suggesting if it is secreted, it accomplishes this through an alternative route from the standard secretory pathway (Hanson and Lemaitre, 2020; Hedengren et al., 2000). *Drosophila* encode other antimicrobial peptide families with structural similarity to these N- and C-terminal Attacin domains: the Drosocins and Diptericins respectively. Drosocin is a proline-rich peptide that also binds to bacterial DnaK, like the firebug AMP Pyrrhocoricin or bumblebee Abaecin (Zahn and Straeter, 2013). This antibacterial potency of Drosocin requires O-glycosylation at its Threonine residue, as Drosocin lacking this O-glycosylation has severely reduced in vitro activity (Bikker et al., 2006; Bulet et al., 1996). Diptericins meanwhile encode Glycine-rich proteins, which like the Attacin G-rich domain, have a structure that remains unresolved. Recent predictions by AlphaFold (Jumper et al., 2021) suggest a beta sheet folding structure, however whether this is true in the presence of a lipid bilayer, or alongside post-translation modifications is unknown. What is known is that the Glycine-rich domain must be contiguous for function, as artificial cleavage and co-culture of the domain as

two smaller peptides causes a loss of antimicrobial activity (Cudic et al., 1999). Finally two classical *Drosophila* antifungal peptides are Metchnikowin and Drosomycin. Metchnikowin (named after Élie Metchnikoff) encodes a small Proline-rich peptide with in vitro activity displayed against *Neurospora crassa* fungi (Levashina et al., 1995). The proposed mechanism of action for Metchnikowin is mediated through targeting of the fungal iron-sulfur subunit of succinate-coenzyme Q reductase, which inhibits succinate dehydrogenase activity of certain fungal species (Moghaddam et al., 2017). *Metchnikowin* is somewhat unique as it is transcriptionally regulated by both the Toll pathway upon natural infection by fungi, and the Imd pathway upon systemic infection by Gram-negative bacteria (De Gregorio et al., 2002; Schlamp et al., 2021; Troha et al., 2018). Meanwhile Drosomycin displays activity against various filamentous fungi in vitro (Fehlbaum et al., 1994; Simon et al., 2008).

These various AMP families are major components of the hemolymph following infection. It has been estimated that Drosomycin and Drosocin reach up to 100μM and 40μM concentrations respectively, though other AMPs typically settle between 0.5-10μM at their peak concentrations (Ferrandon et al., 1998; Imler and Bulet, 2005; Lemaitre and Hoffmann, 2007). However, these estimates sometimes rely on passing mentions by authors in early studies without providing robust evidence (Fehlbaum et al., 1994; Imler and Bulet, 2005), and little validation of AMP concentrations has been performed in the modern era. Nevertheless, it is clear that AMP concentrations extend well into the micromolar range, and the massive induction of AMPs was even used to demonstrate that the profile of *Drosophila* hemolymph changes visibly after infection in MALDI-TOF proteomic assays (MALDI-TOF: matrix assisted laser deionization time of flight (Uttenweiler-Joseph et al., 1998)). This study revealed that so marked was the induction of AMPs that immune-induced peaks corresponding to the known *Drosophila* AMPs could be resolved clearly, and these peptides remained abundant in the hemolymph sometimes out to 2-3 weeks after infection. Notably this study also highlighted that a number of other immune-induced molecules were highly prevalent in the *Drosophila* hemolymph that likely reached concentrations rivalling that of the classical AMPs. These molecules were termed "*Drosophila* Immune-induced Molecules" (DIMs or IMs) and numbered IM1-24 according to their mass. Later proteomic studies clarified the source genes for a few of these IMs (Levy et al., 2004; Liu et al., 2006; Verleyen et al., 2006), though many of these genes remained uncharacterized (encoding 16 of the 24 IMs) including two where the gene and the sequence of the IM remains unknown (IM7, IM22). Following the pioneering period of AMP characterization in the 1980s-1990s, the attention of the research community turned towards the exciting and burgeoning field of innate immune signalling. It became a core focus of *Dro-*

sophila immune research to determine the genes involved in the core signalling pathways (Lemaitre and Hoffmann, 2007). Perhaps alongside factors like the demotivating rejection of Magainin development for clinical use (Moore, 2003), the purpose of AMPs in *Drosophila* research was transformed, being used almost exclusively as readouts of infection (Hanson and Lemaitre, 2020). It would not be until 2015, two decades after the characterization of the last *Drosophila* AMP (Metchnikowin in 1995 (Levashina et al., 1995)), that another IM family would be formally investigated. The finding was striking: many of the classic *Drosophila* IMs belonged to a multigene family that was essential for Toll-mediated defence against infection. These peptides were named Bomanins in honour of Hans Boman's pioneering work by the group of Steve Wasserman (Clemmons et al., 2015). The 12 Bomanins are typified by a conserved 16-residue sequence involving a CXXC motif that forms the basis of a Cysteine-bridge disulphide bond. What was surprising about the Bomanins is that flies lacking 10 of 12 Bomanin genes suffer a susceptibility to infection by Gram-positive bacteria and fungi broadly mirroring that of mutants lacking the Toll signalling pathway entirely, yet Bomanins do not display antimicrobial activity in vitro (Clemmons et al., 2015; Lindsay et al., 2018). The broad in vivo requirement of Bomanins for defence against all clades of Gram-positive bacteria and fungi may suggest that these peptides somehow potentiate other effectors of the Toll-mediated defence against infection rather than contribute direct antimicrobial activity. Perhaps suggesting that Bomanins interact with some host target, loss of the gene *Bombardier* prevents Bomanin secretion, and is associated with reduced tolerance to infection (Lin et al., 2019). Specifically, the authors find that *Bomanins* are absent in the hemolymph of *Bombardier* mutants after infection, coupled with susceptibility to infection mimicking *Bomanin* mutant flies. Next Lin et al. (Lin et al., 2019) elegantly showed that activation of the immune response by heat-killed *M. luteus* bacteria in *Bombardier* mutants leads to complete mortality just days after challenge, but this can be rescued by co-occuring deletion of *Bomanin* genes. The authors suggest that Bomanin buildup in the fat body leads to autotoxicity, which implies that Bomanins are not innocuous in host tissue, and somehow disrupt fat body function when they fail to get secreted. However it could still be that Bomanins have some direct microbicidal function that in vitro conditions have not yet recovered (Lindsay et al., 2018).

The discovery of the Bomanins reinvigorated the quest to understand the logic of the *Drosophila* effector response. The Bomanins revealed that there was a surprising complexity to the *Drosophila* Toll response that appears to be independent of the classical AMPs. The next IMs to be described were named Daisho, a reference to their length and position in the genome being similar in proportion to the two short swords that Japanese samurai carried (Cohen et

al., 2020). Loss of the two Daisho genes leads to susceptibility to specific fungi, notably *Fusarium* species. Use of FLAG-tagged Daisho peptides also revealed specific binding of Daisho to *Fusarium* hyphae ex vivo. The Daisho peptides are likely to be bona fide antifungal peptides, however the killing ability and concentrations required for Daisho peptides remains to be determined. The description of the Bomanin and Daisho gene families resolved just six of the remaining 16 IMs from the 1998 MALDI-TOF investigation (Uttenweiler-Joseph et al., 1998). Ten of these IMs remain to be investigated.

1.5.4 AMP evolution, polymorphisms, and loss-of-function mutations

Early work on AMPs was enabled by protein-based assays of antimicrobial activity. However the logic at the time was that AMP multigene families were broadly antimicrobial in vitro, and so they likely functioned in somewhat redundant fashions (Lazzaro, 2008; Lemaitre and Hoffmann, 2007). As such, disruption of only one AMP gene was unlikely to yield any noteworthy effect on immune defence, as related genes would cover for its absence. This issue was compounded by the short size of AMP genes that was almost never disrupted by random mutagenesis approaches. At the same time, the early 2000s saw an explosion of *Drosophila* genetic tools, allowing an unparalleled experimental toolkit to manipulate gene expression in tissue-specific fashions. Major tool generation efforts (using mutagens like ethyl methylsuflonate (Rutschmann and Hoebe, 2008), RNA interference libraries (Dietzl et al., 2007), or the construction of P-element insertion toolkits (Bellen et al., 2011; Ryder et al., 2004)) failed to yield ready-made tools for complex AMP investigations, but provided a wide variety of genetic manipulations that made it possible dissect immune signalling epistasis. Accordingly, the next decade witnessed major advances in the understanding of the Toll and Imd signalling pathways. At the same time, evolutionary studies recovered patterns of AMP duplication and loss that suggested individual AMP genes were unlikely to be important for host defence, and rather the cumulative product of the AMP response to infection was the key to successful host defence (Deng et al., 2009; Hedengren et al., 2000; Lazzaro, 2008; Quesada et al., 2005). This notion came to be known as the AMP cocktail approach, proposing that AMPs acted synergistically to kill invading microbes. Subsequent in vitro investigations only further validated this mindset as mixing of AMPs yields synergistic killing curves in antimicrobial assays (Yan and Hancock, 2001; Yu et al., 2016). However one surprising finding has initiated a recent paradigm shift in the way we think about AMP activity. In 2015, Unckless et al. (Unckless et al., 2015) published a study investigating the effect of nutritional status on host defence using the bacterium *Providencia rettgeri* as their infectious model. Their approach was to screen a ge-

netically diverse pool of fly strains established from wild-caught flies and perform a genome-wide association study (GWAS) to highlight genetic loci that determined defence in the interaction between nutrition and *P. rettgeri* infection. However a remarkable signal emerged instead: a single nucleotide polymorphism causing a Serine-Arginine change in residue 69 of the mature Diptericin A peptide explained most of the variation in defence against *P. rettgeri* infection regardless of nutritional state. More striking yet, a second mutation was found that caused a premature stop, which results in an even greater susceptibility (suggesting the susceptible Arginine allele was not completely non-functional). This Serine/Arginine polymorphism has evolved repeatedly in other fly species through natural selection (specifically balancing selection (Unckless et al., 2016)), and two additional residues (Glutamine and Asparagine) are found instead in Diptericin proteins of outgroup flies (Hanson et al., 2016; Unckless et al., 2016). This intriguing result presented a stark contrast to the notion that the AMP response encompassed many redundant peptides with broad-spectrum activity, instead suggesting that minor differences in AMP sequence could have important consequences on activity. Such polymorphisms are in fact quite common in AMPs of animals, but have had little functional investigation to date (Chapman et al., 2019; Halldórsdóttir and Árnason, 2015; Hellgren and Sheldon, 2011; Hollox and Armour, 2008; Tennessen and Blouin, 2008; Unckless and Lazzaro, 2016). The implication of these evolutionary patterns in both vertebrates and invertebrates alike rather suddenly gained a new significance.

The research was therefore at the right place and time for a re-emergence of AMP investigation. Bomanins revealed that immune effectors were not always simple antimicrobials, while the specificity of the Diptericin S69R polymorphism in defence against *P. rettgeri* indicated that even antimicrobials with simple mechanisms (like disrupting cell wall integrity) might not be as generalist and redundant as previously thought. At the same time, the advent of CRISPR/Cas9 genetic engineering ushered in a new era of genetic manipulation offering an unprecedented level of precision (Ledford and Callaway, 2020). The challenges of the previous era could be addressed, and a systematic deletion of AMP genes became possible. In the wake of these Bomanin and Diptericin findings, it was clear that the prevailing wisdom governing the *Drosophila* antimicrobial response lacked the subtlety needed to explain how the fly defends itself against infection. Patterns in vitro could not be used to fully understand the behaviour of AMPs in vivo.

1.6 Objectives of the present PhD thesis

The goal of this PhD thesis is to characterize the function of *Drosophila* antimicrobial peptides in an in vivo context. This is investigated in two main ways: i) a systematic deletion of the classical *Drosophila* AMPs to confirm their contribution to defence, and ii) the characterization of the remaining undescribed IMs of the *Drosophila* immune response first identified in 1998 (Levy et al., 2004; Uttenweiler-Joseph et al., 1998).

Prior to this thesis work, very few studies had investigated the role of AMPs in defence against infection in vivo. An early and elegant genetic study overexpressed *Drosophila* AMPs in a Toll, Imd deficient background, revealing the potential for specific AMPs to rescue the susceptibility of immune deficient flies (Tzou et al., 2002). This provided a powerful in vivo demonstration of the potential for AMPs to protect the fly against infection. Unckless et al. (Unckless et al., 2016, 2015) similarly screened a panel of wild-type flies via GWAS for interactions between diet and nutrition, and so the discovery of the *Diptericin A* - *P. rettgeri* interaction was incidental. My study published in eLife in 2019 (chapter 2) was the first systematic characterization of the in vivo importance of AMPs in defence against a diverse array of microbes (Hanson et al., 2019a). This study confirmed the important role of antibacterial peptides in the defense against Gram-negative bacteria. A surprising finding was the lack of susceptibility of compound *Drosophila* AMP mutants to infection by Gram-positive bacteria. A second surprising finding was the degree to which specific single AMP genes contribute to defence against infection by certain microbes, including the important contribution of Diptericins and a complete lack of role of five other AMP families in defence against *P. rettgeri*.

The second objective of this PhD work is to identify and characterize the remaining *Drosophila* IMs. The conclusion of my 2019 study revealed that individual genes could play highly specific and important roles in defence. To understand the reason(s) that Toll or Imd mutants succumbed to a given pathogen, it became clear that we would need a more complete picture of the immune effector response. To this end, I first focused my attention on the Toll regulated gene *Baramicin A* that encodes many of the previously uncharacterized IMs (chapter 3). Mutations in this gene cause a marked susceptibility to certain fungi, and *Baramicin A* peptides display antifungal activity in vitro. This led us to propose that *Baramicin* should be added to the list of *Drosophila* antimicrobial peptide genes. I also realized that the immune-induced molecule IM22 was encoded by *Baramicin A* (sequence never determined). Alongside these explorations, peptidomic analysis of the AMP mutants inadvertently revealed that compound AMP deficient flies lacked the immune-induced molecule IM7 (sequence never determined).

Ultimately we realized that IM7 is the C-terminal peptide encoded by the *Drosocin* gene. Use of mutations affecting either Drosocin, IM7, or both, reveals their unique roles against different bacteria. This study also investigates a polymorphism in IM7, which was in fact the reason that its sequence remained hidden for so long. The alternate allele of this polymorphism causes a significant susceptibility to the bacterium *Providencia burhodogranariea*. At the same time, I also investigated the role of *Drosophila Diptericin B* in defence against infection, a gene that has received far less attention than its daughter gene *Diptericin A*. As it turns out, *Diptericin B* alone is essential for surviving infection by a specific isolate of *Acetobacter* that was isolated from laboratory flies in Lausanne. Thus further dissection of the genes and peptides encoded by classical AMP genes continues to demonstrate remarkable specificities for individual peptides in defence against specific pathogens. Some of these findings are currently unpublished.

1.7 References

Agaisse H, Perrimon N. 2004. The roles of JAK/STAT signaling in Drosophila immune responses. Immunol Rev 198:72–82. doi:10.1111/j.0105-2896.2004.0133.x

An C, Zhang M, Chu Y, Zhao Z. 2013. Serine Protease MP2 Activates Prophenoloxidase in the Melanization Immune Response of Drosophila melanogaster. PLoS ONE 8:e79533. doi:10.1371/journal.pone.0079533

Banerjee U, Girard JR, Goins LM, Spratford CM. 2019. Drosophila as a Genetic Model for Hematopoiesis. Genetics 211:367–417. doi:10.1534/genetics.118.300223

Barber MF, Elde NC. 2014. Escape from bacterial iron piracy through rapid evolution of transferrin. Science 346:1362–1366. doi:10.1126/science.1259329

Bellen HJ, Levis RW, He Y, Carlson JW, Evans-Holm M, Bae E, Kim J, Metaxakis A, Savakis C, Schulze KL, Hoskins RA, Spradling AC. 2011. The Drosophila gene disruption project: Progress using transposons with distinctive site specificities. Genetics 188:731–743. doi:10.1534/genetics.111.126995

Bikker FJ, Kaman-van Zanten WE, de Vries-van de Ruit A-MBC, Voskamp-Visser I, van Hooft PAV, Mars-Groenendijk RH, de Visser PC, Noort D. 2006. Evaluation of the antibacterial spectrum of drosocin analogues. Chem Biol Drug Des 68:148–153. doi:10.1111/j.1747-0285.2006.00424.x

Binggeli O, Neyen C, Poidevin M, Lemaitre B. 2014. Prophenoloxidase Activation Is Required for Survival to Microbial Infections in Drosophila. PLoS Pathogens 10. doi:10.1371/journal.ppat.1004067

Bosco-Drayon V, Poidevin M, Boneca IG, Narbonne-Reveau K, Royet J, Charroux B. 2012. Peptidoglycan Sensing by the Receptor PGRP-LE in the Drosophila Gut Induces Immune Responses to Infectious Bacteria and Tolerance to Microbiota. Cell Host & Microbe 12:153–165. doi:10.1016/j.chom.2012.06.002

Buchon N, Poidevin M, Kwon H-M, Guillou A, Sottas V, Lee B-L, Lemaitre B. 2009. A single modular serine protease integrates signals from pattern-recognition receptors upstream of the Drosophila Toll pathway. Proc Natl Acad Sci U S A 106:12442–12447. doi:10.1073/pnas.0901924106

Bulet P, Urge L, Ohresser S, Hetru C, Otvos L. 1996. Enlarged scale chemical synthesis and range of activity of drosocin, an O-glycosylated antibacterial peptide of Drosophila. European Journal of Biochemistry 238:64–69. doi:10.1111/j.1432-1033.1996.0064q.x

Busse MS, Arnold CP, Towb P, Katrivesis J, Wasserman SA. 2007. A κB sequence code for pathway-specific innate immune responses. EMBO Journal 26:3826–3835. doi:10.1038/sj.emboj.7601798

Cai H, Holleufer A, Simonsen B, Schneider J, Lemoine A, Gad HH, Huang Jingxian, Huang Jieqing, Chen D, Peng T, Marques JT, Hartmann R, Martins NE, Imler J-L. 2020. 2’3’-cGAMP triggers a STING- and NF-κB–dependent broad antiviral response in Drosophila. Sci Signal 13:eabc4537. doi:10.1126/scisignal.abc4537

Cao C, Cogni R, Barbier V, Jiggins FM. 2017. Complex Coding and Regulatory Polymorphisms in a Restriction Factor Determine the Susceptibility of Drosophila to Viral Infection. Genetics 206:2159–2173. doi:10.1534/genetics.117.201970

Carboni A, Hanson MA, Lindsay SA, Wasserman SA, Lemaitre B. 2021. Cecropins contribute to Drosophila host defence against fungal and Gram-negative bacterial infection (preprint). Immunology. doi:10.1101/2021.05.06.442783

Carré-Mlouka A, Gaumer S, Gay P, Petitjean AM, Coulondre C, Dru P, Bras F, Dezélée S, Contamine D. 2007. Control of sigma virus multiplication by the ref(2)P gene of Drosophila melanogaster: an in vivo study of the PB1 domain of Ref(2)P. Genetics 176:409–419. doi:10.1534/genetics.106.063826

Casteels P, Ampe C, Riviere L, van Damme J, ELICONE C, Fleming M, Jacobs F, Tempst P. 1990. Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (Apis mellifera). European Journal of Biochemistry 187:381–386. doi:10.1111/j.1432-1033.1990.tb15315.x

Casteels-Josson K, Capaci T, Casteels P, Tempst P. 1993. Apidaecin multipeptide precursor structure: a putative mechanism for amplification of the insect antibacterial response. The EMBO journal 12:1569–78. doi:10.1002/j.1460-2075.1993.tb05801.x

Carton Y. 2019. Innate immunity: from Louis Pasteur to Jules Hoffmann.

Cerenius L, Lee BL, Söderhäll K. 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends in Immunology 29:263–271. doi:10.1016/j.it.2008.02.009

Chakrabarti S, Dudzic JP, Li X, Collas EJ, Boquete JP, Lemaitre B. 2016. Remote Control of Intestinal Stem Cell Activity by Haemocytes in Drosophila. PLoS Genetics 12. doi:10.1371/journal.pgen.1006089

Chapman JR, Hill T, Unckless RL. 2019. Balancing selection drives maintenance of genetic variation in Drosophila antimicrobial peptides. Genome Biology and Evolution 11:2691–2701. doi:https://doi.org/10.1093/gbe/evz191

Choe K-M, Lee H, Anderson KV. 2005. Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor. Proceedings of the National Academy of Sciences 102:1122–1126. doi:10.1073/pnas.0404952102

Christensen B, Fink J, Merrifield RB, Mauzerall D. 1988. Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. Proc Natl Acad Sci U S A 85:5072–5076. doi:10.1073/pnas.85.14.5072

Clark RI, Tan SWS, Péan CB, Roostalu U, Vivancos V, Bronda K, Pilátová M, Fu J, Walker DW, Berdeaux R, Geissmann F, Dionne MS. 2013. MEF2 is an in vivo immune-metabolic switch. Cell 155:435–447. doi:10.1016/j.cell.2013.09.007

Clemmons AW, Lindsay SA, Wasserman SA. 2015. An Effector Peptide Family Required for Drosophila Toll-Mediated Immunity. PLoS Pathogens 11. doi:10.1371/journal.ppat.1004876

Cohen LB, Lindsay SA, Xu Y, Lin SJH, Wasserman SA. 2020. The Daisho Peptides Mediate Drosophila Defense Against a Subset of Filamentous Fungi. Front Immunol 11:9. doi:10.3389/fimmu.2020.00009

Cottrell TR, Doering TL. 2003. Silence of the strands: RNA interference in eukaryotic pathogens. doi:10.1016/S0966-842X(02)00004-5

Cudic M, Bulet P, Hoffmann R, Craik DJ, Otvos L. 1999. Chemical synthesis, antibacterial activity and conformation of diptericin, an 82-mer peptide originally isolated from insects. European Journal of Biochemistry 266:549–558. doi:10.1046/j.1432-1327.1999.00894.x

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. 2002. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. EMBO Journal 21:2568–2579. doi:10.1093/emboj/21.11.2568

Deng X-J, Yang W-Y, Huang Y-D, Cao Y, Wen S-Y, Xia Q-Y, Xu P. 2009. Gene expression divergence and evolutionary analysis of the drosomycin gene family in Drosophila melanogaster. J Biomed Biotechnol 2009:315423. doi:10.1155/2009/315423

Dietzl G, Chen D, Schnorrer F, Su K-C, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ. 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448:151–156. doi:10.1038/nature05954

Dimarcq JL, Keppi E, Dunbar B, Lambert J, Reichhart JM, Hoffmann D, Rankine SM, Fothergill JE, Hoffmann JA. 1988. Insect immunity. Purification and characterization of a family of novel inducible antibacterial proteins from immunized larvae of the dipteran Phormia terranovae and complete amino-acid sequence of the predominant member, diptericin A. Eur J Biochem 171:17–22. doi:10.1111/j.1432-1033.1988.tb13752.x

Dudzic JP, Hanson MA, Iatsenko I, Kondo S, Lemaitre B. 2019. More Than Black or White: Melanization and Toll Share Regulatory Serine Proteases in Drosophila. Cell Reports 27:1050-1061.e3. doi:10.1016/j.celrep.2019.03.101

Dudzic JP, Kondo S, Ueda R, Bergman CM, Lemaitre B. 2015. Drosophila innate immunity: regional and functional specialization of prophenoloxidases. BMC biology 13:81–97. doi:10.1186/s12915-015-0193-6

Durell SR, Raghunathan G, Guy HR. 1992. Modeling the ion channel structure of cecropin. Biophysical Journal 63:1623–1631. doi:10.1016/S0006-3495(92)81730-7

Efimova SS, Schagina LV, Ostroumova OS. 2014. Channel-Forming Activity of Cecropins in Lipid Bilayers: Effect of Agents Modifying the Membrane Dipole Potential. Langmuir 30:7884–7892. doi:10.1021/la501549v

Ekengren S, Hultmark D. 2001. A Family of Turandot-Related Genes in the Humoral Stress Response of Drosophila. Biochemical and Biophysical Research Communications 284:998–1003. doi:10.1006/bbrc.2001.5067

Ekengren S, Hultmark D. 1999. Drosophila cecropin as an antifungal agent. Insect Biochemistry and Molecular Biology 29:965–972. doi:10.1016/S0965-1748(99)00071-5

El Chamy L, Leclerc V, Caldelari I, Reichhart J-M. 2008. Sensing of “danger signals” and pathogen-associated molecular patterns defines binary signaling pathways “upstream” of Toll. Nat Immunol 9:1165–1170. doi:10.1038/ni.1643

Engström Y, Kadalayil L, Sun SC, Samakovlis C, Hultmark D, Faye I. 1993. kappa B-like motifs regulate the induction of immune genes in Drosophila. J Mol Biol 232:327–333. doi:10.1006/jmbi.1993.1392

Ertürk-Hasdemir D, Broemer M, Leulier F, Lane WS, Paquette N, Hwang D, Kim C-H, Stöven S, Meier P, Silverman N. 2009. Two roles for the Drosophila IKK complex in the activation of Relish and the induction of antimicrobial peptide genes. Proc Natl Acad Sci U S A 106:9779–9784. doi:10.1073/pnas.0812022106

Faye I, Lindberg BG. 2016. Towards a paradigm shift in innate immunity—seminal work by Hans G. Boman and co-workers. Philosophical Transactions of the Royal Society B: Biological Sciences. doi:10.1098/rstb.2015.0303

Fehlbaum P, Bulet P, Michaut L, Lagueux M, Broekaert WF, Hetru C, Hoffmann JA. 1994. Insect immunity: Septic injury of drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. Journal of Biological Chemistry 269:33159–33163.

Fernando MDA, Kounatidis I, Ligoxygakis P. 2014. Loss of Trabid, a New Negative Regulator of the Drosophila Immune-Deficiency Pathway at the Level of TAK1, Reduces Life Span. PLoS Genetics 10. doi:10.1371/journal.pgen.1004117

Ferrandon D, Jung AC, Criqui MC, Lemaitre B, Uttenweiler-Joseph S, Michaut L, Reichhart JM, Hoffmann JA. 1998. A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. EMBO Journal 17:1217–1227. doi:10.1093/emboj/17.5.1217

Froy O, Gurevitz M. 2003. Arthropod and mollusk defensins – evolution by exon-shuffling. Trends in Genetics 19:684–687. doi:10.1016/j.tig.2003.10.010

Ganz T, Selsted ME, Szklarek D, Harwig SS, Daher K, Bainton DF, Lehrer RI. 1985. Defensins. Natural peptide antibiotics of human neutrophils. Journal of Clinical Investigation 76:1427–1435. doi:10.1172/JCI112120

Gerdol M, Schmitt P, Venier P, Rocha G, Rosa RD, Destoumieux-Garzón D. 2020. Functional Insights From the Evolutionary Diversification of Big Defensins. Front Immunol 11:758. doi:10.3389/fimmu.2020.00758

Giovannini MG, Poulter L, Gibson BW, Williams DH. 1987. Biosynthesis and degradation of peptides derived from Xenopus laevis prohormones. Biochemical Journal 243:113–120. doi:10.1042/bj2430113

Gold KS, Brückner K. 2015. Macrophages and cellular immunity in Drosophila melanogaster. Semin Immunol 27:357–368. doi:10.1016/j.smim.2016.03.010

Goto A, Okado K, Martins N, Cai H, Barbier V, Lamiable O, Troxler L, Santiago E, Kuhn L, Paik D, Silverman N, Holleufer A, Hartmann R, Liu J, Peng T, Hoffmann JA, Meignin C, Daeffler L, Imler JL. 2018. The Kinase IKKβ Regulates a STING- and NF-κB-Dependent Antiviral Response Pathway in Drosophila. Immunity 49:225-234.e4. doi:10.1016/j.immuni.2018.07.013

Gottar M, Gobert V, Matskevich AA, Reichhart JM, Wang C, Butt TM, Belvin M, Hoffmann JA, Ferrandon D. 2006. Dual Detection of Fungal Infections in Drosophila via Recognition of Glucans and Sensing of Virulence Factors. Cell 127:1425–1437. doi:10.1016/j.cell.2006.10.046

Gottar M, Gobert V, Michel T, Belvin M, Duyk G, Hoffmann JA, Ferrandon D, Royet J. 2002. The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature 416:640–644. doi:10.1038/nature734

Gratia JP. 2000. André Gratia: a forerunner in microbial and viral genetics. Genetics 156:471–476. doi:10.1093/genetics/156.2.471

Habayeb MS, Ekengren SK, Hultmark D. 2006. Nora virus, a persistent virus in Drosophila, defines a new picorna-like virus family. J Gen Virol 87:3045–3051. doi:10.1099/vir.0.81997-0

Halldórsdóttir K, Árnason E. 2015. Trans-species polymorphism at antimicrobial innate immunity cathelicidin genes of Atlantic cod and related species. PeerJ 3:e976. doi:10.7717/peerj.976

Hancock REW, Haney EF, Gill EE. 2016. The immunology of host defence peptides: Beyond antimicrobial activity. doi:10.1038/nri.2016.29

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

Hanson MA, Hamilton PT, Perlman SJ. 2016. Immune genes and divergent antimicrobial peptides in flies of the subgenus Drosophila. BMC evolutionary biology 16:228. doi:10.1186/s12862-016-0805-y

Hanson MA, Lemaitre B. 2020. New insights on Drosophila antimicrobial peptide function in host defense and beyond. Curr Opin Immunol 62:22–30. doi:10.1016/j.coi.2019.11.008

Hanson MA, Lemaitre B, Unckless RL. 2019b. Dynamic Evolution of Antimicrobial Peptides Underscores Trade-Offs Between Immunity and Ecological Fitness. Frontiers in Immunology 10:2620. doi:doi: 10.3389/fimmu.2019.02620

Hedengren M, Borge K, Hultmark D. 2000. Expression and evolution of the Drosophila attacin/diptericin gene family. Biochemical and biophysical research communications 279:574–81. doi:10.1006/bbrc.2000.3988

Hellgren O, Sheldon BC. 2011. Locus-specific protocol for nine different innate immune genes (antimicrobial peptides: β-defensins) across passerine bird species reveals within-species coding variation and a case of trans-species polymorphisms. Molecular Ecology Resources 11:686–692. doi:10.1111/j.1755-0998.2011.02995.x

Hill T, Unckless RL. 2020. Recurrent evolution of high virulence in isolated populations of a DNA virus. eLife 9:e58931. doi:10.7554/eLife.58931

Holleufer A, Winther KG, Gad HH, Ai X, Chen Y, Li L, Wei Z, Deng H, Liu J, Frederiksen NA, Simonsen B, Andersen LL, Kleigrewe K, Dalskov L, Pichlmair A, Cai H, Imler J-L, Hartmann R. 2021. Two cGAS-like receptors induce antiviral immunity in Drosophila. Nature. doi:10.1038/s41586-021-03800-z

Hollox EJ, Armour JAL. 2008. Directional and balancing selection in human beta-defensins. BMC Evolutionary Biology 8. doi:10.1186/1471-2148-8-113

Hultmark D, Engström A, Andersson K, Steiner H, Bennich H, Boman HG. 1983. Insect immunity. Attacins, a family of antibacterial proteins from Hyalophora cecropia. The EMBO Journal. doi:10.1002/j.1460-2075.1983.tb01465.x

Hutchings MI, Truman AW, Wilkinson B. 2019. Antibiotics: past, present and future. Current Opinion in Microbiology 51:72–80. doi:10.1016/j.mib.2019.10.008

Iatsenko I, Kondo S, Mengin-Lecreulx D, Lemaitre B. 2016. PGRP-SD, an Extracellular Pattern-Recognition Receptor, Enhances Peptidoglycan-Mediated Activation of the Drosophila Imd Pathway. Immunity 45:1013–1023. doi:10.1016/j.immuni.2016.10.029

Iatsenko I, Marra A, Boquete J-P, Peña J, Lemaitre B. 2020. Iron sequestration by transferrin 1 mediates nutritional immunity in Drosophila melanogaster. Proc Natl Acad Sci USA 117:7317–7325. doi:10.1073/pnas.1914830117

Igaki T. 2009. Correcting developmental errors by apoptosis: lessons from Drosophila JNK signaling. Apoptosis 14:1021–1028. doi:10.1007/s10495-009-0361-7

Imler J-L, Bulet P. 2005. Antimicrobial peptides in Drosophila: structures, activities and gene regulation. Chemical immunology and allergy 86:1–21. doi:10.1159/000086648

Imler JL, Hoffmann JA. 2001. Toll receptors in innate immunity. Trends Cell Biol 11:304–311. doi:10.1016/s0962-8924(01)02004-9

Issa N, Guillaumot N, Lauret E, Matt N, Schaeffer-Reiss C, Van Dorsselaer A, Reichhart JM, Veillard F. 2018. The Circulating Protease Persephone Is an Immune Sensor for Microbial Proteolytic Activities Upstream of the Drosophila Toll Pathway. Molecular Cell 69:648-663.e7. doi:10.1016/j.molcel.2018.01.029

Jousset F-X, Bergoin M, Revet B. 1977. Characterization of the Drosophila C Virus. Journal of General Virology 34:269–283. doi:10.1099/0022-1317-34-2-269

Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, Bridgland A, Meyer C, Kohl SAA, Ballard AJ, Cowie A, Romera-Paredes B, Nikolov S, Jain R, Adler J, Back T, Petersen S, Reiman D, Clancy E, Zielinski M, Steinegger M, Pacholska M, Berghammer T, Bodenstein S, Silver D, Vinyals O, Senior AW, Kavukcuoglu K, Kohli P, Hassabis D. 2021. Highly accurate protein structure prediction with AlphaFold. Nature. doi:10.1038/s41586-021-03819-2

Jünger MA, Rintelen F, Stocker H, Wasserman JD, Végh M, Radimerski T, Greenberg ME, Hafen E. 2003. The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol 2:20. doi:10.1186/1475-4924-2-20

Kaneko T, Goldman WE, Mellroth P, Steiner H, Fukase K, Kusumoto S, Harley W, Fox A, Golenbock D, Silverman N. 2004. Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway. Immunity 20:637–649. doi:10.1016/s1074-7613(04)00104-9

Kaneko T, Yano T, Aggarwal K, Lim J-H, Ueda K, Oshima Y, Peach C, Erturk-Hasdemir D, Goldman WE, Oh B-H, Kurata S, Silverman N. 2006. PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. Nat Immunol 7:715–723. doi:10.1038/ni1356

Kanost MR, Jiang H. 2015. Clip-domain serine proteases as immune factors in insect hemolymph. Current Opinion in Insect Science 11:47–55. doi:10.1016/j.cois.2015.09.003

Kleino A, Silverman N. 2019. Regulation of the Drosophila Imd pathway by signaling amyloids. Insect Biochemistry and Molecular Biology 108:16–23. doi:10.1016/j.ibmb.2019.03.003

Kragol G, Lovas S, Varadi G, Condie BA, Hoffmann R, Otvos L. 2001. The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. Biochemistry 40:3016–3026. doi:10.1021/bi002656a

Kramer JM, Slade JD, Staveley BE. 2008. foxo is required for resistance to amino acid starvation in Drosophila. Genome 51:668–672. doi:10.1139/G08-047

Kurata S. 2014. Peptidoglycan recognition proteins in Drosophila immunity. Dev Comp Immunol 42:36–41. doi:10.1016/j.dci.2013.06.006

Lambert J, Keppi E, Dimarcq JL, Wicker C, Reichhart JM, Dunbar B, Lepage P, Van Dorsselaer A, Hoffmann J, Fothergill J. 1989. Insect immunity: isolation from immune blood of the dipteran Phormia terranovae of two insect antibacterial peptides with sequence homology to rabbit lung macrophage bactericidal peptides. Proc Natl Acad Sci U S A 86:262–266. doi:10.1073/pnas.86.1.262

Lazzaro BP. 2008. Natural selection on the Drosophila antimicrobial immune system. doi:10.1016/j.mib.2008.05.001

Lazzaro BP, Zasloff M, Rolff J. 2020. Antimicrobial peptides: Application informed by evolution. Science 368. doi:10.1126/science.aau5480

Ledford H, Callaway E. 2020. Pioneers of revolutionary CRISPR gene editing win chemistry Nobel. Nature 586:346–347. doi:10.1038/d41586-020-02765-9

Lemaitre B, Hoffmann J. 2007. The Host Defense of Drosophila Melanogaster. Annual Review of Immunology 25:697–743. doi:10.1146/annurev.immunol.25.022106.141615

Lemaitre B, Kromer-Metzger E, Michaut L, Nicolas E, Meister M, Georgel P, Reichhart JM, Hoffmann JA. 1995. A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense. Proc Natl Acad Sci U S A 92:9465–9469. doi:10.1073/pnas.92.21.9465

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86:973–983. doi:10.1016/s0092-8674(00)80172-5

Lemaitre B, Reichhart JM, Hoffmann JA. 1997. Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proceedings of the National Academy of Sciences of the United States of America 94:14614–9. doi:10.1073/pnas.94.26.14614

Leulier F, Parquet C, Pili-Floury S, Ryu J-H, Caroff M, Lee W-J, Mengin-Lecreulx D, Lemaitre B. 2003. The Drosophila immune system detects bacteria through specific peptidoglycan recognition. Nat Immunol 4:478–484. doi:10.1038/ni922

Levashina EA, Langley E, Green C, Gubb D, Ashburner M, Hoffmann JA, Reichhart JM. 1999. Constitutive activation of toll-mediated antifungal defense in serpin-deficient Drosophila. Science 285:1917–1919. doi:10.1126/science.285.5435.1917

Levashina EA, Ohresser S, Bulet P, Reichhart J -M, Hetru C, Hoffmann JA. 1995. Metchnikowin, a Novel Immune-Inducible Proline-Rich Peptide from Drosophila with Antibacterial and Antifungal Properties. European Journal of Biochemistry 233:694–700. doi:10.1111/j.1432-1033.1995.694_2.x

Levy F, Rabel D, Charlet M, Bulet P, Hoffmann JA, Ehret-Sabatier L. 2004. Peptidomic and proteomic analyses of the systemic immune response of Drosophila. Biochimie 86:607–616. doi:10.1016/j.biochi.2004.07.007

Li X, Rommelaere S, Kondo S, Lemaitre B. 2020. Renal Purge of Hemolymphatic Lipids Prevents the Accumulation of ROS-Induced Inflammatory Oxidized Lipids and Protects Drosophila from Tissue Damage. Immunity 52:374-387.e6. doi:10.1016/j.immuni.2020.01.008

Lim J-H, Kim M-S, Kim H-E, Yano T, Oshima Y, Aggarwal K, Goldman WE, Silverman N, Kurata S, Oh B-H. 2006. Structural Basis for Preferential Recognition of Diaminopimelic Acid-type Peptidoglycan by a Subset of Peptidoglycan Recognition Proteins. Journal of Biological Chemistry 281:8286–8295. doi:10.1074/jbc.M513030200

Lin SJH, Fulzele A, Cohen LB, Bennett EJ, Wasserman SA. 2019. Bombardier Enables Delivery of Short-Form Bomanins in the Drosophila Toll Response. Front Immunol 10:3040. doi:10.3389/fimmu.2019.03040

Lindsay SA, Lin SJH, Wasserman SA. 2018. Short-Form Bomanins Mediate Humoral Immunity in Drosophila. J Innate Immun 10:306–314. doi:10.1159/000489831

Lindsay SA, Wasserman SA. 2014. Conventional and non-conventional Drosophila Toll signaling. Developmental and Comparative Immunology 42. doi:10.1016/j.dci.2013.04.011

Liu F, Baggerman G, D'Hertog W, Verleyen P, Schoofs L, Wets G. 2006. In Silico Identification of New Secretory Peptide Genes in Drosophila melanogaster. Molecular & Cellular Proteomics 5:510–522. doi:10.1074/mcp.M400114-MCP200

Lizcano JM, Alrubaie S, Kieloch A, Deak M, Leevers SJ, Alessi DR. 2003. Insulin-induced Drosophila S6 kinase activation requires phosphoinositide 3-kinase and protein kinase B. Biochem J 374:297–306. doi:10.1042/BJ20030577

Longdon B, Wilfert L, Jiggins F. 2012. The Sigma Viruses of DrosophilaRhabdoviruses: Molecular Taxonomy, Evolution, Genomics, Ecology, Host-Vector Interactions, Cytopathology and Control. Caister Academic Press.

Ludtke SJ, He K, Heller WT, Harroun TA, Yang L, Huang HW. 1996. Membrane Pores Induced by Magainin. Biochemistry 35:13723–13728. doi:10.1021/bi9620621

Martins NE, Faria VG, Nolte V, Schlotterer C, Teixeira L, Sucena E, Magalhaes S. 2014. Host adaptation to viruses relies on few genes with different cross-resistance properties. Proceedings of the National Academy of Sciences 111:5938–5943. doi:10.1073/pnas.1400378111

Meister M, Lagueux M. 2003. Drosophila blood cells. Cell Microbiol 5:573–580. doi:10.1046/j.1462-5822.2003.00302.x

Misof B, Liu S, Meusemann K, Peters RS, Donath A, Mayer C, Frandsen PB, Ware J, Flouri T, Beutel RG, Niehuis O, Petersen M, Izquierdo-Carrasco F, Wappler T, Rust J, Aberer AJ, Aspock U, Aspock H, Bartel D, Blanke A, Berger S, Bohm A, Buckley TR, Calcott B, Chen J, Friedrich F, Fukui M, Fujita M, Greve C, Grobe P, Gu S, Huang Y, Jermiin LS, Kawahara AY, Krogmann L, Kubiak M, Lanfear R, Letsch H, Li Y, Li Z, Li J, Lu H, Machida R, Mashimo Y, Kapli P, McKenna DD, Meng G, Nakagaki Y, Navarrete-Heredia JL, Ott M, Ou Y, Pass G, Podsiadlowski L, Pohl H, von Reumont BM, Schutte K, Sekiya K, Shimizu S, Slipinski A, Stamatakis A, Song W, Su X, Szucsich NU, Tan M, Tan X, Tang M, Tang J, Timelthaler G, Tomizuka S, Trautwein M, Tong X, Uchifune T, Walzl MG, Wiegmann BM, Wilbrandt J, Wipfler B, Wong TKF, Wu Q, Wu G, Xie Y, Yang S, Yang Q, Yeates DK, Yoshizawa K, Zhang Q, Zhang R, Zhang W, Zhang Y, Zhao J, Zhou C, Zhou L, Ziesmann T, Zou S, Li Y, Xu X, Zhang Y, Yang H, Wang J, Wang J, Kjer KM, Zhou X. 2014. Phylogenomics resolves the timing and pattern of insect evolution. Science 346:763–767. doi:10.1126/science.1257570

Moghaddam M-RB, Gross T, Becker A, Vilcinskas A, Rahnamaeian M. 2017. The selective antifungal activity of Drosophila melanogaster metchnikowin reflects the species-dependent inhibition of succinate–coenzyme Q reductase. Sci Rep 7:8192. doi:10.1038/s41598-017-08407-x

Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ. 2020. Antimicrobial host defence peptides: functions and clinical potential. Nat Rev Drug Discov. doi:10.1038/s41573-019-0058-8

Moore A. 2003. The big and small of drug discovery. Biotech versus pharma: advantages and drawbacks in drug development. EMBO Rep 4:114–117. doi:10.1038/sj.embor.embor748

Moore KS, Wehrli S, Roder H, Rogers M, Forrest JN, McCrimmon D, Zasloff M. 1993. Squalamine: an aminosterol antibiotic from the shark. Proceedings of the National Academy of Sciences 90:1354–1358. doi:10.1073/pnas.90.4.1354

Musselman LP, Fink JL, Grant AR, Gatto JA, Tuthill BF, Baranski TJ. 2018. A Complex Relationship between Immunity and Metabolism in Drosophila Diet-Induced Insulin Resistance. Mol Cell Biol 38. doi:10.1128/MCB.00259-17

Myllymäki H, Valanne S, Rämet M. 2014. The Drosophila imd signaling pathway. J Immunol 192:3455–3462. doi:10.4049/jimmunol.1303309

Mylonakis E, Podsiadlowski L, Muhammed M, Vilcinskas A. 2016. Diversity, evolution and medical applications of insect antimicrobial peptides. Philosophical transactions of the Royal Society of London Series B, Biological sciences 371:169–184. doi:10.1098/rstb.2015.0290

Nakamoto M, Moy RH, Xu J, Bambina S, Yasunaga A, Shelly SS, Gold B, Cherry S. 2012. Virus Recognition by Toll-7 Activates Antiviral Autophagy in Drosophila. Immunity 36:658–667. doi:10.1016/j.immuni.2012.03.003

Neyen C, Poidevin M, Roussel A, Lemaitre B. 2012. Tissue- and ligand-specific sensing of gram-negative infection in drosophila by PGRP-LC isoforms and PGRP-LE. J Immunol 189:1886–1897. doi:10.4049/jimmunol.1201022

O'Farrell F, Wang S, Katheder N, Rusten TE, Samakovlis C. 2013. Two-Tiered Control of Epithelial Growth and Autophagy by the Insulin Receptor and the Ret-Like Receptor, Stitcher. PLoS Biol 11:e1001612. doi:10.1371/journal.pbio.1001612

Palmer WH, Medd NC, Beard PM, Obbard DJ. 2018. Isolation of a natural DNA virus of Drosophila melanogaster, and characterisation of host resistance and immune responses. PLoS Pathog 14:e1007050. doi:10.1371/journal.ppat.1007050

Park JM, Brady H, Ruocco MG, Sun H, Williams DA, Lee SJ, Kato T, Richards N, Chan K, Mercurio F, Karin M, Wasserman SA. 2004. Targeting of TAK1 by the NF-κB protein Relish regulates the JNK-mediated immune response in Drosophila. Genes and Development 18:584–594. doi:10.1101/gad.1168104

Peng S, Yang M, Sun RN, Liu Y, Wang W, Xi Q, Gong H, Chen C. 2018. Mechanism of actions of Oncocin, a proline-rich antimicrobial peptide, in early elongation revealed by single-molecule FRET. doi:10.1007/s13238-017-0495-2

Pili-Floury S, Leulier F, Takahashi K, Saigo K, Samain E, Ueda R, Lemaitre B. 2004. In Vivo RNA Interference Analysis Reveals an Unexpected Role for GNBP1 in the Defense against Gram-positive Bacterial Infection in Drosophila Adults. Journal of Biological Chemistry 279:12848–12853. doi:10.1074/jbc.M313324200

Quesada H, Ramos-Onsins SE, Aguad?? M. 2005. Birth-and-death evolution of the Cecropin multigene family in Drosophila. Journal of Molecular Evolution 60:1–11. doi:10.1007/s00239-004-0053-4

Rabel D, Charlet M, Ehret-Sabatier L, Cavicchioli L, Cudic M, Otvos L, Bulet P. 2004. Primary Structure and in Vitro Antibacterial Properties of the Drosophila melanogaster Attacin C Pro-domain. Journal of Biological Chemistry 279:14853–14859. doi:10.1074/jbc.M313608200

Rahnamaeian M, Cytryńska M, Zdybicka-Barabas A, Vilcinskas A. 2016. The functional interaction between abaecin and pore-forming peptides indicates a general mechanism of antibacterial potentiation. Peptides 78:17–23. doi:10.1016/j.peptides.2016.01.016

Reichhart JM, Meister M, Dimarcq JL, Zachary D, Hoffmann D, Ruiz C, Richards G, Hoffmann JA. 1992. Insect immunity: developmental and inducible activity of the Drosophila diptericin promoter. EMBO J 11:1469–1477.

Rizki TM, Rizki RM. 1992. Lamellocyte differentiation in Drosophila larvae parasitized by Leptopilina. Developmental & Comparative Immunology 16:103–110. doi:10.1016/0145-305X(92)90011-Z

Rolff J, Schmid-Hempel P. 2016. Perspectives on the evolutionary ecology of arthropod antimicrobial peptides. Philosophical Transactions of the Royal Society B: Biological Sciences 371. doi:10.1098/rstb.2015.0297

Roote J, Prokop A. 2013. How to Design a Genetic Mating Scheme: A Basic Training Package for Drosophila Genetics. G3: Genes|Genomes|Genetics 3:353–358. doi:10.1534/g3.112.004820

Rutschmann S, Hoebe K. 2008. Dissecting innate immunity by germline mutagenesis. Immunology 123:459–468. doi:10.1111/j.1365-2567.2007.02786.x

Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, Drummond J, Webster J, Gubb D, Gunton N, Johnson G, O'Kane CJ, Huen D, Sharma P, Asztalos Z, Baisch H, Schulze J, Kube M, Kittlaus K, Reuter G, Maroy P, Szidonya J, Rasmuson-Lestander Å, Ekström K, Dickson B, Hugentobler C, Stocker H, Hafen E, Lepesant JA, Pflugfelder G, Heisenberg M, Mechler B, Serras F, Corominas M, Schneuwly S, Preat T, Roote J, Russell S. 2004. The DrosDel collection: A set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster. Genetics 167:797–813. doi:10.1534/genetics.104.026658

Sanchez Bosch P, Makhijani K, Herboso L, Gold KS, Baginsky R, Woodcock KJ, Alexander B, Kukar K, Corcoran S, Jacobs T, Ouyang D, Wong C, Ramond EJV, Rhiner C, Moreno E, Lemaitre B, Geissmann F, Brückner K. 2019. Adult Drosophila Lack Hematopoiesis but Rely on a Blood Cell Reservoir at the Respiratory Epithelia to Relay Infection Signals to Surrounding Tissues. Developmental Cell 51:1–17. doi:https://doi.org/10.1016/j.devcel.2019.10.017

Schlamp F, Delbare SYN, Early AM, Wells MT, Basu S, Clark AG. 2021. Dense time-course gene expression profiling of the Drosophila melanogaster innate immune response. BMC Genomics 22:304. doi:10.1186/s12864-021-07593-3

Selsted ME, Novotny MJ, Morris WL, Tang YQ, Smith W, Cullor JS. 1992. Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. J Biol Chem 267:4292–4295.

Selsted ME, Szklarek D, Lehrer RI. 1984. Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes. Infection and Immunity 45:150–154.

Shelly S, Lukinova N, Bambina S, Berman A, Cherry S. 2009. Autophagy Is an Essential Component of Drosophila Immunity against Vesicular Stomatitis Virus. Immunity 30:588–598. doi:10.1016/j.immuni.2009.02.009

Simon A, Kullberg BJ, Tripet B, Boerman OC, Zeeuwen P, Van Der Ven-Jongekrijg J, Verweij P, Schalkwijk J, Hodges R, Van Der Meer JWM, Netea MG. 2008. Drosomycin-like defensin, a human homologue of Drosophila melanogaster drosomycin with antifungal activity. Antimicrobial Agents and Chemotherapy 52:1407–1412. doi:10.1128/AAC.00155-07

Slavik KM, Morehouse BR, Ragucci AE, Zhou W, Ai X, Chen Y, Li L, Wei Z, Bähre H, König M, Seifert R, Lee ASY, Cai H, Imler J-L, Kranzusch PJ. 2021. cGAS-like receptors sense RNA and control 3'2'-cGAMP signalling in Drosophila. Nature 597:109–113. doi:10.1038/s41586-021-03743-5

Steiner H, Hultmark D, Engström \AA, Bennich H, Boman HG. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292:246–248. doi:10.1038/292246a0

Tanji T, Yun E-Y, Ip YT. 2010. Heterodimers of NF-κB transcription factors DIF and Relish regulate antimicrobial peptide genes in Drosophila. Proceedings of the National Academy of Sciences 107:14715–14720. doi:10.1073/pnas.1009473107

Tattikota SG, Cho B, Liu Y, Hu Y, Barrera V, Steinbaugh MJ, Yoon S-H, Comjean A, Li F, Dervis F, Hung R-J, Nam J-W, Ho Sui S, Shim J, Perrimon N. 2020. A single-cell survey of Drosophila blood. eLife 9:e54818. doi:10.7554/eLife.54818

Tennessen JA, Blouin MS. 2008. Balancing selection at a frog antimicrobial peptide locus: Fluctuating immune effector alleles? Molecular Biology and Evolution 25:2669–2680. doi:10.1093/molbev/msn208

Tettweiler G. 2005. Starvation and oxidative stress resistance in Drosophila are mediated through the eIF4E-binding protein, d4E-BP. Genes & Development 19:1840–1843. doi:10.1101/gad.1311805

Toda H, Williams JA, Gulledge M. 2019. A sleep-inducing gene, nemuri , links sleep and immune function in Drosophila 515:509–515.

Troha K, Im JH, Revah J, Lazzaro BP, Buchon N. 2018. Comparative transcriptomics reveals CrebA as a novel regulator of infection tolerance in D. melanogaster. PLoS Pathog 14:e1006847. doi:10.1371/journal.ppat.1006847

Tzou P, Ohresser S, Ferrandon D, Capovilla M, Reichhart JM, Lemaitre B, Hoffmann JA, Imler JL. 2000. Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity 13:737–748. doi:10.1016/S1074-7613(00)00072-8

Tzou P, Reichhart J-M, Lemaitre B. 2002. Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants. Proceedings of the National Academy of Sciences of the United States of America 99:2152–2157. doi:10.1073/pnas.042411999

Unckless RL, Howick VM, Lazzaro BP. 2016. Convergent Balancing Selection on an Antimicrobial Peptide in Drosophila. Current Biology 26:257–262. doi:10.1016/j.cub.2015.11.063

Unckless RL, Lazzaro BP. 2016. The potential for adaptive maintenance of diversity in insect antimicrobial peptides. Philosophical Transactions of the Royal Society B: Biological Sciences 371:20150291. doi:10.1098/rstb.2015.0291

Unckless RL, Rottschaefer SM, Lazzaro BP. 2015. The Complex Contributions of Genetics and Nutrition to Immunity in Drosophila melanogaster. PLoS Genetics 11. doi:10.1371/journal.pgen.1005030

Uttenweiler-Joseph S, Moniatte M, Lagueux M, Van Dorsselaer a, Hoffmann J a, Bulet P. 1998. Differential display of peptides induced during the immune response of Drosophila: a matrix-assisted laser desorption ionization time-of-flight mass spectrometry study. Proceedings of the National Academy of Sciences of the United States of America 95:11342–11347. doi:10.1073/pnas.95.19.11342

Valanne S, Kleino A, Myllymäki H, Vuoristo J, Rämet M. 2007. Iap2 is required for a sustained response in the Drosophila Imd pathway. Dev Comp Immunol 31:991–1001. doi:10.1016/j.dci.2007.01.004

Vanha-Aho L-M, Kleino A, Kaustio M, Ulvila J, Wilke B, Hultmark D, Valanne S, Rämet M. 2012. Functional characterization of the infection-inducible peptide Edin in Drosophila melanogaster. PLoS One 7:e37153. doi:10.1371/journal.pone.0037153

Vaz F, Kounatidis I, Covas G, Parton RM, Harkiolaki M, Davis I, Filipe SR, Ligoxygakis P. 2019. Accessibility to Peptidoglycan Is Important for the Recognition of Gram-Positive Bacteria in Drosophila. Cell Reports 27:2480-2492.e6. doi:10.1016/j.celrep.2019.04.103

Verleyen P, Baggerman G, D'Hertog W, Vierstraete E, Husson SJ, Schoofs L. 2006. Identification of new immune induced molecules in the haemolymph of Drosophila melanogaster by 2D-nanoLC MS/MS. Journal of Insect Physiology 52:379–388. doi:10.1016/j.jinsphys.2005.12.007

Volchenkov R, Sprater F, Vogelsang P, Appel S. 2012. The 2011 Nobel Prize in physiology or medicine. Scandinavian Journal of Immunology 75:1–4. doi:10.1111/j.1365-3083.2011.02663.x

Wang S, Tsarouhas V, Xylourgidis N, Sabri N, Tiklová K, Nautiyal N, Gallio M, Samakovlis C. 2009. The tyrosine kinase Stitcher activates Grainy head and epidermal wound healing in Drosophila. Nat Cell Biol 11:890–895. doi:10.1038/ncb1898

Woodcock KJ, Kierdorf K, Pouchelon CA, Vivancos V, Dionne MS, Geissmann F. 2015. Macrophage-Derived upd3 Cytokine Causes Impaired Glucose Homeostasis and Reduced Lifespan in Drosophila Fed a Lipid-Rich Diet. Immunity 42:133–144. doi:10.1016/j.immuni.2014.12.023

Yan H, Hancock REW. 2001. Synergistic interactions between mammalian antimicrobial defense peptides. Antimicrobial Agents and Chemotherapy 45:1558–1560. doi:10.1128/AAC.45.5.1558-1560.2001

Yu G, Baeder DY, Regoes RR, Rolff J. 2016. The More The Better? Combination Effects of Antimicrobial Peptides. Antimicrobial Agents and Chemotherapy AAC.02434-15. doi:10.1128/AAC.02434-15

Zahn M, Straeter N. 2013. Crystal structure of the substrate binding domain of E.coli DnaK in complex with the C-terminal part of drosocin (residues 12 to 19). doi:10.2210/pdb4EZR/pdb

Zanetti M, Gennaro R, Romeo D. 1995. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett 374:1–5. doi:10.1016/0014-5793(95)01050-o

Zasloff M. 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci U S A 84:5449–5453. doi:10.1073/pnas.84.15.5449

Zeidler MP, Bach EA, Perrimon N. 2000. The roles of the Drosophila JAK/STAT pathway. Oncogene 19:2598–2606. doi:10.1038/sj.onc.1203482

1.8 Figures chapter 1

1.8.1 Main figures

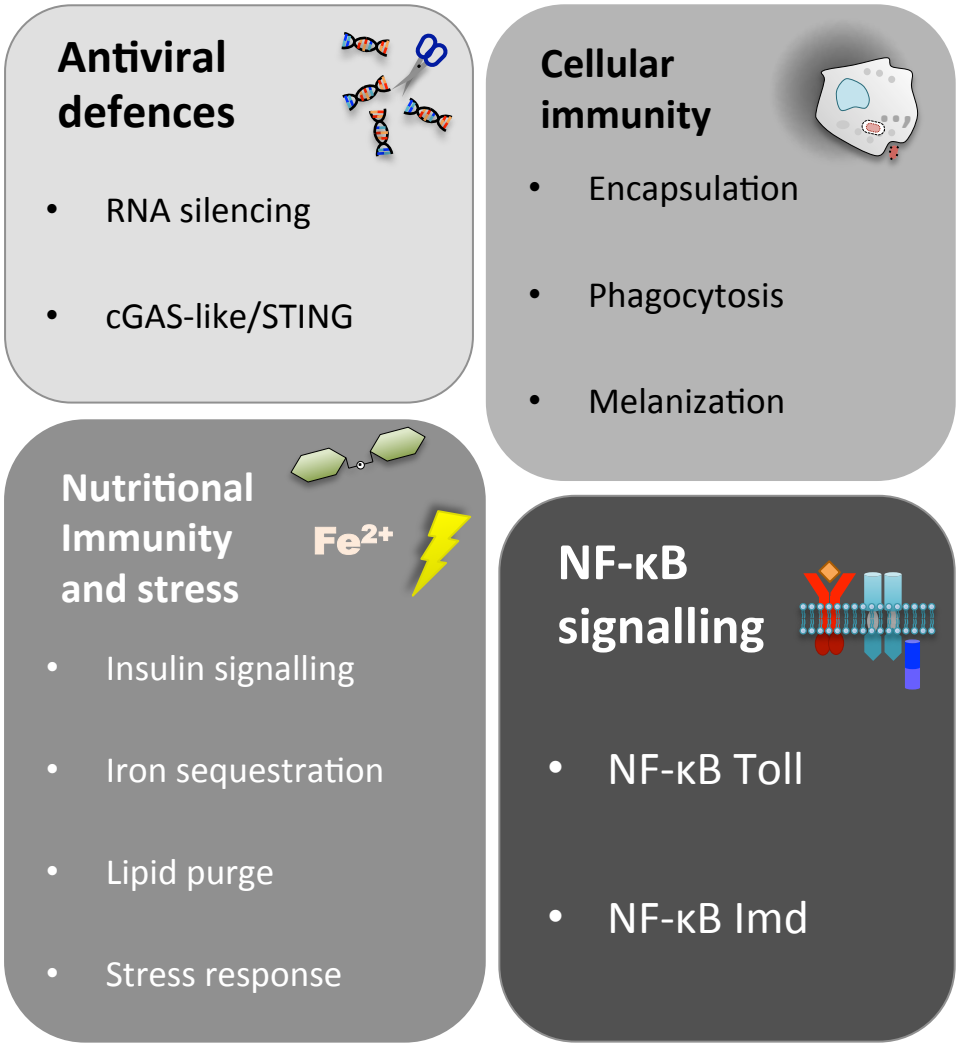

Figure 1.1: major categories of innate immune mechanisms.

Antiviral defences are intrinsic cellular defences. Cellular immunity refers to the systemic immune response coordinated by *Drosophila* blood cells (hemocytes). Nutritional immunity and stress responses involve changes to the blood (hemolymph) compartment that do not directly kill invading pathogens, but limit pathogen growth and prepare the host for the metabolic requirements of mounting the systemic immune response. NF-κB signalling is one of the most significant contributors to the systemic immune response, regulating hundreds of genes specifically induced upon infection that directly combat invading microbes.

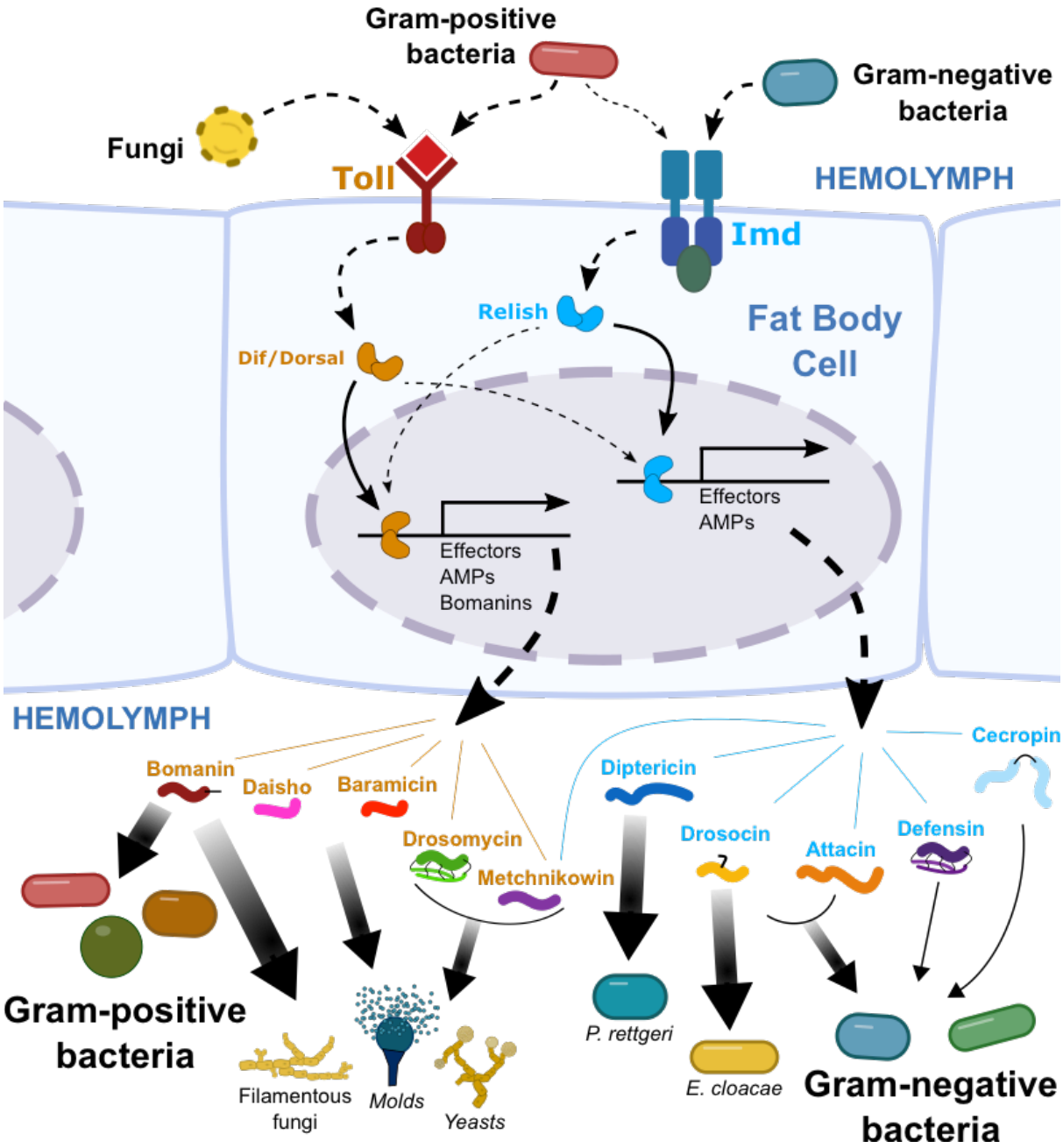

Figure 1.2: the Toll and Imd NF-κB signalling pathways regulate specific subsets of AMP genes that confer defence against microbes that principally trigger those pathways.

Bomanins are thus far the only major AMP-like effector family that has a robust effect on survival after Gram-positive bacterial infection. Two recently-described AMP/AMP-like families now called Daisho and Baramicin mediate defence against certain fungi, alongside the contributions of Drosomycin and Metchnikowin. Metchnikowin in particular also receives an important input from Imd signalling after systemic infection by Gram-negative bacteria. The Diptericin and Drosocin gene families have been shown to individually contribute to defence against *Providencia rettgeri* and *Enterobacter cloacae* respectively, and also contribute alongside the Attacin, Defensin, and Cecropin gene families in defence against Gram-negative bacteria more generally.

Chapter 2 Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach

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2.1 Abstract

Antimicrobial peptides (AMPs) are host-encoded antibiotics that combat invading microorganisms. These short, cationic peptides have been implicated in many biological processes, primarily involving innate immunity. In vitro studies have shown AMPs kill bacteria and fungi at physiological concentrations, but little validation has been done in vivo. We utilized CRISPR gene editing to delete most known immune-inducible AMPs of *Drosophila,* namely: 4 Attacins, 2 Diptericins, Drosocin, Drosomycin, Metchnikowin and Defensin. Using individual and multiple knockouts, including flies lacking all 10 of these AMP genes, we characterize the in vivo function of individual and groups of AMPs against diverse bacterial and fungal pathogens. We found that *Drosophila* AMPs act primarily against Gram-negative bacteria and fungi, contributing either additively or synergistically. We also describe remarkable specificity wherein certain AMPs contribute the bulk of microbicidal activity against specific pathogens, providing functional demonstrations of highly specific AMP-pathogen interactions in an in vivo setting.

2.2 Introduction

While innate immune mechanisms were neglected during the decades where adaptive immunity captured most of the attention, they have become central to our understanding of immunology. Recent emphasis on innate immunity has, however, mostly focused on the first two phases of the immune response: microbial recognition and associated downstream signaling pathways. In contrast, how innate immune effectors individually or collectively contribute to host resistance has not been investigated to the same extent. The existence of multiple effectors that redundantly contribute to host resistance has hampered their functional characterization by genetic approaches (Lemaitre and Hoffmann, 2007). The single mutation methodology that still prevails today has obvious limits in the study of immune effectors, which often belong to large gene families. As such, our current understanding of the logic underlying the roles of immune effectors is only poorly defined. As a consequence, the key parameters that influence host survival associated with a successful immune response are not well characterized. In this paper, we harnessed the power of the CRISPR gene editing approach to study the function of *Drosophila* antimicrobial peptides in host defence both individually and collectively.

Antimicrobial peptides (AMPs) are small, cationic, usually amphipathic peptides that contribute to innate immune defence in plants and animals (Guaní-Guerra et al., 2010; Imler and Bulet, 2005; Rolff and Schmid-Hempel, 2016). They display potent antimicrobial activity *in vitro* by disrupting negatively-charged microbial membranes, but AMPs can also target specific microbial processes (Kragol et al., 2001; Park et al., 1998; Rahnamaeian et al., 2015). Their expression is induced to very high levels upon challenge to provide microbicidal concentrations in the μM range. Numerous studies have revealed unique roles that AMPs may play in host physiology including anti-tumour activity (Kuroda et al., 2015; Suttmann et al., 2008), inflammation in aging (Cao et al., 2013; Kounatidis et al., 2017; Lezi et al., 2018a), involvement in memory (Barajas-azpeleta et al., 2018a; Bozler et al., 2017), mammalian immune signaling (Tjabringa et al., 2003; Van Wetering et al., 2002), wound-healing (Chung et al., 2017; Tokumaru et al., 2005), regulation of the host microbiota (Login et al., 2011a; Mergaert et al., 2017a), tolerance to oxidative stress (Zhao et al., 2011; Zheng et al., 2007), and of course microbicidal activity (Imler and Bulet, 2005b; Lemaitre and Hoffmann, 2007b; Wimley, 2010). The fact that AMP genes are immune inducible and expressed at high levels has led to the common assumption they play a vital role in the innate immune response (Duneau et al., 2017). However, little is known in most cases about how AMPs individually or collectively contribute to animal host defence. *In vivo* functional analysis of AMPs has been hampered by the sheer number and small size of these genes, making them difficult to mutate with traditional genetic tools (but *e.g.* see (Hoeckendorf et al., 2012; Nakatsuji et al., 2016)).

Since the first animal AMPs were discovered in silk moths (Steiner et al., 1981a), insects and particularly *Drosophila melanogaster* have emerged as a powerful model for characterizing their function. There are currently seven known families of inducible AMPs in *D. melanogaster*. Their activities have been determined either *in vitro* by using peptides directly purified from flies or produced in heterologous systems, or deduced by comparison with homologous

peptides isolated in other insect species: Drosomycin and Metchnikowin show antifungal activity (Fehlbaum et al., 1994b; Levashina et al., 1995b); Cecropins (four inducible genes) and Defensin have both antibacterial and some antifungal activities (Cociancich et al., 1993; Ekengren and Hultmark, 1999b; Hultmark et al., 1980; Tzou et al., 2002a); and Drosocin, Attacins (four genes) and Diptericins (two genes) primarily exhibit antibacterial activity (Åsling et al., 1995; Bulet et al., 1996b; Cudic et al., 1999b; Hedengren et al., 2000b; Kragol et al., 2001c). In *Drosophila*, these AMPs are produced either locally at various surface epithelia in contact with environmental microbes (Bischoff et al., 2006; Gendrin et al., 2009; Zaidman-Rémy et al., 2006), or secreted systemically into the hemolymph, the insect blood. During systemic infection, these 14 antimicrobial peptides are strongly induced in the fat body, an organ analogous to the mammalian liver.

The systemic production of AMPs is regulated at the transcriptional level by two NF-κB pathways, the Toll and Imd pathways, which are activated by different classes of microbes. The Toll pathway is predominantly responsive to Gram-positive bacteria and fungi, and accordingly plays a major role in defence against these microbes. In contrast, the Imd pathway is activated by Gram-negative bacteria and a subset of Gram-positive bacteria with DAP-type peptidoglycan, and mutations affecting this pathway cause profound susceptibility to Gram-negative bacteria (De Gregorio et al., 2002a; Lemaitre et al., 1997a). However, the expression pattern of AMP genes is complex as each gene is expressed with different kinetics and can often receive transcriptional input from both pathways (De Gregorio et al., 2002a; Leulier et al., 2000). This ranges from *Diptericin*, which is tightly regulated by the Imd pathway, to *Drosomycin*, whose expression is mostly regulated by the Toll pathway (Lemaitre et al., 1997a), except at surface epithelia where *Drosomycin* is under the control of Imd signaling (Ferrandon et al., 1998b). While a critical role of AMPs in *Drosophila* host defence is supported by transgenic flies overexpressing a single AMP (Tzou et al., 2002a), the specific contributions of each of these AMPs has not been tested. Indeed loss-of-function mutants for most AMP genes were not previously available due to their small size, making them difficult to mutate before the advent of CRISPR/Cas9 technology. Despite this, the great susceptibility to infection of mutants with defective Toll and Imd pathways is commonly attributed to the loss of the AMPs they regulate, though these pathways control hundreds of genes awaiting characterization (De Gregorio et al., 2002a). Strikingly, Clemmons *et al*. (Clemmons et al., 2015b) recently reported that flies lacking a set of uncharacterized Toll-responsive peptides (named Bomanins) succumb to infection by Gram-positive bacteria and fungi at rates similar to *Toll*-deficient mutants (Clemmons et al., 2015b). This provocatively suggests that Bomanins, and not AMPs, might be the predominant effectors downstream of the Toll pathway; yet synthesized Bomanins do not display antimicrobial activity *in vitro* (Lindsay et al., 2018b). Thus, while today the fly represents one of the best-characterized animal immune systems, the contribution of AMPs as immune effectors is poorly defined as we still do not understand why Toll and Imd pathway mutants succumb to infection.

In this paper, we took advantage of recent gene editing technologies to delete most of the known immune inducible AMP genes of *Drosophila*. Using single and multiple knockouts, as well as a variety of bacterial and fungal pathogens, we have characterized the *in vivo* function of individual and groups of antimicrobial peptides. We reveal that AMPs can play highly spe-

cific roles in defence, being vital for surviving certain infections yet dispensable against others. We highlight key interactions amongst immune effectors and pathogens and reveal to what extent these defence peptides act in concert or alone.

2.3 Results

Generation and characterization of AMP mutants

We generated null mutants for 10 of the 14 known *Drosophila* antimicrobial peptide genes that are induced upon systemic infection. These include five single gene mutations affecting *Defensin* (*Def*^{SK3}), *Attacin C* (*AttC*^{Mi}), *Metchnikowin* (*Mtk*^{R1}), *Attacin D* (*AttD*^{SK1}) and *Drosomycin* (*Drs*^{R1}), respectively, and two small deletions removing both *Diptericins DptA* and *DptB* (*Dpt*^{SK1}), or the gene cluster containing *Drosocin,* and *Attacins AttA* and *AttB* (*Dro-AttAB*^{SK2}). The function of *Cecropins* was not assessed in this manuscript. All mutations/deletions were made using the CRISPR editing approach with the exception of *Attacin C*, which was disrupted by insertion of a *Minos* transposable element (Bellen et al., 2011b), and the *Drosomycin* and *Metchnikowin* deletions generated by homologous recombination (Fig. 1A and Fig. S1). To disentangle the role of *Drosocin* and *AttA/AttB* in the *Dro-AttAB*^{SK2} deletion, we also generated an individual *Drosocin* mutant (*Dro*^{SK4}); for complete information, see Figure S1. We then isogenized these mutations for at least seven generations into the *w*^{1118} DrosDel isogenic genetic background (Ryder et al., 2004a) (*iso w*^{1118}). Then, we recombined these seven independent mutations into a background lacking these 10 inducible AMPs referred to as "*ΔAMPs*." *ΔAMPs* flies were viable and showed no morphological defects. To confirm the absence of AMPs in our *ΔAMPs* background, we performed a MALDI-TOF analysis of hemolymph from both unchallenged and immune-challenged flies infected by a mixture of *Escherichia coli* and *Micrococcus luteus*. This analysis revealed the presence of peaks induced upon challenge corresponding to AMPs in wild-type but not *ΔAMPs* flies. Importantly it also confirmed that induction of most other immune-induced molecules (IMs)(Uttenweiler-Joseph et al., 1998b), was unaffected in *ΔAMPs* flies (Fig. 1B). Of note, we failed to observe two IMs, IM7 and IM21, in our *ΔAMPs* flies, suggesting that these unknown peptides are secondary products of AMP genes. We further confirmed that Toll and Imd NF-κB signaling pathways were intact in *ΔAMPs* flies by measuring the expression of target genes of these pathways (Fig. 1C-D). This demonstrates that *Drosophila* AMPs are not signaling molecules required for Toll or Imd pathway activity. We also assessed the role of AMPs in the melanization response, wound clotting, and hemocyte populations. After clean injury, *ΔAMPs* flies survive as wild-type (Fig. 1 supplement A). We found no defect in melanization (χ^2, p = .34, Fig. 1 supplement B) as both adults and larvae strongly melanize the cuticle following clean injury, (Fig. 1 supplement C). Furthermore, we visualized the formation of clot fibers *ex vivo* using the hanging drop assay and PNA staining (Scherfer et al., 2004) in hemolymph of both wild-type and *ΔAMPs* larvae (Fig. 1 supplement D). Hemocyte counting (*i.e.* crystal cells, FACS) did not reveal any deficiency in hemocyte populations of *ΔAMPs* larvae (Fig. 1 supplement E, F, and not shown). Altogether, our study suggests that *Drosophila* AMPs are primarily immune effectors, and not regulators of innate immunity.

AMPs are essential for combating Gram-negative bacterial infection

We used these *ΔAMPs* flies to explore the role that AMPs play in defence against pathogens during systemic infection. We first focused our attention on Gram-negative bacterial infections, which are combatted by Imd pathway-mediated defence in *Drosophila* (Lemaitre and Hoffmann, 2007b). We challenged wild-type and *ΔAMPs* flies with six different Gram-negative bacterial species, using inoculation doses (given as OD600) selected such that at least some wild-type flies were killed. In our survival experiments, we also include Oregon R (*OR-R*) as an alternate wild-type for comparison, and *Relish* mutants *(Rel^{E20})* that lack a functional Imd response and are known to be very susceptible to this class of bacteria (Hedengren et al., 1999) (Fig. 2). Globally, *ΔAMPs* flies were extremely susceptible to all Gram-negative pathogens tested (Fig. 2, light blue plots). The susceptibility of AMP-deficient flies to Gram-negative bacteria largely mirrored that of Rel^{E20} flies. For all Gram-negative infections tested, *ΔAMPs* flies show a higher bacterial count at 18 hours post-infection (hpi) indicating that AMPs actively inhibit bacterial growth, as expected of 'antimicrobial peptides' (Fig. 2 supplement A). Use of GFP-expressing bacteria show that bacterial growth in *ΔAMPs* flies radiates from the wound site until spreading systemically (Fig. 2 supplement B,C). Collectively, the use of AMP-deficient flies reveals that AMPs are major players in resistance to Gram-negative bacteria, and likely constitute an essential component of the Imd pathway's contribution for survival against these germs.

Bomanins and to a lesser extent AMPs contribute to resistance against Gram-positive bacteria and fungi

Previous studies have shown that resistance to Gram-positive bacteria and fungi in *Drosophila* is mostly mediated by the Toll pathway, although the Imd pathway also contributes to some extent (Lemaitre et al., 1997a; Leulier et al., 2000; Rutschmann et al., 2000; Tanji et al., 2007). Moreover, a deletion removing ten uncharacterized Bomanins ($Bom^{\Delta 55C}$) induces a strong susceptibility to both Gram-positive bacteria and fungi (Clemmons et al., 2015b), suggesting that Bomanins are major players downstream of Toll in the defence against these germs. This prompted us to explore the role of antimicrobial peptides in defence against Gram-positive bacteria and fungi. We additionally included *spätzle* mutant flies (spz^{rm7}) lacking Toll signaling as susceptible controls. We first challenged wild-type and *ΔAMPs* flies with two lysine-type (*E. faecalis, S. aureus*) and two DAP-type (*B. subtilis, L. innocua*) peptidoglycan-containing Gram-positive bacterial species. We observed that *ΔAMPs* flies display only weak or no increased susceptibility to infection with these Gram-positive bacterial species, as *ΔAMPs* survival rates were closer to the wild-type than to spz^{rm7} mutants lacking a functional Toll pathway (Fig. 2, orange plots), with the exception of *S. aureus*. Meanwhile, $Bom^{\Delta 55C}$ mutants consistently phenocopied spz^{rm7} flies, confirming the important contribution of these peptides in defence against Gram-positive bacteria (Clemmons et al., 2015b).

Next, we monitored the survival of *ΔAMPs* to the yeast *Candida albicans*, the opportunistic fungus *Aspergillus fumigatus* and two entomopathogenic fungi, *Beauveria bassiana,* and *Metarhizium anisopliae.* For the latter two, we used a natural mode of infection by spreading spores on the cuticle (Lemaitre et al., 1997a). *ΔAMPs* flies were more susceptible to fungal

infections with *B. bassiana, A. fumigatus,* and *C. albicans*, but not *M. anisopliae* (Fig. 2, yellow plots). In all instances, $Bom^{\Delta 55C}$ mutants were as or more susceptible to fungal infection than *ΔAMPs* flies, approaching *Toll*-deficient mutant levels. Collectively, our data demonstrate that AMPs are major immune effectors in defence against Gram-negative bacteria and have a less essential role in defence against bacteria and fungi.

A combinatory approach to explore AMP interactions

The impact of the *ΔAMPs* deletion on survival could be due to the action of certain AMPs having a specific effect, or more likely due to the combinatory action of co-expressed AMPs. Indeed, cooperation of AMPs to potentiate their microbicidal activity has been suggested by numerous *in vitro* approaches (Mohan et al., 2014; Rahnamaeian et al., 2015c; Yu et al., 2016b), but rarely in an *in vivo* context (Zanchi et al., 2017). Having shown that AMPs as a whole significantly contribute to fly defence, we next explored the contribution of individual peptides to this effect. To tackle this question in a systematic manner, we performed survival analyses using fly lines lacking one or several AMPs, focusing on pathogens with a range of virulence that we previously showed to be sensitive to the action of AMPs. This includes the yeast *C. albicans* and the Gram-negative bacterial species *P. burhodogranariea, P. rettgeri, Ecc15,* and *E. cloacae*. Given seven independent AMP mutations, over 100 combinations of mutants are possible, making a systematic analysis of AMP interactions a logistical nightmare. Therefore, we designed an approach that would allow us to characterize their contributions to defence by deleting groups of AMPs. To this end, we generated three groups of combined mutants: A) flies lacking *Defensin* (Group A); *Defensin* is regulated by Imd signalling but is primarily active against Gram-positive bacteria in vitro (Imler and Bulet, 2005). B) Flies lacking three antibacterial and structurally related AMP families: the Proline-rich *Drosocin* and the Proline- and Glycine-rich *Diptericins* and *Attacins* (Group B, regulated by the Imd pathway). C) Flies lacking the two antifungal peptide genes *Metchnikowin* and *Drosomycin* (Group C, mostly regulated by the Toll pathway). We then combined these three groups to generate flies lacking AMPs from groups A and B (AB), A and C (AC), or B and C (BC). Finally, flies lacking all three groups are our *ΔAMPs* flies, which are highly susceptible to a number of infections. By screening these seven genotypes as well as individual mutants, we were able to assess potential interactions between AMPs of different groups, as well as decipher the function of individual AMPs.

Drosomycin and Metchnikowin additively contribute to defence against the yeast C. albicans

We first applied this AMP-groups approach to infections with the relatively avirulent yeast *C. albicans*. Previous studies have shown that Toll, but not Imd, contributes to defence against this fungus (Glittenberg et al., 2011; Gottar et al., 2006). Thus, we suspected that the two antifungal peptides, Drosomycin and Metchnikowin, could play a significant role in the susceptibility of *ΔAMPs* flies to this yeast. Consistent with this, Group C flies lacking *Metchnikowin* and *Drosomycin* were more susceptible to infection ($p < .001$ relative to *iso* w^{1118}) with a survival rate similar to *ΔAMPs* flies (Fig. 3A). Curiously, AC-deficient flies that also lack *Defensin* survived better than Group C-deficient flies (Log-Rank p=0.014). We have no

explanation for this interaction, but this could be due to i) a better canalization of the immune response by preventing the induction of ineffective AMPs, ii) complex biochemical interactions amongst the AMPs involved affecting either the host or pathogen, or iii) differences in genetic background generated by additional recombination. We then investigated the individual contributions of *Metchnikowin* and *Drosomycin* to survival to *C. albicans*. We found that both Mtk^{R1} and Drs^{R1} individual mutants were somewhat susceptible to infection, but notably only *Mtk; Drs* compound mutants reached *ΔAMPs* levels of susceptibility (Fig. 3B). This co-occurring loss of resistance appears to be primarily additive (Mutant, Cox Hazard Ratio (HR), p-value: Mtk^{R1}*, HR = +1.17, p = .008;* Drs^{R1}*, HR = +1.85, p < .001; Mtk*Drs, HR = -0.80, p =.116*). We observed that Group C deficient flies eventually succumb to uncontrolled *C. albicans* growth by monitoring yeast titre, indicating that these AMPs indeed act by suppressing yeast growth (Fig. 3C)**.**

In conclusion, our study provides an *in vivo* validation of the potent antifungal activities of Metchnikowin and Drosomycin (Fehlbaum et al., 1994b; Levashina et al., 1995b), and highlights a clear example of additive cooperation of AMPs.

AMPs synergistically contribute to defence against P. burhodogranariea

We next analyzed the contribution of AMPs in resistance to infection with the moderately virulent Gram-negative bacterium *P. burhodogranariea*. We found that Group B mutants lacking *Drosocin*, the two *Diptericins*, and the four *Attacins*, were as susceptible to infection as *ΔAMPs* flies (Fig. 4A), while flies lacking the antifungal peptides Drosomycin and Metchnikowin (Toll-regulated, Group C) resisted the infection as wild-type. Flies lacking *Defensin* (Group A) showed an intermediate susceptibility, but behave as wild-type in the additional absence of Toll Group C peptides (Group AC). Thus, we again observed a better survival rate with the co-occurring loss of Group A and C peptides (see possible explanation above). In this case, Group A flies were susceptible while AC flies were not.

Following the observation that Group B flies were as susceptible as *ΔAMPs* flies, we sought to better decipher the contribution of each Group B AMP to resistance to *P. burhodogranariea*. We observed that mutants for *Drosocin* alone (Dro^{SK4}), or the *DiptericinA/B* deficiency were not susceptible to this bacterium (Fig. 4B). We additionally saw no marked susceptibility of *Drosocin-Attacin A/B* deficient flies, nor *Attacin C* or *Attacin D* mutants (not shown). Interestingly, we found that compound mutants lacking *Drosocin* and *Attacins A, B, C,* and *D* (Fig. 4B: '*ΔDro, ΔAtt*'), or *Drosocin* and *Diptericins DptA* and *DptB* ('*ΔDro, ΔDpt*') displayed an intermediate susceptibility. Only the Group B mutants lacking *Drosocin*, all *Attacins*, and both *Diptericins* (*ΔDro, ΔAtt, ΔDpt)* phenocopied *ΔAMPs* flies (Fig. 4B), with synergistic statistical interactions observed upon co-occurring loss of *Attacins* and *Diptericins* (*ΔAtt*ΔDpt*: HR = +1.45, p < .001). By 6hpi, bacterial titres of individual flies already showed significant differences in the most susceptible genotypes (Fig. 4C), though these differences were reduced by 18hpi likely owing to the high chronic load *P. burhodogranariea* establishes in surviving flies (Duneau et al., 2017); also see Fig. 2 supplement A.

Collectively, the use of various compound mutants reveals that several Imd-responsive AMPs,

notably Drosocin, Attacins, and Diptericins, jointly contribute to defence against *P. burhodogranariea* infection. A strong susceptibility of Group B flies was also observed upon infection with *Ecc15*, another Gram-negative bacterium commonly used to infect flies (Neyen et al., 2014) (Fig. 4 supplement B).

Diptericins alone contribute to defence against *P. rettgeri*

We continued our exploration of AMP interactions using our AMP groups approach with the fairly virulent *P. rettgeri* (strain Dmel), a strain isolated from wild-caught *Drosophila* hemolymph (Juneja and Lazzaro, 2009a). We were especially interested by this bacterium as previous studies (Unckless et al., 2016b, 2015b) have shown a correlation between susceptibility to *P. rettgeri* and a polymorphism in the *Diptericin A* gene pointing to a specific AMP-pathogen interaction. Use of compound mutants revealed only loss of Group B AMPs was needed to reach the susceptibility of *ΔAMPs* and Rel^{E20} flies (Fig. 5A). Use of individual mutant lines however revealed a pattern overtly different from that *P. burhodogranariea,* as the sole *Diptericin A/B* deficiency caused susceptibility similar to Group B, *ΔAMPs,* and Rel^{E20} flies (Fig. 5B,C). We further confirmed this susceptibility using a *DptA RNAi* construct (Fig. 5 supplement A, B). Moreover, flies carrying the Dpt^{SK1} mutation over a deficiency (*Df(2R)Exel6067*) were also highly susceptible to *P. rettgeri* (Fig. 5D). Interestingly, flies that were heterozygotes for Dpt^{SK1} or the *Df(2R)Exel6067* that have only one copy of the two *Diptericins* were markedly susceptible to infection with *P. rettgeri* (Fig. 5D). This indicates that a full transcriptional output of *Diptericin* is required over the course of the infection to resist *P. rettgeri* infection (Fig. 5E). Altogether, our results suggest that only the *Diptericin* gene family, amongst the many AMPs regulated by the Imd pathway, provides the full AMP-based contribution to defence against this bacterium. To test this hypothesis, we generated a fly line lacking all the AMPs except *DptA* and *DptB* ($\Delta AMPs^{+Dpt}$). Strikingly, $\Delta AMPs^{+Dpt}$ flies have the same survival rate as wild-type flies, further emphasizing the specificity of this interaction (Fig. 5B). Bacterial counts confirm that the susceptibility of these *Diptericin* mutants arises from an inability of the host to suppress bacterial growth (Fig. 5C).

Collectively, our study shows that *Diptericins* are critical to resist *P. rettgeri*, while they play an important but less essential role in defence against *P. burhodogranariea* infection. We were curious whether *Diptericin*'s major contribution to defence observed with *P. rettgeri* could be generalized to other members of the genus *Providencia*. An exclusive role for *Diptericins* was also found for the more virulent *P. stuartii* (Fig. 5 supplement C), but not for other *Providencia* species tested (*P. burhodogranariea, P. alcalifaciens, P. sneebia, P. vermicola*) (data not shown).

Drosocin is critical to resist infection with *E. cloacae*

In the course of our exploration of AMP-pathogen interactions, we identified another highly specific interaction between *E. cloacae* and Drosocin. Use of compound mutants revealed that alone, Group B flies were already susceptible to *E. cloacae*. Meanwhile, Group AB flies additionally lacking *Defensin* reached *ΔAMPs* levels of susceptibility, while Group A and Group C flies resisted as wild-type (Figure 6A). The high susceptibility of Group AB flies results from a synergistic statistical interaction amongst Group A (Defensin) and Group B peptides in defence against *E. cloacae* (*A*B*, HR =+2.55, p=0.003).

We chose to further explore the AMPs deleted in Group B flies, as alone this genotype already displayed a strong susceptibility. Use of individual mutant lines revealed that mutants for *Drosocin* alone (Dro^{SK4}) or the *Drosocin-Attacin A/B* deficiency ($Dro\text{-}AttAB^{SK2}$), but not *AttC, AttD, nor* Dpt^{SK1} (not shown), recapitulate the susceptibility observed in Group B flies (Fig. 6B). At 18hpi, both Dro^{SK4} and *ΔAMPs* flies had significantly higher bacterial loads compared to wild-type flies, while Rel^{E20} mutants were already moribund with much higher bacterial loads (Fig. 6C). Indeed, the deletion of *Drosocin* alone drastically alters the fly's ability to control the otherwise avirulent *E. cloacae* upon inoculations using OD=200 (~39,000 bacteria, Fig. 6A-C) or even OD=10 (~7,000 bacteria, Fig. 6 supplement A).

We confirmed the high susceptibility of *Drosocin* mutant flies to *E. cloacae* in various contexts: transheterozygote flies carrying Dro^{SK4} over a *Drosocin* deficiency *(Df(2R)BSC858)* that also lacks flanking genes including *AttA* and *AttB* ((Fig. 6D), the Dro^{SK4} mutations in an alternate genetic background (*yw*, Fig. 6E), and, *Drosocin RNAi* (Fig. 6 supplement B,C). Thus, we recovered two highly specific AMP-pathogen interactions: Diptericins are essential to combat *P. rettgeri* infection, while *Drosocin* is paramount to surviving *E. cloacae* infection.

2.4 Discussion

A combinatory approach to study AMPs

Despite the recent emphasis on innate immunity, little is known on how immune effectors contribute individually or collectively to host defence, exemplified by the lack of in depth *in vivo* functional characterization of *Drosophila* AMPs. Taking advantage of new gene editing approaches, we developed a systematic mutation approach to study the function of *Drosophila* AMPs. With seven distinct mutations, we were able to generate a fly line lacking 10 AMPs that are known to be strongly induced during the systemic immune response. A striking first finding is that *ΔAMPs* flies were perfectly healthy and have an otherwise wild-type immune response. This indicates that in contrast to mammals (Van Wetering et al., 2002), *Drosophila* AMPs are not likely to function as signaling molecules. While AMPs are expressed in various tissues, our systemic infection model bypasses epithelial defences and focuses on the systemic immune response. Using this infection model, we found that most flies lacking a single AMP family exhibited a higher susceptibility to certain pathogens consistent with their *in vitro* activity and a previous study constitutively expressing individual peptides (Tzou et al., 2002a). We found activity of Diptericins against *P. rettgeri*, Drosocin against *E. cloacae*, Drosomycin and Metchnikowin against *C. albicans*, and Defensin against *P. burhodogranariea*. (Fig. 4 supplement A). In most cases, the susceptibility of single mutants was slight, and the contribution of individual AMPs could be revealed only when combined to other AMP mutations as illustrated by the susceptibility of *Drosocin, Attacin,* and *Diptericin* combined mutants to *P. burhodogranariea*. Thus, the use of compound rather than single mutations provides a better strategy to decipher the contribution of AMPs to host defence.

AMPs and Bomanins are essential contributors to Toll and Imd pathway mediated host defence

The Toll and Imd pathways provide a paradigm of innate immunity, illustrating how two distinct pathways link pathogen recognition to distinct but overlapping sets of downstream immune effectors (Buchon et al., 2014; Lemaitre and Hoffmann, 2007b). However, a method of deciphering the contributions of the different downstream effectors to the specificity of these pathways remained out of reach, as mutations in these immune effectors were lacking. Our study shows that AMPs contribute greatly to resistance to Gram-negative bacteria. Consistent with this, *ΔAMPs* flies are almost as susceptible as Imd-deficient mutants to most Gram-negative bacteria. In contrast, flies lacking AMPs were only slightly more susceptible to Gram-positive bacteria and fungal infections compared to wild-type flies, and this susceptibility rarely approached the susceptibility of *Bomanin* mutants. It is possible that additional loss of *Cecropins* would further increase the sensitivity of *ΔAMPs* flies to bacteria or fungi. This may be due to the cell walls of Gram-negative bacteria being thinner and more fluid than the rigid cell walls of Gram-positive bacteria (Fayaz et al., 2010), consequently making Gram-negative bacteria more prone to the action of pore-forming cationic peptides. It would be interesting to know if the specificity of AMPs to primarily combatting Gram-negative bacteria is also true in other species.

Based on our study and Clemmons et al. (Clemmons et al., 2015b), we can now explain the susceptibility of Toll and Imd mutants at the level of the effectors, as we show that mutations affecting Imd-pathway responsive antibacterial peptide genes are highly susceptible to Gram-negative bacteria while the Toll-responsive targets Drosomycin, Metchnikowin, and especially the Bomanins, confer resistance to fungi and Gram-positive bacteria. Thus, the susceptibility of these two pathways to different sets of microbes not only reflects specificity at the level of recognition, but can now also be translated to the activities of downstream effectors. It remains to be seen how Bomanins contribute to the microbicidal activity of immune-induced hemolymph, as attempts to synthesize Bomanins have not revealed direct antimicrobial activity (Lindsay et al., 2018b). It should also be noted that many putative effectors downstream of Toll and Imd remain uncharacterized, and so could also contribute to host defence beyond AMPs and Bomanins.

AMPs act additively and synergistically to suppress bacterial growth in vivo

In the last few years, numerous *in vitro* studies have focused on the potential for synergistic interactions of AMPs in microbial killing (Chen et al., 2005; Nuding et al., 2014; Rahnamaeian et al., 2015c; Stewart et al., 2014; Yan and Hancock, 2001; Yu et al., 2016b; Zanchi et al., 2017; Zdybicka-Barabas et al., 2012a; Zerweck et al., 2017). Our collection of AMP mutant fly lines placed us in an ideal position to investigate AMP interactions in an *in vivo* setting. While Toll-responsive AMPs (Group C: Metchnikowin, Drosomycin) additively contributed to defence against the yeast *C. albicans*, we found that certain combinations of AMPs have synergistic contributions to defence against *P. burhodogranariea*. Synergistic loss of resistance may arise in two general fashions: first, co-operation of AMPs using similar mechanisms of action may breach a threshold microbicidal activity whereupon pathogens are no longer able to resist. This may be the case for the synergistic effect of Diptericins and Attacins against *P. burhodogranariea*, as only co-occurring loss of both these related glycine-rich peptide families (Hedengren et al., 2000b) led to complete loss of resistance. Alternatively, synergy may arise

due to complementary mechanisms of action, whereupon one AMP potentiates the other AMP's ability to act. For instance, the action of the bumblebee AMP Abaecin, which binds to the molecular chaperone DnaK to inhibit bacterial DNA replication, is potentiated by the presence of the pore-forming peptide Hymenoptaecin (Rahnamaeian et al., 2016b). *Drosophila* Drosocin is highly similar to Abaecin and the related peptide Apidecin, including O-glycosylation of a critical threonine residue (Hanson et al., 2016; Imler and Bulet, 2005), and thus likely acts in a similar fashion. Furthermore, *Drosophila* Attacin C is maturated into both a glycine-rich peptide and a Drosocin-like peptide called MPAC (Rabel et al., 2004b). As such, co-occuring loss of Drosocin, MPAC, and other possible MPAC-like peptides encoded by the Attacin/Diptericin superfamily may be responsible for the synergistic loss of resistance in *Drosocin, Attacin, Diptericin* combined mutants.

AMPs can act with great specificity against certain pathogens

It is commonly thought that the innate immune response lacks the specificity of the adaptive immune system, which mounts directed defences against specific pathogens. Accordingly for innate immunity, the diversity of immune-inducible AMPs can be justified by the need for generalist and/or co-operative mechanisms of microbial killing. However, an alternate explanation may be that innate immunity expresses diverse AMPs in an attempt to hit the pathogen with a "silver bullet:" an AMP specifically attuned to defend against that pathogen. Here, we provide a demonstration in an *in vivo* setting that such a strategy may actually be employed by the innate immune system. Remarkably we recovered not just one, but two examples of exquisite specificity in our laborious but relatively limited assays.

Diptericin has previously been highlighted for its important role in defence against *P. rettgeri* (Unckless et al., 2016b), but it was previously unknown whether other AMPs may confer defence in this infection model. Astoundingly, flies mutant for the other inducible AMPs resisted *P. rettgeri* infection as wild-type, while only *Diptericin* mutants succumbed to infection. This means that Diptericins do not co-operate with other AMPs in defence against *P. rettgeri*, and are solely responsible for defence in this specific host-pathogen interaction. Moreover, $+/Dpt^{SK1}$ heterozygote flies were nonetheless extremely susceptible to infection, demonstrating that a full transcriptional output over the course of infection is required to effectively prevent pathogen growth. A previous study has shown that ~7hpi appears to be the critical time point at which *P. rettgeri* either grows unimpeded or the infection is controlled (Duneau et al., 2017). This time point correlates with the time at which the *Diptericin* transcriptional output is in full-force (Lemaitre et al., 1997a). Thus, a lag in the transcriptional response in $Dpt^{SK1}/+$ flies likely prevents the host from reaching a competent Diptericin concentration, indicating that *Diptericin* expression level is a key factor in successful host defence.

We also show that *Drosocin* is specifically required for defence against *E. cloacae*. This striking finding validates previous biochemical analyses showing Drosocin *in vitro* activity against several Enterobacteriaceae, including *E. cloacae* (Bulet et al., 1996b). As *ΔAMPs* flies are more susceptible than *Drosocin* single mutants, other AMPs also contribute to Drosocin-mediated control of *E. cloacae*. As highlighted above, Drosocin is similar to other Proline-rich AMPs (*e.g.* Abaecin, Pyrrhocoricin) that have been shown to target bacterial DnaK (Kragol et al., 2001b;

Rahnamaeian et al., 2015c). Alone, these peptides still penetrate bacteria cell walls through their uptake by bacterial permeases (Narayanan et al., 2014; Rahnamaeian et al., 2016b). Thus, while Drosocin would benefit from the presence of pore-forming toxins to enter bacterial cells (Rahnamaeian et al., 2016b), the veritable "stake to the heart" is likely the plunging of Drosocin itself into vital bacterial machinery.

On the role of AMPs in host defence

It has often been questioned why flies should need so many AMPs (Lemaitre and Hoffmann, 2007; Rolff and Schmid-Hempel, 2016; Unckless and Lazzaro, 2016). A common idea, supported by *in vitro* experiments (Rahnamaeian et al., 2015c; Yan and Hancock, 2001; Zdybicka-Barabas et al., 2012a), is that AMPs work as cocktails, wherein multiple effectors are needed to kill invading pathogens. However, we find support for an alternative hypothesis that suggests AMP diversity may be due to highly specific interactions between AMPs and subsets of pathogens that they target. Burgeoning support for this idea also comes from recent evolutionary studies that show *Drosophila* and vertebrate AMPs experience positive selection (Chapman et al., 2018; Halldórsdóttir and Árnason, 2015; Hanson et al., 2016; Hellgren and Sheldon, 2011; Sackton, 2018; Tennessen and Blouin, 2008; Unckless et al., 2016; Unckless and Lazzaro, 2016), a hallmark of host-pathogen evolutionary conflict. Our functional demonstrations of AMP-pathogen specificity, using naturally relevant pathogens (Cox and Gilmore, 2007; Juneja and Lazzaro, 2009a), suggest that such specificity is fairly common, and that certain AMPs can act as the arbiters of life or death upon infection by certain pathogens. This stands in contrast to the classical view that the AMP response contains such redundancy that single peptides should have little effect on organism-level immunity (Rolff and Schmid-Hempel, 2016; Tzou et al., 2000; Unckless et al., 2015; Unckless and Lazzaro, 2016). Nevertheless, it seems these immune effectors play non-redundant roles in defence.

By providing a long-awaited *in vivo* functional validation for the role of AMPs in host defence, we also pave the way for a better understanding of the functions of immune effectors. Future studies may investigate the role of AMPs in epithelial immunity, notably in the gut, genitalia, and malpighian tubules. Our approach of using multiple compound mutants, now possible with the development of new genome editing approaches, was especially effective to decipher the logic of immune effectors. Understanding the role of AMPs in innate immunity holds great promise for the development of novel antibiotics (Chung et al., 2017; Mahlapuu et al., 2016; Mylonakis et al., 2016b), insight into autoimmune diseases (Gilliet and Lande, 2008; Kumar et al., 2016; Schluesener et al., 1993; Sun et al., 2015), and given their potential for remarkably specific interactions, perhaps in predicting key parameters that predispose individuals or populations to certain kinds of infections (Chapman et al., 2018; Unckless et al., 2015; Unckless and Lazzaro, 2016). Finally, our set of isogenized *AMP* mutant lines provides long-awaited tools to decipher the role of AMPs not only in immunity, but also in the various roles that AMPs may play in aging, neurodegeneration, anti-tumour activity, regulation of the microbiota and more, where disparate evidence has pointed to their involvement.

2.5 Materials and Methods

Drosophila genetics and mutant generation

The DrosDel(Ryder et al., 2004a) isogenic w^{1118} (*iso* w^{1118}) wild type was used as a genetic background for mutant isogenization. Alternate wild-types used throughout include Oregon R (*OR-R*), w^{1118} from the Vienna Drosophila Resource Centre, and the Canton-S isogenic line Exelexis w^{1118}, which was kindly provided by Brian McCabe. $Bom^{\Delta 55C}$ mutants were generously provided by Steven Wasserman, and $Bom^{\Delta 55C}$ was isogenized into the *iso* w^{1118} background. Rel^{E20} and spz^{rm7} *iso* w^{1118} flies were provided by Luis Teixeira(Ferreira et al., 2014a; Hedengren et al., 1999). Prophenoloxidase mutants (*ΔPPO*) are described in Dudzic et al.(Dudzic et al., 2015b). P-element mediated homologous recombination according to Baena-Lopez et al.(Baena-Lopez et al., 2013) was used to generate mutants for *Mtk* (Mtk^{R1}) and *Drs* (Drs^{R1}). Plasmids were provided by Mickael Poidevin. *Attacin C* mutants ($AttC^{Mi}$, #25598), the *Diptericin* deficiency (*Df(2R)Exel6067,* #7549), the *Drosocin* deficiency (*Df(2R)BSC858*, #27928), *UAS-Diptericin RNAi* (Dpt^{RNAi}, #53923), *UAS-Drosocin RNAi* (Dro^{RNAi}, #67223), and *Actin5C-Gal4* (*ActGal4*, #4414) were ordered from the Bloomington stock centre (stock #s included). CRISPR mutations were performed by Shu Kondo according to Kondo and Ueda(Kondo and Ueda, 2013), and full descriptions are given in Figure S1. In brief, flies deficient for *Drosocin, Attacin A,*and *Attacin B (Dro-*$AttAB^{SK2}$*),* and *Diptericin A* and *Diptericin B (*Dpt^{SK1}*)* were produced by gene region deletion specific to those AMPs without affecting other genes. Single mutants for *Defensin* (Def^{SK3}*), Drosocin (*Dro^{SK4}*),* and *Attacin D (*$AttD^{SK1}$*)* are small indels resulting in the production of short (80-107 residues) nonsense peptides. Mutations were isogenized for a minimum of seven generations into the *iso* w^{1118} background prior to subsequent recombination. It should be noted that Group A flies were initially thought to be a double mutant for both *Defensin* and the *Cecropin* cluster, resulting from a combination of Def^{SK3} and a CRISPR-induced *Cecropin* deletion (called Cec^{SK6}). It was subsequently shown that Cec^{SK6} is a complex aberration at the *Cecropin* locus that retains a wild-type copy of the *Cecropin* cluster. This re-arranged *Cecropin* locus does not contribute significantly to the susceptibility of Group A flies, as Group A was not different from Def^{SK3} alone (Log-Rank p=0.818; Figure 4—figure supplement 1A). Thus, group A flies were considered as single Def^{SK3}mutants.

Microbial culture conditions

Bacteria were grown overnight on a shaking plate at 200rpm in their respective growth media and temperature conditions, and then pelleted by centrifugation at 4°C. These bacterial pellets were diluted to the desired optical density at 600nm (OD) as indicated. The following bacteria were grown at 37°C in LB media: *Escherichia coli strain 1106, Salmonella typhimurium, Enterobacter cloacae β12, Providencia rettgeri strain Dmel, Providencia burhodogranariea strain B, Providencia stuartii strain DSM 4539, Providencia sneebia strain Dmel, Providencia alcalifaciens strain Dmel, Providencia vermicola strain DSM 17385, Bacillus subtilis,* and *Staphylococcus aureus. Erwinia carotovora carotovora (Ecc15)* and *Micrococcus luteus* were grown overnight in LB at 29°C. *Enterococcus faecalis* and *Listeria innocua* were cultured in BHI medium at 37°C.

Candida albicans was cultured in YPG medium at 37°C. *Aspergillus fumigatus* was grown at room temperature on Malt Agar, and spores were collected in sterile PBS rinses, pelleted by centrifugation, and then resuspended to the desired OD in PBS. The entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* were grown on Malt Agar at room temperature until sporulation.

Systemic infections and survival

Systemic infections were performed by pricking 3-5 day old adult males in the thorax with a 100 μm thick insect pin dipped into a concentrated pellet of bacteria or fungal spores. Infected flies were subsequently maintained at 25°C for experiments. For infections with *B. bassiana* and *M. anisopliae*, flies were anaesthetized and then shaken on a sporulating plate of fungi for 30s. At least two replicate survival experiments were performed for each infection, with 20-35 flies per vial on standard fly medium without yeast. Survivals were scored twice daily, with additional scoring at sensitive time points. Comparisons of *iso* w^{1118} wild-type to *ΔAMPs* mutants were made using a Cox-proportional hazard (CoxPH) model, where independent experiments were included as covariates, and covariates were removed if not significant ($p > .05$). Direct comparisons were performed using Log-Rank tests in Prism 7 software. The effect size and direction is included as the CoxPH hazard ratio (HR) where relevant, with a positive effect indicating increased susceptibility. CoxPH models were used to test for synergistic contributions of AMPs to survival in R 3.4.4. Total sample size (N) is given for each experiment as indicated.

Quantification of microbial load

The native *Drosophila* microbiota does not readily grow overnight on LB, allowing for a simple assay to estimate bacterial load. Flies were infected with bacteria at the indicated OD as described, and allowed to recover. At the indicated time post-infection, flies were anaesthetized using CO_2 and surface sterilized by washing them in 70% ethanol. Ethanol was removed, and then flies were homogenized using a Precellys™ bead beater at 6500rpm for 30 seconds in LB broth, with 300ul for individual samples, or 500uL for pools of 5-7 flies. These homogenates were serially diluted and 150uL was plated on LB agar. Bacterial plates were incubated overnight, and colony-forming units (CFUs) were counted manually. Statistical analyses were performed using One-way ANOVA with Sidak's correction. P-values are reported as < 0.05 = *, < 0.01 = **, and < 0.001 = ***. For *C. albicans*, BiGGY agar was used instead to select for *Candida* colonies from fly homogenates.

Gene expression by qPCR

Flies were infected by pricking flies with a needle dipped in a pellet of either *E. coli* or *M. luteus* (OD600 = 200), and frozen at -20°C 6h and 24h post-infection respectively. Total RNA was then extracted from pooled samples of five flies each using TRIzol reagent, and re-suspended in MilliQ dH_2O. Reverse transcription was performed using 0.5 micrograms total RNA in 10 μl reactions using PrimeScript RT (TAKARA) with random hexamer and oligo dT primers. Quan-

titative PCR was performed on a LightCycler 480 (Roche) in 96-well plates using Applied Biosystems™ SYBR™ Select Master Mix. Values represent the mean from three replicate experiments. Error bars represent one standard deviation from the mean. Primers used in this study can be found in Table S1. Statistical analyses were performed using one-way ANOVA with Tukey post-hoc comparisons. P-values are reported as not significant = ns, < 0.05 = *, < 0.01 = **, and < 0.001 = ***. qPCR primers and sources(Mark A Hanson et al., 2016; Iatsenko et al., 2016; Kounatidis et al., 2017) are included in Table S1.

MALDI-TOF peptide analysis

Two methods were used to collect hemolymph from adult flies: in the first method, pools of five adult females were pricked twice in the thorax and once in the abdomen. Wounded flies were then spun down with 15µL of 0.1% trifluoroacetic acid (TFA) at 21000 RCF at 4°C in a mini-column fitted with a 10µm pore to prevent contamination by circulating hemocytes. These samples were frozen at -20°C until analysis, and three biological replicates were performed with 4 technical replicates. In the second method, approximately 20nL of fresh hemolymph was extracted from individual adult males using a Nanoject, and immediately added to 1µL of 1% TFA, and the matrix was added after drying. Peptide expression was visualized as described in Üttenweiller-Joseph et al.(Uttenweiler-Joseph et al., 1998b). Both methods produced similar results, and representative expression profiles are given.

Melanization and hemocyte characterization, image acquisition

Melanization assays(Dudzic et al., 2018) and peanut agglutinin (PNA) clot staining(Scherfer et al., 2004) was performed as previously described. In brief, flies or L3 larvae were pricked, and the level of melanization was assessed at the wound site. We used FACS sorting to count circulating hemocytes. For sessile crystal cell visualization, L3 larvae were cooked in dH_2O at 70°C for 20 minutes, and crystal cells were visualized on a Leica DFC300FX camera using Leica Application Suite and counted manually.

2.6 Author contributions and acknowledgements

Author contributions:

MAH, AD and BL designed the study. MAH and AD performed DrosDel isogenization and recombination. MP and SK supplied critical reagents. MAH performed the experiments, and CC provided experimental support. MAH and BL analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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2.7 References

Literature cited:

Åsling B, Dushay MS, Hultmark D. 1995. Identification of early genes in the Drosophila immune response by PCR-based differential display: the Attacin A gene and the evolution of attacin-like proteins. Insect Biochem Mol Biol 25:511–518. doi:10.1016/0965-1748(94)00091-C

Baena-Lopez LA, Alexandre C, Mitchell A, Pasakarnis L, Vincent J-P. 2013. Accelerated homologous recombination and subsequent genome modification in Drosophila. Development 140:4818–4825. doi:10.1242/dev.100933

Barajas-azpeleta R, Wu J, Gill J, Welte R. 2018. Antimicrobial peptides modulate long-term memory. PLoS Genet 1–26. doi:10.1371/journal.pgen.1007440

Bellen HJ, Levis RW, He Y, Carlson JW, Evans-Holm M, Bae E, Kim J, Metaxakis A, Savakis C, Schulze KL, Hoskins RA, Spradling AC. 2011. The Drosophila gene disruption project: Progress using transposons with distinctive site specificities. Genetics 188:731–743. doi:10.1534/genetics.111.126995

Bischoff V, Vignal C, Duvic B, Boneca IG, Hoffmann JA, Royet J. 2006. Downregulation of the Drosophila immune response by peptidoglycan- recognition proteins SC1 and SC2. PLoS Pathog 2:0139–0147. doi:10.1371/journal.ppat.0020014

Bozler J, Kacsoh BZ, Chen H, Theurkauf WE, Weng Z, Bosco G. 2017. A systems level approach to temporal expression dynamics in Drosophila reveals clusters of long term memory genes. PLoS Genet 13. doi:10.1371/journal.pgen.1007054

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

Bulet P, Urge L, Ohresser S, Hetru C, Otvos L. 1996. Enlarged scale chemical synthesis and range of activity of drosocin, an O-glycosylated antibacterial peptide of Drosophila. Eur J Biochem 238:64–69. doi:10.1111/j.1432-1033.1996.0064q.x

Cao Y, Chtarbanova S, Petersen AJ, Ganetzky B. 2013. Dnr1 mutations cause neurodegeneration in Drosophila by activating the innate immune response in the brain. Proc Natl Acad Sci 110:E1752–E1760. doi:10.1073/pnas.1306220110
Chapman JR, Hill T, Unckless RL. 2019. Balancing selection drives maintenance of genetic variation in Drosophila antimicrobial peptides. Genome Biol Evol 11:2691–2701. doi:https://doi.org/10.1093/gbe/evz191
Chen X, Niyonsaba F, Ushio H, Okuda D, Nagaoka I, Ikeda S, Okumura K, Ogawa H. 2005. Synergistic effect of antibacterial agents human β-defensins, cathelicidin LL-37 and lysozyme against Staphylococcus aureus and Escherichia coli. J Dermatol Sci 40:123–132. doi:10.1016/j.jdermsci.2005.03.014
Chung EMC, Dean SN, Propst CN, Bishop BM, Van Hoek ML. 2017. Komodo dragon-inspired synthetic peptide DRGN-1 promotes wound-healing of a mixed-biofilm infected wound. npj Biofilms Microbiomes 3. doi:10.1038/s41522-017-0017-2
Clemmons AW, Lindsay SA, Wasserman SA. 2015. An Effector Peptide Family Required for Drosophila Toll-Mediated Immunity. PLoS Pathog 11. doi:10.1371/journal.ppat.1004876
Cociancich S, Ghazi A, Hetru C, Hoffmann JA, Letellier L. 1993. Insect defensin, an inducible antibacterial peptide, forms voltage- dependent channels in Micrococcus luteus. J Biol Chem 268:19239–19245.
Cox CR, Gilmore MS. 2007. Native microbial colonization of Drosophila melanogaster and its use as a model of Enterococcus faecalis pathogenesis. Infect Immun 75:1565–1576. doi:10.1128/IAI.01496-06
Cudic M, Bulet P, Hoffmann R, Craik DJ, Otvos L. 1999. Chemical synthesis, antibacterial activity and conformation of diptericin, an 82-mer peptide originally isolated from insects. Eur J Biochem 266:549–558. doi:10.1046/j.1432-1327.1999.00894.x
De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. 2002. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. EMBO J 21:2568–2579. doi:10.1093/emboj/21.11.2568
Dudzic JP, Hanson MA, Iatsenko I, Kondo S, Lemaitre B. 2018. More than black and white: complex relationships involving serine proteases regulate the Toll pathway and the melanization response in Drosophila. bioRxiv. doi:https://doi.org/10.1101/383257
Dudzic JP, Kondo S, Ueda R, Bergman CM, Lemaitre B. 2015. Drosophila innate immunity: regional and functional specialization of prophenoloxidases. BMC Biol 13:81–97. doi:10.1186/s12915-015-0193-6
Duneau D, Ferdy JB, Revah J, Kondolf H, Ortiz GA, Lazzaro BP, Buchon N. 2017. Stochastic variation in the initial phase of bacterial infection predicts the probability of survival in D. melanogaster. Elife 6. doi:10.7554/eLife.28298
Ekengren S, Hultmark D. 1999. Drosophila cecropin as an antifungal agent. Insect Biochem Mol Biol 29:965–972. doi:10.1016/S0965-1748(99)00071-5
Fayaz AM, Balaji K, Girilal M, Yadav R, Kalaichelvan PT, Venketesan R. 2010. 15 Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. Nanomedicine 6:103–9. doi:10.1016/j.nano.2009.04.006
Fehlbaum P, Bulet P, Michaut L, Lagueux M, Broekaert WF, Hetru C, Hoffmann JA. 1994. Insect immunity: Septic injury of drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. J Biol Chem 269:33159–33163.
Ferrandon D, Jung AC, Criqui MC, Lemaitre B, Uttenweiler-Joseph S, Michaut L,

Reichhart JM, Hoffmann JA. 1998. A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. EMBO J 17:1217–1227. doi:10.1093/emboj/17.5.1217
Ferreira ÁG, Naylor H, Esteves SS, Pais IS, Martins NE, Teixeira L. 2014. The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral Infection in Drosophila. PLoS Pathog 10. doi:10.1371/journal.ppat.1004507
Gendrin M, Welchman DP, Poidevin M, Hervé M, Lemaitre B. 2009. Long-range activation of systemic immunity through peptidoglycan diffusion in Drosophila. PLoS Pathog 5. doi:10.1371/journal.ppat.1000694
Gilliet M, Lande R. 2008. Antimicrobial peptides and self-DNA in autoimmune skin inflammation. Curr Opin Immunol. doi:10.1016/j.coi.2008.06.008
Glittenberg MT, Silas, SukritGlittenberg, M. T., Silas, S., MacCallum, D. M., Gow, N. a R., & Ligoxygakis P. 2011. Wild-type Drosophila melanogaster as an alternative model system for investigating the pathogenicity of Candida albicans. Dis Model Mech 4:504–514. doi:10.1242/dmm.006619
Gottar M, Gobert V, Matskevich AA, Reichhart JM, Wang C, Butt TM, Belvin M, Hoffmann JA, Ferrandon D. 2006. Dual Detection of Fungal Infections in Drosophila via Recognition of Glucans and Sensing of Virulence Factors. Cell 127:1425–1437. doi:10.1016/j.cell.2006.10.046
Guaní-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM. 2010. Antimicrobial peptides: General overview and clinical implications in human health and disease. Clin Immunol 135:1–11. doi:10.1016/j.clim.2009.12.004
Halldórsdóttir K, Árnason E. 2015. Trans-species polymorphism at antimicrobial innate immunity cathelicidin genes of Atlantic cod and related species. PeerJ 3:e976. doi:10.7717/peerj.976
Hanson MA, Hamilton PT, Perlman SJ. 2016. Immune genes and divergent antimicrobial peptides in flies of the subgenus Drosophila. BMC Evol Biol 16:228. doi:10.1186/s12862-016-0805-y
Hedengren M, Åsling B, Dushay MS, Ando I, Ekengren S, Wihlborg M, Hultmark D. 1999. Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Mol Cell 4:827–837. doi:10.1016/S1097-2765(00)80392-5
Hedengren M, Borge K, Hultmark D. 2000. Expression and evolution of the Drosophila attacin/diptericin gene family. Biochem Biophys Res Commun 279:574–81. doi:10.1006/bbrc.2000.3988
Hellgren O, Sheldon BC. 2011. Locus-specific protocol for nine different innate immune genes (antimicrobial peptides: β-defensins) across passerine bird species reveals within-species coding variation and a case of trans-species polymorphisms. Mol Ecol Resour 11:686–692. doi:10.1111/j.1755-0998.2011.02995.x
Hoeckendorf A, Stanisak M, Leippe M. 2012. The saposin-like protein SPP-12 is an antimicrobial polypeptide in the pharyngeal neurons of Caenorhabditis elegans and participates in defence against a natural bacterial pathogen. Biochem J 445:205–212. doi:10.1042/BJ20112102
Hultmark D, Steiner H, Rasmuson T, Boman HG. 1980. Insect Immunity. Purification and Properties of Three Inducible Bactericidal Proteins from Hemolymph of Immunized Pupae of Hyalophora cecropia. Eur J Biochem 106:7–16. doi:10.1111/j.1432-1033.1980.tb05991.x
Iatsenko I, Kondo S, Mengin-Lecreulx D, Lemaitre B. 2016. PGRP-SD, an Extracellular Pattern-Recognition Receptor, Enhances Peptidoglycan-Mediated Activation of the Drosophila Imd Pathway. Immunity 45:1013–1023.

doi:10.1016/j.immuni.2016.10.029
Imler J-L, Bulet P. 2005. Antimicrobial peptides in Drosophila: structures, activities and gene regulation. Chem Immunol Allergy 86:1–21. doi:10.1159/000086648
Juneja P, Lazzaro BP. 2009. Providencia sneebia sp. nov. and Providencia burhodogranariea sp. nov., isolated from wild Drosophila melanogaster. Int J Syst Evol Microbiol 59:1108–1111. doi:10.1099/ijs.0.000117-0
Kondo S, Ueda R. 2013. Highly Improved gene targeting by germline-specific Cas9 expression in Drosophila. Genetics 195:715–721. doi:10.1534/genetics.113.156737
Kounatidis I, Chtarbanova S, Cao Y, Hayne M, Jayanth D, Ganetzky B, Ligoxygakis P. 2017. NF-κB Immunity in the Brain Determines Fly Lifespan in Healthy Aging and Age-Related Neurodegeneration. Cell Rep 19:836–848. doi:10.1016/j.celrep.2017.04.007
Kragol G, Lovas S, Varadi G, Condie BA, Hoffmann R, Otvos L. 2001. The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. Biochemistry 40:3016–3026. doi:10.1021/bi002656a
Kumar DKV, et al. … Wells CL. 2016. Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. Sci Transl Med 8:340ra72. doi:10.1126/scitranslmed.aaf1059
Kuroda K, Okumura K, Isogai H, Isogai E. 2015. The Human Cathelicidin Antimicrobial Peptide LL-37 and Mimics are Potential Anticancer Drugs. Front Oncol 5. doi:10.3389/fonc.2015.00144
Lemaitre B, Hoffmann J. 2007. The Host Defense of Drosophila Melanogaster. Annu Rev Immunol 25:697–743. doi:10.1146/annurev.immunol.25.022106.141615
Lemaitre B, Reichhart JM, Hoffmann JA. 1997. Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc Natl Acad Sci U S A 94:14614–9. doi:10.1073/pnas.94.26.14614
Leulier F, Rodriguez A, Khush RS, Abrams JM, Lemaitre B. 2000. The Drosophila caspase Dredd is required to resist Gram-negative bacterial infection. EMBO Rep 1:353–358. doi:10.1093/embo-reports/kvd073
Levashina EA, Ohresser S, Bulet P, Reichhart J -M, Hetru C, Hoffmann JA. 1995. Metchnikowin, a Novel Immune-Inducible Proline-Rich Peptide from Drosophila with Antibacterial and Antifungal Properties. Eur J Biochem 233:694–700. doi:10.1111/j.1432-1033.1995.694_2.x
Lezi E, Zhou T, Koh S, Chuang M, Sharma R, Pujol N, Chisholm AD, Eroglu C, Matsunami H, Yan D. 2018. An Antimicrobial Peptide and Its Neuronal Receptor Regulate Dendrite Degeneration in Aging and Infection. Neuron 97:125-138.e5. doi:10.1016/j.neuron.2017.12.001
Lindsay SA, Lin SJH, Wasserman SA. 2018. Short-Form Bomanins Mediate Humoral Immunity in Drosophila. doi:10.1159/000489831
Login FH, Balmand S, Vallier A, Vincent-Monégat C, Vigneron A, Weiss-Gayet M, Rochat D, Heddi A. 2011. Antimicrobial peptides keep insect endosymbionts under control. Science (80-) 334:362–365. doi:10.1126/science.1209728
Mahlapuu M, Håkansson J, Ringstad L, Björn C. 2016. Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. Front Cell Infect Microbiol 6. doi:10.3389/fcimb.2016.00194
Mergaert P, Kikuchi Y, Shigenobu S, Nowack ECM. 2017. Metabolic Integration of

Bacterial Endosymbionts through Antimicrobial Peptides. Trends Microbiol. doi:10.1016/j.tim.2017.04.007
Mohan KVK, Rao SS, Gao Y, Atreya CD. 2014. Enhanced antimicrobial activity of peptide-cocktails against common bacterial contaminants of ex vivo stored platelets. Clin Microbiol Infect 20. doi:10.1111/1469-0691.12326
Mylonakis E, Podsiadlowski L, Muhammed M, Vilcinskas A. 2016. Diversity, evolution and medical applications of insect antimicrobial peptides. Philos Trans R Soc Lond B Biol Sci 371:169–184. doi:10.1098/rstb.2015.0290
Nakatsuji T, Chen TH, Two AM, Chun KA, Narala S, Geha RS, Hata TR, Gallo RL. 2016. Staphylococcus aureus Exploits Epidermal Barrier Defects in Atopic Dermatitis to Trigger Cytokine Expression. J Invest Dermatol 136:2192–2200. doi:10.1016/j.jid.2016.05.127
Narayanan S, Modak JK, Ryan CS, Garcia-Bustos J, Davies JK, Roujeinikova A. 2014. Mechanism of escherichia coli resistance to pyrrhocoricin. Antimicrob Agents Chemother 58:2754–2762. doi:10.1128/AAC.02565-13
Neyen C, Bretscher AJ, Binggeli O, Lemaitre B. 2014. Methods to study Drosophila immunity. Methods 68:116–128. doi:10.1016/j.ymeth.2014.02.023
Nuding S, Frasch T, Schaller M, Stange EF, Zabel LT. 2014. Synergistic effects of antimicrobial peptides and antibiotics against clostridium difficile. Antimicrob Agents Chemother 58:5719–5725. doi:10.1128/AAC.02542-14
Park CB, Kim HS, Kim SC. 1998. Mechanism of action of the antimicrobial peptide buforin II: Buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem Biophys Res Commun 244:253–257. doi:10.1006/bbrc.1998.8159
Rabel D, Charlet M, Ehret-Sabatier L, Cavicchioli L, Cudic M, Otvos L, Bulet P. 2004. Primary Structure and in Vitro Antibacterial Properties of the Drosophila melanogaster Attacin C Pro-domain. J Biol Chem 279:14853–14859. doi:10.1074/jbc.M313608200
Rahnamaeian M, Cytry ska M, Zdybicka-Barabas A, Dobslaff K, Wiesner J, Twyman RM, Zuchner T, Sadd BM, Regoes RR, Schmid-Hempel P, Vilcinskas A. 2015. Insect antimicrobial peptides show potentiating functional interactions against Gram-negative bacteria. Proc R Soc B Biol Sci 282:20150293–20150293. doi:10.1098/rspb.2015.0293
Rahnamaeian M, Cytryńska M, Zdybicka-Barabas A, Vilcinskas A. 2016. The functional interaction between abaecin and pore-forming peptides indicates a general mechanism of antibacterial potentiation. Peptides 78:17–23. doi:10.1016/j.peptides.2016.01.016
Rolff J, Schmid-Hempel P. 2016. Perspectives on the evolutionary ecology of arthropod antimicrobial peptides. Philos Trans R Soc B Biol Sci 371. doi:10.1098/rstb.2015.0297
Rutschmann S, Jung AC, Hetru C, Reichhart JM, Hoffmann JA, Ferrandon D. 2000. The Rel protein DIF mediates the antifungal but not the antibacterial host defense in Drosophila. Immunity 12:569–580. doi:10.1016/S1074-7613(00)80208-3
Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, Drummond J, Webster J, Gubb D, Gunton N, Johnson G, O'Kane CJ, Huen D, Sharma P, Asztalos Z, Baisch H, Schulze J, Kube M, Kittlaus K, Reuter G, Maroy P, Szidonya J, Rasmuson-Lestander Å, Ekström K, Dickson B, Hugentobler C, Stocker H, Hafen E, Lepesant JA, Pflugfelder G, Heisenberg M, Mechler B, Serras F, Corominas M, Schneuwly S, Preat T, Roote J, Russell S. 2004. The DrosDel collection: A set of P-element insertions for generating custom chromosomal aberrations

in Drosophila melanogaster. Genetics 167:797–813. doi:10.1534/genetics.104.026658
Sackton TB. 2018. Comparative genomics and transcriptomics of host-pathogen interactions in insects: evolutionary insights and future directions. Curr Opin Insect Sci 31:106–113. doi:10.1016/J.COIS.2018.12.007
Scherfer C, Karlsson C, Loseva O, Bidla G, Goto A, Havemann J, Dushay MS, Theopold U. 2004. Isolation and characterization of hemolymph clotting factors in Drosophila melanogaster by a pullout method. Curr Biol 14:625–629. doi:10.1016/j.cub.2004.03.030
Schluesener HJ, Radermacher S, Melms A, Jung S. 1993. Leukocytic antimicrobial peptides kill autoimmune T cells. J Neuroimmunol 47:199–202. doi:10.1016/0165-5728(93)90030-3
Steiner H, Hultmark D, Engström Å, Bennich H, Boman HG. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292:246–248. doi:10.1038/292246a0
Stewart SE, Kondos SC, Matthews AY, D'Angelo ME, Dunstone MA, Whisstock JC, Trapani JA, Bird PI. 2014. The perforin pore facilitates the delivery of cationic cargos. J Biol Chem 289:9172–9181. doi:10.1074/jbc.M113.544890
Sun J, Furio L, Mecheri R, van der Does AM, Lundeberg E, Saveanu L, Chen Y, van Endert P, Agerberth B, Diana J. 2015. Pancreatic β-Cells Limit Autoimmune Diabetes via an Immunoregulatory Antimicrobial Peptide Expressed under the Influence of the Gut Microbiota. Immunity 43:304–317. doi:10.1016/j.immuni.2015.07.013
Suttmann H, Retz M, Paulsen F, Harder J, Zwergel U, Kamradt J, Wullich B, Unteregger G, Stockle M, Lehmann J. 2008. Antimicrobial peptides of the Cecropin-family show potent antitumor activity against bladder cancer cells. BMC Urol 8:5. doi:10.1186/1471-2490-8-5
Tanji T, Hu X, Weber ANR, Ip YT. 2007. Toll and IMD Pathways Synergistically Activate an Innate Immune Response in Drosophila melanogaster. Mol Cell Biol 27:4578–4588. doi:10.1128/MCB.01814-06
Tennessen JA, Blouin MS. 2008. Balancing selection at a frog antimicrobial peptide locus: Fluctuating immune effector alleles? Mol Biol Evol 25:2669–2680. doi:10.1093/molbev/msn208
Tjabringa GS, Aarbiou J, Ninaber DK, Drijfhout JW, Sorensen OE, Borregaard N, Rabe KF, Hiemstra PS. 2003. The Antimicrobial Peptide LL-37 Activates Innate Immunity at the Airway Epithelial Surface by Transactivation of the Epidermal Growth Factor Receptor. J Immunol 171:6690–6696. doi:10.4049/jimmunol.171.12.6690
Tokumaru S, Sayama K, Shirakata Y, Komatsuzawa H, Ouhara K, Hanakawa Y, Yahata Y, Dai X, Tohyama M, Nagai H, Yang L, Higashiyama S, Yoshimura A, Sugai M, Hashimoto K. 2005. Induction of Keratinocyte Migration via Transactivation of the Epidermal Growth Factor Receptor by the Antimicrobial Peptide LL-37. J Immunol 175:4662–4668. doi:10.4049/jimmunol.175.7.4662
Tzou P, Ohresser S, Ferrandon D, Capovilla M, Reichhart JM, Lemaitre B, Hoffmann JA, Imler JL. 2000. Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity 13:737–748. doi:10.1016/S1074-7613(00)00072-8
Tzou P, Reichhart J-M, Lemaitre B. 2002. Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants. Proc Natl Acad Sci U S A 99:2152–2157. doi:10.1073/pnas.042411999

Unckless RL, Howick VM, Lazzaro BP. 2016. Convergent Balancing Selection on an Antimicrobial Peptide in Drosophila. Curr Biol 26:257–262. doi:10.1016/j.cub.2015.11.063

Unckless RL, Lazzaro BP. 2016. The potential for adaptive maintenance of diversity in insect antimicrobial peptides. Philos Trans R Soc B Biol Sci 371:20150291. doi:10.1098/rstb.2015.0291

Unckless RL, Rottschaefer SM, Lazzaro BP. 2015. The Complex Contributions of Genetics and Nutrition to Immunity in Drosophila melanogaster. PLoS Genet 11. doi:10.1371/journal.pgen.1005030

Uttenweiler-Joseph S, Moniatte M, Lagueux M, Van Dorsselaer a, Hoffmann J a, Bulet P. 1998. Differential display of peptides induced during the immune response of Drosophila: a matrix-assisted laser desorption ionization time-of-flight mass spectrometry study. Proc Natl Acad Sci U S A 95:11342–11347. doi:10.1073/pnas.95.19.11342

Van Wetering S, Mannesse-Lazeroms SPG, Van Sterkenburg MAJA, Hiemstra PS. 2002. Neutrophil defensins stimulate the release of cytokines by airway epithelial cells: Modulation by dexamethasoneInflammation Research. pp. 8–15. doi:10.1007/PL00000282

Wimley WC. 2010. Describing the mechanism of antimicrobial peptide action with the interfacial activity model. ACS Chem Biol. doi:10.1021/cb1001558

Yan H, Hancock REW. 2001. Synergistic interactions between mammalian antimicrobial defense peptides. Antimicrob Agents Chemother 45:1558–1560. doi:10.1128/AAC.45.5.1558-1560.2001

Yu G, Baeder DY, Regoes RR, Rolff J. 2016. The More The Better? Combination Effects of Antimicrobial Peptides. Antimicrob Agents Chemother AAC.02434-15. doi:10.1128/AAC.02434-15

Zaidman-Rémy A, Hervé M, Poidevin M, Pili-Floury S, Kim MS, Blanot D, Oh BH, Ueda R, Mengin-Lecreulx D, Lemaitre B. 2006. The Drosophila Amidase PGRP-LB Modulates the Immune Response to Bacterial Infection. Immunity 24:463–473. doi:10.1016/j.immuni.2006.02.012

Zanchi C, Johnston PR, Rolff J. 2017. Evolution of defence cocktails: Antimicrobial peptide combinations reduce mortality and persistent infection. Mol Ecol 26:5334–5343. doi:10.1111/mec.14267

Zdybicka-Barabas A, Mak P, Klys A, Skrzypiec K, Mendyk E, Fiołka MJ, Cytryńska M. 2012. Synergistic action of Galleria mellonella anionic peptide 2 and lysozyme against Gram-negative bacteria. Biochim Biophys Acta - Biomembr 1818:2623–2635. doi:10.1016/j.bbamem.2012.06.008

Zerweck J, Strandberg E, Kukharenko O, Reichert J, Bürck J, Wadhwani P, Ulrich AS. 2017. Molecular mechanism of synergy between the antimicrobial peptides PGLa and magainin 2. Sci Rep 7. doi:10.1038/s41598-017-12599-7

Zhao HW, Zhou D, Haddad GG. 2011. Antimicrobial peptides increase tolerance to oxidant stress in Drosophila melanogaster. J Biol Chem 286:6211–6218. doi:10.1074/jbc.M110.181206

Zheng Y, Niyonsaba F, Ushio H, Nagaoka I, Ikeda S, Okumura K, Ogawa H. 2007. Cathelicidin LL-37 induces the generation of reactive oxygen species and release of human alpha-defensins from neutrophils. Br J Dermatol 157:1124–1131. doi:10.1111/j.1365-2133.2007.08196.x

2.8 Figures chapter 2

2.8.1 Main Figures

Figures 2.1-2.6 and supplemental figures are as presented in the published article.

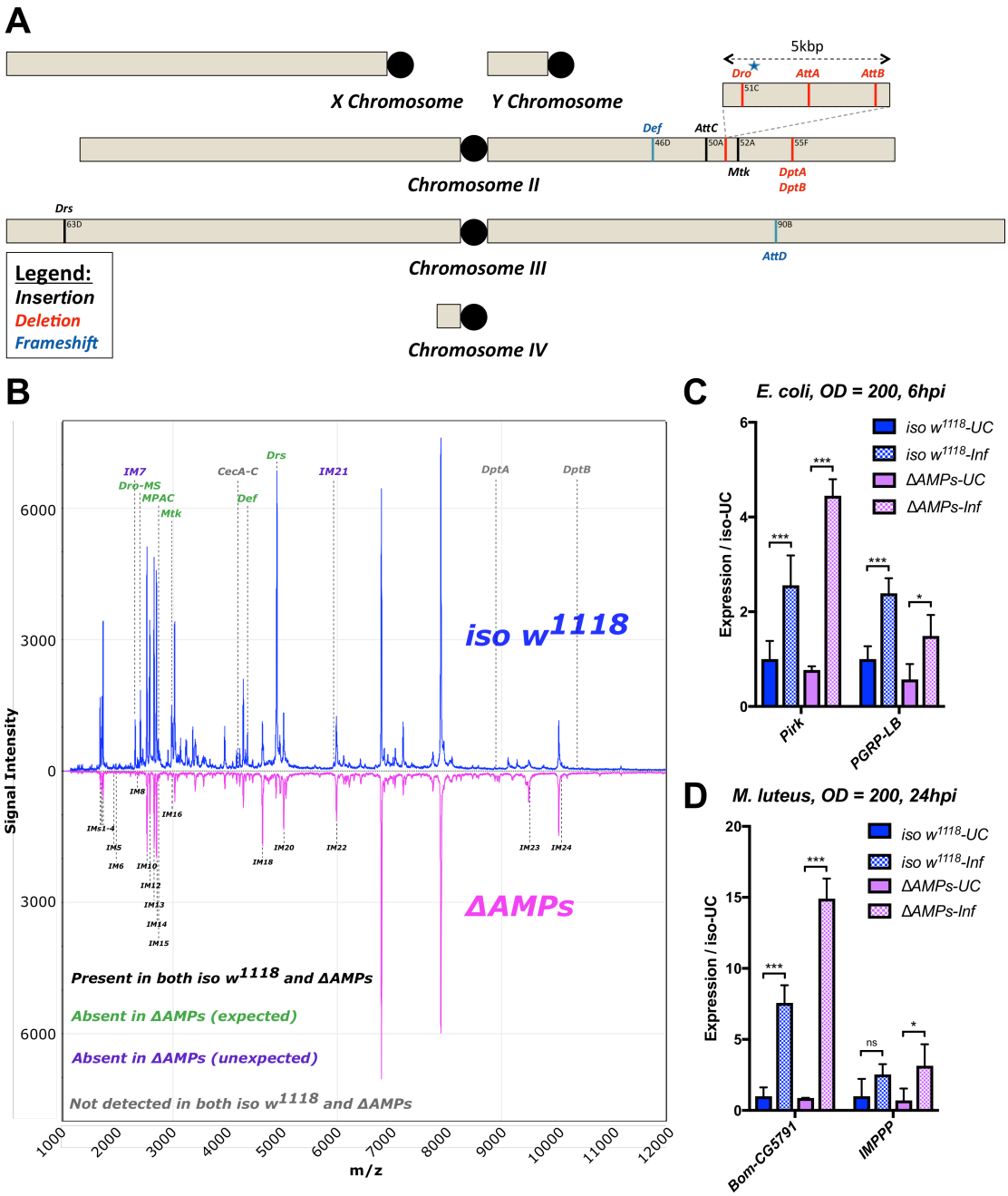

Figure 1 : Description of *AMP* mutants. (**A**) Chromosomal locations of *AMP* genes that were deleted. Each mutation is color-coded with the mutagenic agent: black, a *Minos* insertion or homologous recombination, red, CRISPR-CAS9-mediated deletion, and blue CRISPR CAS9 mediated indel causing a nonsense peptide. (**B**) A representative MALDI-TOF analysis of hemolymph samples from immune-challenged (1:1 *E. coli* and *M. luteus* at OD600 = 200) *iso w*^{1118} and *ΔAMPs* flies as described in Uttenweiler-Joseph et al. (1998). No AMP-derived products were detected in the hemolymph samples of *ΔAMPs* flies. No signals for IM7, nor IM21 were observed in the hemolymph samples of *ΔAMPs* mutants suggesting that these uncharacterized immune-induced molecules are the products of AMP genes. The Imd pathway (**C**) and Toll pathway (**D**) are functional and respond to immune challenge in *ΔAMPs* flies. We used alternate readouts to monitor the Toll and Imd pathways: *pirk* and *PGRP-LB* for Imd pathway and *CG5791 (Bomanin)* and *IMPPP* for Toll signaling (De Gregorio et al., 2002; Hanson et al., 2016). UC = unchallenged, Inf = infected. hpi = hours post-infection. Expression normalized with *iso w*^{1118}*-UC* set to a value of 1.

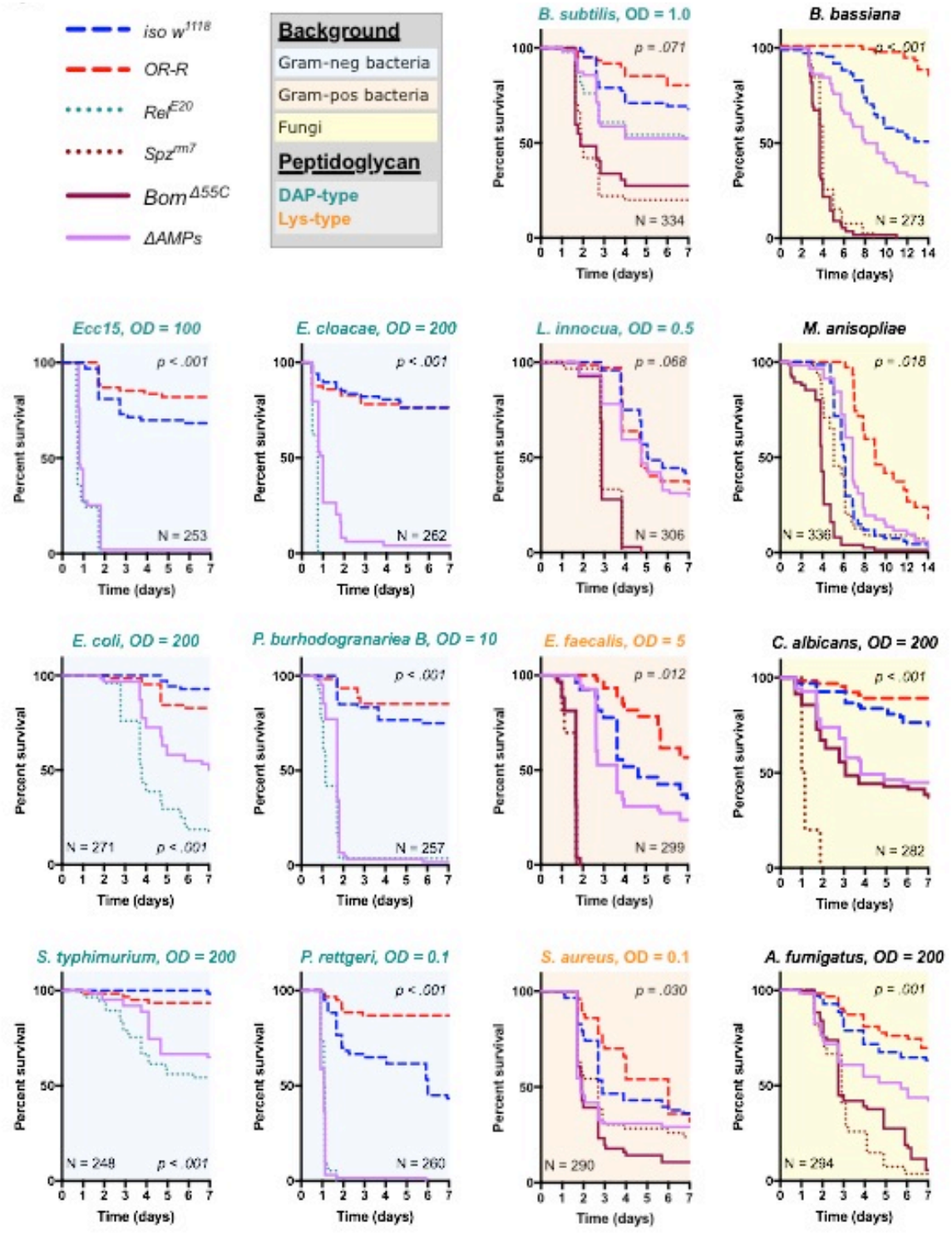

Figure 2: Survival of *ΔAMPs* flies to diverse microbial challenges. Control lines for survival experiments included two wild-types (*w;Drosdel* (*iso w*^{1118}) and Oregon R (*OR-R*) as an alternate wild-type), mutants for the Imd response (*Rel*^{E20}), mutants for Toll signaling (*spz*^{rm7}), and mutants for Bomanins (*Bom*$^{\Delta 55C}$). *ΔAMPs* flies are extremely susceptible to infection with Gram-negative bacteria (blue backgrounds). Unexpectedly, *ΔAMPs* flies were not markedly susceptible to infection with Gram-positive bacteria (orange backgrounds), while *Bom*$^{\Delta 55C}$ flies were extremely susceptible, often mirroring *spz*^{rm7} mutants. This pattern of *Bom*$^{\Delta 55C}$ susceptibility held true for fungal infections (yellow backgrounds). *ΔAMPs* flies are somewhat susceptible to fungal infections, but the severity shifts with different fungi. Pellet densities are reported for all systemic infections in OD at 600 nm. p-Values are given for *ΔAMPs* flies compared to *iso w*^{1118} using a Cox-proportional hazards model. N = total number of flies in experiments. A full description of p-values relative to *iso w*^{1118} can be found in Figure 2—source data 1.

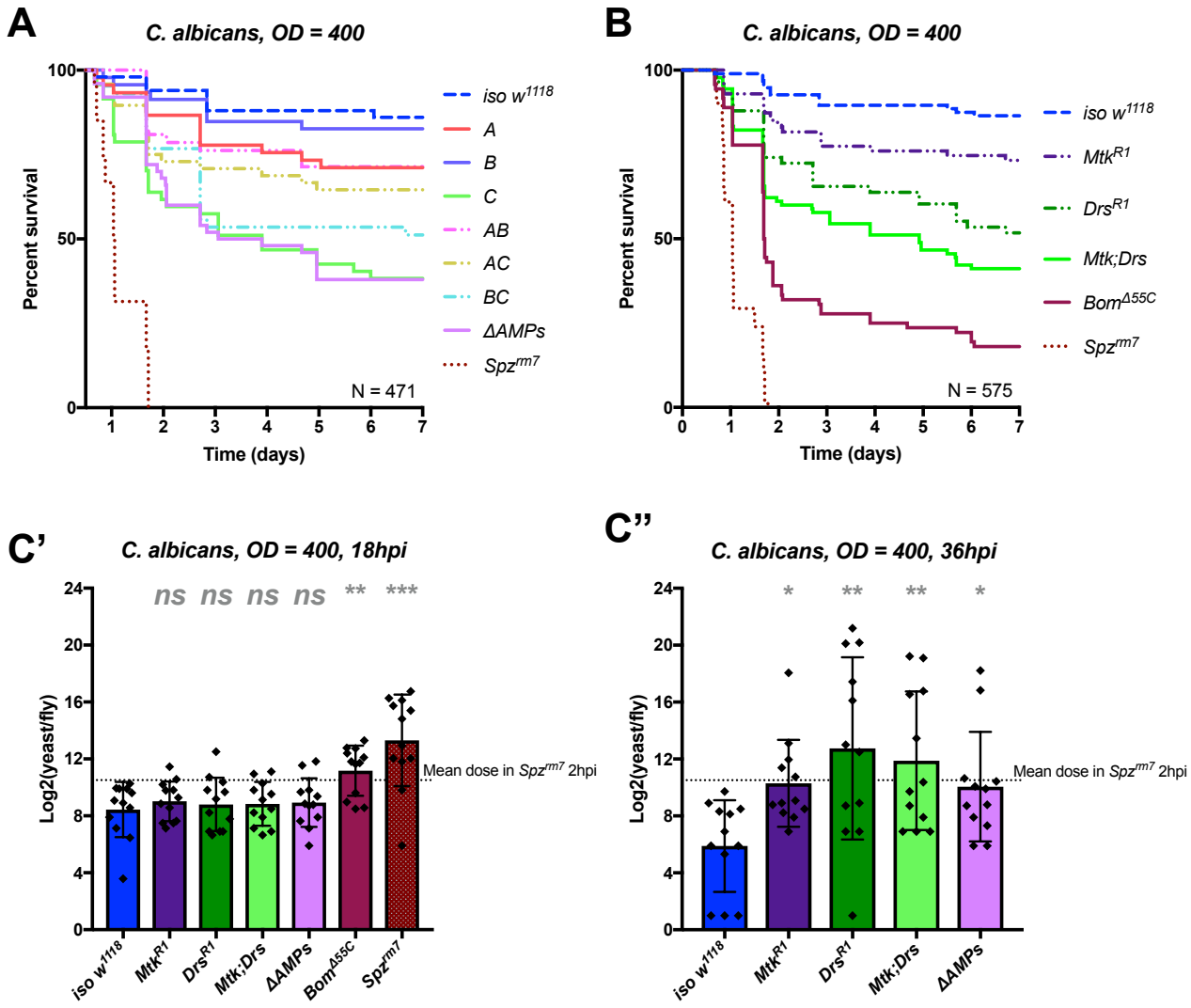

Figure 3: Identification of AMPs involved in the susceptibility of *ΔAMPs* flies to *C. albicans*. (A) Survival of mutants for groups of AMPs reveals that loss of only Toll-responsive Group C peptides (Metchnikowin and Drosomycin) is required to recapitulate the susceptibility of *ΔAMPs* flies. Co-occurring loss of groups A and C has a net protective effect (*A*C*: HR = −1.71, p=0.002). (B) Further dissection of Group C mutations reveals that both Metchnikowin and Drosomycin contribute to resist *C. albicans* survival (p=0.008 and p<0.001, respectively). The interaction of Metchnikowin and Drosomycin was not different from the sum of their individual effects (*Mtk*Drs*: HR = −0.80, p=0.116). (C) Fungal loads of individual flies at 18 hpi. At this time point, $Bom^{\Delta 55C}$ mutants and spz^{rm7} flies have already failed to constrain *C. albicans* growth (C'). Fungal titres at 36hpi (C''), a time point closer to mortality for many AMP mutants, show that some AMP mutants fail to control fungal load, while wild-type flies consistently controlled fungal titre. One-way ANOVA: not significant = *ns*, p<0.05 = *, p<0.01 = **, and p<0.001 = *** relative to $iso\ w^{1118}$.

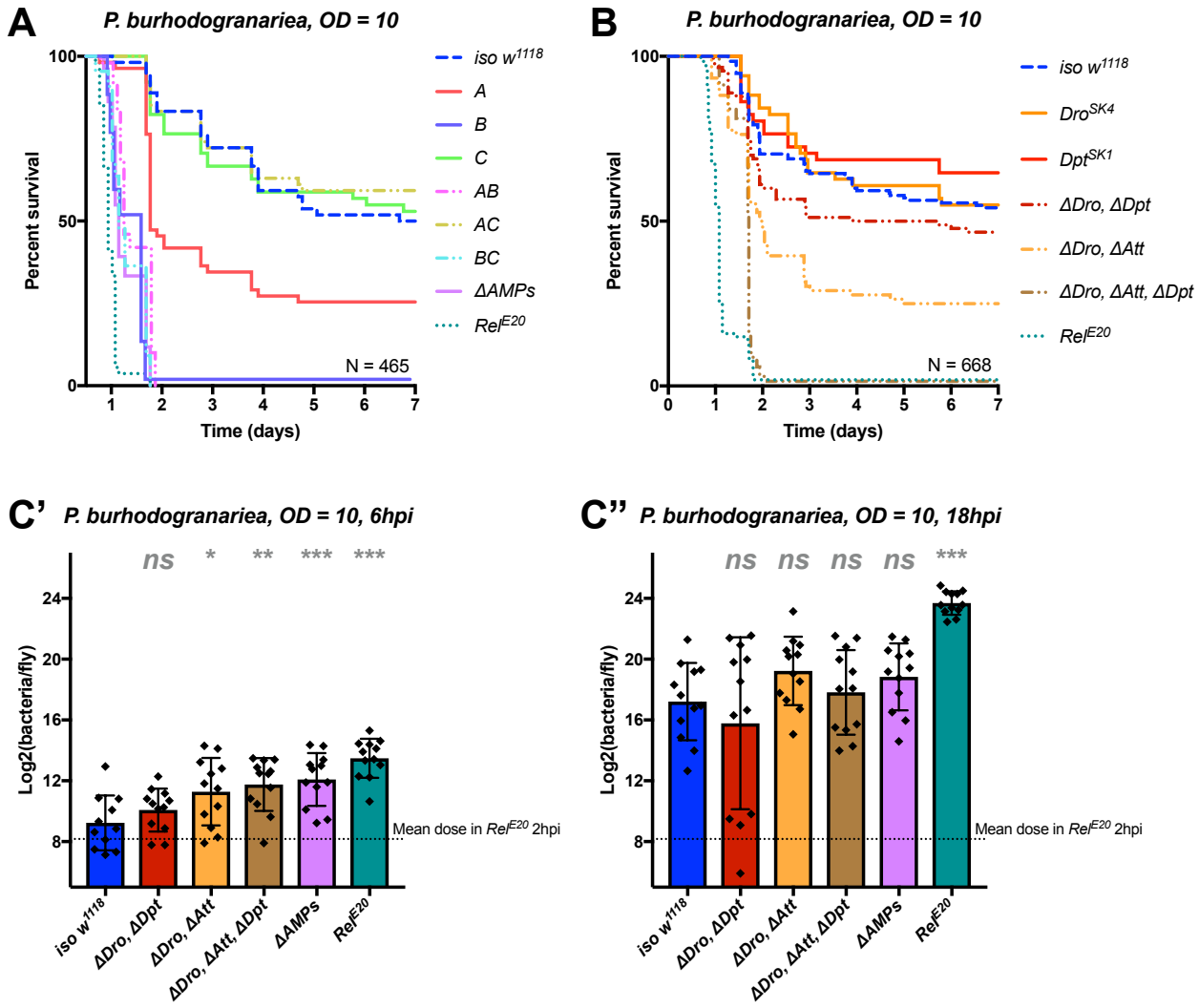

Figure 4: Identification of AMPs involved in the susceptibility of *ΔAMPs* flies to *P. burhodogranariea*. (A) Survival of mutants for groups of AMPs reveals that loss of Imd-responsive Group B peptides (Drosocin, Attacins, and Diptericins) recapitulates the susceptibility of *ΔAMPs* flies. Loss of the Group A peptide Defensin also resulted in strong susceptibility (p<0.001) (and see Figure 4—figure supplement 1). (B) Further dissection of AMPs deleted in Group B reveals that only the loss of all Drosocin, Attacin, and Diptericin gene families leads to susceptibility similar to *ΔAMPs* flies. Simultaneous loss of *Attacins* and *Diptericins* results in a synergistic loss of resistance (*ΔAtt*ΔDpt*: HR =+1.45, p<0.001). (C) Bacterial loads of individual flies at 6 hpi (C'). At this time point, most AMP mutants had significantly higher bacterial loads compared to wild-type flies. At 18 hpi (C''), differences in bacterial load are reduced, likely owing to the high chronic load *P. burhodogranariea* establishes even in surviving flies (Duneau et al., 2017). Meanwhile Rel^{E20} flies succumb ~18 hr earlier than *ΔAMPs* flies in survival experiments, and already have significantly higher loads. One-way ANOVA: not significant = *ns*, p<0.05 = *, p<0.01 = **, and p<0.001 = *** relative to $iso\ w^{1118}$.

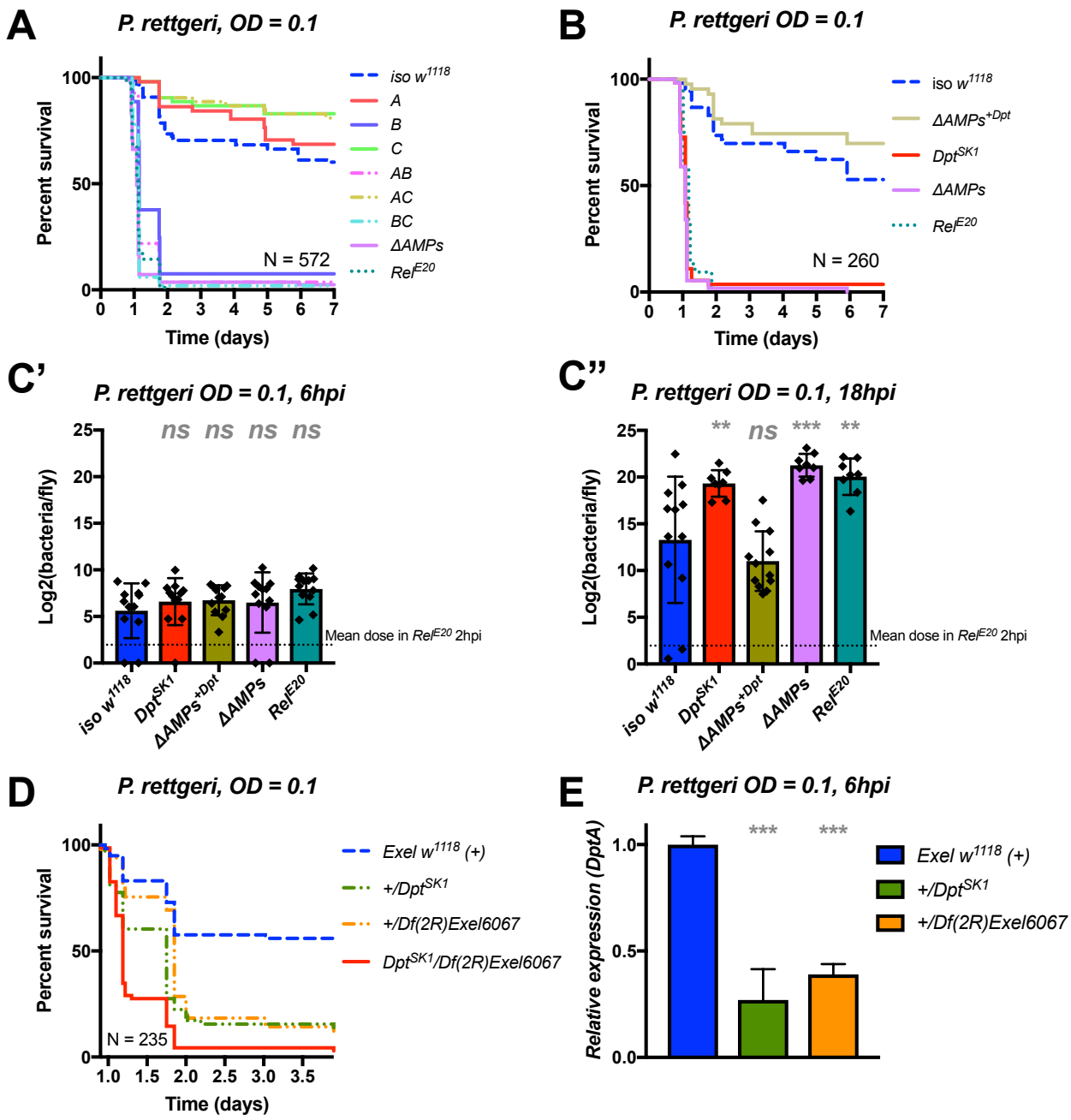

Figure 5: Identification of AMPs involved in the susceptibility of *ΔAMPs* flies to *P. rettgeri*. (A) Survival of mutants for groups of AMPs reveals that only loss of Imd-responsive Group B peptides (Drosocin, Attacins, and Diptericins) recapitulates the susceptibility of *ΔAMPs* flies. (B) Further dissection of the mutations affected in Group B reveals that only the loss of Diptericins (Dpt^{SK1}) leads to susceptibility similar to *ΔAMPs* flies. Remarkably, flies lacking all other AMPs ($ΔAMPs^{+Dpt}$) resist as wild-type. (C) Bacterial loads of individual flies are similar at 6hpi (C'), but by 18hpi (C''), *Dpt* mutants and Rel^{E20} flies have all failed to control *P. rettgeri* growth. (D) Heterozygote flies for Dpt^{SK1} and a deficiency including the *Diptericins* and flanking genes (*Df(2R)Exel6067*) recapitulates the susceptibility of *Diptericin* mutants. Intriguingly, heterozygotes with one functional copy of the Diptericins (*+/*Dpt^{SK1} or *+/Df(2R)Exel6067*) are nonetheless highly susceptible to infection. (E) *Diptericin A* transcriptional output is strongly reduced in heterozygotes 6 hpi compared to wild-type flies. One-way ANOVA: not significant = *ns*, p<0.05 = *, p<0.01 = **, and p<0.001 = *** relative to *iso* w^{1118}.

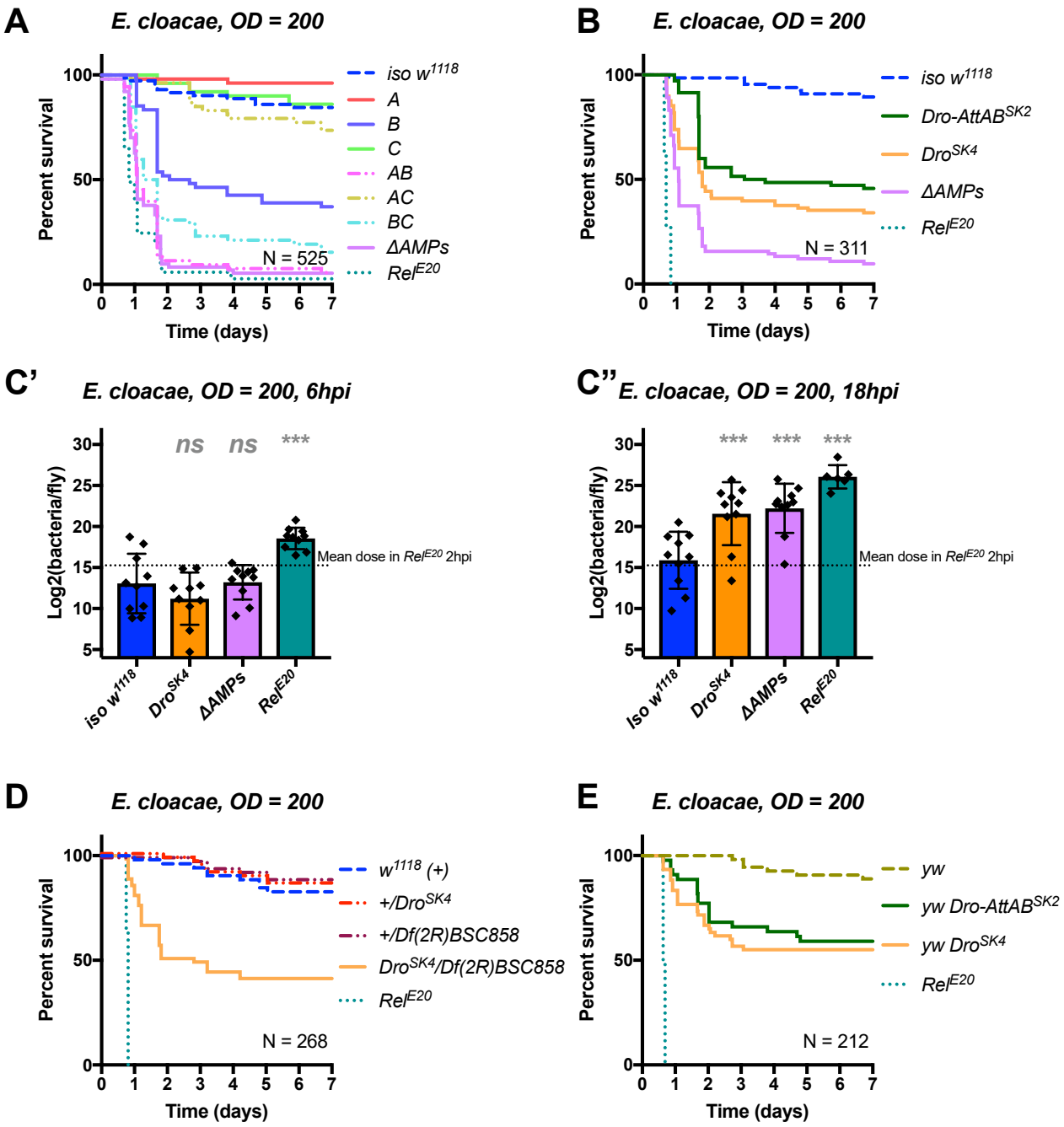

Figure 6: Identification of AMPs involved in the susceptibility of *ΔAMPs* flies to *E.cloacae*. (A) Survival of mutants for groups of AMPs reveals that loss of Imd-responsive Group B peptides (Drosocin, Attacins, and Diptericins) results in a strong susceptibility to infection ($p<0.001$), while loss of Group A or C peptides alone resists as wild-type ($p>0.1$ each). Group AB flies were as susceptible as *ΔAMPs* flies, and we observed a synergistic interaction between Group A and B mutations (A*B: HR =+2.55, $p=0.003$). (B) Further dissection of the mutations in Group B revealed that loss of *Drosocin* alone (Dro^{SK4}), or a deficiency lacking both *Drosocin* and *Attacins AttA* and *AttB* ($Dro\text{-}AttAB^{SK2}$) recapitulates the susceptibility of Group B flies. (C) By 18hpi, bacterial loads in individual *Drosocin* mutants or Rel^{E20} flies are significantly higher than wild-type. (D) Heterozygote flies for Dro^{SK4} and *Df(2R)BSC858* (a deficiency removing *Drosocin, Attacins AttA* and *AttB*, and other genes) are strongly susceptible to *E. cloacae* infection. (E) *Drosocin* mutants in an alternate genetic background (*yw*) are susceptible to *E. cloacae*. One-way ANOVA: not significant = *ns*, and $p<0.001$ = *** relative to $iso\ w^{1118}$.

2.8.2 Supplementary Figures and Tables

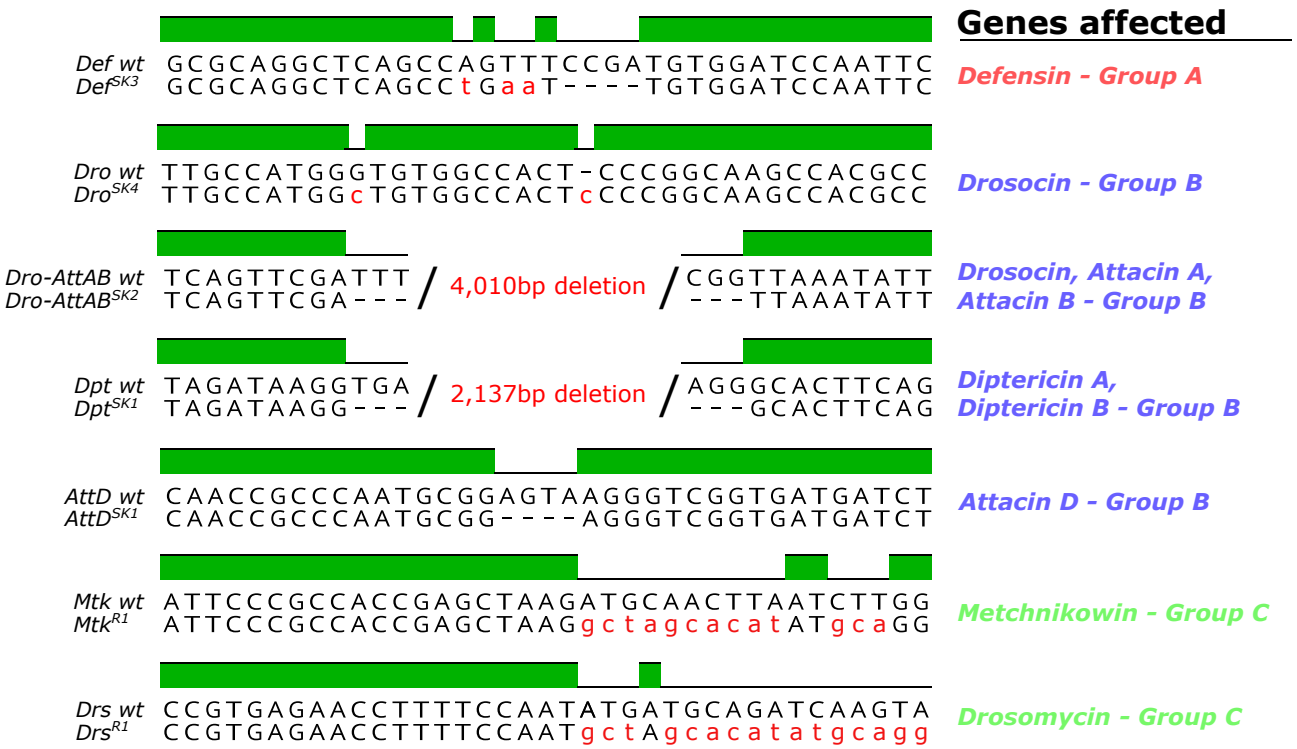

Figure 1 supplement 1: Genetic description of mutations generated in this study. The *Mtk*^{R1} and *Drs*^{R1} mutations entirely replaced the CDS with an insert from the piHR vector. Non-synonymous nucleotides in mutants are given in red. Mutations are listed according to groups in Figures 3–6 (discussed later).

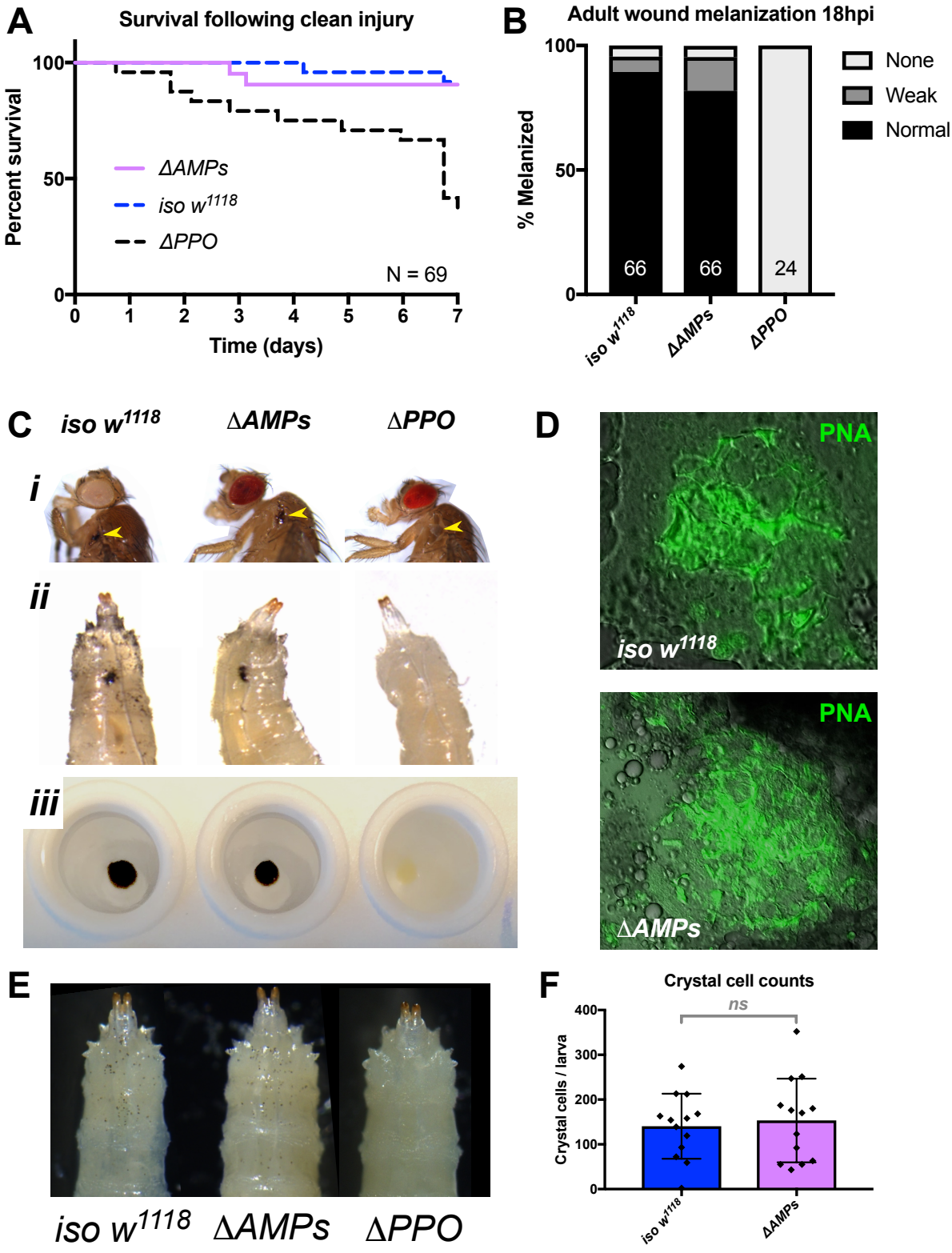

Figure 1 supplement 2: *ΔAMPs* flies have otherwise wild-type immune reactions. (A) *ΔAMPs* flies survive clean injury like wild-type flies, while *ΔPPO* mutants deficient for melanization have reduced survival over time. (B) *ΔAMPs* flies melanize the cuticle similar to wild-type flies following pricking (χ^2 = 2.14, p=0.34). Melanization categories (None, Weak, Normal) were as described in Dudzic et al (Dudzic et al., 2018). Sample sizes (n) are included in each bar. (C) Melanization in *iso w*^{1118}, *ΔAMPs*, and *ΔPPO* flies of the cuticle in adults (*i,* yellow arrowheads), larvae (*ii,* melanized wounds), and larval hemolymph (*iii*). (D) To investigate clotting ability, we used the hanging drop assay (Scherfer et al., 2004) with *ΔAMPs* larval hemolymph and visualized clot fibers with PNA staining (green). Both *iso w*^{1118} and *ΔAMPs* hemolymph produced visible clot fibres measured after 20 min. Hemocyte populations are normal in *ΔAMPs* flies, including crystal cell distribution (E) and number (F).

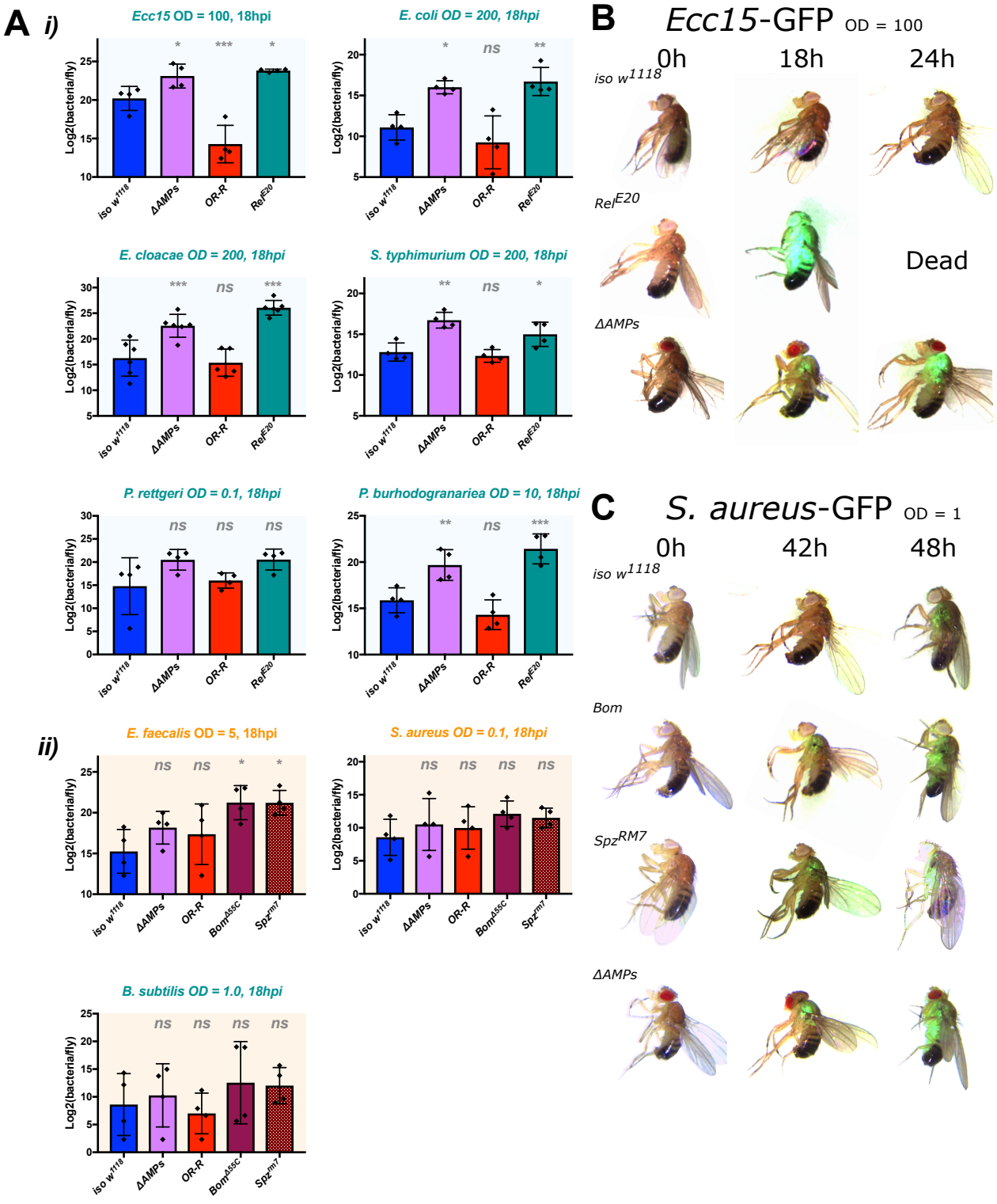

Figure 2 supplement 1: *ΔAMPs* flies fail to suppress Gram-negative bacterial growth. Colony counts were performed on pooled samples (five flies) for bacteria amenable to LB agar, a medium that avoids overnight growth of the host microbiota. (A) For Gram-negative bacterial infections, *ΔAMPs* flies have significantly higher bacterial loads compared to *iso* w^{1118} at 18 hr post-infection (hpi) (i). This is not true for any of the Gram-positive bacteria tested (*ii*), while *spz*^{rm7} mutants carried higher bacterial loads, significantly so in *E. faecalis* infections. Gram-negative (B) and Gram-positive (C) infections with GFP-labelled bacteria spread from the wound site systemically in all genotypes tested. Thus, *ΔAMPs* fly mortality is likely not due to tissue-specific colonization by invading bacteria, but rather a failure to suppress bacterial growth first locally, and then systemically. One-way ANOVA: not significant = *ns*, p<0.05 = *, p<0.01 = **, and p<0.001 = *** relative to *iso* w^{1118}.

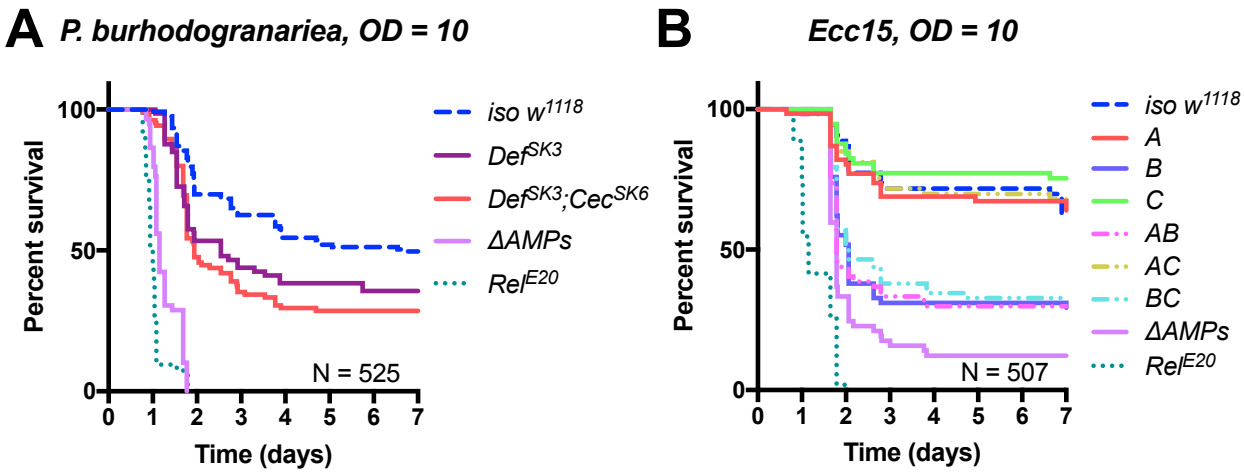

Figure 4 supplement 1: Further dissecting effects of AMP groups. (**A**) Group A flies (here labelled Def^{SK3}; Cec^{SK6}) have an abberant Cecropin locus (Cec^{SK6}), but this contributes little to survival compared to Def^{SK3} mutants (p=0.818). Def^{SK3} flies are susceptible to *P. burhodogranariea* (Log-Rank p=0.022). (**B**) Upon infection with the Gram-negative *Ecc15*, Group B peptides (Drosocin, Attacins and Diptericins) explain the bulk of mortality, but additional loss of other peptides in *ΔAMPs* flies leads to increased mortality (Log-Rank p=0.013).

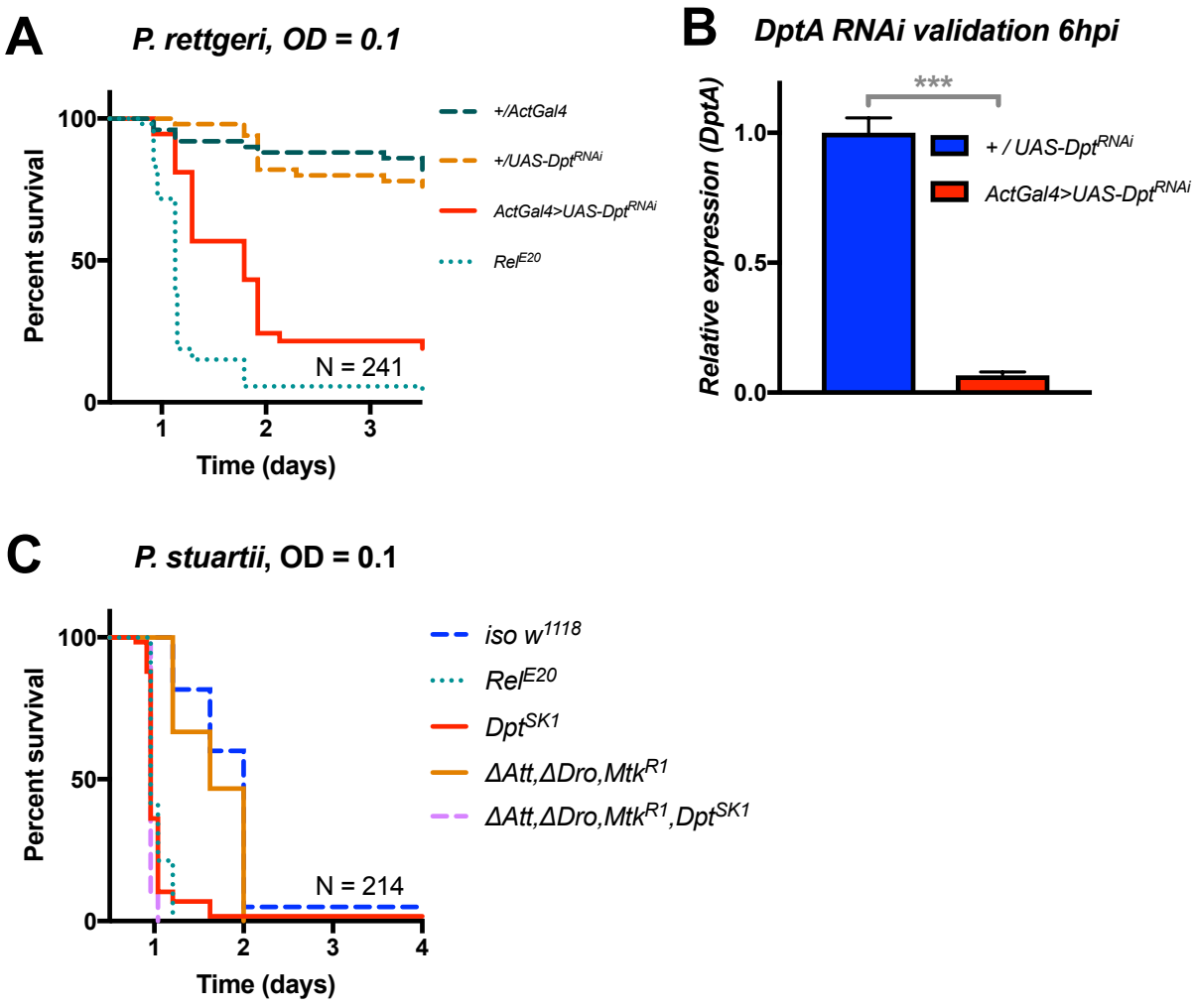

Figure 5 supplement 1: Additional validation of the role of *Diptericin* in resistance to *Providencia*. (**A**) Silencing of *Diptericin by RNAi* leads to higher susceptibility to *P. rettgeri* infection (p<0.001). (**B**) Validation of the *Diptericin* RNAi construct 6 hpi. C) Mutants lacking multiple peptides (Attacins, Drosocin, and Metchnikowin) succumb to *P. stuartii* infection as wild-type ('*ΔAtt, ΔDro, Mtk*^{R1}'), while Diptericin mutation alone (*Dpt*^{SK1}) or combined ('*ΔAtt, ΔDro, Mtk*^{R1}, *Dpt*^{SK1}') leads to a susceptibility similar to *Rel*^{E20} mutants. This pattern of survival was similar to the pattern observed with *P. rettgeri*. One-way ANOVA: p<0.001 = ***.

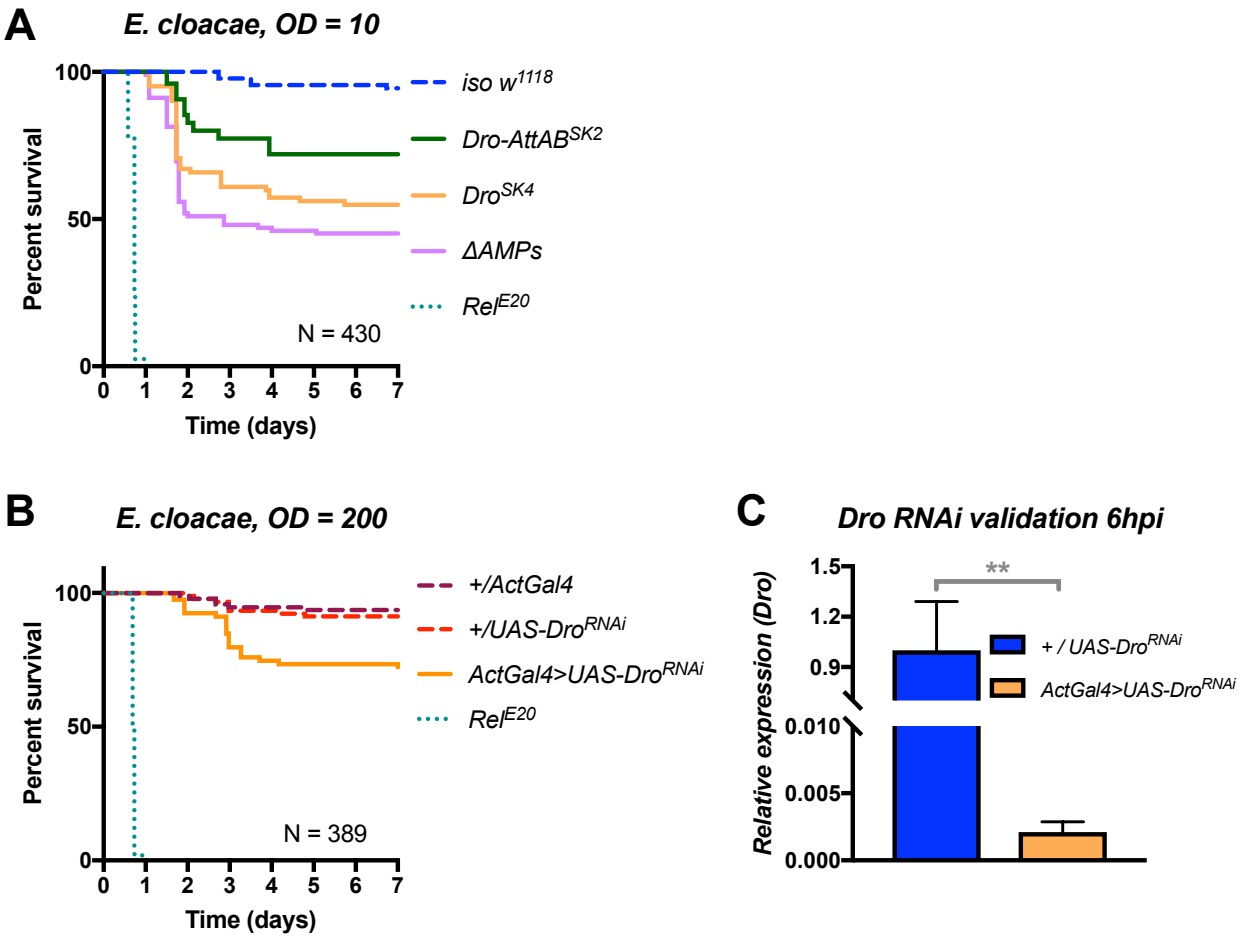

Figure 6 supplement 1: (A) *Drosocin* mutant susceptibility remains even at a lower dose (OD = 10,~7000 bacteria/fly), while *Rel*^{E20} flies succumb rapidly regardless of initial dose. (B) Silencing of *Drosocin by RNAi* leads to significant mortality from *E. cloacae* infection (p<0.001). (C) Validation of the *Drosocin* RNAi construct 6hpi.

Table S1 : Primers used in this study.

Primers used for tracking mutations

Gene(s)	Mutation	Name	Sequence
Def	Def^{SK3}	DefSK3new F	AGG CTC AGC CTG AAT TGT GG
		DefSK R	TGG TAA GTC GCT AAC GCT AAT
AttC	$AttC^{Mi}$	AttCKO F1	CTT GGG CTG CAG ATT GTT
		AttCKO R1	GCC AAC GAT GAC CAC AAT
Dro	Dro^{SK4}	DroSK4 F	GGC TGT GGC CAC TCC CC
		DroSK4 R	GTG TCA ACG AAA AGT TTG CAC
Dro, AttA, AttB	$Dro\text{-}AttAB^{SK2}$	DroAttCas F	TTG CCT TCA GTC GCC TAT
		DroAttCas R	TCA TTG AGT GGG ATC GAA
Mtk	Mtk^{R1}	MetchKO F1	CTG GCC ACA ATC GGT TAT
		mCher R1	AAG CGC ATG AAC TCC TTG
DptA, DptB	Dpt^{SK1}	Dpt-DptB-120 F	CCT CGT TTA AGA AAG ATC
		Dpt-DptB+254 R	GGT GGG TCT GTA AAC TTG GAT GAC GAG
Drs	Drs^{R1}	DrsKO F1	GCG TCC CAG TCA AAG GTA
		mCher R1	AAG CGC ATG AAC TCC TTG
AttD	$AttD^{SK1}$	dAttD F2	CGC CCA ATG CGG AGG GT
		dAttD R	TGG CGT TGA GGT TGA GAT
CecA1, CecA2, CecB, CecC	Cec^{SK6}	CecShu F2	CCG ACT TAG AAA GAT AGA
		CecShu R2	CCA CCC TGG GAA AGT GTA

Primers used for qPCR

Gene	Source	Name	Sequence
DptA	Hanson et al. (2016)	DptA-HanF	ATG CCC GAC GAC ATG ACC AT
		DptA-HanR	TTG TCG GTG GTC CAC ACC TT
Drs	Bruno Lemaitre	Drom-F	CGTGAGAACCTTTTCCAATATGAT
		Drom-R	TCCCAGGACCACCAGCAT
Dro	Hanson et al. (2016)	Dro-161F	ACTGGCCATCGAGGATCACC
		Dro-246R	TCTCCGCGGTATGCACACAT
CG5791	Hanson et al. (2016)	CG5791-70F	CTGATCGGCGCTCATCCCAG
		CG5791-187R	GGGATGAGGAGAAGCTGCGG
IMPPP	This study	IMPPP 230F	GGTGAGCATGTGTACACCGA
		IMPPP 331R	GGCGGAAAAATTGGGACCAC
Pirk	Kounatidis et al. (2017)	Pirk F	CGATGACGAGTGCTCCAC
		Pirk R	TGCTGCCCAGGTAGATCC
PGRP-LB	Iatsenko et al. (2016)	PGRP-LB F	GGACATGCAGGACTTCCA
		PGRP-LB R	GGTTCTCCAATCTCCGAT
Rp49/RpL32	Bruno Lemaitre	RpL32 F	GCC GCT TCA AGG GAC AGT ATC TG
		RpL32 R	AAA CGC GGT TCT GCA TGA G

Chapter 3 The *Drosophila Baramicin* polypeptide gene protects against fungal infection

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3.1 Abstract

The fruit fly *Drosophila melanogaster* combats microbial infection by producing a battery of effector peptides that are secreted into the hemolymph. Technical difficulties prevented the investigation of these short effector genes until the recent advent of the CRISPR/CAS era. As a consequence, many putative immune effectors remain to be formally described, and exactly how each of these effectors contribute to survival is not well characterized. Here we describe a novel *Drosophila* antifungal peptide gene that we name *Baramicin A*. We show that *BaraA* encodes a precursor protein cleaved into multiple peptides via furin cleavage sites. *BaraA* is strongly immune-induced in the fat body downstream of the Toll pathway, but also exhibits expression in other tissues. Importantly, we show that flies lacking *BaraA* are viable but susceptible to the entomopathogenic fungus *Beauveria bassiana*. Consistent with *BaraA* being directly antimicrobial, overexpression of *BaraA* promotes resistance to fungi and the IM10-like peptides produced by *BaraA* synergistically inhibit growth of fungi in vitro when combined with a membrane-disrupting antifungal. Surprisingly, *BaraA* mutant males but not females display an erect wing phenotype upon infection. Here, we characterize a new antifungal immune effector downstream of Toll signalling, and show it is a key contributor to the *Drosophila* antimicrobial response.

3.2 Author Summary

The ways that animals combat infection involve complex molecular pathways that are triggered upon microbial challenge. While a great deal is known about which pathways are key to a successful defence response, far less is known about exactly what elements of that response are critical to combat a given infection. Using the fruit fly – a genetic workhorse of Biology – we recently showed that a class of host-encoded antibiotics called "antimicrobial peptides" are essential for defence against bacterial infection, but do not contribute as strongly to defence against fungi. However a number of fly immune peptides remain uncharacterized, possibly explaining this gap in our understanding of the fly antifungal defence. Here we describe a novel antifungal peptide gene of fruit flies, and show that it is a major contributor to the fly antifungal defence response. We also found that this gene seems to regulate a behaviour that flies perform after infection, paralleling exciting recent findings that these genes are involved in neurological processes. Collectively, we clarify a key part of the fly antifungal defence, and contribute an important piece to help explain the logical organization of the immune defence against microbial infection.

3.3 Introduction

The innate immune response provides the first line of defence against pathogenic infection. This reaction is usually divided into three stages: i) the recognition of pathogens through dedicated pattern recognition receptors, ii) the activation of conserved immune signalling pathways and iii) the production of immune effectors that target invading pathogens (Kurz and Ewbank, 2003; Lemaitre and Hoffmann, 2007b). The study of invertebrate immune systems has led to key observations of broad relevance, such as the discovery of phagocytosis (Kaufmann, 2008), antimicrobial peptides (AMPs) (Steiner et al., 1981b), and the implication of Toll receptors in metazoan immunity (Bruno Lemaitre et al., 1996). Elucidating immune mechanisms, genes, and signalling pathways has greatly benefited from investigations in the fruit fly *Drosophila melanogaster*, which boasts a large suite of molecular and genetic tools for manipulating the system. One of the best-characterized immune reactions of *Drosophila* is the systemic immune response. This reaction involves the fat body (an analog of the mammalian liver) producing immune effectors that are secreted into the hemolymph. In *Drosophila*, two NF-κB signalling pathways, the Toll and Imd pathways, regulate most inducible immune effectors: the Toll pathway is predominantly activated in response to infection by Gram-positive bacteria and fungi (Bruno Lemaitre et al., 1996; Lemaitre et al., 1997a), while the immune-deficiency pathway (Imd) responds to the DAP-type peptidoglycan most commonly found in Gram-negative bacteria and a subset of Gram-positive bacteria (Lemaitre et al., 1995b). These two signalling pathways regulate a transcriptional program that results in the massive syn-

thesis and secretion of humoral effector peptides (Lemaitre et al., 1997a; Uttenweiler-Joseph et al., 1998b). Accordingly, mutations affecting the Toll and Imd pathways cause extreme susceptibilities to systemic infection that reflect the important contribution of these pathways to host defence. The best-characterized immune effectors downstream of these pathways are antimicrobial peptides (AMPs). AMPs are small and often cationic peptides that disrupt the membranes of microbes, although some have more specific mechanisms (Lazzaro et al., 2020). Multiple AMP genes belonging to seven well-characterized families are induced upon systemic infection (Hanson and Lemaitre, 2020). However transcriptomic analyses have revealed that the systemic immune response encompasses far more than just the canonical AMPs. Many uncharacterized genes encoding small secreted peptides are induced to high levels downstream of the Toll and Imd pathways, pointing to the role for these peptides as immune effectors (De Gregorio et al., 2002b). In parallel, MALDI-TOF analyses of the hemolymph of infected flies revealed the induction of 24 peaks – mostly corresponding to uncharacterized peptides – that were named "IMs" for Immune-induced Molecules (IM1-IM24) (Uttenweiler-Joseph et al., 1998b). Many of the genes that encode these components of the immune peptidic secretome had remained unexplored until recently. This is mainly due to the fact that these IMs belong to large gene families of small genes that were not typically disrupted using random mutagenesis (Hanson and Lemaitre, 2020; Lin et al., 2020).

The CRISPR/Cas9 gene editing approach now allows the necessary precision to delete small genes, singly or in groups, providing the opportunity to dissect effector peptide functions. In 2015 a family of 12 related IM-encoding genes, unified under the name *Bomanins*, were shown to function downstream of Toll. Importantly, a deletion removing 10 out of the 12 Bomanins revealed their potent contribution to defence against both Gram-positive bacteria and fungi (Clemmons et al., 2015b). While Bomanins contribute significantly to Toll-mediated defence, their molecular functions are still unknown and it is unclear if they are directly antimicrobial (Lindsay et al., 2018a). Two other IMs encoding IM4 and IM14 (renamed *Daisho1* and *Daisho2*, respectively) were shown to contribute downstream of Toll to resistance against *Fusarium* fungi. Interestingly, Daisho peptides bind to fungal hyphae, suggesting direct antifungal activity (Cohen et al., 2020). Finally a systematic knock-out analysis of *Drosophila* AMPs revealed that they play an important role in defence against Gram-negative bacteria and some fungi, but surprisingly little against Gram-positive bacteria (Hanson et al., 2019a). An unforeseen finding from these recent studies is the high degree of AMP-pathogen specificity: this is perhaps best illustrated by the specific requirement for *Diptericin*, but not other AMPs, in defence against *Providencia rettgeri* (Hanson et al., 2019a; Unckless et al., 2016b). Collectively,

these studies in *Drosophila* reveal that immune effectors can be broad or specific in mediating host-pathogen interactions. Understanding the logic of the *Drosophila* effector response will thus require a careful dissection of the remaining uncharacterized immune induced peptides.

Previous studies identified an uncharacterized Toll-regulated gene (*CG18279/CG33470)*, which we rename "*BaraA*" (see below), that encodes several IMs, indicating a role in the humoral response. Here, we have improved the annotation of IMs produced by *BaraA* to include: IM10, IM12 (and its sub-peptide IM6), IM13 (and its sub-peptides IM5 and IM8), IM22, and IM24. Using a *BaraA* reporter, we show that *BaraA* is not only immune-induced in the fat body, but also expressed in the head, and nervous system tissue including the eyes, and ocelli. Importantly, we show that flies lacking *BaraA* are viable but susceptible to specific infections, notably by the entomopathogenic fungus *Beauveria bassiana*. Consistent with this, the IM10-like peptides produced by *BaraA* inhibit fungal growth in vitro when combined with the antifungal Pimaricin. Surprisingly, *BaraA* deficient males also display a striking erect wing behaviour upon infection. Collectively, we identify a new antifungal immune effector downstream of Toll signalling, improving our knowledge of the *Drosophila* antimicrobial response.

3.4 Results

BaraA is regulated by the Toll pathway

A previous microarray study from De Gregorio et al. (De Gregorio et al., 2002b) suggested that *BaraA (CG18279/CG33470)* is primarily regulated by the Toll pathway, with a minor input from the Imd pathway (**Fig.** 1A). Consistent with this, we found several putative NF-κB binding sites upstream of the *BaraA* gene (guided by previous reports (Busse et al., 2007; Copley et al., 2007; Tanji et al., 2010)). Notably there are two putative binding sites for Relish, the transcription factor of the Imd pathway and three putative binding sites for the Dif/Dorsal transcription factors acting downstream of Toll (**Fig.** S1A). We challenged wild-type flies and Imd or Toll pathway mutants (Rel^{E20} and spz^{rm7} respectively) with the yeast *Candida albicans*, the Gram-negative bacterium *Escherichia coli*, or the Gram-positive bacterium *Micrococcus luteus*. RT-qPCR analysis confirms that *BaraA* is abolished in spz^{rm7} flies similar to the Toll-regulated *BomBc3* gene (**Fig.** 1B), but remains highly inducible in Rel^{E20} flies (**Fig.** S1B). Collectively, the expression pattern of *BaraA* is reminiscent of the antifungal peptide gene *Drosomy-*

cin with a primary input by the Toll pathway and a minor input from the Imd pathway (Ferrandon et al., 1998a; Hanson and Lemaitre, 2020).

To further characterize the expression of *BaraA*, we generated a *BaraA-Gal4* transgene in which 1675bp of the *BaraA* promoter sequence is fused to the yeast transcription factor Gal4. Monitoring GFP in *BaraA-Gal4>UAS-mCD8-GFP* flies (referred to as *BaraA>mGFP*) confirms that the *BaraA* reporter is highly induced in the fat body after infection by *M. luteus*, but less so by *E. coli* (**Fig.** 1C). This result is consistent with a recent time course study that found Toll-regulated genes (including *BaraA*) were rapidly induced after injection stimulating the Imd pathway, but this principally Imd-based induction resolves to nearly basal levels within 48 hours (Schlamp et al., 2021) (and see **Fig.** S1B-C). Additionally, larvae pricked with *M. luteus* show a robust GFP signal primarily stemming from the fat body when examined 2hpi (**Fig.** S1D). We also observed a constitutive GFP signal in the headcase of adults (**Fig.** 1D), including the border of the eyes and the ocelli (**Fig.** 1E). Dissection confirmed that the *BaraA* reporter is expressed in brain tissue, including posterior to the central brain furrow in adults and at the posterior of the ventral nervous system in larvae. Other consistent signals include GFP in the wing veins and subcutaneously along borders of thoracic pleura in adults (**Fig.** 1F-G), and in spermatheca of females (**Fig.** S1E). There was also sporadic GFP signal in other tissues that included the larval hindgut, the dorsal abdomen of developing pupae, and the seminal vesicle of males. These expression patterns largely agree with data reported in FlyAtlas1 (wherein *BaraA* is called "*IM10*") (Robinson et al., 2013).

Baramicin A encodes a precursor protein cleaved into multiple peptides

Previous studies using bioinformatics and proteomics have suggested that four highly immune-induced peptides (IM10, IM12, IM13, and IM24) are encoded in tandem as a single polypeptide precursor by *CG33470* (aka *IMPPP/BaraA*) (Levy et al., 2004b; Uttenweiler-Joseph et al., 1998b). Some less-abundant sub-peptides (IM5, IM6, and IM8) are also produced by additional cleavage or degradation of IM12 and IM13 (Levy et al., 2004b). Using a newly generated null mutant ("*ΔBaraA,*" described below and design shown in **Fig.** 2A), we analyzed hemolymph samples of wild-type and *ΔBaraA* flies infected with a bacterial mixture of *E. coli* and *M. luteus* by MALDI-TOF analysis. We confirmed the loss of the seven immune-induced peaks corresponding to IMs 5, 6, 8, 10, 12, 13, and 24 in *ΔBaraA* flies (**Fig.** 2A). We also noticed that an additional immune-induced peak at ~5975 Da was absent in our *BaraA* mutants. Upon re-visiting the original studies that annotated the *Drosophila* IMs, we realized this peak corresponded to IM22, whose sequence was never determined (Levy et al., 2004b;

Uttenweiler-Joseph et al., 1998b) (see supplementary information for details). We subjected hemolymph from infected flies to LC-MS proteomic analysis following trypsin digestion and found that in addition to the known IMs of *BaraA* (IMs 5, 6, 8, 10, 12, 13, and 24), trypsin-digested fragments of the *BaraA* C-terminus peptide were also detectable in the hemolymph (**Fig.** S2). The range of detected fragments did not match the full length of the C-terminus exactly, as the first four residues were absent in our LC-MS data (a truncation not predicted to arise via trypsin cleavage). The *BaraA* C-terminus lacking these four residues has a calculated mass of 5974.5 Da, exactly matching the observed mass of the IM22 peak absent in *BaraA* mutant flies. Furthermore in other *Drosophila* species these four residues are absent, and instead the C-terminus directly follows an RXRR furin cleavage motif (**Fig.** S3A). Therefore IM22 cleavage in other species, even by an alternate cleavage process, should result in the same maturated IM22 domain as found in *D. melanogaster*. Taken together, we conclude that IM22 is the mature form of the BaraA protein C-terminus.

Thus, a single gene, *BaraA*, contributes to one third of the originally described *Drosophila* IMs. These peptides are encoded as a polypeptide precursor interspersed by furin cleavage sites (e.g. RXRR) (**Fig.** 2B). We note that the IM10, IM12 and IM13 peptides are tandem repeats of related peptides, which we collectively refer to as "IM10-like" peptides (**Fig.** S3B). The IM22 peptide also contains a similar motif as the IM10-like peptides (**Fig.** S3A-B), suggesting a related biological activity. We name this gene "*Baramicin A*" (symbol: *BaraA*) for the Japanese idiom Bara Bara (バラバラ), meaning "to break apart;" a reference to the fragmenting structure of the *Baramicin* precursor protein and its many peptidic products.

A BaraA duplication is present in some laboratory stocks

Over the course of our investigation, we realized that *IMPPP (CG18279)* was identical to its neighbour gene *CG33470* owing to a duplication event of the *BaraA* locus present in the *D. melanogaster* reference genome. The exact nature of this duplication is discussed in a separate article (Hanson and Lemaitre, 2021). In brief, the duplication involves the entire *BaraA* gene including over 1kbp of 100% identical promoter sequence, and also the neighbouring sulfatase gene CG30059 and the 3' terminus of the *ATP8A* gene region (**Fig.** 2C). We distinguish the two daughter genes as *BaraA1 (CG33470)* and *BaraA2 (CG18279).* Available sequence data suggests the *BaraA1* and *BaraA2* transcripts are 100% identical. In a separate study, we analyzed the presence of the *BaraA* duplication using a PCR assay spanning the junction of the duplicated region (also see supplementary data file 1). Interestingly, *BaraA*

copy number is variable in common lab strains and wild flies, indicating this duplication event is not fixed in *D. melanogaster* (Hanson and Lemaitre, 2021).

Over-expression of BaraA improves the resistance of immune deficient flies

Imd, Toll deficient flies are extremely susceptible to microbial infection as they fail to induce hundreds of immune genes, including antimicrobial peptides (De Gregorio et al., 2002b). It has been shown that over-expression of even a single AMP can improve the resistance of *Imd, Toll* deficient flies (Tzou et al., 2002b). As such, immune gene over-expression in *Imd, Toll* immune-compromised flies provides a direct assay to test the ability of a gene to contribute to defence independent of other immune effectors. We applied this strategy to *Baramicin A* by generating flies that constitutively express *BaraA* using the ubiquitous *Actin5C-Gal4* driver *(Act-Gal4)* in an immune-deficient Rel^{E20}, spz^{rm7} double mutant background (**Fig.** S4A). In these experiments, we pooled results from both males and females due to the very low availability of homozygous *Rel, spz* adults when combined with *Act-Gal4*. Overall, similar trends were seen in both sexes, and separate male and female survival curves are shown in **Fig.** S4.

Ubiquitous *BaraA* expression marginally improved the survival of *Rel, spz* flies upon infection with *M luteus* bacteria, however there was no effect upon infection with *E. coli* (**Fig.** S4B-C). On the other hand, ubiquitous expression of *BaraA* provided a more pronounced protective effect against infection by a variety of fungal pathogens. This was true upon pricking with *C. albicans* (**Fig.** 3A), or upon natural infections using *Aspergillus fumigatus* or *Neurospora crassa* filamentous fungi (**Fig.** 3B-C). This over-expression study reveals that *BaraA* alone can partially rescue the susceptibility of *Imd, Toll* deficient flies to infection, and points to a more prominent role for *BaraA* in antifungal defence.

IM10-like peptides display antifungal activity in vitro

The Baramicin A gene encodes a polypeptide precursor that ultimately produces multiple mature peptides. However the most prominent *BaraA* products are the 23-residue IM10, 12, and 13 peptides (collectively the “IM10-like” peptides); indeed three IM10-like peptides are produced for every one IM24 peptide (**Fig.** 2B), and IM22 also bears an IM10-like motif (**Fig.** S3). This prompted us to explore the in vitro activity of the BaraA IM10-like peptides as potential AMPs.

We synthesized IM10, IM12, and IM13 and performed in vitro antimicrobial assays with these three IM10-like peptides using a 1:1:1 cocktail with a final concentration of 300μM (100 μM each of IM10, IM12, and IM13). We monitored the microbicidal activity of this peptide cocktail using a protocol adapted from Wiegand et al. (Wiegand et al., 2008). We did not detect any killing activity of our IM10-like peptide cocktail alone against *Pectobacterium carotovora Ecc15* (hereafter *"Ecc15"*), *Enterococcus faecalis,* or *C. albicans*. Previous studies have shown that the microbicidal activities of Abaecin-like peptides, which target the bacterial DNA chaperone *DnaK,* increase exponentially in combination with a membrane disrupting agent (Kragol et al., 2001a; Rabel et al., 2004a; Rahnamaeian et al., 2015a). Inspired by this approach, we next assayed combinations of the IM10-like cocktail with membrane-disrupting antibiotics relevant to tested microbes that should facilitate peptide entry into the cell. We again found no activity of IM10-like peptides against *Ecc15* or *E. faecalis* when co-incubated with a sub-lethal dose of Cecropin or Ampicillin respectively, indicating IM10-like peptides likely do not affect *Ecc15* or *E. faecalis* either alone or in combination with membrane-disrupting antibiotics. However, we observed a synergistic interaction between IM10-like peptides and the commercial antifungal Pimaricin against *C. albicans* (**Fig.** 3D). Co-incubation of the IM10-like cocktail with Pimaricin significantly improved the killing activity of Pimaricin at 16 and 32μg/mL relative to either treatment alone. While not statistically significant, the combination of IM10-like cocktail and Pimaricin also outperformed either the IM10-like cocktail alone or Pimaricin alone across the entire range of Pimaricin concentrations tested.

We next co-incubated dilute preparations of *B. bassiana* strain R444 spores under the same conditions as used previously with *C. albicans*, plated 2μL droplets, and assessed the diameters and corresponding surface area of colonies derived from individual spores after 4 days of growth at 25°C to assess growth rate. We found that neither the IM10-like cocktail nor Pimaricin alone significantly affected surface area relative to a PBS buffer control (Tukey's HSD: $p = 0.656$ and 0.466 respectively). However in combination, the IM10-like cocktail plus Pimaricin led to significantly reduced colony size compared to either treatment alone, corresponding to a 19-29% reduction in surface area relative to controls (**Fig.** 3E, Tukey's HSD: $p < .01$ in all cases). This indicates that incubation with IM10-like peptides and Pimaricin synergistically inhibits *B. bassiana* mycelial growth, revealing an otherwise cryptic antifungal effect of the BaraA IM10-like peptides in vitro.

Overall, we found that IM10-like peptides alone do not kill *C. albicans* yeast or impair *B. bassiana* mycelial growth in vitro. However, IM10-like peptides seem to synergize with the antifungal Pimaricin to inhibit growth of both of these fungi.

BaraA deficient flies broadly resist like wild-type upon bacterial infection

To further characterize *BaraA* function, we generated a null mutation of *BaraA* by replacing the entire *BaraA* locus with a dsRed cassette using CRISPR mediated homology-directed repair with fly stocks that contain only one *BaraA* gene copy (BDSC #2057 and BL51323) (**Fig.** 2A). After isolation, this mutation ($BaraA^{SW1}$) was then backcrossed once to a lab strain of w^{1118} (used in (Clemmons et al., 2015b; Cohen et al., 2020; Lindsay et al., 2018a)) to remove a second site mutation (see materials and methods). The resulting w^{1118}*;* $BaraA^{SW1}$ flies are hereon referred to as *"w; ΔBaraA*." As a consequence of this backcrossing event, *w; ΔBaraA* flies are a mixed genetic background, which we arbitrarily compare to *OR-R* as representative wild-type flies. Finally, the $BaraA^{SW1}$ mutation was isogenized by seven rounds of backcrossing into the w^{1118} *DrosDel* isogenic genetic background (*iso* w^{1118}) (Ryder et al., 2004b) as described in Ferreira et al. (2014) and are hereon referred to as "*iso ΔBaraA*" (Ferreira et al., 2014b). Relevant to this study, both our *OR-R* and *DrosDel iso* w^{1118} wild-type lines contain the *BaraA* duplication and thus have both *BaraA1 and A2* genes, while *w; ΔBaraA* and *iso ΔBaraA* flies lack *BaraA* entirely. In the following experiments, we compare the immune response of both *w; ΔBaraA* and *iso ΔBaraA* to wild-type flies, and focused on phenotypes that were consistent in both genetic backgrounds.

We validated these mutant lines by PCR, qPCR and MALDI-TOF peptidomics (**Fig.** 2A, supplementary data file 1). *BaraA*-deficient flies were viable with no morphological defects. Furthermore, *ΔBaraA* flies have wild-type Toll and Imd signalling responses following infection, indicating that *BaraA* is not required for the activation of these signaling cascades (**Fig.** S5A-C). *BaraA* mutant flies also survive clean injury like wild-type (**Fig.** S5D), and have comparable lifespan to wild-type flies (**Fig.** S5E). We next challenged *BaraA* mutant flies using our two genetic backgrounds with a variety of pathogens. We included susceptible Imd deficient Rel^{E20} flies, Toll deficient spz^{rm7} flies and *Bomanin* deficient $Bom^{\Delta 55C}$ flies as comparative controls. We observed that *BaraA* null flies have comparable resistance as wild-type to infection with the Gram-negative bacteria *Ecc15* and *Providencia burhodogranariea* (**Fig.** S6A-B), or with the Gram-positive bacterium *B. subtilis* (**Fig.** S6C). In contrast, we saw a mild increase in the susceptibility of *w; ΔBaraA* flies to infection by the Gram-positive bacterium *E. faecalis* (HR = +0.73, p = .014). We also saw an early mortality phenotype in *iso ΔBaraA* flies (at 3.5 days, p

< .001), although this was not ultimately statistically significant (**Fig.** S7A; p = .173). This trend of a mild susceptibility was broadly consistent in deficiency crosses and flies ubiquitously expressing *BaraA* RNAi (**Fig.** S7B-C), though none of these sets of survival experiments individually reached statistical significance. Overall, the susceptibility of *BaraA* mutants to *E. faecalis* is mild, but appears consistent using a variety of genetic approaches.

BaraA mutant flies are highly susceptible to Beauveria fungal infection

Entomopathogenic fungi such as *Metarhizium* and *Beauveria* represent an important class of insect pathogens (Lemaitre et al., 1997a). They have the ability to directly invade the body cavity by digesting and crossing through the insect cuticle. The Toll pathway is critical to survive fungal pathogens as it is directly responsible for the expression of *Bomanin*, *Daisho*, *Drosomycin* and *Metchnikowin* antifungal effectors (Clemmons et al., 2015b; Cohen et al., 2020; Fehlbaum et al., 1994a; Hanson et al., 2019a; Levashina et al., 1995a). The fact that i) *BaraA* is Toll-regulated, ii) BaraA IM10-like peptides display antifungal activity in vitro, and iii) *BaraA* overexpression improves the resistance of *Imd, Toll* deficient flies against fungi all point to a role for *BaraA* against fungal pathogens.

We infected *BaraA* mutant and wild-type flies using a septic injury model of *Metarhizium rileyi* strain PHP1705 (Andermatt Biocontrol). spz^{rm7} and $Bom^{\Delta 55C}$ mutant flies were highly susceptible to *M. rileyi* septic injury. Likewise, both *w; ΔBaraA* and *iso ΔBaraA* mutants showed a significant susceptibility to *M. rileyi* septic injury (**Fig.** 4A, HR ≥ 1.0 and p < .05 in both cases). We next rolled flies in sporulating *B. bassiana strain 802* petri dishes. Strikingly, both *w; ΔBaraA* and *iso ΔBaraA* flies displayed a pronounced susceptibility to natural infection with *B. bassiana* (HR = +2.10 or +0.96 respectively, p < .001 for both) (**Fig.** S8A). An increased susceptibility to fungi was also observed using flies carrying the *BaraA* mutation over a deficiency (**Fig.** S8B) or that ubiquitously express *BaraA* RNAi (**Fig.** S8C). Moreover, constitutive *BaraA* expression (*Act-Gal4>UAS-BaraA)* in an otherwise wild-type background improves survival to *B. bassiana 802* relative to *Act-Gal4>OR-R* controls (HR = -0.52, p = .010) (**Fig.** S8D). We next used a preparation of commercial *B. bassiana R444* spores (Andermatt Biocontrol) to perform controlled systemic infections by septic injury with a needle dipped in spore solution. In these experiments we monitored both survival and fungal load using qPCR primers specific to the *B. bassiana* 18S rRNA gene (Zhang et al., 2009). As seen with natural infection, *BaraA* mutants were highly susceptible to *Beauveria* systemic infection (**Fig.** 4B), and suffered increased fungal load by 48 hours after infection (**Fig.** 4C). We also compared the effect of *BaraA* in defence against *B. bassiana* to the effect of deleting two classical antifungal peptide genes of

Drosophila: *Metchnikowin (Mtk)* and *Drosomycin (Drs)*. Use of infection models with very different virulence (septic injury vs. natural infection) suggests that *BaraA* contributes far more strongly to defence against *B. bassiana* compared to the combined effect of *Mtk* and *Drs* (**Fig.** S8E), while *Mtk* and *Drs* did not greatly affect resistance relative to wild-type (HR = +0.15, p > .10).

Finally, we combined the *ΔBaraA* mutation with both a *UAS-BaraA* construct on the 2nd chromosome or our *BaraA-Gal4* driver on the 3rd chromosome to rescue the susceptibility of *BaraA* deficient flies. Supplementing *ΔBaraA* flies with *BaraA* expressed via the *BaraA-Gal4>UAS-BaraA* method restores resistance almost to wild-type levels (**Fig.** 4D). Collectively, our survival analyses point to a role for *BaraA* in defence against entomopathogenic fungi, including *M. rileyi* and especially *B. bassiana*. Consistent with a direct effect of *BaraA* on fungi, *BaraA* mutant susceptibility is correlated with increased proliferation of *B. bassiana*, and heterologous expression of *BaraA* via the Gal4/UAS system rescues the susceptibility of mutants, confirming that mutant susceptibility is caused by the loss of *BaraA*.

BaraA contributes to antifungal defence independent of Bomanins

Use of compound mutants carrying multiple mutations in effector genes has shown that some of them additively contribute to host resistance to infection (Hanson et al., 2019a). Compound deletions of immune genes can also reveal contributions of immune effectors that are not detectable via single mutant analysis (Binggeli et al., 2014; Dudzic et al., 2019; Hanson et al., 2019a). Recent studies have indicated that *Bomanins* play a major role in defence against fungi (Clemmons et al., 2015b; Lindsay et al., 2018a), though their mechanism of action is unknown. It is possible that *Bomanin* activity relies on the presence of *BaraA*, or vice versa. This prompted us to investigate the interaction of *Bomanins* and *BaraA* in defence against fungi. To do this, we recombined the $Bom^{\Delta 55C}$ mutation (that removes a cluster of 10 *Bomanin* genes) with *ΔBaraA.* Furthermore, we used low-virulence models of infection that allowed some *Bomanin* mutant flies to survive, so as to ensure additional mutation of *BaraA* had an opportunity to affect survival if relevant. While natural infection with *Aspergillus fumigatus* did not induce significant mortality in *BaraA* single mutants (**Fig.** S6D-E), we observed that combining *ΔBaraA* and $Bom^{\Delta 55C}$ mutations increases fly susceptibility to this pathogen relative to $Bom^{\Delta 55C}$ alone (HR = -0.46, p = .003; **Fig.** 5A). We next exposed these *ΔBaraA*, $Bom^{\Delta 55C}$, double mutant flies to a low dose natural infection with 30mg of commercial spores of *B. bassiana R444* as this dose allows some *Bomanin* mutant flies to survive. This is equivalent to approximately 60 million spores added to a vial containing 20 flies, many of which are

removed afterwards upon fly grooming. When using this infection method, we found that *BaraA* mutation markedly increases the susceptibility of $Bom^{\Delta 55C}$ mutant flies (HR = -0.89, p < .001), approaching spz^{rm7} susceptibility (**Fig.** 5B).

If BaraA and Bom peptides relied on each other for activity, we would expect no increased susceptibility of double mutants. However *BaraA, Bom* double mutation results in increased susceptibility relative to *Bom* mutation alone. We conclude *BaraA* acts independently of *Bomanins*, agreeing with the ability of heterologous overexpression of *BaraA* to rescue Toll, Imd double mutant flies that are similarly deficient in *Bomanin* production (**Fig.** 3A-C). Alongside a more prominent activity of *BaraA* in defence against *B. bassiana* compared to *Drs* and *Mtk* (**Fig.** S8D), these results suggest *BaraA* improves survival against fungi independent of other effectors of the systemic immune response also using effector mutant analysis, consistent with a direct effect on invading fungi.

ΔBaraA males display an erect wing phenotype upon infection

While performing natural infections with *A. fumigatus*, we observed a high prevalence of *BaraA* mutant flies with upright wings (**Fig.** 6A, **Fig.** S9A), a phenotype similar to the effect of disrupting the gene encoding the "*erect wing"* (*ewg*) transcription factor (DeSimone and White, 1993). Curiously, this erect wing phenotype was most specifically observed in males. Upon further observation, erect wing was observed not only upon *A. fumigatus* infection, but also upon infections with all Gram-positive bacteria and fungi tested, and less so upon clean injury or using Gram-negative bacteria (**S1 Table** and **Fig.** S9B-C). We eventually pursued this striking phenotype further using an *E. faecalis* septic injury model. A greater prevalence of erect wing flies was observed upon infection with live *E. faecalis* (**Fig.** 6B). Strikingly, even injury with heat-killed *E. faecalis* is sufficient to induce erect wing (**Fig.** 6C), collectively indicating that this phenotype is observed in *BaraA* mutants upon Toll pathway stimulation, but does not require a live infection.

Such a phenotype in infected males has never been reported, but is reminiscent of the wing extension behaviour of flies infected by the brain-controlling "zombie" fungus *Entomopthera muscae* (Elya et al., 2018). Intrigued by this phenotype, we further explored its prevalence in other genetic backgrounds. We next confirmed that this phenotype was also observed in other *BaraA*-deficient backgrounds such as *Df(BaraA)/ΔBaraA*; however the penetrance was variable from one background to another (**S1 Table**). Erect wing was also observed in *ΔBaraA*/+ heterozygous flies (*Df(BaraA)/+* or *ΔBaraA*/+), indicating that the lack of

BaraA on one chromosome was sufficient to cause the phenotype (**Fig.** S9D), independent of overall susceptibility to *E. faecalis* (**Fig.** S7B). Moreover, spz^{rm7} flies that lack functional Toll signalling phenocopy *ΔBaraA* flies and display erect wing, but other immune-deficient genotypes such as mutants for the Toll-regulated *Bomanin* effectors ($Bom^{\Delta 55C}$), or Rel^{E20} mutants that lack Imd signalling, did not readily display erect wing (**Fig.** 6B-C, **S1 Table**). Thus the erect wing phenotype is not linked to susceptibility to infection, but rather to loss of *BaraA* alongside stimuli triggering the Toll immune pathway. This phenotype suggests an additional effect of *BaraA* on tissues related to the wing muscle or in the nervous system.

The expression profile of *BaraA* is complex and poorly defined in existing transcriptomic datasets, likely owing to the gene duplication of *BaraA1* and *BaraA2* complicating read mapping (Hanson and Lemaitre, 2021; Schlamp et al., 2021). As *BaraA* is expressed in various tissues including the head/eye, crop, and fat body (**Fig.** 1 and (Robinson et al., 2013)), it is unclear if *BaraA* absence in the brain, neuromusculature, or non-neuronal tissues (such as the fat body) could underlie the predisposal to erect wing. To this end, we used stocks containing both the *ΔBaraA* mutation and either a *UAS-BaraA* construct, *c564-Gal4* constitutive fat body driver, or *BaraA-Gal4* driver, and performed genetic crosses to attempt to rescue the presentation of erect wing upon septic injury with heat-killed *E. faecalis* using the Gal4/UAS system. Surprisingly, constitutive *BaraA* expression in the fat body by *c564-Gal4* rescued erect wing presentation to effectively zero levels (**Fig.** 6D). On the other hand, *ΔBaraA, BaraA-Gal4>UAS-BaraA* flies displayed erect wing (exact genotype as in **Fig.** 4E), similar to *Df(BaraA)/+* and *ΔBaraA*/+ flies (**Fig.** S9D). Indeed, qPCR of *BaraA* expression after infection shows that *BaraA* levels are lower than wild-type in both *ΔBaraA, BaraA-Gal4>UAS-BaraA* (**Fig.** S9E) and *ΔBaraA*/+ transheterozygotes (**Fig.** S10).

Cumulatively, these experiments confirm that loss of *BaraA* results in the erect wing phenotype upon immune stimulus given *BaraA* deficiency, either by mutation or by loss of Toll signalling. This phenotype occurs independent of active infection, and is specifically tied to *BaraA* downstream of Toll signalling. A full transcriptional output of *BaraA* appears to be required to prevent erect wing after infection, as flies with less than wild-type *BaraA* expression are predisposed to displaying erect wing. However priming the hemolymph with BaraA peptides via constitutive expression in the fat body is sufficient to rescue the erect wing phenotype. Importantly, this rescue by fat body driven expression indicates that systemically secreted BaraA peptides mediate this phenotype, and not *BaraA* expression in e.g. neuronal tissue. Taken together, a wild-type induction of *BaraA* is required to prevent erect wing presen-

tation following Toll activation, which can be ameliorated by priming the hemolymph with constitutive *BaraA* expression.

3.5 Discussion

Seven *Drosophila* AMP families were identified in the 1980s-1990s either by homology with AMPs characterized in other insects or owing to their abundant production and microbicidal activities in vitro (Imler and Bulet, 2005a). In the 2000s, genome annotations revealed the existence of many additional paralogous genes from the seven well-defined families of AMPs (Hedengren et al., 2000; Khush and Lemaitre, 2000). At that time, microarray and MALDI-TOF analyses also revealed the existence of many more small immune-induced peptides, which may function as AMPs (Levy et al., 2004b; Uttenweiler-Joseph et al., 1998b). Genetic analyses using loss of function mutations have recently shown that some of these peptides do play an important role in host defence, however key points surrounding their direct microbicidal activities remain unclear. In 2015, *Bomanins* were shown to be critical to host defence using genetic approaches, but to date no activity in vitro has been found (Clemmons et al., 2015b; Lindsay et al., 2018a). The overt susceptibility of Bomanin mutants to most Gram-positive bacteria and fungi also suggests a generalist role in supporting the effectors of Toll, rather than a direct effect on microbes. In addition, two candidate AMPs, Listericin (Goto et al., 2010) and GNBP-like3 (Barajas-azpeleta et al., 2018b), have been shown to inhibit microbial growth upon heterologous expression using S2 cell lines or bacteria respectively. Most recently, Daisho peptides were shown to bind to fungal hyphae ex vivo, and are required for resisting *Fusarium* fungal infection in vivo (Cohen et al., 2020). However the mechanism and direct microbicidal activity of these various peptides at physiological concentrations has not yet been assessed.

In this study, we provide evidence from four separate experimental approaches that support adding *BaraA* products to the list of bona-fide antifungal peptides. First, the *BaraA* gene is strongly induced in the fat body upon infection resulting in abundant peptide production. *BaraA* is also tightly regulated by the Toll pathway, which orchestrates the antifungal response. Second, loss of function study shows that *BaraA* contributes to resistance against fungi. *BaraA* mutation increases susceptibility to *M. rileyi* and *B. bassiana*, and *BaraA* deficient flies suffer increased *B. bassiana* proliferation. Third, the antifungal activity of *BaraA* is independent of other key effectors. Over-expression of *BaraA* in the absence of Toll/Imd inducible peptides increased the resistance of compound *Rel, spz* deficient flies to various fungi includ-

ing *C. albicans*, *A. fumigatus*, and *N. crassa*, and rescues the *ΔBaraA* mutant susceptibility to *B. bassiana*. Additionally, compound gene deletion of both *BaraA and Bomanins* causes greater susceptibility than *Bomanin* mutation alone after *B. bassiana* natural infection. Fourth, and lastly, a cocktail of the *BaraA* IM10-like peptides possesses antifungal activity against *C. albicans* and *B. bassiana* in vitro when co-incubated with the membrane disrupting antifungal Pimaricin.

While it is difficult to estimate the concentration of BaraA peptides in the hemolymph of infected flies, it is expected based on MALDI-TOF peak intensities that the IM10-like peptides should reach concentrations similar to other AMPs (up to 100μM) (Ferrandon et al., 1998a; Hanson and Lemaitre, 2020); our in vitro assays used a peptide cocktail at the upper limit of this range. AMPs are often - but not exclusively – positively charged. This positive charge is thought to recruit these molecules to negatively charged membranes of microbes (Hanson and Lemaitre, 2020). That said, the net charges at pH=7 of the IM10-like peptides are: IM10 +1.1, IM12 +0.1, and IM13 -0.9. Given this range of net charge, IM10-like peptides are not overtly cationic. However some AMPs are antimicrobial without being positively charged, exemplified by human Dermicidin (Steffen et al., 2006) and anionic peptides of Lepidoptera that synergize with membrane-disrupting agents (Zdybicka-Barabas et al., 2012b). More extensive in vitro experiments with additional fungi and alternate membrane-disrupting antifungals (such as other insect or *Drosophila* antifungal peptides) should confirm the range of BaraA peptide activities. Furthermore, the potential activities of IM22 and IM24 should be addressed, which were not included in the present study. Future studies would benefit from testing different in vitro approaches, which might better mimic physiological conditions that could be relevant for BaraA peptide activity.

Our study also reveals that the *Baramicin A* gene alone produces at least 1/3 of the initially reported IMs. In addition to the IM10-like peptides and IM24 that were previously assigned to *BaraA* (Levy et al., 2004b), we show IM22 is encoded by the C terminus of *BaraA*, and is conserved in other *Drosophila* species. The production of multiple IMs encoded as tandem repeats between furin cleavage sites is built-in to the BaraA protein design akin to a "protein operon." Such tandem repeat organization is rare, but not totally unique among AMPs. This structure was first described in the bumblebee AMP Apidaecin (Casteels-Josson et al., 1993), and has since also been found in Drosocin of *Drosophila neotestacea* (Hanson et al., 2016). In *D. melanogaster*, several AMPs are furin-processed including Attacin C and its propeptide MPAC, wherein both parts synergize in killing bacteria (Rabel et al., 2004a). There-

fore, furin cleavage in Attacin C enables the precise co-expression of distinct peptides with synergistic activity. It is interesting to note that IM10-like peptides did not show antifungal activity in the absence of membrane disruption by Pimaricin. An attractive hypothesis is that longer peptides encoded by BaraA such as IM22 and IM24 could contribute to the antifungal activity of *BaraA* by membrane permeabilization, allowing the internalization of IM10-like peptides. However rigorous experimentation is needed to determine the IM10-like mechanism of action. Indeed, the BaraA IM24 peptide is a short Glycine-rich peptide (96 AA) that is positively-charged (charge +2.4 at pH=7). These traits are shared by amphipathic membrane-disrupting AMPs such as Attacins (Hanson and Lemaitre, 2020), however the precise role of the Baramicin IM24 domain is likely complex given the repeated evolution of neural-specific *Baramicins* that preferentially retain the IM24 domain (Hanson and Lemaitre, 2021).

An unexpected observation of our study is the display of an erect wing phenotype by *BaraA* deficient males upon infection. Our study suggests that this phenotype relies on the activation of the Toll pathway in the absence of *BaraA*. Erect wing is also induced by heat-killed bacteria, and is not observed in *Bomanin* or *Relish* mutants, indicating that the erect wing phenotype is not a generic consequence of susceptibility to infection. The *erect wing* gene, whose inactivation causes a similar phenotype, is a transcription factor that regulates synaptic growth in developing neuromuscular junctions (DeSimone and White, 1993). This raises the intriguing hypothesis that immune processes downstream of the Toll ligand Spätzle somehow affect wing neuromuscular junctions, and that *BaraA* modulates this activity. Another puzzling observation is the sexual dimorphism exhibited for this response. Male courtship and aggression displays involve similar wing extension behaviours. Koganezawa et al. (Koganezawa et al., 2010) showed that males deficient for *Gustatory receptor 32a (Gr32a)* failed to unilaterally extend wings during courtship display. *Gr32a*-expressing cells extend into the subesophageal ganglion where they contact mAL, a male-specific set of interneurons involved in unilateral wing display (Koganezawa et al., 2010). One possible explanation for the male specific effects of *BaraA* could be that *BaraA* mediates this effect through interactions with such male-specific neurons. Recent studies have highlighted how NF-κB signalling in the brain is activated by bacterial peptidoglycan (Kurz et al., 2017), and that immune effectors expressed either by fat body surrounding the brain or from within brain tissue itself affect memory formation (Barajas-azpeleta et al., 2018b). Moreover, an AMP of nematodes regulates aging-dependent neurodegeneration through binding to its G-protein coupled receptor, and this pathway is sufficient to trigger motor neuron degeneration following infection (Lezi et al., 2018a). The ability of fat body-derived *BaraA* to rescue the erect wing pheno-

type suggests a similar interplay of the immune response with neuromuscular processes. Future studies characterizing the role of *BaraA* in the erect wing phenotype should provide insight on interactions between systemic immunity and host physiology more generally.

Here we describe a complex immune effector gene that produces multiple peptide products. *BaraA* encodes many of the most abundant immune effectors induced downstream of the Toll signalling pathway. We show that *BaraA* has a pronounced effect on survival after *Beauveria* fungal infection. Moreover, this gene regulates an erect wing behavioural response upon infection. How each peptide contributes to the immune response and/or erect wing behaviour will be informative in understanding the range of effects immune effectors can have on host physiology. This work and others also clarifies how the cocktail of immune effectors produced upon infection acts specifically during innate host defence reactions.

3.6 Materials and Methods

Fly genetics and sequence comparisons

Sequence files were collected from FlyBase (Gramates et al., 2017) and recently-generated sequence data (Hanson et al., 2016; Hill et al., 2019) and comparisons were made using Geneious R10. Putative NF-κB binding sites were annotated using the Relish motif "GGRDNNHHBS" described in Copley et al. (Copley et al., 2007) and a manually curated amalgam motif of "GGGHHNNDVH" derived from common Dif binding sites described previously (Busse et al., 2007; Tanji et al., 2010). Gene expression analyses were performed using primers described in supplementary data file 1, and further microarray validation for *BaraA* expression comes from De Gregorio et al. (De Gregorio et al., 2002b).

The *UAS-BaraA* and *BaraA-Gal4* constructs were generated using the TOPO pENTR entry vector and cloned into the pTW or pBPGUw Gateway vector systems respectively. The *BaraA-Gal4* promoter contains 1675bp upstream of *BaraA1* (but also *BaraA2,* sequence in supplementary information). The *BaraA-Gal4* construct was inserted into the VK33 attP docking site (BDSC line #24871). The $BaraA^{SW1}$ (*ΔBaraA)* mutation was generated using CRISPR with two gRNAs and an HDR vector by cloning 5' and 3' region-homologous arms into the pHD-dsRed vector, and consequently *ΔBaraA* flies express dsRed in their eyes, ocelli, and abdomen. *ΔBaraA* was generated using the Bloomington stocks BL2057 and BL51323 as these backgrounds contain only one copy of the *BaraA* locus. The induction of the immune response in these flies was validated by qPCR and MALDI-TOF proteomics, wherein we discovered an

aberrant *Dso2* locus in these preliminary *BaraA^{SW1}* flies. We thus backcrossed the *BaraA^{SW1}* mutation once with a standard *w^{1118}* background (used in (Clemmons et al., 2015b; Cohen et al., 2020; Lindsay et al., 2018a)) and screened for wild-type *Dso2* before use in any survival experiments. As a consequence, *w; ΔBaraA* flies are considered an arbitrary genetic background with no appropriate wild-type control. We typically used *Oregon-R* (*OR-R*) flies as a representative wild-type that displays similar resistance to bacterial infections (**Fig.** S6). Of note, *ΔBaraA* was also isogenized into the *DrosDel w^{1118}* isogenic background for seven generations before use in isogenic fly experiments as described in Ferreira et al. (Ferreira et al., 2014b). We value the use of both genetic backgrounds to ensure that interpretation of mutant analysis is not biased by genetic background.

A full description of fly stocks used for crosses and in experiments is provided in supplementary data file 2.

Microbe culturing conditions

Bacteria and *C. albicans* yeast were grown to mid-log phase shaking at 200rpm in their respective growth media (Luria Bertani, Brain Heart Infusion, or Yeast extract-Peptone-Glycerol) and temperature conditions, and then pelleted by centrifugation to concentrate microbes. Resulting cultures were diluted to the desired optical density at 600nm (OD) for survival experiments, which is indicated in each figure. The following microbes were grown at 37°C: *Escherichia coli strain 1106* (LB)*, Enterococcus faecalis* (BHI)*,* and *Candida albicans* (YPG)*.* The following microbes were grown at 29°C: *Erwinia carotovora carotovora (Ecc15)* (LB) and *Micrococcus luteus* (LB). For filamentous fungi and molds, *Aspergillus fumigatus* was grown at 37°C, and *Neurospora crassa* and *Beauveria bassiana strain 802* were grown at room temperature on Malt Agar in the dark until sporulation. *Metarhizium rileyi strain PHP1705* and *Beauveria bassiana strain R444* commercial spores were produced by Andermatt Biocontrol, products: Nomu-PROTEC and BB-PROTEC respectively. A summary of microbe strains is provided in supplementary data file 2.

Survival experiments

Survival experiments were performed as previously described (Hanson et al., 2019a), with 20 flies per vial with 2-3 replicate experiments. 3-5 day old males were used in experiments unless otherwise specified. As *Rel, spz* double mutant flies and wild-type backgrounds differ drastically in their immune competence, we selected pathogens, infection routes, and temperatures to provide infection models that could best reveal phenotypes in these dispar-

ate genetic backgrounds. For fungi natural infections, flies were flipped at the end of the first day to remove excess fungal spores from the vials. Otherwise, flies were flipped thrice weekly. Statistical analyses were performed using a Cox proportional hazards (CoxPH) model in R 3.6.3. We report the hazard ratio (HR) alongside p-values as a proxy for effect size in survival experiments. Throughout our analyses, we required $p < .05$ as evidence to report an effect as significant, but note interactions with |HR| near or above 0.5 as potentially important provided p-value approached .05, and tamp down importance of interactions that were significant, but have relatively minor effect size (|HR| less than 0.5) in our discussion of the data.

Erect wing scoring

The erect wing phenotype was scored as the number of flies with splayed wings throughout a distinct majority of the period of observation (30s); if unclear, the vial was monitored an additional 30s. Here we define splayed wings as wings not at rest over the back, but did not require wings to be fully upright; on occasion wings were held splayed outward at ~45° relative to the dorsal view, and often slightly elevated relative to the resting state akin to male aggressive displays. Sometimes only one wing was extended, which occurred in both thoracic pricking and fungi natural infections; these flies were counted as having erect wing. In natural infections, the typical course of erect wing display developed in two fashions at early time points, either: i) flies beginning with wings slightly splayed but not fully upright, or ii) flies constantly flitting their wings outward and returning them to rest briefly, only to flit them outward again for extended periods of time. Shortly after infection, some flies were also observed wandering around with wings beating at a furious pace, which was not counted as erect wing. However at later time points erect wing flies settled more permanently on upright splayed wings. Erect wing measurements were taken daily following infection, and erect wing flies over total flies was converted to a percent. Data points in **Fig.** 6B-D represent % with erect wing in individual replicate experiments with 20-25 flies per vial. Flies stuck in the vial, or where the wings had become sticky or mangled were not included in totals. **S1 Table** reports mean percentages across replicate experiments for all pathogens and genotypes where erect wing was monitored. Days post-infection reported in **S1 Table** were selected as the final day prior to major incidents of mortality. For *E. faecalis* live infections, $Bom^{\Delta 55C}$ and spz^{rm7} erect wing was taken at 1dpi due to major mortality events by 2dpi specifically in these lines.

Erect wing measurements were performed in parallel with survival experiments, which often introduced injury to the thorax below the wing possibly damaging flight muscle. It is unlikely that muscle damage explains differences in erect wing display. First: we noticed

erect wing initially during natural infections with *A. fumigatus*, and observed erect wing upon *B. bassiana R444* and *Metarhizium rileyi PHP1705* natural infections (**S1 Table**). Second: only 1 of 75 total *iso w*^{1118} males displayed erect wing across 4 systemic infection experiments with *E. faecalis*. For comparison: 19 of 80 total *iso ΔBaraA* and 48 of 80 *w; ΔBaraA* flies displayed erect wing (**S1 Table**). Future studies might be better served using an abdominal infection mode, which can have different infection dynamics (Chambers et al., 2014). However we find erect wing display to be robust upon either septic injury or natural infection modes.

IM10-like peptide in vitro activity

The 23-residue Baramicin peptides were synthesized by GenicBio to a purity of >95%, verified by HPLC. An N-terminal pyroglutamate modification was included based on previous peptidomic descriptions of Baramicins IM10, IM12, and IM13 (Verleyen et al., 2006b), which we also detected in our LC-MS data (**Fig.** S2). Peptides were dissolved in DMSO and diluted to a working stock of 1200μM in 0.6% DMSO; the final concentration for incubations was 300μM in 0.15% DMSO. For microbe-killing assays, microbes were allowed to grow to log-growth phase, at which point they were diluted to ~50cells/μL (for *C. albicans* this was OD ≈ 0.01 in our hands). Two μL of culture (~100 cells), and 1μL water or antibiotic was mixed with 1μL of a 1:1:1 cocktail of IM10, IM12, and IM13 peptides to a final concentration of 300μM total peptides; 1μL of water + DMSO (final concentration = 0.15% DMSO) was used as a negative control. These 4μL microbe-peptide solutions were incubated for 24h at 4°C. Microbe-peptide cultures were then diluted to a final volume of 100μL and the entire solution was plated on LB agar or BiGGY agar plates. Colonies were counted manually. For combinatorial assays with bacteria, *C. albicans* yeast, and *B. bassiana R444* spores, peptide cocktails were combined with membrane disrupting antimicrobials effective against relevant pathogens beginning at: 10 μM Cecropin A (Sigma), 500μg/mL ampicillin, or 250μg/mL Pimaricin (commercially available as "Fungin," InVivogen), serially diluted through to 0.1 μM, 0.5μg/mL, and 4μg/mL respectively.

Beauveria bassiana R444 spores were prepared by dissolving ~30mg of spores in 10mL PBS, and then 4μL microbe-peptide solutions were prepared as described for *C. albicans* followed by incubation for 24h at 4°C; this spore density was optimal in our hands to produce distinct individual colonies. Then, 4μL PBS was added to each solution and 2μL droplets were plated on malt agar at 25°C. Colony diameters were measured 4 days after plating by manually analyzing colony diameters in InkScape v0.92. Experimental batches were included as covariates in one-way ANOVA analysis. The initial dataset approached violating Shapiro-Wilk

assumptions of normality (p = 0.061) implemented in R 3.6.3. We subsequently removed four colonies from the analysis, as these outliers had diameters over two standard deviations lower than their respective mean (removed colonies: PBS 15mm, PBS 25mm, IM10-like+Pimaricin 21mm, and a second IM10-like+Pimaricin colony of 21mm); the resulting Shapiro-Wilk p-value = 0.294, and both QQ and residual plots suggested a normal distribution. Final killing activities and colony surface areas were compared by One-way ANOVA with Holm-Sidak multiple test correction (*C. albicans*) and Tukey's honest significant difference multiple test correction (*B. bassiana R444*).

Gene expression analyses

RNA was extracted using TRIzol according to manufacturer's protocol. cDNA was reverse transcribed using Takara Reverse Transcriptase. qPCR was performed using PowerUP mastermix from Applied Biosystems at 60°C using primers listed in supplementary data file 1. Gene expression was quantified using the PFAFFL method (Pfaffl, 2001) with *Rp49* as the reference gene. Statistical analysis was performed by one-way ANOVA with Holm-Sidak's multiple test correction or student's t-test. Error bars represent one standard deviation from the mean.

Proteomic analyses

Raw hemolymph samples were collected from immune-challenged flies for MALDI-TOF proteomic analysis as described in (Cohen et al., 2020; Hanson et al., 2019a). MALDI-TOF proteomic signals were confirmed independently at facilities in both San Diego, USA and Lausanne, CH. In brief, hemolymph was collected by capillary and transferred to 0.1% TFA before addition to acetonitrile universal matrix. Representative spectra are shown. Peaks were identified via corresponding m/z values from previous studies (Levy et al., 2004b; Uttenweiler-Joseph et al., 1998b). Spectra were visualized using mMass, and figures were additionally prepared using Inkscape v0.92.

3.7 Author contributions and acknowledgements

Author contributions:

MAH planned experiments, performed bioinformatic analyses, infection experiments, and in vitro assays. BL supervised the project and MAH and BL wrote the manuscript. LC planned and generated the *BaraA* deletion and performed key descriptive experiments and observa-

tions. AM assisted with infection and in vitro assays. MH, II, and SAW generated and supplied other critical fly stock reagents and provided constructive commentary.

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3.8 References

1. Lemaitre B, Hoffmann J. The Host Defense of Drosophila Melanogaster. Annual Review of Immunology. 2007;25: 697–743. doi:10.1146/annurev.immunol.25.022106.141615
2. Kurz CL, Ewbank JJ. Caenorhabditis elegans: An emerging genetic model for the study of innate immunity. Nature Reviews Genetics. 2003;4: 380–390. doi:10.1038/nrg1067
3. Kaufmann SHE. Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. Nat Immunol. 2008;9: 705–712. doi:10.1038/ni0708-705
4. Steiner H, Hultmark D, Engström \AA, Bennich H, Boman HG. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature. 1981;292: 246–248. doi:10.1038/292246a0
5. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/Cactus controls the potent antifungal response in Drosophila adults. Cell. 1996;86: 973–983. doi:10.1016/S0092-8674(00)80172-5
6. Lemaitre B, Reichhart JM, Hoffmann JA. Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proceedings of the National Academy of Sciences of the United States of America. 1997;94: 14614–9. doi:10.1073/pnas.94.26.14614
7. Lemaitre B, Kromer-Metzger E, Michaut L, Nicolas E, Meister M, Georgel P, et al. A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense. Proc Natl Acad Sci U S A. 1995;92: 9465–9469. doi:10.1073/pnas.92.21.9465
8. Uttenweiler-Joseph S, Moniatte M, Lagueux M, Van Dorsselaer a, Hoffmann J a, Bulet P. Differential display of peptides induced during the immune response of Drosophila: a matrix-assisted laser desorption ionization time-of-

flight mass spectrometry study. Proceedings of the National Academy of Sciences of the United States of America. 1998;95: 11342–11347. doi:10.1073/pnas.95.19.11342
9. Lazzaro BP, Zasloff M, Rolff J. Antimicrobial peptides: Application informed by evolution. Science. 2020;368. doi:10.1126/science.aau5480
10. Hanson MA, Lemaitre B. New insights on Drosophila antimicrobial peptide function in host defense and beyond. Curr Opin Immunol. 2020;62: 22–30. doi:10.1016/j.coi.2019.11.008
11. De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. EMBO Journal. 2002;21: 2568–2579. doi:10.1093/emboj/21.11.2568
12. Lin SJH, Cohen LB, Wasserman SA. Effector specificity and function in Drosophila innate immunity: Getting AMPed and dropping Boms. PLoS Pathog. 2020;16: e1008480. doi:10.1371/journal.ppat.1008480
13. Clemmons AW, Lindsay SA, Wasserman SA. An Effector Peptide Family Required for Drosophila Toll-Mediated Immunity. PLoS Pathogens. 2015;11. doi:10.1371/journal.ppat.1004876
14. Lindsay SA, Lin SJH, Wasserman SA. Short-Form Bomanins Mediate Humoral Immunity in Drosophila. J Innate Immun. 2018;10: 306–314. doi:10.1159/000489831
15. Cohen LB, Lindsay SA, Xu Y, Lin SJH, Wasserman SA. The Daisho Peptides Mediate Drosophila Defense Against a Subset of Filamentous Fungi. Front Immunol. 2020;11: 9. doi:10.3389/fimmu.2020.00009
16. Hanson MA, Dostálová A, Ceroni C, Poidevin M, Kondo S, Lemaitre B. Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. eLife. 2019;8. doi:10.7554/elife.44341
17. Unckless RL, Howick VM, Lazzaro BP. Convergent Balancing Selection on an Antimicrobial Peptide in Drosophila. Current Biology. 2016;26: 257–262. doi:10.1016/j.cub.2015.11.063
18. Busse MS, Arnold CP, Towb P, Katrivesis J, Wasserman SA. A κB sequence code for pathway-specific innate immune responses. EMBO Journal. 2007;26: 3826–3835. doi:10.1038/sj.emboj.7601798
19. Copley RR, Totrov M, Linnell J, Field S, Ragoussis J, Udalova IA. Functional conservation of Rel binding sites in drosophilid genomes. Genome Research. 2007;17: 1327–1335. doi:10.1101/gr.6490707
20. Tanji T, Yun E-Y, Ip YT. Heterodimers of NF-κB transcription factors DIF and Relish regulate antimicrobial peptide genes in Drosophila. Proceedings of the National Academy of Sciences. 2010;107: 14715–14720. doi:10.1073/pnas.1009473107
21. Ferrandon D, Jung AC, Criqui MC, Lemaitre B, Uttenweiler-Joseph S, Michaut L, et al. A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. EMBO Journal. 1998;17: 1217–1227. doi:10.1093/emboj/17.5.1217
22. Schlamp F, Delbare SYN, Early AM, Wells MT, Basu S, Clark AG. Dense time-course gene expression profiling of the Drosophila melanogaster innate immune response. BMC Genomics. 2021;22: 304. doi:10.1186/s12864-021-07593-3
23. Robinson SW, Herzyk P, Dow JAT, Leader DP. FlyAtlas: database of gene expression in the tissues of Drosophila melanogaster. Nucleic Acids Research. 2013;41: D744–D750. doi:10.1093/nar/gks1141

24. Levy F, Rabel D, Charlet M, Bulet P, Hoffmann JA, Ehret-Sabatier L. Peptidomic and proteomic analyses of the systemic immune response of Drosophila. Biochimie. 2004;86: 607–616. doi:10.1016/j.biochi.2004.07.007
25. Hanson MA, Lemaitre B. Extensive duplication and convergent sequence evolution of an antimicrobial peptide gene. bioRxiv; 2021 Feb. doi:10.1101/2021.02.24.432738
26. Tzou P, Reichhart J-M, Lemaitre B. Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants. Proceedings of the National Academy of Sciences of the United States of America. 2002;99: 2152–2157. doi:10.1073/pnas.042411999
27. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols. 2008. doi:10.1038/nprot.2007.521
28. Rabel D, Charlet M, Ehret-Sabatier L, Cavicchioli L, Cudic M, Otvos L, et al. Primary Structure and in Vitro Antibacterial Properties of the Drosophila melanogaster Attacin C Pro-domain. Journal of Biological Chemistry. 2004;279: 14853–14859. doi:10.1074/jbc.M313608200
29. Rahnamaeian M, Cytry ska M, Zdybicka-Barabas A, Dobslaff K, Wiesner J, Twyman RM, et al. Insect antimicrobial peptides show potentiating functional interactions against Gram-negative bacteria. Proceedings of the Royal Society B: Biological Sciences. 2015;282: 20150293–20150293. doi:10.1098/rspb.2015.0293
30. Kragol G, Lovas S, Varadi G, Condie BA, Hoffmann R, Otvos L. The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. Biochemistry. 2001;40: 3016–3026. doi:10.1021/bi002656a
31. Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, Drummond J, et al. The DrosDel collection: A set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster. Genetics. 2004;167: 797–813. doi:10.1534/genetics.104.026658
32. Ferreira ÁG, Naylor H, Esteves SS, Pais IS, Martins NE, Teixeira L. The Toll-dorsal pathway is required for resistance to viral oral infection in Drosophila. PLoS Pathog. 2014;10: e1004507. doi:10.1371/journal.ppat.1004507
33. Levashina EA, Ohresser S, Bulet P, Reichhart J -M, Hetru C, Hoffmann JA. Metchnikowin, a Novel Immune-Inducible Proline-Rich Peptide from Drosophila with Antibacterial and Antifungal Properties. European Journal of Biochemistry. 1995;233: 694–700. doi:10.1111/j.1432-1033.1995.694_2.x
34. Fehlbaum P, Bulet P, Michaut L, Lagueux M, Broekaert WF, Hetru C, et al. Insect immunity: Septic injury of drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. Journal of Biological Chemistry. 1994;269: 33159–33163.
35. Zhang Y, Zhao J, Fang W, Zhang J, Luo Z, Zhang M, et al. Mitogen-Activated Protein Kinase hog1 in the Entomopathogenic Fungus Beauveria bassiana Regulates Environmental Stress Responses and Virulence to Insects. AEM. 2009;75: 3787–3795. doi:10.1128/AEM.01913-08
36. Dudzic JP, Hanson MA, Iatsenko I, Kondo S, Lemaitre B. More Than Black or White: Melanization and Toll Share Regulatory Serine Proteases in Drosophila. Cell Reports. 2019;27: 1050-1061.e3. doi:10.1016/j.celrep.2019.03.101

37. Binggeli O, Neyen C, Poidevin M, Lemaitre B. Prophenoloxidase Activation Is Required for Survival to Microbial Infections in Drosophila. PLoS Pathogens. 2014;10. doi:10.1371/journal.ppat.1004067
38. DeSimone SM, White K. The Drosophila erect wing gene, which is important for both neuronal and muscle development, encodes a protein which is similar to the sea urchin P3A2 DNA binding protein. Mol Cell Biol. 1993;13: 3641–3649. doi:10.1128/MCB.13.6.3641
39. Elya C, Lok TC, Spencer QE, McCausland H, Martinez CC, Eisen M. Robust manipulation of the behavior of Drosophila melanogaster by a fungal pathogen in the laboratory. eLife. 2018;7: e34414. doi:10.7554/eLife.34414
40. Imler J-L, Bulet P. Antimicrobial peptides in Drosophila: structures, activities and gene regulation. Chemical immunology and allergy. 2005;86: 1–21. doi:10.1159/000086648
41. Hedengren M, Borge K, Hultmark D. Expression and evolution of the Drosophila attacin/diptericin gene family. Biochemical and biophysical research communications. 2000;279: 574–81. doi:10.1006/bbrc.2000.3988
42. Khush RS, Lemaitre B. Genes that fight infection: what the Drosophila genome says about animal immunity. Trends Genet. 2000;16: 442–449. doi:10.1016/s0168-9525(00)02095-3
43. Goto A, Yano T, Terashima J, Iwashita S, Oshima Y, Kurata S. Cooperative regulation of the induction of the novel antibacterial Listericin by peptidoglycan recognition protein LE and the JAK-STAT pathway. J Biol Chem. 2010;285: 15731–15738. doi:10.1074/jbc.M109.082115
44. Barajas-azpeleta R, Wu J, Gill J, Welte R. Antimicrobial peptides modulate long-term memory. PLoS Genetics. 2018; 1–26. doi:10.1371/journal.pgen.1007440
45. Steffen H, Rieg S, Wiedemann I, Kalbacher H, Deeg M, Sahl H-G, et al. Naturally processed dermicidin-derived peptides do not permeabilize bacterial membranes and kill microorganisms irrespective of their charge. Antimicrob Agents Chemother. 2006;50: 2608–2620. doi:10.1128/AAC.00181-06
46. Zdybicka-Barabas A, Mak P, Klys A, Skrzypiec K, Mendyk E, Fiołka MJ, et al. Synergistic action of Galleria mellonella anionic peptide 2 and lysozyme against Gram-negative bacteria. Biochim Biophys Acta. 2012;1818: 2623–2635. doi:10.1016/j.bbamem.2012.06.008
47. Casteels-Josson K, Capaci T, Casteels P, Tempst P. Apidaecin multipeptide precursor structure: a putative mechanism for amplification of the insect antibacterial response. The EMBO journal. 1993;12: 1569–78. doi:10.1002/j.1460-2075.1993.tb05801.x
48. Hanson MA, Hamilton PT, Perlman SJ. Immune genes and divergent antimicrobial peptides in flies of the subgenus Drosophila. BMC evolutionary biology. 2016;16: 228. doi:10.1186/s12862-016-0805-y
49. Koganezawa M, Haba D, Matsuo T, Yamamoto D. The Shaping of Male Courtship Posture by Lateralized Gustatory Inputs to Male-Specific Interneurons. Current Biology. 2010;20: 1–8. doi:10.1016/j.cub.2009.11.038
50. Kurz CL, Charroux B, Chaduli D, Viallat-Lieutaud A, Royet J. Peptidoglycan sensing by octopaminergic neurons modulates Drosophila oviposition. Elife. 2017;6. doi:10.7554/eLife.21937
51. Lezi E, Zhou T, Koh S, Chuang M, Sharma R, Pujol N, et al. An Antimicrobial Peptide and Its Neuronal Receptor Regulate Dendrite Degeneration in Aging and Infection. Neuron. 2018;97: 125-138.e5. doi:10.1016/j.neuron.2017.12.001

52. Gramates LS, Marygold SJ, Dos Santos G, Urbano JM, Antonazzo G, Matthews BB, et al. FlyBase at 25: Looking to the future. Nucleic Acids Research. 2017;45: D663–D671. doi:10.1093/nar/gkw1016
53. Hill T, Koseva BS, Unckless RL. The genome of Drosophila innubila reveals lineage-specific patterns of selection in immune genes. Molecular Biology and Evolution. 2019. doi:10.1093/molbev/msz059
54. Chambers MC, Jacobson E, Khalil S, Lazzaro BP. Thorax injury lowers resistance to infection in Drosophila melanogaster. Infect Immun. 2014;82: 4380–4389. doi:10.1128/IAI.02415-14
55. Verleyen P, Baggerman G, D'Hertog W, Vierstraete E, Husson SJ, Schoofs L. Identification of new immune induced molecules in the haemolymph of Drosophila melanogaster by 2D-nanoLC MS/MS. Journal of Insect Physiology. 2006;52: 379–388. doi:10.1016/j.jinsphys.2005.12.007
56. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29: e45. doi:10.1093/nar/29.9.e45
57. Huang J, Lou Y, Liu J, Bulet P, Jiao R, Hoffmann JA, et al. The BaramicinA gene is required at several steps of the host defense against Enterococcus faecalis and Metarhizium robertsii in a septic wound infection model in Drosophila melanogaster. bioRxiv; 2020 Nov. doi:10.1101/2020.11.23.394809
58. Tyler-Cross R, Schirch V. Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. J Biol Chem. 1991;266: 22549–22556.

3.9 Figures and Tables chapter 3

3.9.1 Main figures

Figures 3.1-3.6 and supplemental figures and text are as presented in the published article.

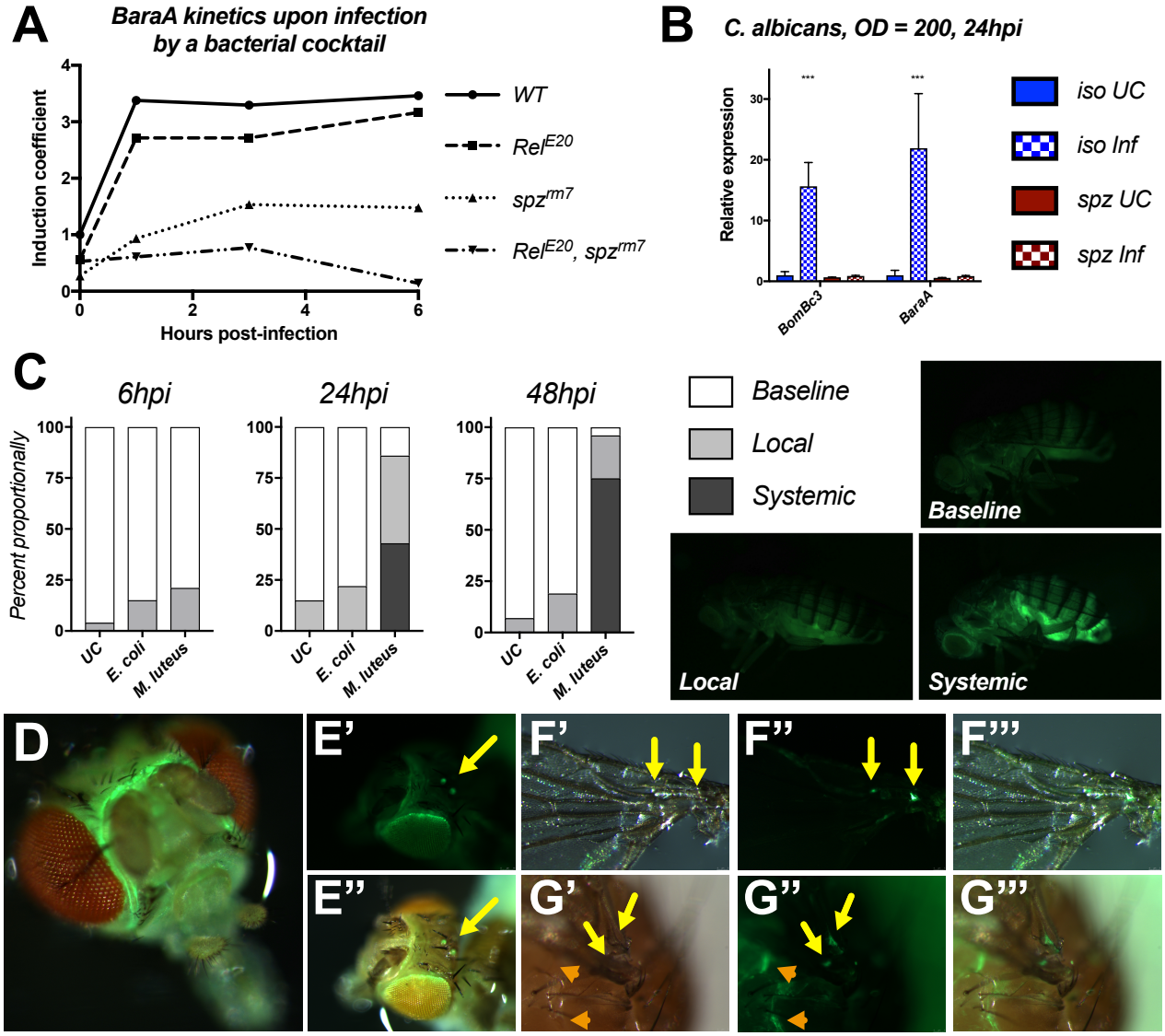


Fig 1. *BaraA* is an immune-induced gene regulated by the Toll pathway. A) Expression profile of *BaraA* upon bacterial challenge by a mixture of *E. coli* and *M. luteus* (from De Gregorio et al. [11]). Induction coefficient reports a Log_{10}-fold calculation then normalized to unchallenged wild-type expression levels (see De Gregorio et al. [11]). **B)** *BaraA* expression profiles in wild-type and spz^{rm7} flies upon septic injury with the yeast *C. albicans*. *BomBc3* is used as an inducible control gene for the Toll pathway. Significance relative to *iso-UC* indicated as *** = $p < .001$. Additional gene expression measurements are shown in S1B and S1C Fig. **C)** Use of a *BaraA* reporter reveals that *BaraA* induction upon infection is primarily driven by the fat body in adults, and results in a strong and systemic GFP signal upon pricking with OD = 200 *M. luteus* (stimulating the Toll pathway), but less so by *E. coli* (stimulating the Imd pathway) 24hpi and 48hpi (χ^2 $p < .001$, N = 82). **D-G)** Baseline *BaraA>mGFP* is highly expressed in the head **(D)**, at the border of the eyes and in the ocelli **(E)**, in the wing veins (**F-G** yellow arrows), and beneath the cuticle in the thorax (**G**, orange arrowheads).

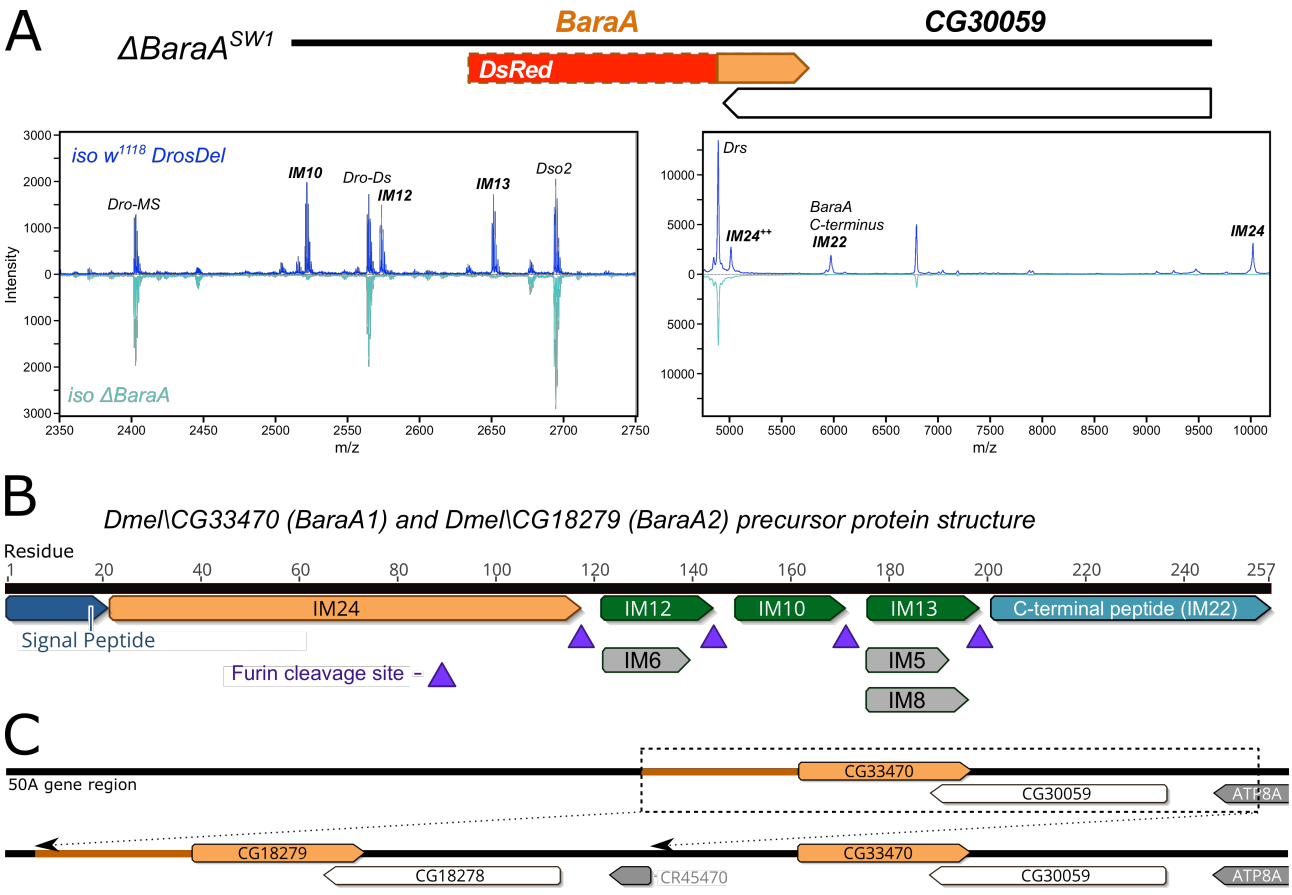


Fig 2. The *BaraA* gene structure. A) MALDI-TOF analysis of hemolymph from *iso w*^{1118} wild-type and *iso ΔBaraA* flies 24 hours post-infection (hpi) confirms that *BaraA* mutants fail to produce the IM10-like and IM24 peptides. *iso ΔBaraA* flies also fail to produce an immune-induced peak at ~5795 Da corresponding to IM22 (the C-terminal peptide of BaraA, see S1 Text). A diagram of the *ΔBaraA*^{SW1} mutation that replaces the N-terminal gene region with a DsRed construct is shown in the bottom right. **B)** The *BaraA* gene encodes a precursor protein that is cleaved into multiple mature peptides at RXRR furin cleavage sites. The sub-peptides IMs 5, 6, and 8 are additional minor cleavage products of IM12 and IM13. IM22 is additionally cleaved following its GIND motif (S2 Fig and S3A). **C)** There is a *BaraA* locus duplication event present in the Dmel_R6 reference genome. This duplication is not fixed in laboratory stocks and wild-type flies [25]. The *ΔBaraA* mutation was generated in a background with only one *BaraA* copy.

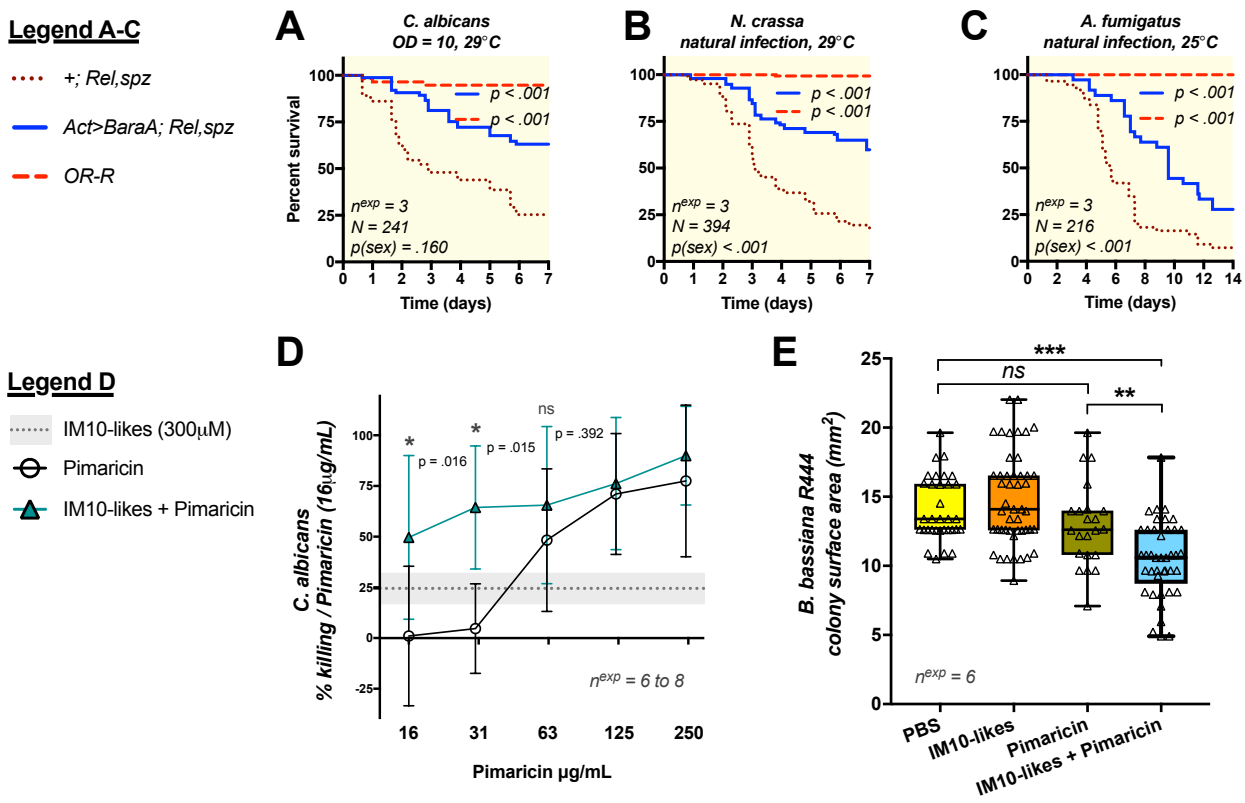


Fig 3. Overexpression of *BaraA* partially rescues the susceptibility of *Rel, spz* flies against fungi and BaraA IM10-like peptides inhibit fungal growth in vitro. A-C) Overexpression of *BaraA* (*Act>BaraA)* rescues the susceptibility of *Rel*, *spz* flies upon systemic infection with *C*. *albicans* **(A)**, or natural infection with either *N*. *crassa* or *A*. *fumigatus* **(B-C)**. Survivals represent pooled results from males and females (see S4 Fig for sex-specific survival curves). **D)** A 300μM cocktail of the three IM10-like peptides improves the killing activity of the antifungal Pimaricin against *C*. *albicans* yeast. Error bars and the shaded area (IM10-likes alone) represent ±1 standard deviation from the mean. Killing activity (%) was compared against no-peptide controls, then normalized to the activity of Pimaricin alone. **E)** The IM10-like peptide cocktail also synergizes with Pimaricin (250μg/mL) to inhibit mycelial growth of *B*. *bassiana strain R444*. The diameters of individual colonies of *B*. *bassiana* were assessed after four days of growth at 25°C after peptide treatment, and surface area calculated as πr^2.

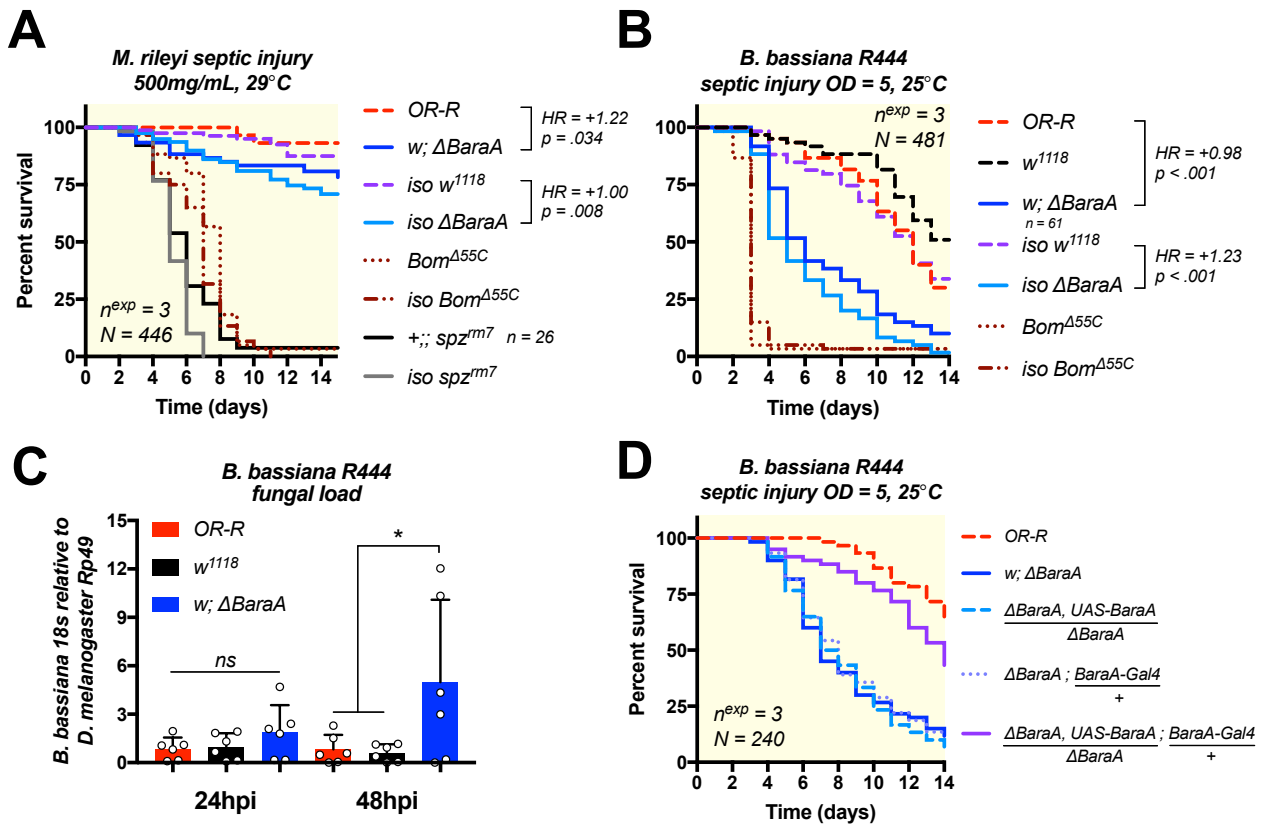


Fig 4. *ΔBaraA* flies are susceptible to fungal infection. A) *BaraA* mutants in two genetic backgrounds (here called *w* or *iso*) display a significant susceptibility to septic injury with *M. rileyi*. **B-C)** Increased susceptibility of *ΔBaraA* flies upon septic injury with *B. bassiana R444* **(B)** correlates with increased fungal load 48hpi **(C)**. **D)** Heterologous expression of *BaraA* via combination of the *BaraA-Gal4* and *UAS-BaraA* constructs rescues the susceptibility of *BaraA* mutant females to *B. bassiana* infection.

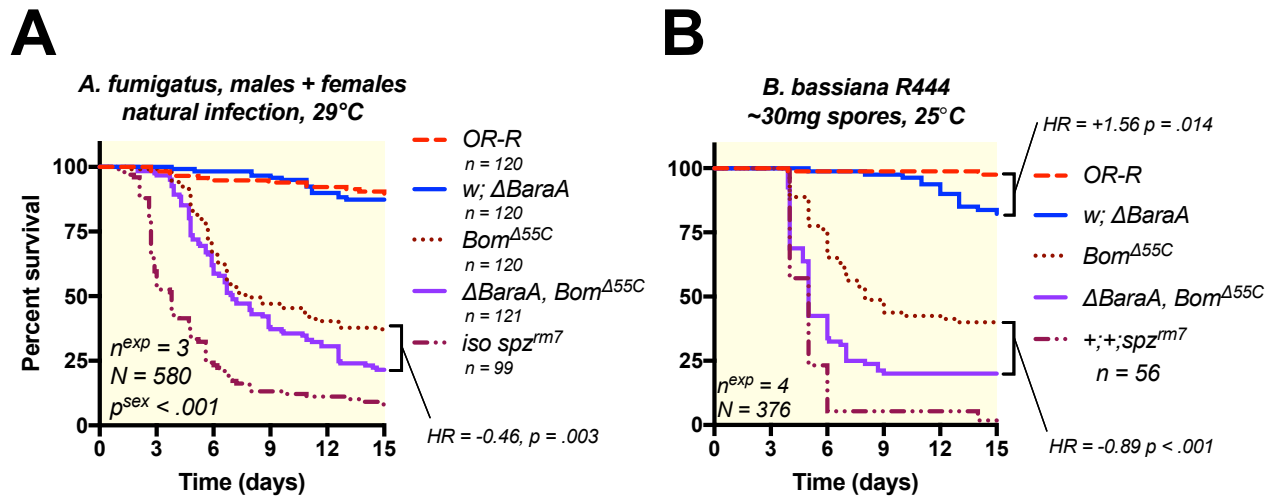


Fig 5. *BaraA* contributes to antifungal defence independent of other effectors. A) *ΔBaraA, Bom*$^{\Delta 55C}$ double mutant flies were more susceptible than either mutation alone to natural infection with *A. fumigatus* (see S6D and S6E Fig for sex-specific survival curves). **B)** *ΔBaraA, Bom*$^{\Delta 55C}$ double mutant flies were similarly more susceptible than individual mutants when given a mild (30mg of spores) *Beauveria* natural infection using *B. bassiana R444*.

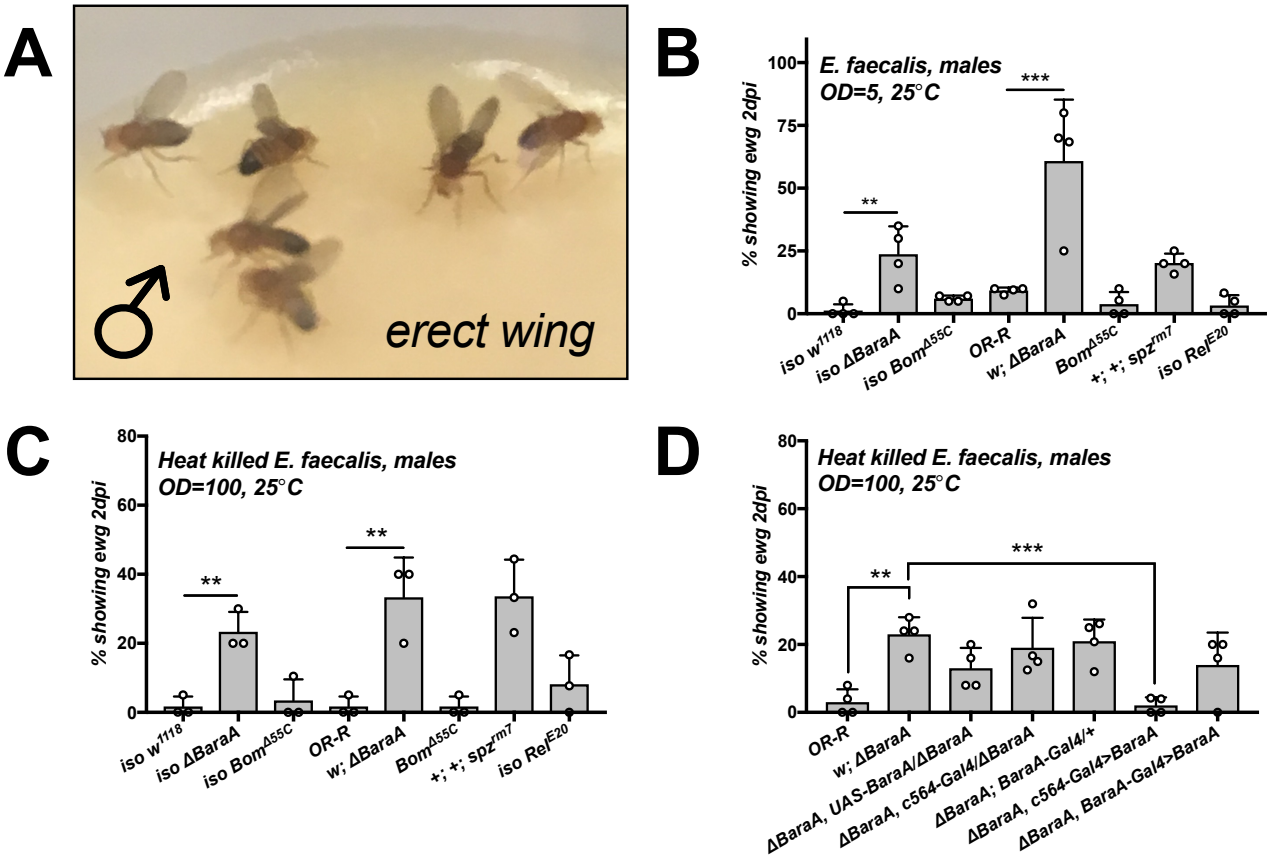
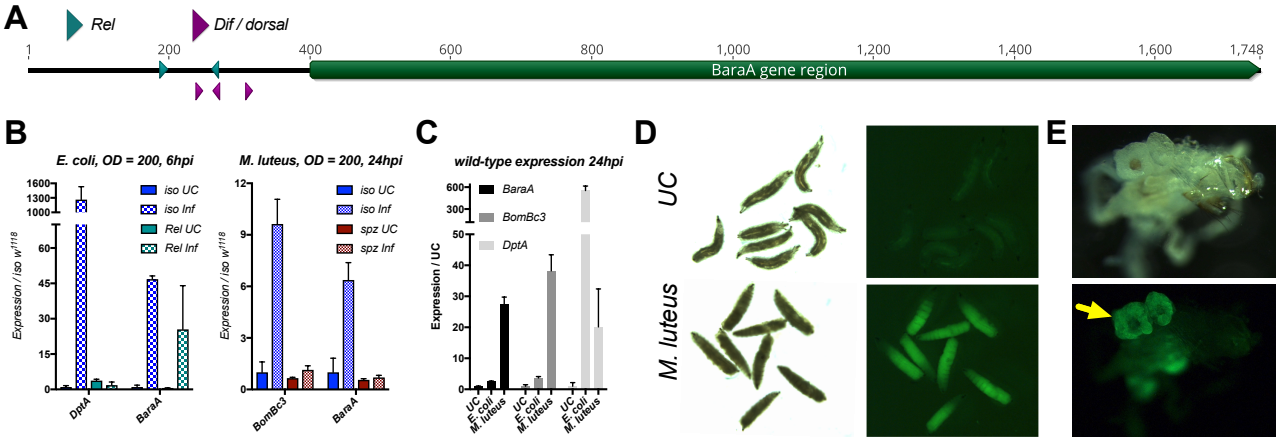
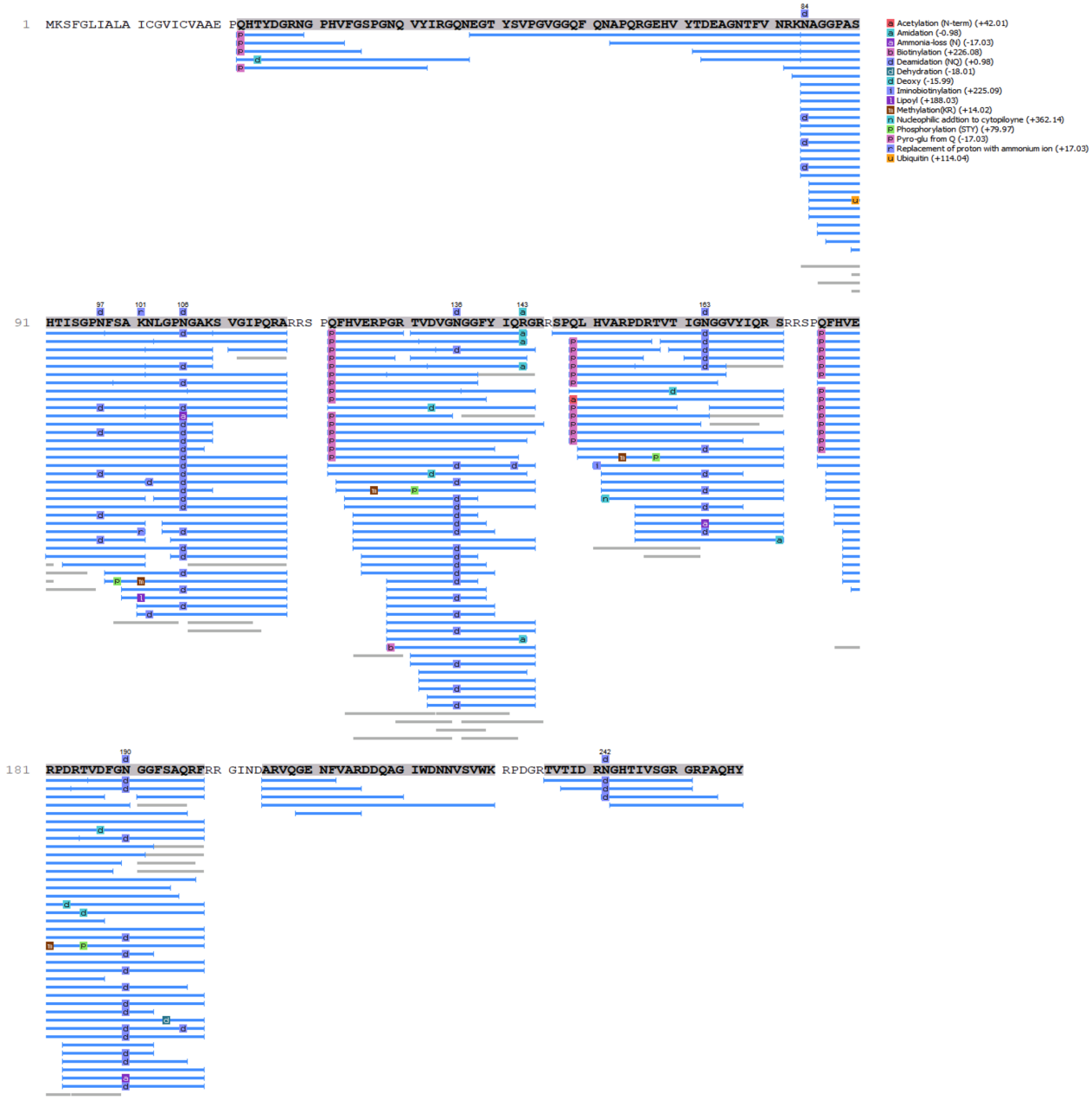


Fig 6. *ΔBaraA* males display an erect wing phenotype upon infection. A) *ΔBaraA* males displaying erect wing six days after *A. fumigatus* natural infection. **B-C)** spz^{rm7} and *ΔBaraA* males, but not $Bom^{\Delta 55C}$ or Rel^{E20} flies display the erect wing phenotype upon septic injury with live **(B)** or heat-killed *E. faecalis* **(C)**. **D)** The presentation of erect wing in *ΔBaraA* flies is rescued by c564-Gal4 ubiquitous expression of *BaraA*. Barplots show the percentage of flies displaying erect wing following treatment, with individual data points reflecting replicate experiments. Asterisks indicate one-way ANOVA significance relative to reference *w; ΔBaraA* flies (**, and *** = p < .01, and.001 respectively). Erect wing frequency after additional challenges are shown in S9 Fig and S1 Table.

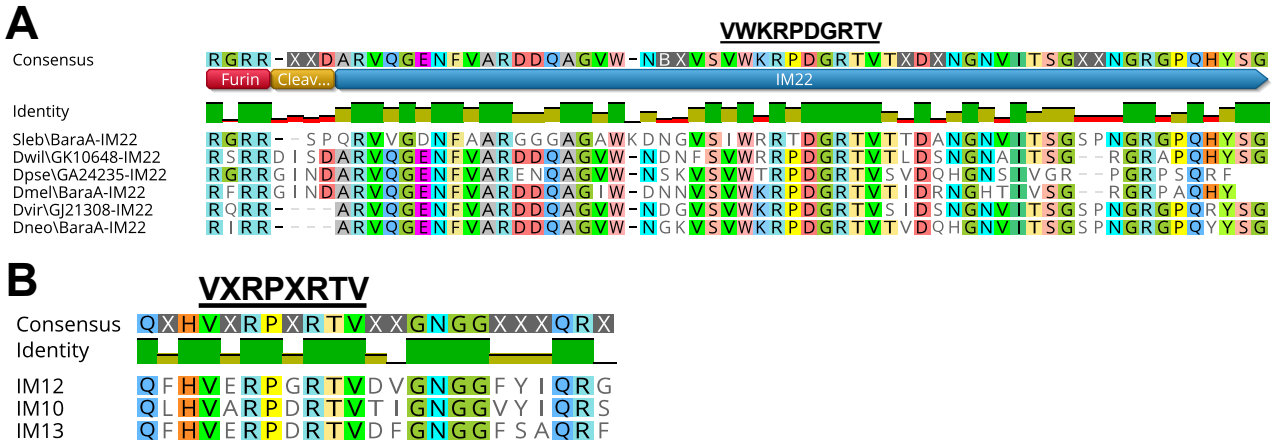
3.9.2 Supplementary figures and tables



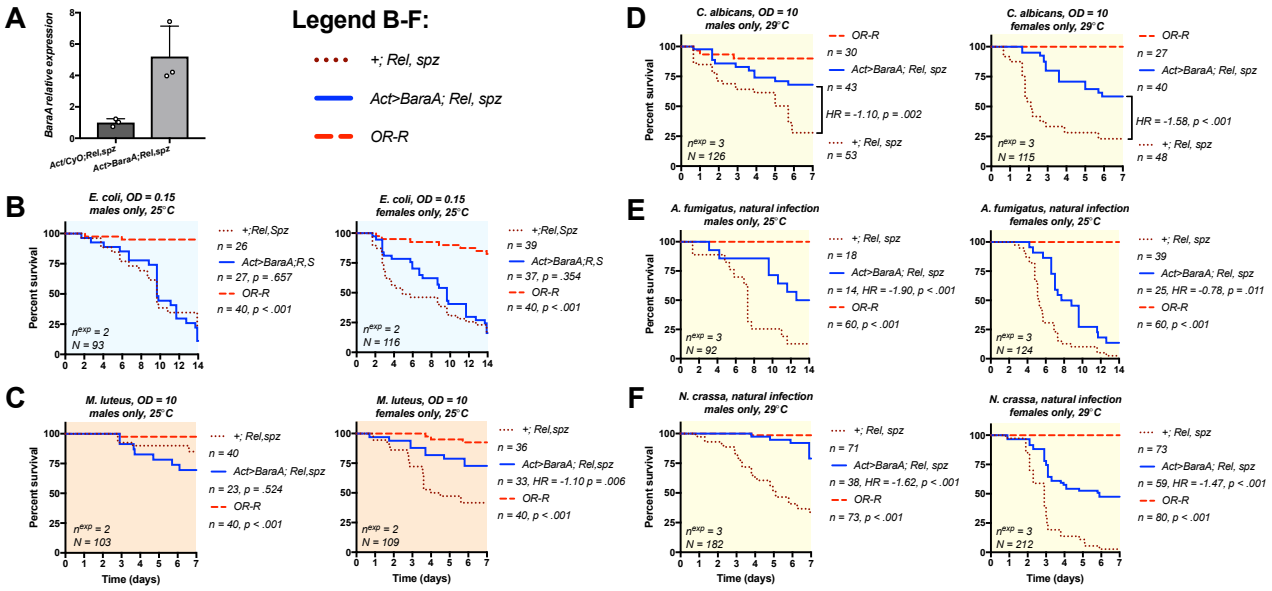
S1 Fig. Supplemental *BaraA* expression patterns. A) 400bp of upstream sequence from *BaraA* annotated with putative *Rel* or *Dif/dl* binding sites (included in S1 Data). B) Expression of *BaraA in wild-type* and *spz*^{rm7} *flies* following injury with the Gram-negative bacterium *E. coli* or the Gram-positive bacterium *M. luteus*. As seen in a previous microarray (Fig 1A), basal *BaraA* expression is depressed in *Rel*^{E20} flies, but is nevertheless highly induced upon infection, likely representing the *BaraA* response to injury. C) In a separate set of experiments, *BaraA* returns to near-baseline levels of expression by 24hpi using *E. coli*. Meanwhile *BaraA* remained induced after pricking with *M. luteus*, mirroring the Toll-regulated *BomBc3* but not the Imd-regulated *DptA*. D) The *BaraA>mGFP* reporter line shows a robust induction of GFP 2hpi upon pricking with *M. luteus* in larvae. E) Expression of *BaraA>mGFP* in the spermatheca of females (yellow arrow). Representative images shown.



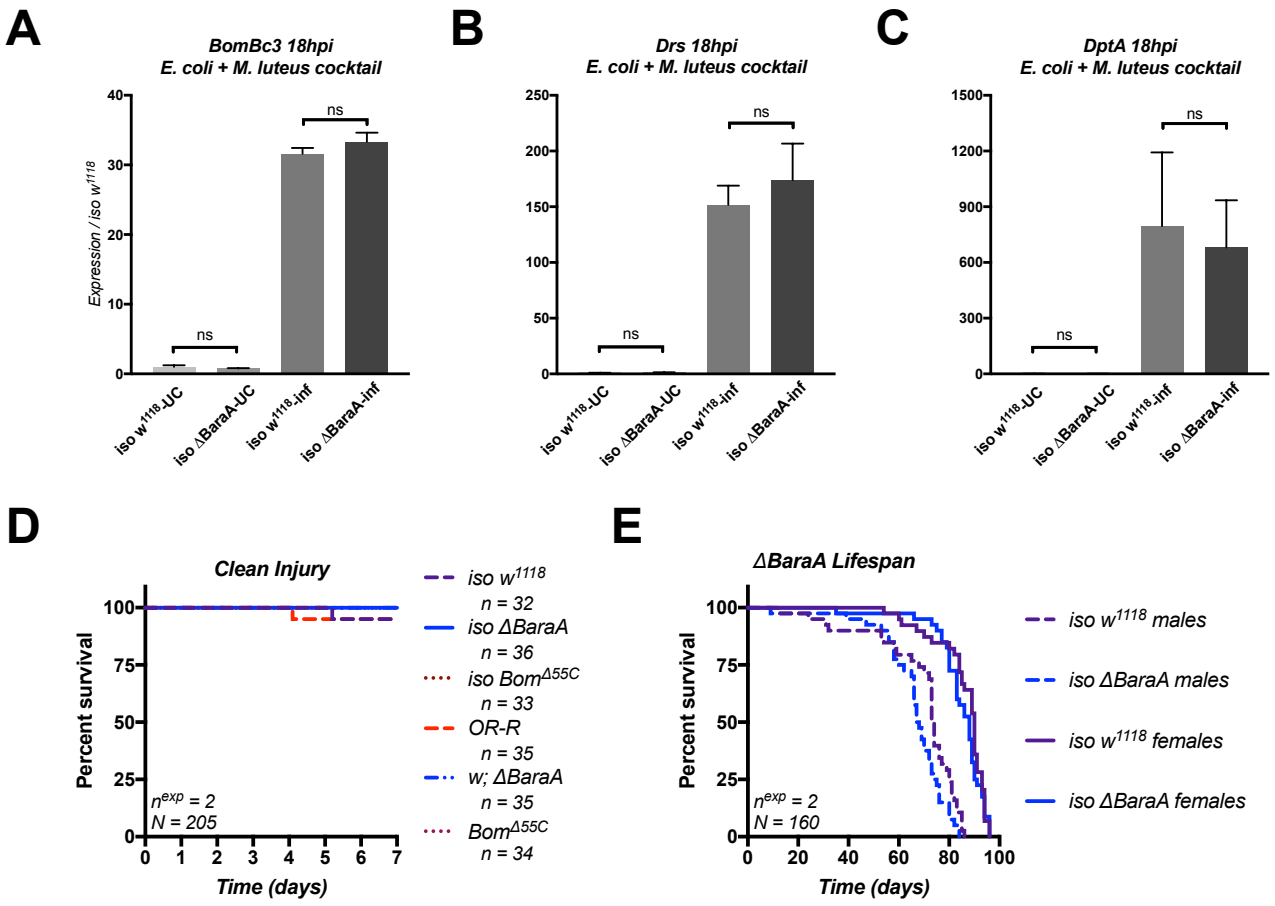
S2 Fig. LCMS coverage of trypsin-digested and detected BaraA peptides aligned to the protein coding sequence. Detected peptide fragments (blue bars) cover the whole precursor protein barring furin site-associated motifs. Additionally, two peptide fragments are absent: i) the first 4 residues of the C-terminus ("GIND," not predicted *a priori*), and ii) the C-terminus peptide's "RPDGR" motif, which is predicted as a degradation product of Trypsin cleavage and whose size is beyond the minimum range of detection. Without the GIND motif, the mass of the contiguous C-terminus is 5974.5 Da, matching the mass observed by MALDI-TOF for IM22 (Fig 2A). The N-terminal Q residues of IM10, IM12, IM13, and IM24 are pyroglutamate-modified, as described previously [24]. The Asparagine residues of IM10-like peptides are sometimes deamidated, likely as a consequence of our 0.1% TFA sample collection method as "NG" motifs are deamidated in acidic conditions [58].



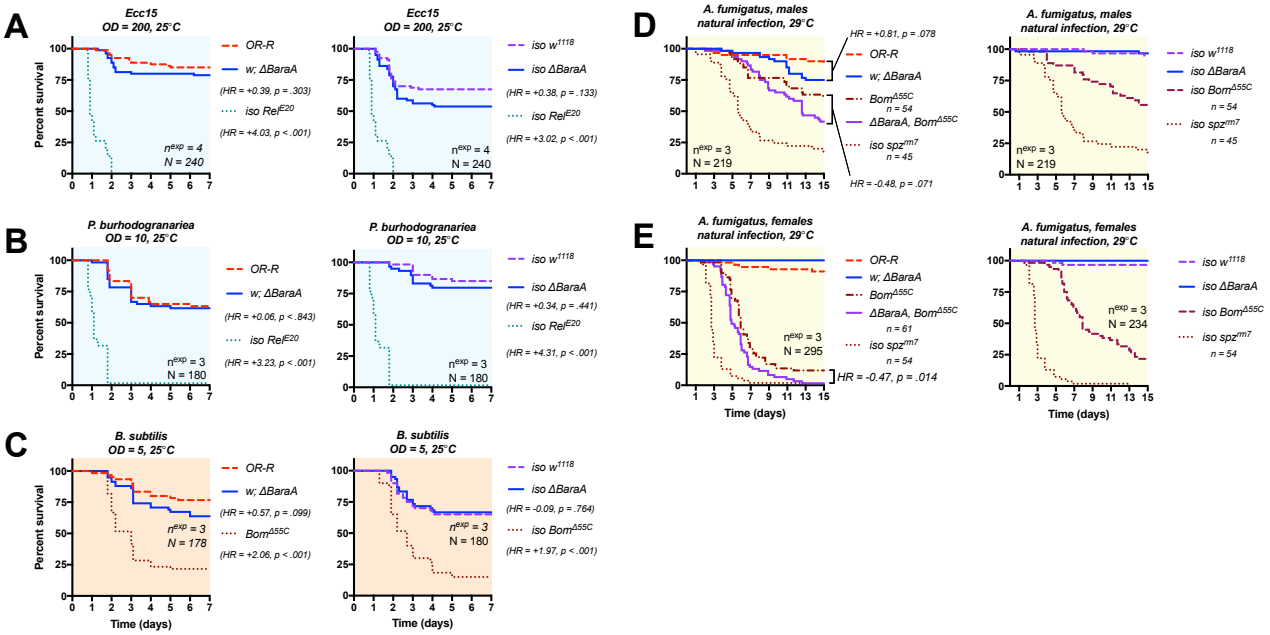
S3 Fig. Alignments of BaraA peptide motifs. A) Aligned IM22 peptides of *Drosophila Baramicin A-like* genes, with the IM10-like 'VWKRPDGRTV' motif noted. The GIND residues at the N-terminus are cleaved off in *Dmel\BaraA* by an unknown process, and this subsequent peptide is similarly cleaved following RXRR furin cleavage sites in subgenus Drosophila flies. As a consequence, the mature IM22 peptide is predicted to be the same across species even when different cleavage mechanisms are utilized. **B)** Alignment of the three IM10-like peptides of *D. melanogaster BaraA* with the "VXRPXRTV" motif noted. The residue 8 polymorphism of either G (IM12) or D (IM10, IM13) has evolved repeatedly in outgroup flies [25], indicating it is likely key for IM10-like peptide activity.



S4 Fig. Over-expression of *BaraA* partially rescues *Rel, spz* double mutant susceptibility to infection in both males and females. A) Validation of the *UAS-BaraA* construct in the *Rel, spz* background. Flies were unchallenged. B) Overexpressing *BaraA* did not improve the survival of *Rel, spz* flies upon *E. coli* infection. C) Overexpressing *BaraA* only marginally improves survival of *Rel, spz* females, but not males, upon *M. luteus* infections. Infections using a higher dose (OD = 100) tended to kill 100% of *Rel, spz* flies regardless of sex or expression of *BaraA*, suggesting that if *BaraA* overexpression does affect susceptibility to *M. luteus*, this effect is possible within only a narrow window of *M. luteus* concentration. D-F) Overexpressing *BaraA* improves survival of *Rel, spz* male and female flies upon injury with *C. albicans* (D) or natural infection with *A. fumigatus* (E) and *N. crassa* (F). P-values are shown for each biological sex in an independent CoxPH model not including the other sex relative to *Rel, spz* as a reference.

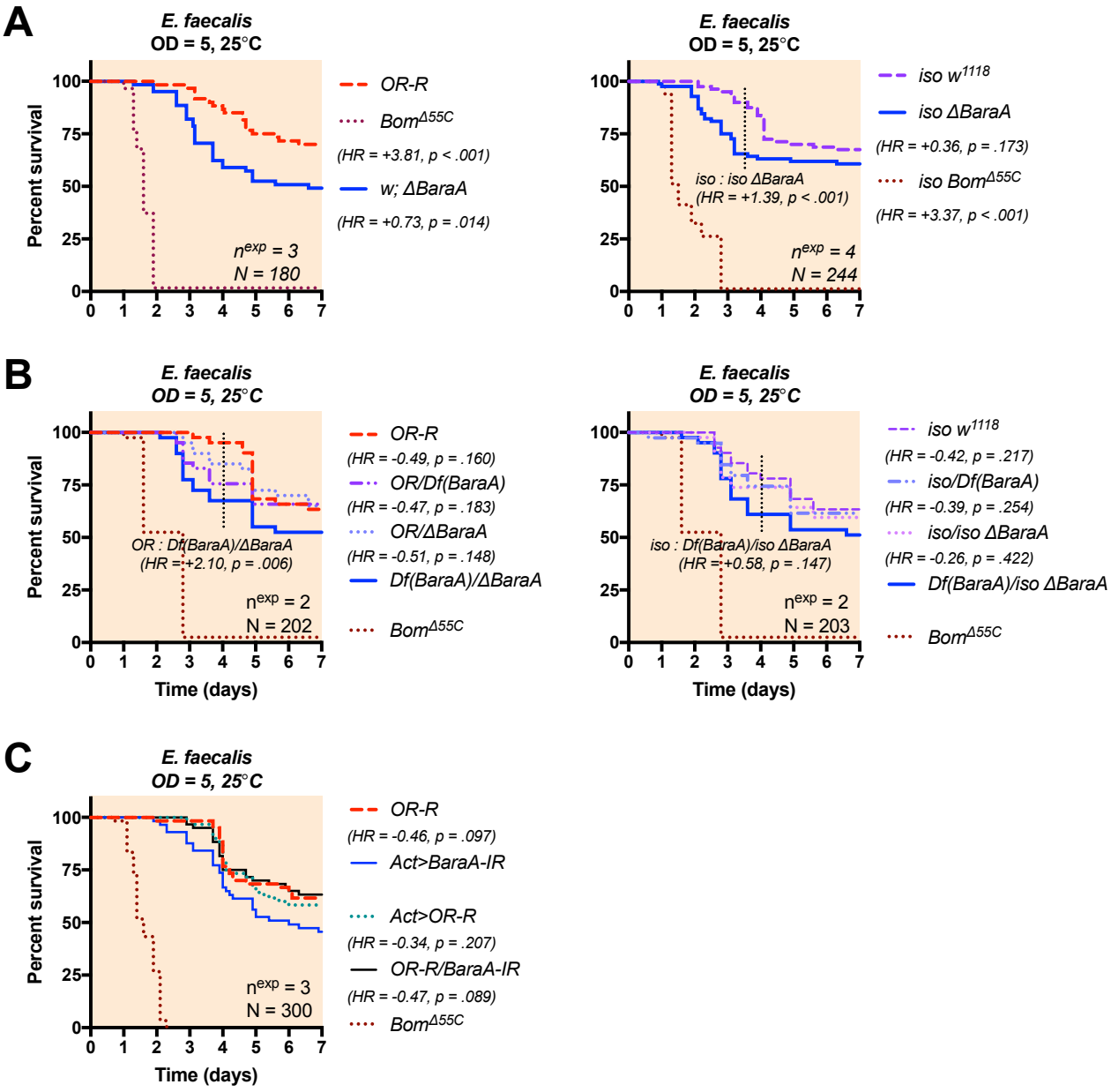


S5 Fig. RT-qPCR shows that the expression of *BomBc3* (A) *Drs* (B) and *DptA* (C) is wild-type 18hpi in *iso ΔBaraA* flies. D) *BaraA* mutants survive clean injury like wild-type flies. E) *iso ΔBaraA* flies have similar lifespan compared with the *iso w*^{1118} wild-type (males + females, *iso* vs. *iso ΔBaraA*: HR = 0.26, p = .118)

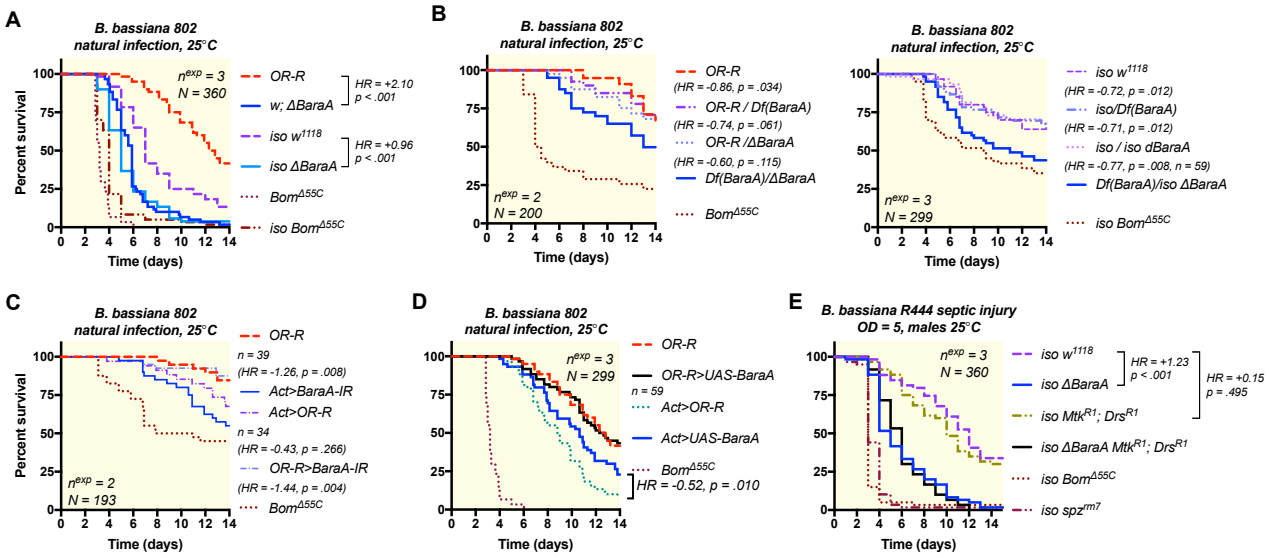


S6 Fig. **Additional survivals using *ΔBaraA* flies in two distinct genetic backgrounds upon infection by a diversity of microbes.** A-B) No significant susceptibility of *ΔBaraA* flies to *Ecc15* (A), *P. burhodogranariea* (B), or *B. subtilis* (C), bacterial infections. D-E) *w;*

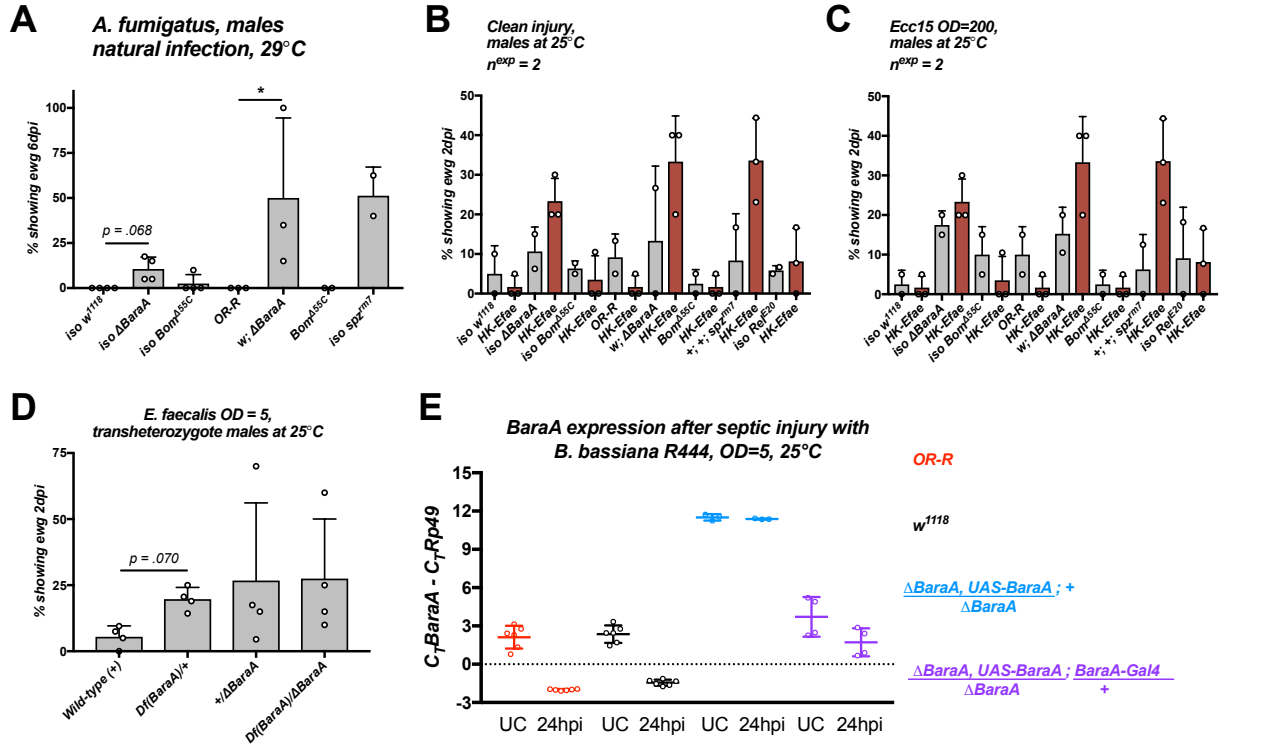
ΔBaraA males were slightly susceptible to *A. fumigatus* natural infection (HR > 0.5, p = .078), but not females, nor isogenic flies. Additional infections using *ΔBaraA*, $Bom^{\Delta 55C}$ double mutant flies reveals that *BaraA* mutation increases the susceptibility of $Bom^{\Delta 55C}$ flies in both males and females (cumulative curves shown in Fig 5A). Blue backgrounds = Gram-negative bacteria, orange backgrounds = Gram-positive bacteria, yellow backgrounds = fungi.



S7 Fig. Survival analysis suggests a minor contribution of *BaraA* to defence against infection by *E. faecalis*. **A)** *w; ΔBaraA* but not *iso ΔBaraA* flies are significantly susceptible to *E. faecalis*. However we note that *iso ΔBaraA* flies suffer an earlier mortality than *iso* w^{1118} wild-type controls that is highly significant if the experiment is artificially censored at 3.5 days (dotted line and associated statistics). **B)** Crosses with a genomic deficiency (*Df(BaraA)*) leads to increased susceptibility in both the *w* background and isogenic DrosDel background, with *Df(BaraA)/ΔBaraA* flies suffering the greatest mortality in either crossing scheme. Both deficiency crosses yielded an earlier susceptibility in *BaraA*-deficient flies (shown with dotted black lines), however neither experiment ultimately reached statistical significance. **C)** *BaraA* RNAi flies (*Act>BaraA-IR*) suffered greater mortality than *Act>OR-R* or *OR-R/BaraA-IR* controls, but this was not statistically significant at α = .05; p-values reported are comparisons to *Act>BaraA-IR* flies.

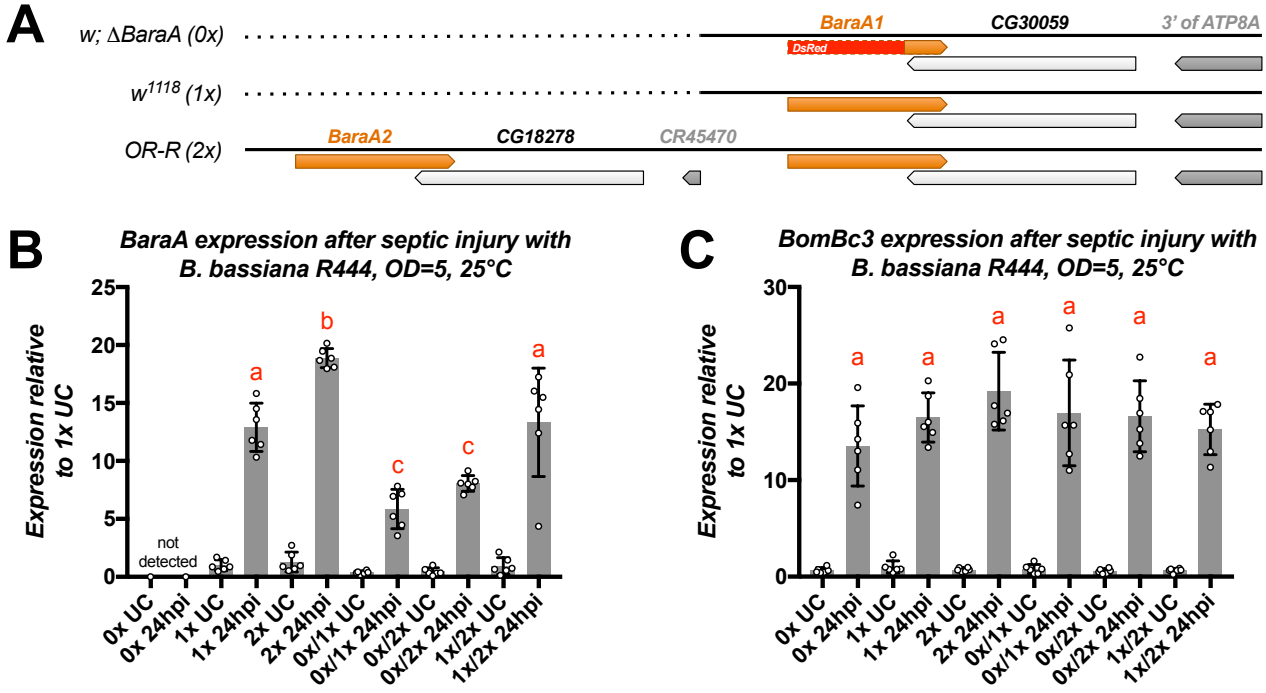


S8 Fig. Additional survival analyses reveal a consistent contribution of *BaraA* to defence against infection by *B. bassiana*. A) *BaraA* mutants in both backgrounds are highly susceptible to natural infection with the entomopathogenic fungus *B. bassiana 802*. B) Crossing with a genomic deficiency (*Df(BaraA)*) leads to increased susceptibility of *Df(BaraA)/ΔBaraA* flies for both the *w* background and isogenic DrosDel background relative to wild-type controls ($p < .05$) upon *B. bassiana 802* natural infection. C) *Act>BaraA-IR* flies were more susceptible than the *OR-R* wild-type ($p = .008$) and *OR>BaraA-IR* ($p = .004$), although not significantly different from our *Act>OR-R* control ($p = .266$). D) Overexpressing *BaraA* (*Act>UAS-BaraA*) improved survival against *B. bassiana 802* relative to *Act>OR-R* controls (HR = -0.52, $p = 0.010$). E) *BaraA* alone contributes to survival against *B. bassiana* to a far greater extent than the two canonical antifungal peptide genes *Mtk* and *Drs*, which in fact had little effect on survival outcome.



S9 Fig. Frequency of erect wing display following additional challenges. A) Erect wing occurs in flies given natural infection with *A. fumigatus*, wherein flies do not readily succumb to infection (S6D Fig) and no thoracic injury was introduced. B-C) Erect

wing frequencies 2dpi after clean injury (B), or *Ecc15* septic injury (C). The erect wing frequencies of flies pricked by HK-*E. faecalis* (Fig 6C) are included in brown to facilitate direct comparison with the frequency observed upon Toll pathway activation. D) The frequency of erect wing display is increased following *E. faecalis* septic injury in *ΔBaraA/+* or *Df(BaraA)/+* flies. Data points are pooled from *w; ΔBaraA* and *iso ΔBaraA* crosses after *E. faecalis* infections shown in S7A Fig and data in S1 Table. E) C_T*BaraA*-C_T*Rp49* (ΔC_T) non-normalized expression of the *BaraA-Gal4>UAS-BaraA* method to better visualize expression level differences. This Gal4/UAS approach rescues *BaraA* expression in *ΔBaraA* flies, though not quite to wild-type levels. A very low level of expression was observed in *ΔBaraA, UAS-BaraA/ΔBaraA* flies without the Gal4 (indicating a tiny level of UAS leakiness), while *BaraA* was never detected in *w; ΔBaraA* flies. Differences in this ΔC_T y-axis effectively equate to Log2 expression differences. The level of *BaraA* induction in these *ΔBaraA, BaraA-Gal4>UAS-BaraA* was ~3.3x the unchallenged state by 24hpi.



S10 Fig. *ΔBaraA/+* transheterozygotes suffer significantly reduced *BaraA* expression. A) Schematic detailing the *BaraA* loci of genotypes used in transheterozygote crosses. B-C) *BaraA* (B) and *BomBc3* (C) expression after *B. bassiana* pricking in *BaraA* homozygous or heterozygous flies. Transheterozygotes with one mutant locus have significantly reduced *BaraA* expression. Intriguingly, *OR-R* flies (homozygous for 2 gene copies) have higher *BaraA* expression levels compared to w^{1118} (1 gene copy) after infection (B), which appears to be unrelated to the activation of the Toll response generally as *BomBc3* levels were comparable across genotypes (C). Instead, *OR-R* flies seemingly reach a slightly greater absolute expression (S9E Fig). Statistically significant differences at 24hpi are indicated by red letters, to facilitate complex multiple comparisons (one-way ANOVA with Holm-Sidak's multiple test correction). Genotypes with the same letter group are not significantly different from each other. In all cases, no significant differences were observed amongst unchallenged flies.

S1 Table. Erect wing frequencies from various infection experiments. Following initial erect wing observations after *A. fumigatus* natural infection, we scored erect wing frequency in all subsequent survival experiments. Data represent the mean % of males displaying erect wing ± one standard deviation. n exp = number of replicate experiments performed, and dpi ewg taken = days post-infection where erect wing data were recorded. We additionally performed natural infections with *Metarhizium rileyi* that generally did not cause significant mortality even in *ΔBaraA*, $Bom^{\Delta 55C}$ double mutant males, but nevertheless induced erect wing specifically in *ΔBaraA* males and spz^{rm7} controls. Bacterial infections were performed by septic injury, while fungal challenges were either natural infections (NI) performed by rolling flies in spores or septic injuries as indicated. Underlying data are included in S5 Data.

Treatment			***Percent (%) males showing ewg***									
Pathogen	**n exp**	**dpi ewg taken**	***iso DrosDel***	***iso ΔBaraA***	***iso*** $Bom^{\Delta 55C}$	***iso*** spz^{rm7}	***OR-R***	***w; ΔBaraA***	$Bom^{\Delta 55C}$	***+; +;*** spz^{rm7}	***iso*** Rel^{E20}	$Bom^{\Delta 55C}$***, ΔBaraA***
Clean Injury	2	2	5.0 ± 7.1	10.6 ± 6.2	6.3 ± 1.9		9.2 ± 5.9	13.3 ± 18.9	2.6 ± 3.7	8.3 ± 11.2	5.8 ± 1.2	
P. burhodogranariea	3	2	1.7 ± 2.9	5.5 ± 0.4			2.0 ± 3.4	16.4 ± 5.8			3.0 ± 5.2	
Ecc15	2	2	2.5 ± 3.5	17.5 ± 3.5	10.0 ± 7.1		10.0 ± 7.1	15.3 ± 6.7	2.5 ± 3.5	6.3 ± 8.8		
HK-E. faecalis (OD100)	3	2	1.7 ± 2.9	23.3 ± 5.8	3.5 ± 6.1		1.7 ± 2.9	33.3 ± 11.6	1.7 ± 2.9	33.6 ± 10.1	8.1 ± 8.3	
E. faecalis (OD5)	4	2	1.3 ± 2.5	23.8 ± 11.1	6.1 ± 1.2		10.5 ± 3.2	60.9 ± 24.4	3.8 ± 4.8	20.2 ± 3.8	3.4 ± 4.1	
B. subtilis	1	2	0.0	30.0	5.0		25.0	40.0	5.0			
A. fumigatus	4 or indicated	6	0.0 ± 0.0	10.6 ± 6.6	2.5 ± 5.0	51.3 ± 15.9 (n=2)	0.0 ± 0.0 (n=3)	50 ± 44.5 (n=3)	0.0 ± 0.0 (n=2)			5.0 ± 7.1 (n=2)
B. bassiana 802 (sporulating plate)	3	3	10.0 ± 0.0	31.5 ± 24.5	11.5 ± 16.0							
B. bassiana R444 (NI)	3 or indicated	5	1.67 ± 2.58 (n=6)	13.44 ± 8.13 (n=6)	3.48 ± 4.17 (n=6)		1.7 ± 1.0	15.0 ± 8.6	0.0 ± 0.0	22.2 ± 15.6		14.3 ± 10.0
B. bassiana R444 (septic injury)	3	2	1.7 ± 2.9	16.7 ± 5.8	13.7 ± 6.4	16.1 ± 11.2						
M. rileyi PHP1705 (NI)	4 or indicated	5	0.0 ± 0.0 (n=2)	15.0 ± 7.1 (n=2)	2.8 ± 3.9 (n=2)		2.8 ± 3.3	18.1 ± 13.8	0.0 ± 0.0	11.2 ± 8.8		23.2 ± 11.9
M. rileyi PHP1705 (septic injury)	3 or indicated	3				23.5 (n=1)	6.7 ± 2.9	15.9 ± 9.0	2.0 ± 3.4	57.1 (n=1)		12.4 ± 2.3

3.9.3 Supplementary text

Identification of the BaraA C-terminus as IM22 from Uttenweiler-Joseph et al.

In 1998, Uttenweiler-Joseph et al. (Uttenweiler-Joseph et al., 1998b) described 24 immune-induced molecules by MALDI-TOF and informed predictions suggested that *BaraA* could encodes several of them (Levy et al., 2004b). We generated a knock out mutant for the *BaraA* gene ($BaraA^{SW1}$), which we validated by MALDI-TOF peptidomic analysis. Strikingly, we noticed an immune-induced peak at ~5981 Da in Linear mode collections that is absent in *ΔBaraA* flies (**Fig.** 2A); this mass closely resembled the 5984 Da estimated mass of IM22 from Uttenweiler-Joseph et al. (Uttenweiler-Joseph et al., 1998b), for which sequence was never determined. We took the Linear masses reported for then-unknown IMs from Uttenweiler-Joseph et al. (Uttenweiler-Joseph et al., 1998b) and post-hoc generated a standard curve with now-confirmed mass values from Levy et al. (Levy et al., 2004b). Our post-hoc standard curve corrects the mass of IM22 as found in Uttenweiler-Joseph et al. (Uttenweiler-Joseph et al., 1998b) to be 5973.5 Da. Using the same approach with our own linear data we find a mass of 5975.1 Da for our 5981 Da peak (supplementary data file 3). With LCMS proteomics, we confirmed that the *BaraA* C-terminus is cleaved to remove 4 N-terminal residues, which should produce a putative 5974.5 Da peptide (**Fig** S2). Together these observations indicate the *BaraA* C-terminus encodes the following 53-residue mature peptide, matching the estimated mass of IM22: ARVQGENFVA RDDQAGIWDN NVSVWKRPDG RTVTIDRNGH TIVSGRGRPA QHY.

The *BaraA* gene is therefore involved in the production of over one third of the classical *Drosophila* IMs from Uttenweiler-Joseph et al. (Uttenweiler-Joseph et al., 1998b), including: IM5, 6, 8, 10, 12, 13, 20 (doubly-charged IM24 (Levy et al., 2004b)), 22, and 24.

Sequence of the BaraA-Gal4 promoter construct

The following 1675bp sequence was cloned from the DrosDel isogenic background into the pBPGUw vector to drive a downstream Gal4 gene, and inserted into the VK33 attP docking site using BDSC line #24871:

Dif/dorsal binding site **(bold)***:* **GGGHHNNDVH**

Rel binding site (underline): GGRDNNHHBS

>iso_DrosDel_BaraA_promoter-Gal4
CTGCTACTCCTCTACACATTCGACTCCTTCGCCTTGCTGGCTG-
GAAAAAATTTTCATAATTATGTGGGTGCCGCGCACACGGAGGTCCCGACGGAT-
TCGAAGTATCCGAAGGATTCGAAAGGAAAACAACGCACGAGCACCACGGCCAACTGAT-
TTAAATGCAATTGCACTGAAGTATTTTGTTTGGCGAACGAAGCTGGATGAAA-
TAGGGGGGTGTGGGGTTTTCTATTGAGACATCTGCACGTGCAACCGGAAACATCCGAAGA-
GAACAGCACAGGCCGGGCTACGCCGGGCAATTTCTTTTCATTTGCCAAGGTGTTGAGTT-
GCACCAACATTCGACATCGACGTGGCCAGAAGCCAACAAAAGCCAA-
GAGCCAAACCCCTTTTTGTGGTCACAAGTGTCGTCTATTTGTCGTGGGCATCTTGGGCAC-
CTTGGGCATCCTCGACATCCTTGCCATTTTGGTCTGGCCAAGACAAACAACCAG-
CAAATTTAGTGTATTTTGTGCATTTTTAAAATTGTCCAAATTTATGTGACAC-
GCTGCGCCAATTGATCAGATTAAATAAACATGAGGCCAAGCGAATCGAATTTGGCTTCAC-
CAAGAAGACAATGCAGTCTGTATTCAAATGGGTGGGCGCATCCACCAA-
GCGGTGAATACAGTGACCGCTCGCTATAATGGAC-
GGTCAGGTGTTACTTTAACTTAAAAAAATATGTAACAAATCTTATCAAGTTTGAAA-
TAGATTGAAATAGATTTGGTTATTGCATTCGAAAGATATATATTAAATTCGAATATTCCAA-
GAAATTTCATGAGAATGTCACTTATGTCATGAGATTATATTAACGTACGAA-
TAAACAATGTATTTTCCAAAATTAAAAATAAAATTTAATTTAATTACGCAGTAC-
CTTTACACTATCAGTCGGAGGTAATAACTCATATAATTAGATTAGCATTAGATTTTAAA-
GCGAAAAACACTTAAAAGCTGAAATTATTAGACAACACTCTTAAATTAGTCGAGCTGATA-
TATAGCCTCAAGTTTTGCTTAAATCCAAAGATAAAGGAATGCCTTCAAAAATATATTTT-
GTTTTATACCAAGTGACAGCAGAGAATGGGGTTGCAATATCTTAAAAGAGTTTCAC-
TTAGCCAATATTTACTGCCATTGTTGGCCACCAAATAGTAGCAACCAGAGACTTCCAGGAA-
TATATTCTCGTGTCAAATGCAATCCACTTTAAATGCAACTATCTGGCGGCTAA-
GAAAACCCGACAGTTTGATTCAAGTCGACGAAACAATATAAGCACGTGCTAAATAAAGA-
GACCTATGCAGTTAATACTCTTGTCATATTATAATATAATTTAGTGACATAAGTTGCATGG-
TATACGAGTACTGAACAAGTTATGGCAGCTTTTCCAAATAAGCGATCACATATTCCGCGG-
GATGATGGGTGGATTTCTAGCATATGTG-
GATGCTTAATGGCTTATTGC**GGGTCAGGGC**GGCGCAATCTGTTC**AGAAATTCCC**GAAC-
GCACACCCATTTCAGATCAGATTGTGACGTTTT**GGGAAATTCT**TGACGATCGGTG-
TAAACAAGCTCAGCAACCAGATTCGATGGCTATTTGCCGGCTATAAATACTAGAAAC-
CATTCGATTGCACTCAGTTGAAGCTGGGCTCTGGAACAGATCACA

Chapter 4 Innate immune specificity via antimicrobial peptide gene duplication

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4.1 Abstract

Antimicrobial peptides (AMPs) are immune effectors key to defence against infection. We recently generated various AMP mutations in *Drosophila* affecting both single genes and AMPs in combination. In that study, we observed both additive and synergistic effects of AMP deletion on survival, confirming in vitro observations. However a surprising result was the highly specific interaction between single AMPs and certain microbes. An earlier study highlighted a Serine/Arginine polymorphism in *Diptericin (Dpt)* that greatly affected defence against *Providencia rettgeri* bacteria. We complimented this study by showing that deletion of multiple other AMP families does not cause any increase in susceptibility to *P. rettgeri*, while deleting the two fly *Diptericins* (*DptA, DptB*) already causes a susceptibility rivalling classic immune deficient flies. However to date, there has been no investigation on the individual role of the two *Drosophila Diptericin* genes in defence. Here, we have used null mutations in *DptA* and *DptB* to better characterize the role of these genes. We confirm that *DptA* is the main contributor to resistance against *P. rettgeri,* but find *DptB* could play a minor role. Surprisingly, we observed that *DptB* but not *DptA* is critical to resist infection with a specific isolate of the fly gut microbe *Acetobacter*. Flies lacking *DptB* are as susceptible to systemic infection by this *Acetobacter* as flies lacking the Imd pathway entirely, while deletion of other AMPs has little effect. Thus the two *Diptericins* have highly specific activities against two distinct microbes. Our study reveals contrasting specificity of two innate immune effectors following gene duplication.

4.2 Introduction

The ability to rapidly combat a pathogenic invasion is critical to organism health and survival. Animals sense "non-self" microbial products through a variety of signalling cascades, and in turn produce a battery of effector molecules that initiate the host defence response. Chief amongst these molecules are antimicrobial peptides, host-encoded antibiotics that combat invading pathogens (Hanson and Lemaitre, 2020; Lazzaro et al., 2020; Mookherjee et al., 2020). AMPs are small generally cationic peptides that combat pathogens and shape the microbiota in both plants and animals (Hacquard et al., 2017; Login et al., 2011; Mergaert et al., 2017; Nyholm and McFall-Ngai, 2021). In *Drosophila*, AMP genes from eight families and also a number of uncharacterized peptides are produced by the fat body to combat systemic microbial infection. This response is transcriptionally regulated by two NF-κB signalling pathways: the Toll and Imd pathways, which share similarities with mammalian TLR and TNF alpha signalling (Lemaitre and Hoffmann, 2007).

Use of compound mutant flies lacking many AMP genes has confirmed that AMPs contribute significantly to survival downstream of the Toll and Imd pathways (Carboni et al., 2021; Hanson et al., 2019a). Contrary to previous assumptions, these studies revealed that *Drosophila* AMPs are not simple generalist antibiotics. This is illustrated by the specific role of Diptericin against infection by *Providencia rettgeri* bacteria, as flies specifically lacking *Diptericins* succumb to infection with this bacterium like Imd pathway mutants (Hanson et al., 2019a). In contrast, other AMP families contribute almost nothing to resistance against *P. rettgeri*. Moreover, a Serine/Arginine polymorphism in *Diptericin A* (S69R) found in wild *Drosophila* populations greatly impacts resistance to infection by *P. rettgeri*, wherein Arginine is an immune-poor allele with regards to *P. rettgeri* (Unckless et al., 2016). This suggests an evolutionary constraint on *Diptericin* imposed by *P. rettgeri*, and reveals a truly remarkable specificity: one effector of the AMP response basically acts alone in defence against *P. rettgeri*.

The *Drosophila* genome encodes two *Diptericin genes* (*DptA* and *DptB*), which are clustered together in the genome on chromosome 2R. Comparison with other *Drosophila* species indicates that the ancestral Drosophilid *Diptericin* gene looked like *DptB*, and that the *DptA*/*DptB* duplication happened right before the diversification of the genus *Drosophila* (Hanson et al., 2016). There are a few notable differences between the *DptA* and *DptB* genes: the *DptB* gene in *D. melanogaster* encodes Glutamine (Q) at its S69R homologous site, but also differs from *DptA* as it encodes a propeptide similar to the antibacterial propeptide of *Attacin C* (Hanson et al., 2019; Hedengren et al., 2000; Rabel et al., 2004). Additionally, the sequence of Drosophilid DptA and DptB differ markedly, with consensus sequence comparisons revealing only ~40% similarity at the protein level (Hanson et al., 2016). Previous studies have pointed to a prominent role of *DptA* in defence against *P. rettgeri*. However these studies did not investigate the contribution of *DptB* to host defence, including its role after infection by *P. rettgeri*.

Using specific mutation, here we analyse the respective roles of *DptA* and *DptB* in *Drosophila* host defense. Our study confirms previous findings that show *DptA* is critical in defence against *P. rettgeri*. Surprisingly, we also uncovered a highly specific requirement for *DptB* in defence against systemic infection by a *Drosophila* laboratory isolate of *Acetobacter,* which is a core member of the gut microbiota (Marra et al., 2021). Thus, *Drosophila* encodes two *Dipteri-*

cins that have highly specific activity against two different bacterial species. Our study provides not only a fascinating example of immune novelty generated by gene duplication, but also highlights two sides of the same coin, which reveal an unexpectedly high degree of specificity of effector genes of the innate immune system.

4.3 Results

Diptericin A is specifically required against P. rettgeri

Previous studies revealed a prominent role of *DptA* against *P. rettgeri*, but did not address the role of *DptB*. To fill this gap, we generated isogenic fly lines lacking either *DptA* ($DptA^{\Delta 822}$) or *DptB* ($DptB^{KO}$), and use flies carrying a small genomic deficiency deleting both *Dpt* genes (Dpt^{SK1}). Considering the relevance of the S69R DptA polymorphism to *P. rettgeri* (Unckless et al., 2016), we also prepared an isogenic stock with an Arginine allele ($DptA^{S69R}$), and used our DrosDel isogenic background as our representative Serine-encoding strain (referred to here as $DptA^{S69}$). Of note, the $DptB^{KO}$ mutation was generated previously (Barajas-azpeleta et al., 2018), and its *Diptericin* locus encodes a Serine allele at *DptA* (Fig. 4.1A).

We next performed systemic infections with *P. rettgeri* using these *Diptericin* variant flies to determine the contribution of each allele or mutation in defence. Our findings with isogenic flies corroborate those of Unckless et al. (Unckless et al., 2016) showing that flies with the Arginine allele ($DptA^{S69R}$) have a very poor defence against *P. rettgeri* compared to flies with a Serine allele ($DptA^{S69}$). Flies with the *DptA* premature stop ($DptA^{\Delta 822}$) fully recapitulated the susceptibility of Dpt^{SK1} flies lacking both *DptA* and *DptB* ($DptA^{\Delta 822} : Dpt^{SK1}$, $p = 0.867$), with susceptibility approaching Imd-deficient Rel^{E20} mutants (Fig. 4.1B). Intriguingly, flies lacking *DptB* ($DptB^{KO}$) were also susceptible to infection by *P. rettgeri* ($p < .001$ compared to $DptA^{S69}$), but less than either the $DptA^{S69R}$ or $DptA^{\Delta 822}$ flies affected only in *DptA* (Fig. 4.1B). We confirmed these susceptibilities were caused by uncontrolled bacterial proliferation in the fly (Fig. 4.1C). As the $DptB^{KO}$ mutation was first generated in a different genetic background before isogenization into our DrosDel background, we could not exclude the possibility that $DptB^{KO}$ susceptibility to *P. rettgeri* was caused by a cis-genetic background effect on *DptA* expression. Indeed, upon inspection we realized that *DptA* expression in $DptB^{KO}$ flies reached only ~57% of the $DptA^{S69}$ wild-type level at 7hpi (Fig. S4.1A); *DptB* was not detected in $DptB^{KO}$ flies (Fig. S4.1B). Measuring the expression of other AMPs confirmed that this reduced expression was specific to *DptA* and does not reflect a lower overall immune induction in these flies (Fig. S4.1C-D). This *DptA* expression in $DptB^{KO}$ flies nevertheless reflects an increase in levels of *DptA* transcript of many hundreds of times, but could explain some of the susceptibility of $DptB^{KO}$ flies. However, we also note that knockdown of *DptB* using *Actin-Gal4* (*Act>DptB-IR*) leads to reduced survival compared to controls (Fig. 4.1D, $p < .05$ in all comparisons with *Act>DptB-IR*). Thus it is unclear what extent of the susceptibility of $DptB^{KO}$ flies relies on reduced *DptA* expression and what extent is caused by deletion of *DptB*.

Regardless, we confirm previous findings that *DptA* contributes most significantly to defence against *P. rettgeri*, including a specific competence of the Serine allele compared to the Arginine allele. Our results also suggest that *DptB* could contribute somewhat to defence against *P. rettgeri*, albeit to a lesser extent compared to *DptA*.

Diptericin B is very specifically required for defence against an Acetobacter strain

We previously found that a strain of *Acetobacter* related to *A. aceti/A. nitrogenifigens (ML04.1* from (Erkosar et al., 2017)) grew out of control in the microbiota of AMP mutant flies, and killed AMP mutants upon systemic infection (Marra et al., 2021). Intrigued by the susceptibility of AMP mutants to this typically mutualistic microbiome member, we dissected the susceptibility of *ΔAMP14* flies to this infection model using our systematic AMP mutant approach (Hanson et al., 2019a).

Incredibly, we narrowed down the susceptibility of *ΔAMP14* flies to just a single gene: *DptB* (Fig. 4.2A and Fig. S4.2). *DptB* deficient flies, either by $DptB^{KO}$ or Dpt^{SK1} mutation, suffered 100% mortality after infection with kinetics almost mirroring *ΔAMP14* and Rel^{E20} flies. Meanwhile $DptA^{\Delta 822}$ flies and *ΔAMP8* flies lacking five other AMP gene families resisted like wild-type. Moreover, we noticed that flies susceptible to *Acetobacter sp. ML04.1* display a severe bloating phenotype by 3dpi pre-empting mortality, and this was again specific to only *DptB* deficient flies (Fig. S4.2B-C). When we infected flies with another *Acetobacter (A. pomorum* (Roh et al., 2008; Ryu et al., 2008)), we did not find any specific susceptibility of AMP mutants or even Rel^{E20} flies lacking Imd signalling (Fig. 4.2B). Flies infected by *A. pomorum* also never showed the severe bloating phenotype.

Thus we find a highly specific role for *DptB* in defence against *Acetobacter sp. ML04.1.* This specific role of *DptB* parallels the requirement of *DptA* in defence against *P. rettgeri*. In contrast, AMPs in general are largely irrelevant to defence against *A. pomorum* (Fig. 4.2B). We previously showed that *Diptericins* are dispensable in defence against *Providencia burhodogranariea,* but can contribute to defence against *P. burhodogranariea* alongside other microbes (Hanson et al., 2019a). Thus these highly specific interactions are not generalizable even to related microbes.

4.4 Discussion

Many host defence peptides belong to large and rapidly-evolving gene families. Gene duplication can play a major role in the evolution of biological novelty. However, there are important considerations regarding the mechanistic process through which gene duplication can generate distinct function. Neofunctionalization (acquisition of new function by a daughter gene) and subfunctionalization (copies retaining only specific roles of the parent gene) have been proposed as important processes driving the retention of duplicate genes (Assis and Bachtrog, 2013; He and Zhang, 2005). On short evolutionary time scales, gene duplication may simply be a way to increase the produced quantity of an immune effector upon induction. For instance, individual genes of the *Drosophila Cecropin* gene family hardly vary in sequence (Quesada et al., 2005). On the other hand, novel function can arise by changes in gene regulation, tissue specificity, or changes to the coding sequence. These theoretical considerations require formal and concrete examples to confirm this theory applies to evolution of immune novelty. It has previously been shown that *Drosophila Diptericins* diverged rapidly after the duplication event producing *DptA*, leading to three distinct clades of gene sequence across only two genetic loci (Hanson et al., 2016). Here, we report how the duplication of the AMP gene *Diptericin* enabled highly specific roles for the two daughter genes in defence. Our study

confirms the specific role of *DptA* and its S69R polymorphism in the defence against *P. rettgeri* using a controlled genetic background. We suggest a possible contribution of *DptB* to defence against *P. rettgeri*, though this will benefit from further validation. Surprisingly, we also found that *DptB*, but not *DptA*, has a very specific activity against a specific strain of *Acetobacter*. We did not observe bloating during infections with *A. pomorum* or any *Dpt*-microbe interaction screened in Hanson et al. (Hanson et al., 2019a). Thus this bloating phenotype is not a generic consequence of *DptB* deletion, nor an instrinsic property of *Acetobacter* species. Instead this bloating and survival effect is specific to *Acetobacter sp. ML04.1* and uniquely seen in *DptB* deficient flies. In the future, it will be interesting to determine what host or bacterial factors underlie this cachexia-like (Saavedra and Perrimon, 2019) phenotype.

The presence of the two *D. melanogaster Diptericins* has allowed flies to produce specific responses to certain pathogens. Further study should clarify if the distinct antibacterial activities of these two peptides are caused by: **i)** the polymorphic residue at position 69/56 of the respective DptA/DptB mature peptides, **ii)** if the presence of a pro-domain in *DptB* might contribute to this *Acetobacter* phenotype, or **iii)** if other overt sequence changes amongst the *Diptericin* clades (Hanson et al., 2016) affect their spectrum of antibacterial activity. Importantly, the specificity in defence of *DptB* for *Acetobacter sp. ML04.1* and *DptA* for *P. rettgeri* may confirm a long-presumed but never demonstrated logic guiding the evolution of AMP sequence. The polymorphisms frequently seen in AMPs can arise from countervailing evolutionary selection, suggesting trade-offs in defence against one ecologically relevant microbe versus another. It is remarkable that a single residue in the *Diptericin* protein is the key to adapting this AMP for defence against *P. rettgeri*. In this regard, the Serine and Glutamine residues present in *D. melanogaster DptA* and *DptB* are common across fly *Diptericins*, and a convergent Glutamine/Asparagine trans-species polymorphism is present in both *Drosophila DptB* genes across the phylogeny (Hanson et al., 2016), and Tephritid fruit flies that feed on ecological niches also associated with *Acetobacter* (Hanson et al., 2019b; Kounatidis et al., 2009). This suggests that AMP gene duplications offer evolutionary toolkits for specialization of defence against ecologically relevant microbes. This trend supports our recent study that suggested ecology drives AMP gene evolution, where plant-parasitic flies with presumably reduced infectious pressure were more likely to lose AMP gene families over evolutionary timescales (Hanson et al., 2019b). Indeed AMP copy number variation is common in both invertebrates (Bulmer and Crozier, 2004; Hanson et al., 2016; Sackton et al., 2017; Vilcinskas et al., 2013; Wang and Zhu, 2011), and vertebrates alike (Halldórsdóttir and Árnason, 2015; Hellgren and Sheldon, 2011; Hollox and Armour, 2008; Patil et al., 2005; Zou et al., 2007), and humans and mice have over 30 and 50 *Defensin* genes respectively, which encode various polymorphisms (Amid et al., 2009; Linzmeier and Ganz, 2005; Schutte et al., 2002).

In this light, our use of systematic AMP mutant combinations has previously highlighted the prominent role of *Drosocin* in defence against *E. cloacae* (Hanson et al., 2019a), recovered and emphasized the specific susceptibility of *DptA* mutant flies to *P. rettgeri*, and has now allowed the identification of *DptB* as a key determinant in controlling *Acetobacter sp. ML04.1*. Systematic investigation with loss of function AMP mutants is therefore a robust method to highlight important AMP-microbe interactions. This approach can be used to highlight genes for investigations with population genetic data, which can recursively focus GWAS experiments for

AMP-microbe interactions of interest. The future of AMP-inspired antibiotic development should also pay careful attention to the importance of sequence divergence and important polymorphisms on microbicidal activity (Hanson and Lemaitre, 2020; Lazzaro et al., 2020). While the short generation time and genetic tractability of *Drosophila* allowed us to generate these combinations relatively quickly, this approach is tasking but feasible in other model organisms in the CRISPR era. Systematic gene deletions in other models could implicate certain AMPs in disease, and focus future studies to ask if AMP duplications and differences in gene sequence are significant risk factors for infectious syndromes. It may be that the genome does not simply encode multitudinous redundant peptides, but rather maintains similar peptides that are highly specialized for defence against specific microbes. In fruit flies such an idea is not an exception, but rather appears to be one of the rules.

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4.5 Materials and Methods

Fly genetics

Genetic variants were isogenized into the DrosDel isogenic background over 7 generations as described in (Ferreira et al., 2014). The specific mutations studied here were sourced as follows: isogenic *DptA*^{S69R} flies generated here encode a *Diptericin* locus originally isolated from strain DGRP-38 (see DGRP genetic variant: 2R_14753589_SNP). The isogenic *DptA*$^{\Delta 822}$ flies generated here encode a premature stop in their *DptA* gene isolated from strain DGRP-822 (DGRP genetic variant: 2R_14753502_SNP), which was previously associated with very poor immune defence against *P. rettgeri* (Unckless et al., 2016). The *DptB*^{KO} mutation was generated in Barajas-Azpeleta et al. (Barajas-azpeleta et al., 2018) in a Canton S background prior to our isogenization into the DrosDel background. The *Actin5C-Gal4* (*Act-Gal4*) stock is the same as used previously (Hanson et al., 2019a), and *UAS-DptB-IR* flies were Bloomington stock #28975. Sequence comparisons were made using Geneious R10.

Gene expression analysis

Gene expression was performed using primers described previously (Hanson et al., 2019b, 2019b, 2016) with PowerUP SYBR Green Master Mix. The PFAFFL method was used for qPCR quantification with *Rp49* as the reference gene (Pfaffl, 2001). RNA was extracted using TRIzol according to manufacturer's protocol. cDNA was reverse transcribed using Takara Reverse Transcriptase. Statistical analysis was performed by one-way ANOVA with Holm-Sidak's multiple test correction. Error bars represent one standard deviation from the mean.

Microbe culturing conditions

Bacteria were grown to mid-log phase shaking at 200rpm in their respective growth media (Luria Bertani, MRS+Mannitol) and temperature conditions, and then pelleted by centrifugation to concentrate microbes. Resulting cultures were diluted to the desired optical density at 600nm (OD) for survival experiments, which is indicated in each figure. The following microbes were grown at 37°C: *Providencia rettgeri* (LB). The following microbes were grown at 29°C: *Acetobacter sp. ML04.1 (MRS+Mannitol*) and *Acetobacer pomorum*.

Survival experiments and microbial load measurements

Survival experiments were performed as previously described (Hanson et al., 2019a), with 20 flies per vial with replicate experiment numbers listed. ~5 day old males were used in experiments unless otherwise specified. Flies were flipped thrice weekly. Statistical analyses were performed using a Cox proportional hazards (CoxPH) model in R 3.6.3. Bloating was recorded by eye with a binomial outcome (bloated or not bloated). Only severe bloating that caused white cuticle to peek through the black abdominal tergites of males was recorded as bloated. Microbial loads were measured by homogenizing 5 flies in a bead beater (6500rpm) for 30 seconds in 500µL LB medium. Homogenates were then serially diluted and plated on LB or MRS+Mannitol overnight for CFU quantification.

4.6 References

Amid C, Rehaume LM, Brown KL, Gilbert JG, Dougan G, Hancock RE, Harrow JL. 2009. Manual annotation and analysis of the defensin gene cluster in the C57BL/6J mouse reference genome. *BMC Genomics* **10**:606. doi:10.1186/1471-2164-10-606

Assis R, Bachtrog D. 2013. Neofunctionalization of young duplicate genes in Drosophila. *Proceedings of the National Academy of Sciences* **110**:17409–17414. doi:10.1073/pnas.1313759110

Barajas-azpeleta R, Wu J, Gill J, Welte R. 2018. Antimicrobial peptides modulate long-term memory. *PLoS Genetics* 1–26. doi:10.1371/journal.pgen.1007440

Bulmer MS, Crozier RH. 2004. Duplication and diversifying selection among termite antifungal peptides. *Molecular Biology and Evolution* **21**:2256–2264. doi:10.1093/molbev/msh236

Carboni A, Hanson MA, Lindsay SA, Wasserman SA, Lemaitre B. 2021. Cecropins contribute to *Drosophila* host defence against fungal and Gram-negative bacterial infection (preprint). Immunology. doi:10.1101/2021.05.06.442783

Erkosar B, Kolly S, van der Meer JR, Kawecki TJ. 2017. Adaptation to Chronic Nutritional Stress Leads to Reduced Dependence on Microbiota in Drosophila melanogaster. *mBio* **8**:mBio.01496-17, e01496-17. doi:10.1128/mBio.01496-17

Ferreira ÁG, Naylor H, Esteves SS, Pais IS, Martins NE, Teixeira L. 2014. The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral Infection in Drosophila. *PLoS Pathogens* **10**. doi:10.1371/journal.ppat.1004507

Hacquard S, Spaepen S, Garrido-Oter R, Schulze-Lefert P. 2017. Interplay Between Innate Immunity and the Plant Microbiota. *Annu Rev Phytopathol* **55**:565–589. doi:10.1146/annurev-phyto-080516-035623

Halldórsdóttir K, Árnason E. 2015. Trans-species polymorphism at antimicrobial

innate immunity cathelicidin genes of Atlantic cod and related species. *PeerJ* **3**:e976. doi:10.7717/peerj.976
Hanson MA, Dostálová A, Ceroni C, Poidevin M, Kondo S, Lemaitre B. 2019a. Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. *eLife* **8**. doi:10.7554/elife.44341
Hanson MA, Hamilton PT, Perlman SJ. 2016. Immune genes and divergent antimicrobial peptides in flies of the subgenus Drosophila. *BMC evolutionary biology* **16**:228. doi:10.1186/s12862-016-0805-y
Hanson MA, Lemaitre B. 2020. New insights on Drosophila antimicrobial peptide function in host defense and beyond. *Curr Opin Immunol* **62**:22–30. doi:10.1016/j.coi.2019.11.008
Hanson MA, Lemaitre B, Unckless RL. 2019b. Dynamic Evolution of Antimicrobial Peptides Underscores Trade-Offs Between Immunity and Ecological Fitness. *Frontiers in Immunology* **10**:2620. doi:doi: 10.3389/fimmu.2019.02620
He X, Zhang J. 2005. Rapid subfunctionalization accompanied by prolonged and substantial neofunctionalization in duplicate gene evolution. *Genetics* **169**:1157–1164. doi:10.1534/genetics.104.037051
Hedengren M, Borge K, Hultmark D. 2000. Expression and evolution of the Drosophila attacin/diptericin gene family. *Biochemical and biophysical research communications* **279**:574–81. doi:10.1006/bbrc.2000.3988
Hellgren O, Sheldon BC. 2011. Locus-specific protocol for nine different innate immune genes (antimicrobial peptides: β-defensins) across passerine bird species reveals within-species coding variation and a case of trans-species polymorphisms. *Molecular Ecology Resources* **11**:686–692. doi:10.1111/j.1755-0998.2011.02995.x
Hollox EJ, Armour JAL. 2008. Directional and balancing selection in human beta-defensins. *BMC Evolutionary Biology* **8**. doi:10.1186/1471-2148-8-113
Kounatidis I, Crotti E, Sapountzis P, Sacchi L, Rizzi A, Chouaia B, Bandi C, Alma A, Daffonchio D, Mavragani-Tsipidou P, Bourtzis K. 2009. Acetobacter tropicalis is a major symbiont of the olive fruit fly (Bactrocera oleae). *Appl Environ Microbiol* **75**:3281–3288. doi:10.1128/AEM.02933-08
Lazzaro BP, Zasloff M, Rolff J. 2020. Antimicrobial peptides: Application informed by evolution. *Science* **368**. doi:10.1126/science.aau5480
Lemaitre B, Hoffmann J. 2007. The Host Defense of Drosophila Melanogaster. *Annual Review of Immunology* **25**:697–743. doi:10.1146/annurev.immunol.25.022106.141615
Linzmeier RM, Ganz T. 2005. Human defensin gene copy number polymorphisms: Comprehensive analysis of independent variation in α- and β-defensin regions at 8p22-p23. *Genomics* **86**:423–430. doi:10.1016/j.ygeno.2005.06.003
Login FH, Balmand S, Vallier A, Vincent-Monégat C, Vigneron A, Weiss-Gayet M, Rochat D, Heddi A. 2011. Antimicrobial peptides keep insect endosymbionts under control. *Science* **334**:362–365. doi:10.1126/science.1209728
Marra A, Hanson MA, Kondo S, Erkosar B, Lemaitre B. 2021. *Drosophila* antimicrobial peptides and lysozymes regulate gut microbiota composition and abundance (preprint). Microbiology. doi:10.1101/2021.03.19.436153
Mergaert P, Kikuchi Y, Shigenobu S, Nowack ECM. 2017. Metabolic Integration of Bacterial Endosymbionts through Antimicrobial Peptides. doi:10.1016/j.tim.2017.04.007
Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ. 2020. Antimicrobial

host defence peptides: functions and clinical potential. *Nat Rev Drug Discov*. doi:10.1038/s41573-019-0058-8

Nyholm SV, McFall-Ngai MJ. 2021. A lasting symbiosis: how the Hawaiian bobtail squid finds and keeps its bioluminescent bacterial partner. *Nat Rev Microbiol* **19**:666–679. doi:10.1038/s41579-021-00567-y

Patil AA, Cai Y, Sang Y, Blecha F, Zhang G. 2005. Cross-species analysis of the mammalian β-defensin gene family: presence of syntenic gene clusters and preferential expression in the male reproductive tract. *Physiological Genomics* **23**:5–17. doi:10.1152/physiolgenomics.00104.2005

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**:e45. doi:10.1093/nar/29.9.e45

Quesada H, Ramos-Onsins SE, Aguad?? M. 2005. Birth-and-death evolution of the Cecropin multigene family in Drosophila. *Journal of Molecular Evolution* **60**:1–11. doi:10.1007/s00239-004-0053-4

Rabel D, Charlet M, Ehret-Sabatier L, Cavicchioli L, Cudic M, Otvos L, Bulet P. 2004. Primary Structure and in Vitro Antibacterial Properties of the Drosophila melanogaster Attacin C Pro-domain. *Journal of Biological Chemistry* **279**:14853–14859. doi:10.1074/jbc.M313608200

Roh SW, Nam Y-D, Chang H-W, Kim K-H, Kim M-S, Ryu J-H, Kim S-H, Lee W-J, Bae J-W. 2008. Phylogenetic characterization of two novel commensal bacteria involved with innate immune homeostasis in Drosophila melanogaster. *Appl Environ Microbiol* **74**:6171–6177. doi:10.1128/AEM.00301-08

Ryu J-H, Kim S-H, Lee H-Y, Bai JY, Nam Y-D, Bae J-W, Lee DG, Shin SC, Ha E-M, Lee W-J. 2008. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. *Science* **319**:777–782. doi:10.1126/science.1149357

Saavedra P, Perrimon N. 2019. Drosophila as a Model for Tumor-Induced Organ Wasting. *Adv Exp Med Biol* **1167**:191–205. doi:10.1007/978-3-030-23629-8_11

Sackton TB, Lazzaro BP, Clark AG, Wittkopp P. 2017. Rapid expansion of immune-related gene families in the house fly, musca domestica. *Molecular Biology and Evolution*. doi:10.1093/molbev/msw285

Schutte BC, Mitros JP, Bartlett JA, Walters JD, Jia HP, Welsh MJ, Casavant TL, McCray PB. 2002. Discovery of five conserved beta -defensin gene clusters using a computational search strategy. *Proc Natl Acad Sci U S A* **99**:2129–2133. doi:10.1073/pnas.042692699

Unckless RL, Howick VM, Lazzaro BP. 2016. Convergent Balancing Selection on an Antimicrobial Peptide in Drosophila. *Current Biology* **26**:257–262. doi:10.1016/j.cub.2015.11.063

Vilcinskas A, Mukherjee K, Vogel H. 2013. Expansion of the antimicrobial peptide repertoire in the invasive ladybird Harmonia axyridis. *Proceedings of the Royal Society B: Biological Sciences*. doi:10.1098/rspb.2012.2113

Wang Y, Zhu S. 2011. The defensin gene family expansion in the tick Ixodes scapularis. *Developmental and Comparative Immunology*. doi:10.1016/j.dci.2011.03.030

Zou J, Mercier C, Koussounadis A, Secombes C. 2007. Discovery of multiple beta-defensin like homologues in teleost fish. *Molecular Immunology* **44**:638–647. doi:10.1016/j.molimm.2006.01.012

4.7 Figures chapter 4

4.7.1 Main figures

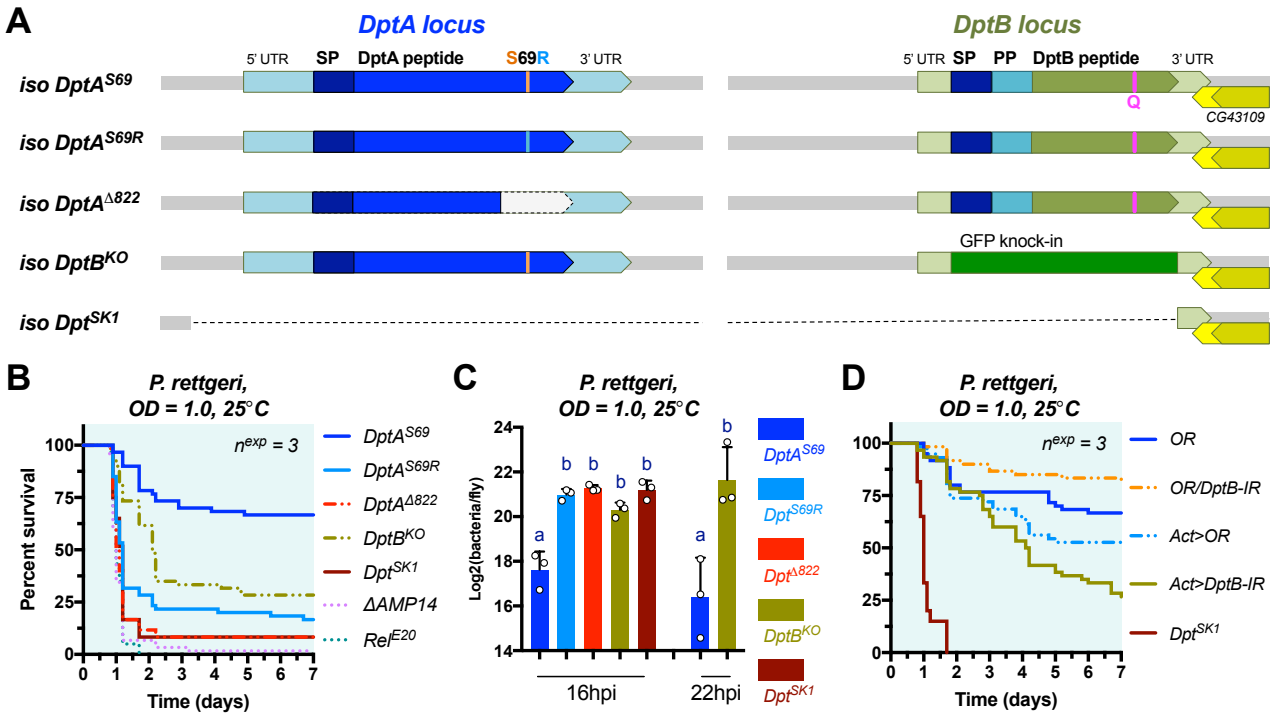

Figure 4.1: Contribution of *DptA* and *DptB* to defence against *P. rettgeri*.

A) Schematic of *Diptericin* mutant loci used in this study. The two Diptericin genes are located in tandem on Chromosome 2R (cytogenetic map 55F). Amino acid residues at the key polymorphic site are annotated. *DptB* encodes a secreted propeptide (PP) not found in *DptA*. B) Survival analysis of isogenic *Diptericin* mutant flies upon infection by *P. rettgeri*. $DptA^{\Delta 822}$ flies mirror the susceptibility of Dpt^{SK1} mutants, while $DptA^{S69R}$ flies that encode Arginine are markedly more susceptible than flies encoding Serine. $DptB^{KO}$ flies are also highly susceptible to infection. However endogenous *DptA* expression reaches only ~57% of *iso* w^{1118} wild-type in these $DptB^{KO}$ flies at 7 hours post-infection (approximate time of control in Duneau et al. (2017)), confounding the ability to attribute $DptB^{KO}$ susceptibility to loss of *DptB* entirely, or lesser *DptA* expression compared to our $DptA^{S69}$ wild-type (Fig. S1). C) Bacterial loads confirm that fly susceptibility is caused by an inability to suppress *P. rettgeri* growth by 16hpi, which is further exaggerated by 22hpi in $DptB^{KO}$ flies. D) *DptB* knockdown supports a minor role for *DptB* in defence against *P. rettgeri* ($p < .05$ in all comparisons to *Act>DptB-IR*).

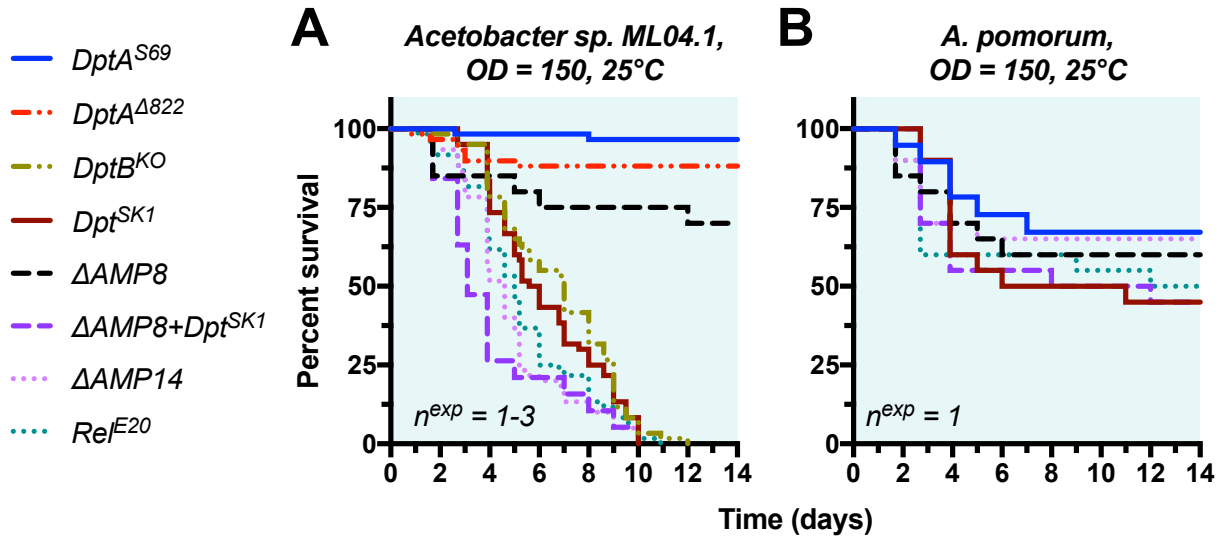

Figure 4.2: The *Diptericin B* gene alone dictates the susceptibility of AMP mutant flies to *Acetobacter sp. ML04.1* systemic infection.

A) Systematic AMP mutant combinations reveal a specific susceptibility only in flies deficient for *DptB* either by $DptB^{KO}$ or Dpt^{SK1} mutations, including *ΔAMP14* and Rel^{E20} flies. *ΔAMP8* represents the combined loss of *Defensin*, *Drosocin*, *Attacin*, *Metchnikowin*, and *Drosomycin* genes (8 genes), and *ΔAMP8+*Dpt^{SK1} flies are additionally missing the two *Diptericin* genes (Note: only one experiment so far for *ΔAMP8* and *ΔAMP8+*Dpt^{SK1} treatments. B) *Diptericins* and in fact AMPs in general are dispensible after systemic infection by *A. pomorum* (note: only one experiment so far).

4.7.2 Supplementary figures

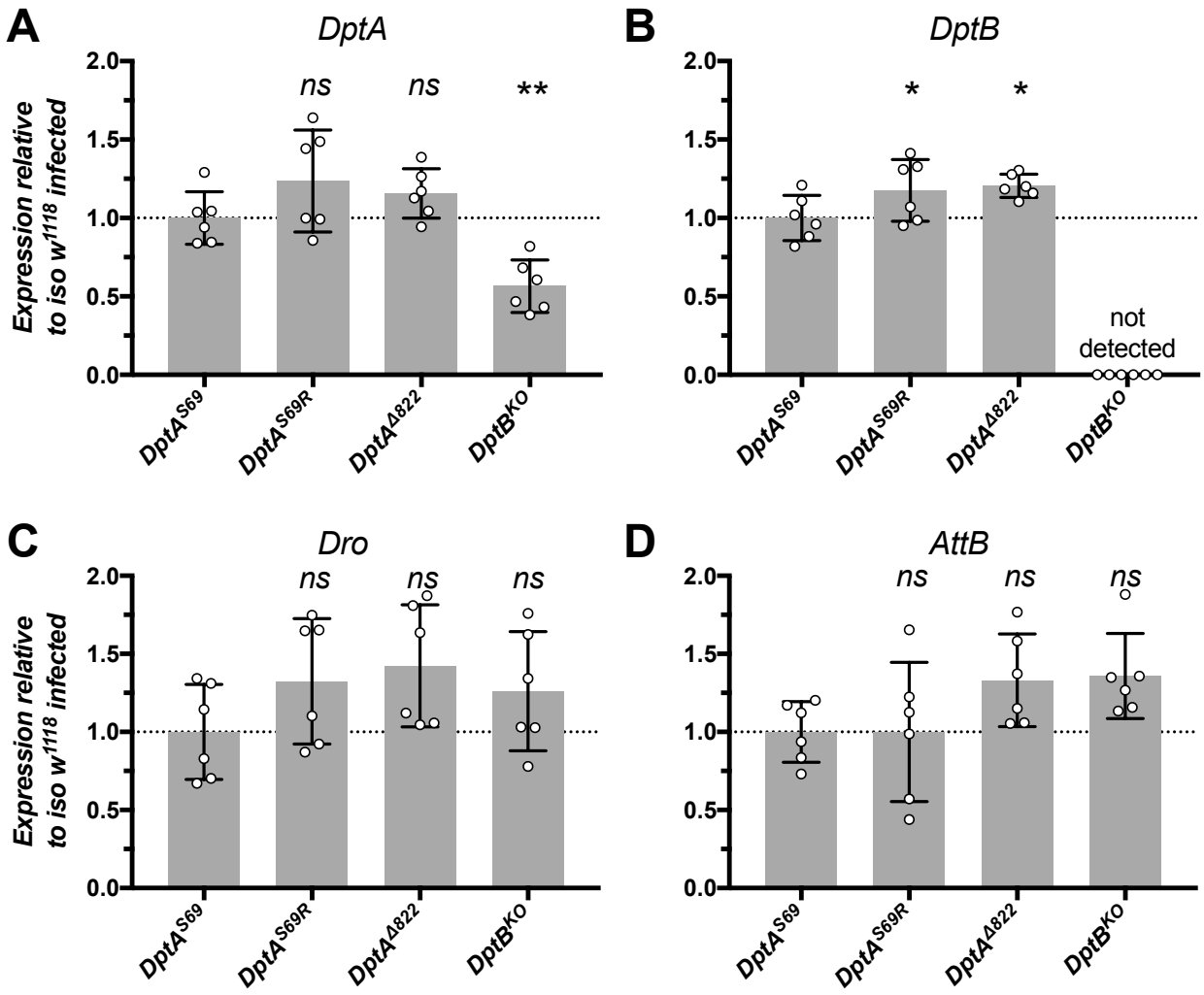

Figure S4.1: AMP expression 7 hours after infection with *P. rettgeri*, and normalized to the *iso w*^{1118} infected state in isogenic flies carrying the various *Diptericin* variants. A) *DptB*^{KO} flies show notably reduced *DptA* induction reaching only ~57% of *iso w*^{1118} wild-type expression. These trends were confirmed using two different sets of *DptA* primers to ensure the effect was robust to primer binding site choice. B) *DptB* expression in mutant flies. C-D) *Drosocin* (C) and *Attacin B* (D) expression is wild-type in all mutant backgrounds. Sanger sequencing of ~1500bp of the *DptA* promoter did not find any off-site mutations in *DptB*^{KO} flies relative to *DptA*^{S69} flies. This might suggest some element in the *DptB* gene region itself has a regulatory effect on *DptA* expression.

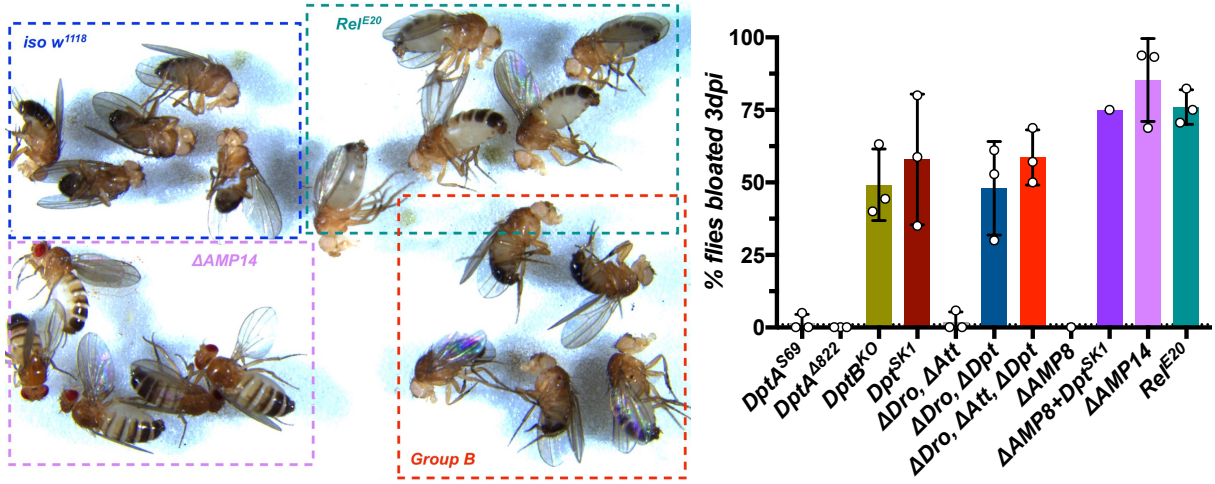

Figure S4.2: Two days post-infection by *Acetobacter sp. ML04.1*, Rel^{E20}, *ΔAMP14*, and some Group B flies present with a severe bloating phenotype, but *iso* w^{1118} wild-type flies do not (left picture). At a later time (3 days post-infection) this effect is more prominent, and specifically found only in flies lacking *DptB* (right bar plots). Flies specifically lacking *DptB* also typically presented with a more prominent bacterial film in the vial, suggesting bacterial growth in the fly after septic injury translates to bacterial deposition into the food over the course of infection.

Chapter 5 Drosophila immunity: The Drosocin locus encodes two antibacterial peptides with distinct activities

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5.1 Abstract

Antimicrobial peptides (AMPs) are key players in innate defence against infection in plants and animals. In *Drosophila*, a large array of immune peptides contribute to host-defence downstream of the Toll and Imd NF-κB pathways. Previous studies using single and compound AMP mutations confirmed that AMPs can additively or synergistically contribute to combat pathogens in vivo. However these studies also revealed a high degree of specificity wherein one AMP can play a major role for combatting a specific pathogen. We recently uncovered a specific importance of *Drosocin* for defence against *Enterobacter cloacae*. Here, we show that the *Drosocin* locus *(CG10816)* is more complex than previously described, as it produces two peptides from a precursor via furin cleavage: the previously described Drosocin peptide and a novel peptide corresponding to the uncharacterized Immune-induced Molecule 7 that we name "Buletin". The existence of a naturally occurring polymorphism (T52A) in the CG10816 precursor protein masked the identification of this peptide previously. Using mutations differently affecting the production of these two *CG10816* gene products, we show that Drosocin, but not Buletin contributes to the *CG10816*-mediated defence against *E. cloacae* described previously. Strikingly, we observed the opposite in defence against *Providencia burhodogranariea,* where deletion of Drosocin has no effect, while deletion of Buletin significantly increases susceptibility to infection. Moreover, a Threonine/Alanine polymorphism in Buletin determines defence against *P. burhodogranariea*. Collectively, our study identifies a novel antibacterial peptide derived from the *CG10816 Drosocin* locus contributing to *Drosophila* host defence, and reveals the high degree of specificity of the two *CG10816*-derived peptides. This reinforces recent findings on the high specificity of *Drosophila* AMPs against pathogenic microbes, and how AMP polymorphisms can greatly affect host susceptibility. The existence of multiple highly specific AMP-pathogen interactions seems likely to be a general feature of innate immune effectors, challenging the prevailing view that innate effectors provide a successful defence owing to broad and generalist activities.

5.2 Introduction

The ability to rapidly combat pathogens is critical to organism health and survival. Organisms sense natural enemies through pattern recognition receptors, triggering the activation of core immune signalling pathways. These pathways regulate the expression of immune effectors that provide a first line of innate defence. The activation of one or several pathways upon infection leads to the production of many effector molecules. It was generally thought that innate immune effectors act together as a cocktail to kill microbes. However recent studies have challenged this view revealing an unexpected high degree of specificity in the host effector response to infection (Hanson and Lemaitre, 2020; Lazzaro et al., 2020; Lin et al., 2020).

Chief amongst immune effectors are antimicrobial peptides, host-encoded antibiotics that combat invading pathogens (Hanson and Lemaitre, 2020; Lazzaro et al., 2020; Mookherjee et al., 2020). Insects, and particularly the genetically tractable model *Drosophila,* have been especially fruitful in identifying and characterizing AMP potency and function (Hanson et al., 2019a; Rolff and Schmid-Hempel, 2016; Steiner et al., 1981). In *Drosophila*, systemic infection triggers the expression of a battery of antimicrobial peptides, which are secreted into the hemolymph by the fat body to transform this compartment into a potent microbicidal environment. This systemic AMP response is tightly regulated by two NF-κB signalling pathways: the Toll and Imd pathways. These two pathways are similar to mammalian TLR and TNF alpha signalling (Lemaitre and Hoffmann, 2007), and are differentially activated by different classes of microbes. The Toll pathway is predominantly instigated after sensing infection by Gram-positive bacteria and fungi, while the Imd pathway is especially responsive to Gram-negative bacteria and some Gram-positive bacteria with DAP-type peptidoglycan (Lemaitre et al., 1997, 1996, 1995). Both the Toll and Imd pathways are key in the expression of AMPs, and while some genes respond with specificity to certain pathways, many genes are co-regulated during the systemic immune response (De Gregorio et al., 2002; Lemaitre et al., 1997).

In *Drosophila*, several families of host defence peptides contribute downstream of Toll and Imd. This includes the Cecropin, Attacin, Diptericin, Defensin, Metchnikowin, Daisho, Baramicin, Bomanin, and Drosocin gene families (discussed in (Cohen et al., 2020; Hanson et al., 2021; Hanson and Lemaitre, 2020)). How these immune effectors contribute individually or collectively to host defence is a major challenge. Use of single and compounds mutants have revealed that defence against some pathogens relies on the collective contributions of multiple AMP families. However recent studies have also revealed how single defence peptide fami-

lies can play highly specific and important roles during infection. In one case, *Diptericins* are the critical AMP family for surviving infection by *Providencia rettgeri* bacteria. This specificity is so remarkable that flies collectively lacking five other AMP gene families nevertheless resist *P. rettgeri* infection like wild-type (Hanson et al., 2019a), while even a single amino acid change in one *Diptericin* gene can cause pronounced susceptibility to *P. rettgeri* (Unckless et al., 2016). Studies on Toll effector genes such as *Bomanins*, *Daishos*, or *Baramicin A* have also found deletion of single gene families can cause strong susceptibilities against specific fungi (Cohen et al., 2020; Hanson et al., 2021), or mediate general defences against broad pathogen types (Clemmons et al., 2015; Lindsay et al., 2018). Lastly, loss of the gene *Drosocin* already causes a specific and pronounced susceptibility to infection by *Enterobacter cloacae (Hanson et al., 2019a)*, agreeing with Drosocin peptide in vitro activity (Bulet et al., 1996). Unlike the example with Diptericins and *P. rettgeri*, other AMPs also contribute to defence against *E. cloacae* (Carboni et al., 2021).

One aspect of AMP genes that is often ignored is that their precursor proteins can encode both their principle antimicrobial peptide, but also additional propeptide products processed by furin cleavage (Hanson et al., 2021). The *Drosocin* gene *(CG10816)* is one such example, as *CG10816* encodes both the Drosocin mature peptide, but also a C-terminus that has not yet been characterized (Hedengren et al., 2000). Intriguingly, the *CG10816* C-terminus has been highlighted previously in a population genetic study, which showed that a Threonine/Alanine polymorphism is segregating in wild flies (Lazzaro and Clark, 2003). Such polymorphisms are common in AMP genes, and are proposed to reflect host-pathogen coevolutionary selection (Chapman et al., 2019; Hanson et al., 2019b; Unckless and Lazzaro, 2016). Thus it is possible that the CG10816 protein C-terminus is not inert, and could contribute to host defence alongside its more famous sister peptide Drosocin.

Here we investigate the role of the different peptide products of the Drosocin-encoding gene *CG10816*. For clarity of discussion, we will use the shorthand Drc (with a “c”, and not italics) to refer to the mature Drosocin peptide, and whenever possible, we will use *CG10816* to refer to the *Drosocin* gene (common shorthand *Dro*, with an “o”). We found that the *CG10816* C-terminus in fact encodes one of the *Drosophila* Immune-induced Molecules (IMs) first identified in 1998: IM7. Using a new mutation affecting only the Drc product but not IM7, we show that these two peptides contribute independently to defence against different microbes. We rename the IM7 peptide Buletin (Btn), and show that Btn contributes to defence against *Providencia burhodogranariea* independent of Drc, which confers no advantage against this bacte-

rium in vivo. Moreover, the less-common Threonine allele of the T52A polymorphism in Btn causes a similar level of susceptibility to *P. burhodogranariea* as Btn deletion. We therefore uncover a striking example where an AMP-encoding gene produces two peptides with distinct activities, and also an example of an AMP polymorphism significantly affecting defence against a specific microbe. Both observations contribute to a growing body of evidence that AMP products can have highly specific and important contributions to defence independent of other immune effectors.

5.3 Results

The Drosocin gene CG10816 encodes IM7

Previous MALDI-TOF proteomic analyses of hemolymph samples from infected *Drosophila* revealed several Immune-induced Molecules (IMs) (Uttenweiler-Joseph et al., 1998). These molecules were annotated as IM1-IM24 according to their mass, and over time each of these IMs was associated with a host defence peptide gene, notably including various AMP genes and also Bomanin defence peptides (Clemmons et al., 2015; Cohen et al., 2020; Hanson et al., 2021; Levy et al., 2004). At this point, only one of the 24 original IMs remains unknown: IM7. Previous efforts were unable to link this 2307 Da peak to a gene in the *Drosophila* reference genome. However during our studies, we noticed that IM7 was absent in flies lacking multiple AMP families (Carboni et al., 2021; Hanson et al., 2019a). We repeated these MALDI-TOF proteomic experiments with hemolymph samples from flies carrying systematic combinations of AMP mutations, ultimately honing in on the *Drosocin* gene *CG10816*. Two independent *CG10816/Dro* mutants (Dro^{SK4} and $Dro\text{-}AttAB^{SK2}$) both lack IM7 in MALDI-TOF peptidomic analysis (Fig. 5.1A-B).

CG10816/Dro was initially identified as a single ORF gene encoding the Drc peptide. Drc is an O-glycosylated Proline-rich peptide that binds bacterial DnaK/Hsp70 similar to other insect AMPs (Bikker et al., 2006; Bulet et al., 1996; Kragol et al., 2001; Rahnamaeian et al., 2016). Mature Drc requires O-glycosylation for activity, which involves the biochemical linking of either mono- (MS), di- (DS), or rarely tri-saccharide (TS) groups to the Threonine at position 11 of the Drc peptide (Bulet et al., 1996; Levy et al., 2004). These different O-glycosylations yield peptides with different mature masses of 2401, 2564, and 2767 Da (Drc-MS, -DS, and -TS respectively). Unmodified Drc peptide has an expected mass of 2199 Da, which is not an intuitive match for the 2307 Da peak of IM7, even considering other glycosylations. This suggests that another element of the *CG10816/Dro* gene encodes IM7.

IM7 is the C-terminus of CG10816, previously masked by a polymorphism

Previous analysis noted that Drc was the *CG10816/Dro* N-terminal peptide cleaved from a precursor protein (Hedengren et al., 2000), but no functional study of the C-terminus has been performed to date. Serendipitously, while generating *CG10816/Dro* mutants using CRISPR-Cas9 we recovered a complex aberrant locus (Dro^{SK3}) that deletes 11 residues of the mature Drc peptide, including its critical O-glycosylated Threonine (Fig. 5.1A). However the Dro^{SK3} deletion later continues in the same reading frame, including the RVRR furin cleavage site and C-terminus. Thus we suspected that the C-terminal peptide would be secreted normally in Dro^{SK3} flies. When we ran MALDI-TOF analysis on immune-induced hemolymph from Dro^{SK3} flies, we recovered a signal that all-but confirmed the identity of the *CG10816* C-terminus: Dro^{SK3} flies lacked the Drc-MS and Drc-DS peaks, but the 2307 Da peak corresponding to IM7 remained immune-inducible (Fig. 5.1B).

It is puzzling that IM7 could not be annotated to the *CG10816/Dro* gene given that the nucleotide sequence has been known for decades, and the C-terminus was previously noted (Hedengren et al., 2000). To this point, Lazzaro and Clark (Lazzaro and Clark, 2003) previously described a polymorphism in the *CG10816/Dro* gene encoding either a Threonine or Alanine at residue 52 of the precursor protein sequence (T52A). The *D. melanogaster* reference genome encodes the Threonine version of this polymorphism. Using the sequence of the reference genome, the *CG10816* C-terminus mature mass would be 2337 Da without considering post-translational modifications. If we instead substitute an Alanine at this site, the predicted mass of the *CG10816* C-terminus becomes 2307 Da, exactly matching the observed mass of IM7. We confirmed that our DrosDel isogenic fly stocks encoded an Alanine allele both by Sanger sequencing and LC-MS proteomics (not shown). We next performed MALDI-TOF on a fly stock known to encode a Threonine in its C-terminus (*DGRP-822*). Exactly matching prediction, *DGRP-822* flies lack the 2307 Da IM7 peak, and instead have a 2337 Da peak that appears after infection (Fig. 5.1B).

Taken together, we confirm that *CG10816* encodes two peptides: Drc and IM7. We also reveal how a naturally occurring polymorphism previously obscured the annotation of IM7 as a *CG10816* gene product. This analysis was greatly facilitated by the use of newly-available AMP mutations. We name this C-terminal peptide Buletin (Btn) after Philippe Bulet, whose dedicated efforts in the 1980s-1990s characterized many of the *Drosophila* AMPs including *Drosocin* (Bulet et al., 1996, 1993; Imler and Bulet, 2005).

Drc, but not Btn, is responsible for CG10816-mediated defence against Enterobacter cloacae

After realizing the *Dro*^{SK3} and *Dro*^{SK4} mutations differently affected the Drc and Btn peptides, we next compared the survival of these various mutants, which differ specifically in the presence or absence of Buletin. We infected these flies with a panel of Gram-negative bacteria: *Acetobacter sp. ML04.1* that can kill AMP mutant flies (Marra et al., 2021), *E. coli 1106* suggested to interact with *Drosocin* (Sanchez Bosch et al., 2019)*, E. cloacae β12* bacteria that *Drosocin* mutants are specifically susceptible to (Hanson et al., 2019a), and *P. burhodogranariea strain B* where *Drosocin* was shown to contribute to defence alongside other AMPs (Hanson et al., 2019a). All experiments were performed in the DrosDel isogenic background isogenized according to Ferreira et al. (Ferreira et al., 2014).

We found that individual *CG10816/Dro* mutants were not overtly susceptible to infection by *E. coli 1106* or *Acetobacter sp. ML04.1* (Fig. S5.1). We could also repeat our previous findings that *Dro*^{SK4} and *Dro-AttAB*^{SK2} flies were highly susceptible to *E. cloacae* infection, causing 40-50% mortality by 3 days after infection using an OD_{600} = 200 pellet. Importantly, use of *Dro*^{SK3} flies that lack Drc but produce Btn confirms that this susceptibility is principally caused by a loss of Drc peptide and not Btn (Fig. 5.2A). Flies lacking both Drc and Btn (*Dro*^{SK4} and *Dro-AttAB*^{SK2}) were only slightly more susceptible than flies lacking Drc alone (*Dro*^{SK3}), which was not statistically significant (*Dro*^{SK4} and *Dro-AttAB*^{SK2} comparisons to *Dro*^{SK3}, $p > .05$ in both cases).

Comparison of mutants lacking Drc, or both Drc and Btn confirms that the *CG10816/Dro*-mediated defence against *E. cloacae* specifically requires the Drc peptide. Meanwhile flies that produce Btn but lack Drc nevertheless succumb to this infection with mortality rates similar to flies lacking Drc alone. Thus Btn does not seem to contribute to defence against *E. cloacae* infection in a significant way.

Btn but not Drc is important for survival after P. burhodogranariea infection

We previously found that *CG10816/Dro* could contribute to defence against *P. burhodogranariea* synergistically alongside *Diptericins* and *Attacins* (Hanson et al., 2019a). We next assessed the contribution of our different *Dro* gene mutants to defence against *P. burhodogranariea*. To our surprise, the presence or absence of Buletin causes a pronounced survival difference after infection by *P. burhodogranariea*: *Dro*^{SK3} flies survive as wild type, while *Dro*^{SK4} or *Dro-AttAB*^{SK2} flies suffer significantly increased mortality (Fig. 4.2B). This trend is

the opposite of what is observed after infection with *E. cloacae*: Drc does not play an important role in defence against *P. burhodogranariea*, but Btn does. As emphasized by the susceptibility of AMP-deficient *ΔAMP14* and Imd-deficient Rel^{E20} control flies (Fig. 5.2A-B), Btn deficiency explains only part of the susceptibility to *P. burhodogranariea* alongside the contributions of *Diptericin* and *Attacin* genes (shown in (Hanson et al., 2019a)).

So far our study shows that the *CG10816/Dro* locus encodes two host-defence peptides with distinct activities in vivo. Our observation that deletion of alternate peptides causes differential susceptibility to two different bacteria reinforces the notion that innate immune effectors have very specific roles in vivo.

The T52A polymorphism affects Btn activity against P. burhodogranariea in vivo

The existence of a Threonine/Alanine polymorphic residue in Btn in natural fly populations suggests an arms race between Btn and naturally occurring pathogens. The *P. burhodogranariea* strain used in this study was originally isolated from the hemolymph of wild-caught flies (Juneja and Lazzaro, 2009), suggesting it is an ecologically relevant microbe to *D. melanogaster*. This prompted us to investigate the contribution of this polymorphism in defence against *P. burhodogranariea*. We next isolated a Btn-Threonine allele (Btn^{Thr}) and recombined this into the DrosDel isogenic background. We infected isogenic Btn^{Thr} and Btn-Alanine (*iso* w^{1118}) flies with *P. burhodogranariea* to determine if the Btn polymorphism impacts survival. Strikingly, Btn^{Thr} flies suffered a ~20% increase in mortality compared to *iso* w^{1118} flies with Btn^{Ala} (Fig. 5.2C, p = .045). The Cox survival hazard ratio (HR) is a measure of effect size. The HR of Dro^{SK4} vs. Dro^{SK3} flies (Fig. 5.2B) and Btn^{Thr}-*iso* w^{1118} (Fig. 5.2C) is nearly-identical (HRs: Dro^{SK4}-Dro^{SK3} = 0.5903, Btn^{Thr}-*iso* w^{1118}: = 0.5896). Thus the size of effect caused by Btn deletion is effectively equal to the effect of changing the Btn allele from Alanine to Threonine.

We therefore uncover a prominent role of Btn in defence against *P. burhodogranariea*, and reveal that the Btn Threonine/Alanine polymorphism impacts survival against an ecologically relevant pathogen: *P. burhodogranariea*. Collectively we show that the *Drosocin* gene encodes two peptide products, Drc and Btn, which have distinct activities against two different Gram-negative bacteria: *E. cloacae* and *P. burhodogranariea* respectively.

5.4 Discussion

Here we show that the *CG10816/Dro* gene encodes two peptides with distinct activities in vivo. Buletin was not annotated previously as *Drosophila* immune studies commonly used a genetic background encoding the Btn^{Ala} allele, and not the Btn^{Thr} allele found in the reference genome. The gene *CG10816* produces a precursor protein cleaved in two locations: i) a two-residue dipeptidyl peptidase (DP) site that is nibbled off of the N-terminus of Drc, and ii) a furin cleavage motif that separates the Drc and Btn peptides ("RVRR" in *CG10816*). Both cleavage motifs are common in AMP genes, including *Drosophila* Attacins, Defensins, Diptericins, and Baramicins, which all encode mature peptides separated by furin cleavage sites (Hanson et al., 2021; Hanson and Lemaitre, 2020; Hedengren et al., 2000). Buletin-like peptides are found only in *Dro* genes of Melanogaster and Obscura group flies, and not other *Drosophila* species (Fig. S5.2) (Hanson et al., 2016). Thus the Buletin peptide is an evolutionary novelty derived from tinkering with the *Dro* gene's C-terminus. This sequence has apparently evolved for specific activity against bacteria like *P. burhodogranariea*.

The fact that *CG10816* encodes two peptides with distinct specificities is interesting, as peptides produced by a single AMP gene might be expected to act synergistically. Previous studies have shown that Proline-rich peptides (e.g. Abaecin, Pyrrhocoricin, Oncocin) target intracellular bacterial machinery, and synergize with pore-forming partners to kill bacteria in vitro (Peng et al., 2018; Rabel et al., 2004; Rahnamaeian et al., 2016). A similar synergy was recently demonstrated for *Drosocin* in vivo by Carboni et al. (Carboni et al., 2021), where flies lacking *CG10816/Dro* and five other AMP families (collectively *Attacin, Diptericin, Defensin, Metchnikowin,* and *Drosomycin*) survived infection by *E. cloacae* similar to deletion of *CG10816/Dro* alone, but additional mutation of *Cecropin* genes that encode pore-forming peptides caused a complete susceptibility rivalling Imd deficient flies. In this case, the Drc + pore forming peptide synergy relies on more than just *Cecropins*, as deletion of just those two peptide families behaved like Dro^{SK4} mutation alone (Carboni et al., 2021). Thus while specific importance of single genes can be recovered, the dynamics of AMP cocktails in vivo are complex. Buletin also encodes multiple Prolines in a row at its C-terminus (Fig. 5.1A), suggesting it could act like other Proline-rich peptides against intracellular bacterial machinery. In this light, we speculate that the previous synergy of *Dro, Att,* and *Dpt* genes in defence against *P. burhodogranariea* likely derives from a one-two punch involving pore formation by the Attacin and Diptericin peptides, combined with Buletin attacking an intracellular target protein like bacterial DnaK/Hsp70 (Hanson et al., 2019a; Kragol et al., 2001).

It remains to be seen whether Buletin displays antibacterial activity in vitro. As such, it will be interesting to test the specificity of the Btn^{Thr} and Btn^{Ala} variants alone, or alongside pore-forming peptides to determine their MIC and confirm if Btn activity relies on being able to enter the bacterial cytoplasm. AMPs were first identified for their potent microbicidal activity in vitro (Imler and Bulet, 2005). However more recently we have recovered striking specificity of AMPs in defence in vivo that was never predicted from in vitro antimicrobial activity analyses (Cohen et al., 2020; Hanson et al., 2019a; Unckless et al., 2016). While in vitro approaches powerfully demonstrate the potential of AMPs, we are realizing more and more that this is not sufficient to characterize peptide activity in vivo. For instance the activity of azithromycin antibiotic changes 64-fold if tested in standard in vitro conditions or with the addition of 20% human serum (Belanger et al., 2020). Likewise Bomanin peptides do not display activity in vitro, but Bomanin-deficient hemolymph loses Candida-killing activity (Lindsay et al., 2018). These results suggest both in vitro and in vivo approaches shed light on host defence peptide activity.

Here we reveal that a Threonine/Alanine polymorphism in Buletin affects the fly defence against *P. burhodogranariea*. This polymorphism is found in wild populations of *D. melanogaster* (Lazzaro and Clark, 2003), and occurs at the following frequency in the Drosophila Genetic Reference Panel (Mackay et al., 2012): genetic variant 2R_10633648_SNP = 29% Threonine, 64% Alanine, 7% unknown. Thus neither allele is especially rare in the wild. A polymorphism in *Diptericin A* causes a profound susceptibility to defence against *Providencia rettgeri* (Unckless et al., 2016), and similar polymorphisms are found in various AMP genes of flies (Chapman et al., 2019; Unckless and Lazzaro, 2016) and other animals including fish, birds, and humans (Hellgren et al., 2010; Hellgren and Sheldon, 2011; Hollox and Armour, 2008). We now add our study on Buletin and *P. burhodogranariea* to the building evidence that such polymorphisms can have important impacts on microbial control. It will be interesting to reflect on these findings in other systems : could inbreeding in honeybees have fixed a disadvantageous AMP allele contributing to colony collapse disorder (Fürst et al., 2014)? Reduced AMP expression is associated with conditions like psoriasis (Marcinkiewicz and Majewski, 2016), and one study has even proposed polymorphisms in human *beta-Defensins* correlate with atopic dermatitis (Prado-Montes de Oca et al., 2007). Might future work show that alleles in key AMPs correlate with predisposition to infectious syndromes?

Conclusion

Our study contributes to a growing body of literature establishing the *Drosophila* systemic infection model as boasting a uniquely specific interplay of host AMP-pathogen interactions. This model effectively treats the fly hemolymph as a petri dish, where bacterial proliferation can be measured in the absence of specific AMPs. In flies, in vivo study has now revealed multiple instances where in vitro explorations suggested fly AMPs had generalist activities. Such studies would never have predicted the highly specific requirement for only single peptides in defence against specific pathogens. Taking lessons from the fly, it should be of significant interest to characterize the differential activity of AMP polymorphisms in humans and other animals, which could reveal critical risk factors for infectious diseases.

5.5 Materials and Methods

Fly genetics

Genetic variants were isogenized into the DrosDel isogenic background over 7 generations as described in (Ferreira et al., 2014). The specific mutations studied here were sourced as follows: the Dro^{SK3} mutation was generated by CRISPR-Cas9 via gRNA injection as described in (Kondo and Ueda, 2013). The Btn^{Thr} allele used in this study was originally detected in *iso* Def^{SK3} flies from Hanson et al. (Hanson et al., 2019a) by virtue of mutation-specific MALDI-TOF proteomics while screening for possible source genes of IM7. This Btn^{Thr} locus was recombined away from the *iso* Def^{SK3} chromosome via two additional rounds of recombination, and *iso* Btn^{Thr} flies were confirmed to have a wild-type *Defensin* gene by PCR. Accordingly, previous studies using Def^{SK3} individual mutants used stocks with a *CG10816/Dro* Btn^{Thr} locus. This finding does not impact experiments with recombined Def^{SK3} mutants, which were each confirmed to have a DrosDel-like Btn^{Ala} allele. Sequence comparisons were made using Geneious R10.

Microbe culturing conditions

Bacteria were grown to mid-log phase shaking at 200rpm in their respective growth media (Luria Bertani, MRS+Mannitol) and temperature conditions, and then pelleted by centrifugation to concentrate microbes. Resulting cultures were diluted to the desired optical density at 600nm (OD) for survival experiments, which is indicated in each figure. The following microbes were grown at 37°C: *Escherichia coli strain 1106* (LB)*, Providencia rettgeri* (LB)*.* The following microbes were grown at 29°C: *Providencia burhodogranariea* (LB) and *Acetobacter sp. ML04.1 (MRS+Mannitol*).

Survival experiments

Survival experiments were performed as previously described (Hanson et al., 2019a), with 20 flies per vial with 2-3 replicate experiments. ~5 day old males were used in experiments unless otherwise specified. Flies were flipped thrice weekly. Statistical analyses were performed using a Cox proportional hazards (CoxPH) model in R 3.6.3.

Proteomic analyses

Raw hemolymph samples were collected from immune-challenged flies for MALDI-TOF proteomic analysis as described in (Hanson et al., 2019a; Uttenweiler-Joseph et al., 1998). In brief, hemolymph was collected by capillary and transferred to 0.1% TFA before addition to acetonitrile universal matrix. Representative spectra are shown. Peaks were identified via corresponding m/z values from previous studies (Hanson et al., 2021; Levy et al., 2004). Spectra were visualized using mMass, and figures were additionally prepared using Inkscape v0.92.

Author contributions:

MAH performed bioinformatic analyses and planned and performed infection experiments. BL supervised the project and MAH and BL wrote the manuscript. SK generated and supplied *Dro*^{SK3} flies.

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5.6 References

Belanger CR, Lee AH-Y, Pletzer D, Dhillon BK, Falsafi R, Hancock REW. 2020. Identification of novel targets of azithromycin activity against *Pseudomonas aeruginosa* grown in physiologically relevant media. *Proc Natl Acad Sci USA* **117**:33519–33529. doi:10.1073/pnas.2007626117

Bikker FJ, Kaman-van Zanten WE, de Vries-van de Ruit A-MBC, Voskamp-Visser I, van Hooft PAV, Mars-Groenendijk RH, de Visser PC, Noort D. 2006. Evaluation of the antibacterial spectrum of drosocin analogues. *Chem Biol Drug Des* **68**:148–153. doi:10.1111/j.1747-0285.2006.00424.x

Bulet P, Dimarcq JL, Hetru C, Lagueux M, Charlet M, Hegy G, Van Dorsselaer A, Hoffmann JA. 1993. A novel inducible antibacterial peptide of Drosophila carries an O-glycosylated substitution. *J Biol Chem* **268**:14893–14897.

Bulet P, Urge L, Ohresser S, Hetru C, Otvos L. 1996. Enlarged scale chemical syn-

thesis and range of activity of drosocin, an O-glycosylated antibacterial peptide of Drosophila. *European Journal of Biochemistry* **238**:64–69. doi:10.1111/j.1432-1033.1996.0064q.x

Carboni A, Hanson MA, Lindsay SA, Wasserman SA, Lemaitre B. 2021. Cecropins contribute to *Drosophila* host defence against fungal and Gram-negative bacterial infection (preprint). Immunology. doi:10.1101/2021.05.06.442783

Chapman JR, Hill T, Unckless RL. 2019. Balancing selection drives maintenance of genetic variation in Drosophila antimicrobial peptides. *Genome Biology and Evolution* **11**:2691–2701. doi:https://doi.org/10.1093/gbe/evz191

Clemmons AW, Lindsay SA, Wasserman SA. 2015. An Effector Peptide Family Required for Drosophila Toll-Mediated Immunity. *PLoS Pathogens* **11**. doi:10.1371/journal.ppat.1004876

Cohen LB, Lindsay SA, Xu Y, Lin SJH, Wasserman SA. 2020. The Daisho Peptides Mediate Drosophila Defense Against a Subset of Filamentous Fungi. *Front Immunol* **11**:9. doi:10.3389/fimmu.2020.00009

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. 2002. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. *EMBO Journal* **21**:2568–2579. doi:10.1093/emboj/21.11.2568

Ferreira ÁG, Naylor H, Esteves SS, Pais IS, Martins NE, Teixeira L. 2014. The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral Infection in Drosophila. *PLoS Pathogens* **10**. doi:10.1371/journal.ppat.1004507

Fürst M a., McMahon DP, Osborne JL, Paxton RJ, Brown MJF. 2014. Disease associations between honeybees and bumblebees as a threat to wild pollinators. *Nature* **506**:364–366. doi:10.1038/nature12977

Hanson MA, Cohen LB, Marra A, Iatsenko I, Wasserman SA, Lemaitre B. 2021. The Drosophila Baramicin polypeptide gene protects against fungal infection. *PLoS Pathog* **17**:e1009846. doi:10.1371/journal.ppat.1009846

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

Hanson MA, Hamilton PT, Perlman SJ. 2016. Immune genes and divergent antimicrobial peptides in flies of the subgenus Drosophila. *BMC evolutionary biology* **16**:228. doi:10.1186/s12862-016-0805-y

Hanson MA, Lemaitre B. 2020. New insights on Drosophila antimicrobial peptide function in host defense and beyond. *Curr Opin Immunol* **62**:22–30. doi:10.1016/j.coi.2019.11.008

Hanson MA, Lemaitre B, Unckless RL. 2019b. Dynamic Evolution of Antimicrobial Peptides Underscores Trade-Offs Between Immunity and Ecological Fitness. *Frontiers in Immunology* **10**:2620. doi:doi: 10.3389/fimmu.2019.02620

Hedengren M, Borge K, Hultmark D. 2000. Expression and evolution of the Drosophila attacin/diptericin gene family. *Biochemical and biophysical research communications* **279**:574–81. doi:10.1006/bbrc.2000.3988

Hellgren O, Sheldon BC. 2011. Locus-specific protocol for nine different innate immune genes (antimicrobial peptides: β-defensins) across passerine bird species reveals within-species coding variation and a case of trans-species polymorphisms. *Molecular Ecology Resources* **11**:686–692. doi:10.1111/j.1755-0998.2011.02995.x

Hellgren O, Sheldon BC, Buckling A. 2010. In vitro tests of natural allelic variation of innate immune genes (avian ??-defensins) reveal functional differences

in microbial inhibition. *Journal of Evolutionary Biology* **23**:2726–2730. doi:10.1111/j.1420-9101.2010.02115.x
Hollox EJ, Armour JAL. 2008. Directional and balancing selection in human beta-defensins. *BMC Evolutionary Biology* **8**. doi:10.1186/1471-2148-8-113
Imler J-L, Bulet P. 2005. Antimicrobial peptides in Drosophila: structures, activities and gene regulation. *Chemical immunology and allergy* **86**:1–21. doi:10.1159/000086648
Juneja P, Lazzaro BP. 2009. Providencia sneebia sp. nov. and Providencia burhodogranariea sp. nov., isolated from wild Drosophila melanogaster. *International Journal of Systematic and Evolutionary Microbiology* **59**:1108–1111. doi:10.1099/ijs.0.000117-0
Kondo S, Ueda R. 2013. Highly Improved gene targeting by germline-specific Cas9 expression in Drosophila. *Genetics* **195**:715–721. doi:10.1534/genetics.113.156737
Kragol G, Lovas S, Varadi G, Condie BA, Hoffmann R, Otvos L. 2001. The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. *Biochemistry* **40**:3016–3026. doi:10.1021/bi002656a
Lazzaro BP, Clark AG. 2003. Molecular population genetics of inducible antibacterial peptide genes in Drosophila melanogaster. *Mol Biol Evol* **20**:914–923. doi:10.1093/molbev/msg109
Lazzaro BP, Zasloff M, Rolff J. 2020. Antimicrobial peptides: Application informed by evolution. *Science* **368**. doi:10.1126/science.aau5480
Lemaitre B, Hoffmann J. 2007. The Host Defense of Drosophila Melanogaster. *Annual Review of Immunology* **25**:697–743. doi:10.1146/annurev.immunol.25.022106.141615
Lemaitre B, Kromer-Metzger E, Michaut L, Nicolas E, Meister M, Georgel P, Reichhart JM, Hoffmann JA. 1995. A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense. *Proc Natl Acad Sci U S A* **92**:9465–9469. doi:10.1073/pnas.92.21.9465
Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**:973–983. doi:10.1016/s0092-8674(00)80172-5
Lemaitre B, Reichhart JM, Hoffmann JA. 1997. Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proceedings of the National Academy of Sciences of the United States of America* **94**:14614–9. doi:10.1073/pnas.94.26.14614
Levy F, Rabel D, Charlet M, Bulet P, Hoffmann JA, Ehret-Sabatier L. 2004. Peptidomic and proteomic analyses of the systemic immune response of Drosophila. *Biochimie* **86**:607–616. doi:10.1016/j.biochi.2004.07.007
Lin SJH, Cohen LB, Wasserman SA. 2020. Effector specificity and function in Drosophila innate immunity: Getting AMPed and dropping Boms. *PLoS Pathog* **16**:e1008480. doi:10.1371/journal.ppat.1008480
Lindsay SA, Lin SJH, Wasserman SA. 2018. Short-Form Bomanins Mediate Humoral Immunity in Drosophila. *J Innate Immun* **10**:306–314. doi:10.1159/000489831
Mackay TFC, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, Casillas S, Han Y, Magwire MM, Cridland JM, Richardson MF, Anholt RRH, Barrón M, Bess C, Blankenburg KP, Carbone MA, Castellano D, Chaboub L, Duncan L, Har-

ris Z, Javaid M, Jayaseelan JC, Jhangiani SN, Jordan KW, Lara F, Lawrence F, Lee SL, Librado P, Linheiro RS, Lyman RF, Mackey AJ, Munidasa M, Muzny DM, Nazareth L, Newsham I, Perales L, Pu L-L, Qu C, Ràmia M, Reid JG, Rollmann SM, Rozas J, Saada N, Turlapati L, Worley KC, Wu Y-Q, Yamamoto A, Zhu Y, Bergman CM, Thornton KR, Mittelman D, Gibbs RA. 2012. The Drosophila melanogaster Genetic Reference Panel. *Nature* **482**:173–8. doi:10.1038/nature10811

Marcinkiewicz M, Majewski S. 2016. The role of antimicrobial peptides in chronic inflammatory skin diseases. doi:10.5114/pdia.2015.48066

Marra A, Hanson MA, Kondo S, Erkosar B, Lemaitre B. 2021. *Drosophila* antimicrobial peptides and lysozymes regulate gut microbiota composition and abundance (preprint). Microbiology. doi:10.1101/2021.03.19.436153

Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ. 2020. Antimicrobial host defence peptides: functions and clinical potential. *Nat Rev Drug Discov*. doi:10.1038/s41573-019-0058-8

Peng S, Yang M, Sun RN, Liu Y, Wang W, Xi Q, Gong H, Chen C. 2018. Mechanism of actions of Oncocin, a proline-rich antimicrobial peptide, in early elongation revealed by single-molecule FRET. doi:10.1007/s13238-017-0495-2

Prado-Montes de Oca E, García-Vargas A, Lozano-Inocencio R, Gallegos-Arreola MP, Sandoval-Ramírez L, Dávalos-Rodríguez NO, Figuera LE. 2007. Association of β-Defensin 1 Single Nucleotide Polymorphisms with Atopic Dermatitis. *Int Arch Allergy Immunol* **142**:211–218. doi:10.1159/000097023

Rabel D, Charlet M, Ehret-Sabatier L, Cavicchioli L, Cudic M, Otvos L, Bulet P. 2004. Primary Structure and in Vitro Antibacterial Properties of the Drosophila melanogaster Attacin C Pro-domain. *Journal of Biological Chemistry* **279**:14853–14859. doi:10.1074/jbc.M313608200

Rahnamaeian M, Cytryńska M, Zdybicka-Barabas A, Vilcinskas A. 2016. The functional interaction between abaecin and pore-forming peptides indicates a general mechanism of antibacterial potentiation. *Peptides* **78**:17–23. doi:10.1016/j.peptides.2016.01.016

Rolff J, Schmid-Hempel P. 2016. Perspectives on the evolutionary ecology of arthropod antimicrobial peptides. *Philosophical Transactions of the Royal Society B: Biological Sciences* **371**. doi:10.1098/rstb.2015.0297

Sanchez Bosch P, Makhijani K, Herboso L, Gold KS, Baginsky R, Woodcock KJ, Alexander B, Kukar K, Corcoran S, Jacobs T, Ouyang D, Wong C, Ramond EJV, Rhiner C, Moreno E, Lemaitre B, Geissmann F, Brückner K. 2019. Adult Drosophila Lack Hematopoiesis but Rely on a Blood Cell Reservoir at the Respiratory Epithelia to Relay Infection Signals to Surrounding Tissues. *Developmental Cell* **51**:1–17. doi:https://doi.org/10.1016/j.devcel.2019.10.017

Steiner H, Hultmark D, Engström \AA, Bennich H, Boman HG. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **292**:246–248. doi:10.1038/292246a0

Unckless RL, Howick VM, Lazzaro BP. 2016. Convergent Balancing Selection on an Antimicrobial Peptide in Drosophila. *Current Biology* **26**:257–262. doi:10.1016/j.cub.2015.11.063

Unckless RL, Lazzaro BP. 2016. The potential for adaptive maintenance of diversity in insect antimicrobial peptides. *Philosophical Transactions of the Royal Society B: Biological Sciences* **371**:20150291. doi:10.1098/rstb.2015.0291

Uttenweiler-Joseph S, Moniatte M, Lagueux M, Van Dorsselaer a, Hoffmann J a,

Bulet P. 1998. Differential display of peptides induced during the immune response of Drosophila: a matrix-assisted laser desorption ionization time-of-flight mass spectrometry study. *Proceedings of the National Academy of Sciences of the United States of America* **95**:11342–11347. doi:10.1073/pnas.95.19.11342

5.7 Figures chapter 5

5.7.1 Main figures

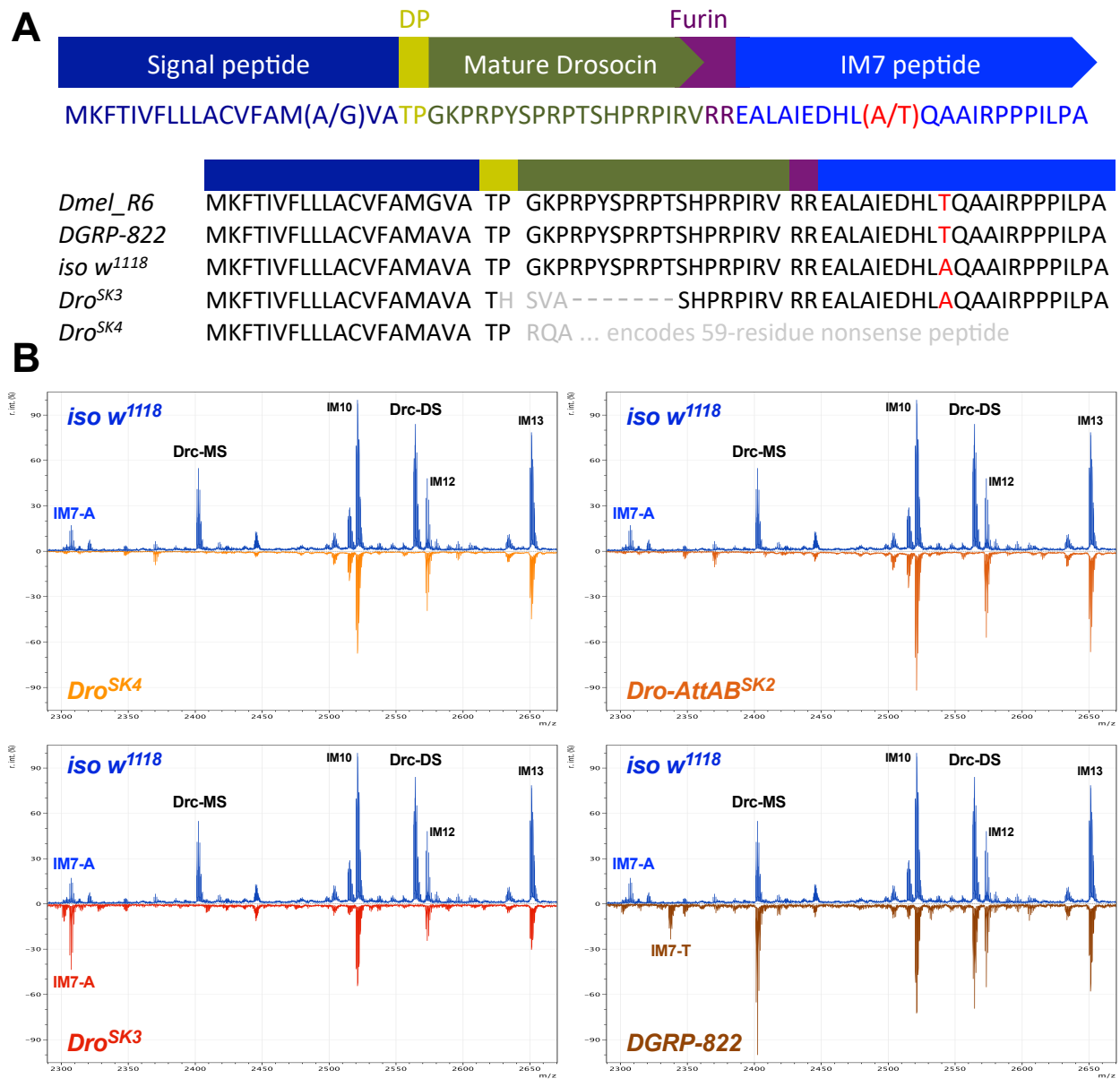

Figure 5.1: The *CG10816/Dro* gene encodes a polypeptide including both Drc and IM7.

A) Overview of the precursor protein structure of the *CG10816/Dro* gene. The T52A polymorphism in IM7 was noted previously (Lazzaro and Clark, 2003). Here we include an alignment of the CG10816 precursor protein in the Dmel_R6 reference genome and sequences from *iso w*^{1118}, *Dro*^{SK3}, *Dro*^{SK4}, and *DGRP-822* flies. B) MALDI-TOF proteomic data from immune-challenged flies shows that both Drc (Dro-MS, Dro-DS) and the 2307 Da peak of IM7 is absent in *Dro*^{SK4} and *Dro-AttAB*^{SK2} flies. The frameshift present in *Dro*^{SK3} removes the Drc peptide, but does not prevent the secretion of IM7. Threonine-encoding IM7 appears in DGRP-822 (2337 Da), alongside loss of the 2307 Da peak.

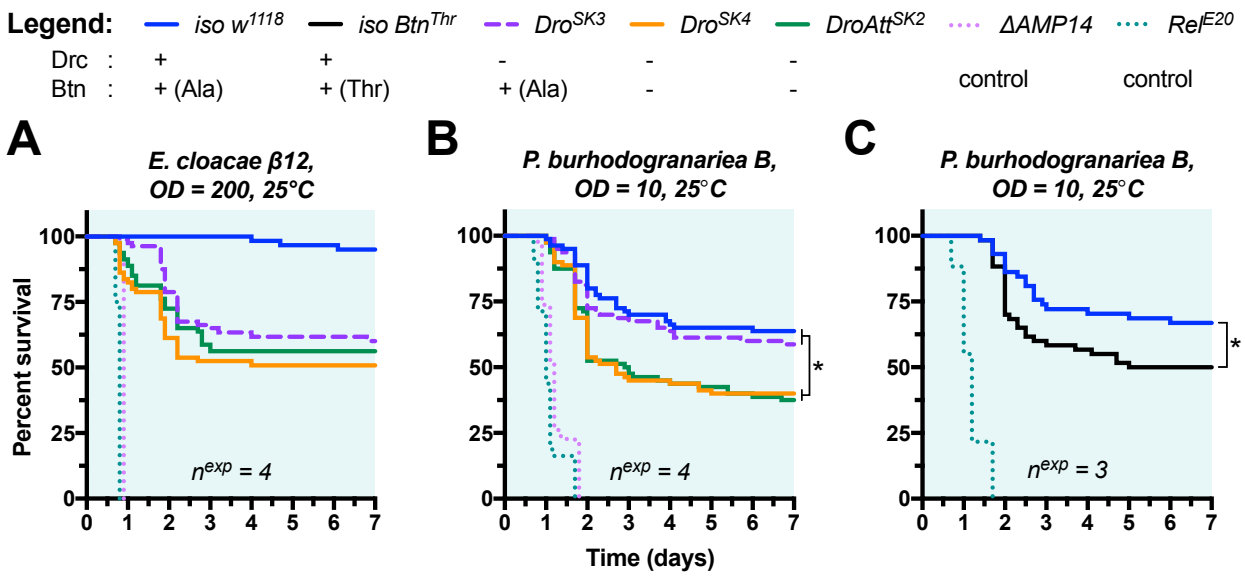

Figure 5.2: The *CG10816/Dro* gene C-terminal peptide IM7 improves survival after infection by *Providencia burhodogranariea*.

A) *Dro*^{SK3} flies succumb to infection by *E. cloacae* slightly later than either *Dro*^{SK4} or *Dro-AttAB*^{SK2} flies that lack both Drc and Btn, with ultimate mortality at comparable levels ($p > .05$ in comparisons between *Dro* mutants). B) *Drosocin* mutants that retain IM7 (*Dro*^{SK3}) survive infection by *Providencia burhodogranariea* better than flies lacking both Drc and Btn (*Dro*^{SK4}, *DroAtt*^{SK2}). C) The Threonine allele of the Btn T52A polymorphism phenocopies flies lacking Btn in defence against *P. burhodogranariea*.

5.7.2 Supplementary figures

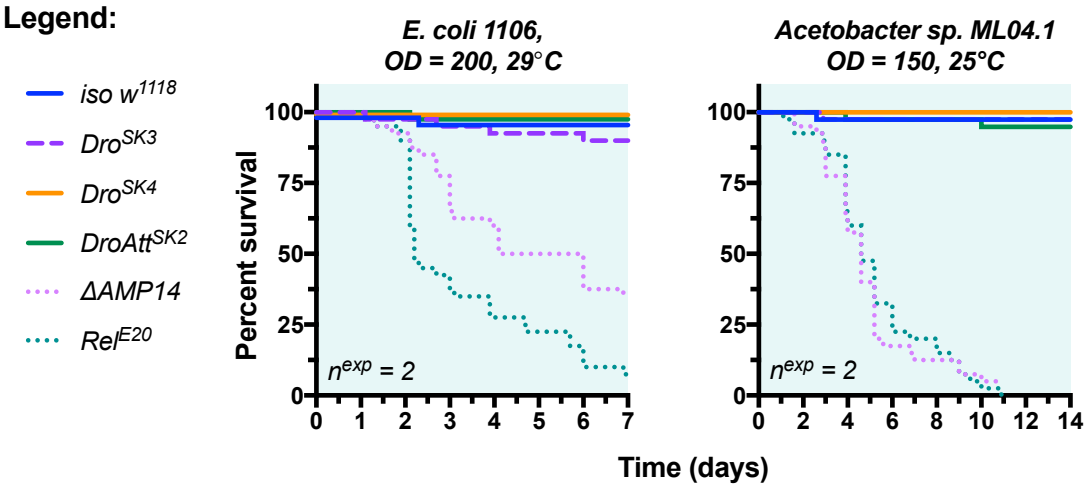

Figure S5. 1: *CG10816/Dro* mutants are not susceptible to *E. coli 1106* or *Acetobacter sp. ML04.1* infection.

ΔAMP14 flies lacking seven AMP gene families including the *CG10816/Dro* gene, and Rel^{E20} mutants deficient for Imd signalling both succumb to these infections.

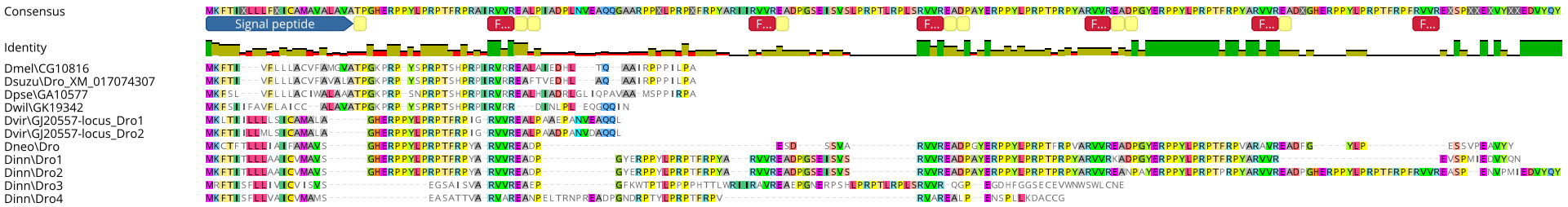

Figure S5. 2: Alignment of Drosocin proteins encoded by various *Drosophila* species.

Buletin-like C-terminus peptides are found in *D. pseudoobscura, D. suzukii,* and *D. melanogaster Dro* genes. In *D. willistoni* and subgenus Drosophila flies, Buletin-like peptides are not found. Uniquely the *D. neotestacea* and *D. innubila Dro* genes encode multiple Drc peptides in tandem between furin cleavage sites (red boxes at top of alignment). These furin sites are usually followed by dipeptidyl peptidase sites (yellow boxes at top of alignment), similar to the tandem repeat structure of honeybee *Apidaecin* and *Drosophila Baramicin*.

Chapter 6 On the future of AMP research

6.1 Understanding the logic of the *Drosophila* immune effector response

The work presented here has validated the previous AMP cocktail model in vivo, with some AMP interactions contributing additively or synergistically to defence against infection. However a surprising finding is the high degree of specificity that certain AMPs have for dictating host-pathogen interactions. Hans Boman's laboratory was founded on the basis of two principle questions : 1) how can an animal survive infection by a bacterium with a 20 minute doubling time, when adaptive immune responses take days to mature? And 2) how do insects survive infection when they have no antibodies or lymphocytes (Carton, 2019; Faye and Lindberg, 2016)? A great deal of progress on these questions has been made since Boman started his lab in 1960. We can list a number of metabolic changes that occur alongside infection to promote survival. However these metabolic shifts generally operate on a principle of preventing microbial growth by caching host resources, rather than on directly killing invading pathogens. We now also have a highly detailed understanding of the core signalling pathways involved in innate immune defence, including the epistatic organization of these pathways. In the 1990s, it seemed likely that AMPs, potent antibiotics regulated by Toll and Imd signalling, explained the microbicidal properties of immune-induced hemolymph. Indeed this thesis confirms that AMPs are critical components of the insect immune response, and AMPs directly explain how insects survive infection without antibodies or lymphocytes – even against bacteria that can divide every twenty minutes. However the assumption that AMPs act broadly and with redundancy is now being challenged, and it is clear that individual effector genes contribute to defence in ways that were never predicted (Hanson and Lemaitre, 2020; Lazzaro et al., 2020; Lin et al., 2020).

It is now apparent that our model of the *Drosophila* effector response is incomplete. Indeed, the work presented here does not explain how effectors contribute to the drastic susceptibility of Toll-deficient flies against Gram-positive bacterial infection beyond the broad requirement of Bomanins. How do Bomanins work? Do they primarily enable other effectors, or are current vitro approaches simply inadequate to recapitulate Bomanin activity in vivo? This is an exciting future avenue to explore, both for its importance in host defence but also to understand potentially core biological principles underlying the microbicidal potency of the innate immune response. Indeed, while *Bomanin* genes are restricted to fruit flies, the mechanism of Bomanin function could be universally applicable and of pharmaceutical interest. While I have both discovered and characterized the source genes of the last two unknown IMs from Uttenweiler-Joseph et al. (Uttenweiler-Joseph et al., 1998) (IM22 in chapter 3 and IM7 in chapter 5), many *Drosophila* immune effectors are awaiting investigation. My work on *Baramicin* did not clarify the activity of IM22 and IM24 (*BaraA* peptides), though in a separate

article I provide evolutionary insight that suggests IM24 is complex and not strictly useful only in an immune context (Hanson and Lemaitre, 2021). Of the original 24 IMs, IM18 (*CG33706*) has a gene annotation but remains undescribed. This gene is encoded in a region annotated as polycistronic with CG10332, obfuscating its expression pattern (e.g. expression is identical for *IM18* and *CG10332* in FlyAtlas (Robinson et al., 2013)). Ongoing work in our lab suggests this gene is ancient (>100 million years old), principally regulated by the Imd pathway, and may be important for defence against bacterial infection (data not shown). It is likely that IM18 is an Imd-regulated AMP with an ancestry as old as Diptericin and with similar expression dynamics.

IM18 is not the only immune-regulated gene that remains to be described. Here I will outline a number of observations on immune effectors and their evolution. Given specific roles for AMPs in vivo, a full knowledge of the fly immune effectors may be needed to explain the fly defence against microbes "X, Y, and Z". In the following sections, I will outline what is known about these genes from the literature and reflect on these findings with regards to AMPs more broadly.

6.2 Immune effector peptides awaiting description

6.2.1 Undescribed mature peptides of known AMP genes

Drosophila AMP processing informs on cryptic mature peptides

First I would like to emphasize that animal AMP genes very commonly encode polypeptides: *Baramicin* is a poster child for this structure, but by no means alone. Many AMPs of insects and other animals have conserved furin cleavage sites (RXRR/RXKR) in their precursor protein directly upstream of their mature peptides (Gerdol et al., 2020; Hanson and Lemaitre, 2020). My work in other *Drosophila* species suggests this furin site is malleable (e.g. RXVR is common in Immigrans-Tripunctata flies (Hanson et al., 2016), and see Fig. S5.2). My work on Baramicin also led to the realization that most of these AMPs possess dipeptidyl peptidase sites (XA/XP motifs) following both their signal peptide and furin cleavage domains, which to my knowledge had never been formally addressed (Hanson et al., 2021). This realization is critical in predicting the mature products of uncharacterized AMP genes, as additional cleavage motifs can now be inferred. Through this understanding, I realized the fly Defensin propeptide is an independent molecule that is indeed secreted into the hemolymph during infection. The Defensin precursor protein encodes a signal peptide, propeptide, furin cleavage site, and mature Defensin peptide in tandem (Fig. 6.1A). The propeptide is annotated as being 32 residues in the June 2nd 2021 release of UniProtKB – (entry P36192: DEFI_DROME). However this annotation fails to consider the Defensin propeptide's dipeptidyl peptidase site at its N-terminus, and also the furin cleavage site at the propeptide's C-terminus. Accounting for these motifs yields a 28-residue peptide with a mass of 3229.5 Da (Fig. 6.1A : VSDVDPIPEDHVLVHEDAHQEVLQHSRQ). I revisited our AMP mutant MALDI data and realized that an immune-induced peak at ~3229 Da is present in wild-type flies, but not our AMP mutants (Fig. 6.1B). I next used Actin-Gal4 to overexpress either *Defensin* or *Baramicin A* as a control, and bled larvae to detect this putative Defensin propeptide by overexpression. As predicted, both the canonical Defensin peptide (4354 Da) and a 3229 Da peak appear unique-

ly in *Act>Def* larvae but not *Act>BaraA* larvae (Fig. 6.1B). Interestingly, in both immune-induced fly hemolymph and *Act>Def* larvae, I also detected an additional 3358 Da peak uniquely lost in AMP mutants and present upon Defensin overexpression (Fig. 6.1B). This suggests this molecule may similarly be produced by Defensin, possibly via a post-translational modification of a *Defensin* gene product. Thus the fly *Defensin* gene produces not just one mature peptide, but two (or perhaps even three), and each is a prominent product in immune-induced hemolymph. Two recent studies attribute anti-cancer properties to the fly *Defensin* gene (Araki et al., 2019; Parvy et al., 2019). Should the Defensin propeptide mediate these effects, any downstream investigation (e.g. therapeutic development) will be misled by the prevailing tunnel vision that focuses on only the famous mature peptide rather than the entirety of the gene's products; to clarify, I have no reason to doubt the fly Defensin mediates these anti-cancer effects, but current evidence extrapolates beyond what is shown. As emphasized by the distinct roles of the Drosocin and Buletin peptides in defence against different bacteria (chapter 5), distinct roles for different AMP gene domains should not be discounted. Fly Defensin has been shown to act against Gram-positive bacteria in vitro, despite a prominent role of the Imd pathway in regulating its expression in vivo (Cociancich et al., 1993; De Gregorio et al., 2002; Dimarcq et al., 1994; Schlamp et al., 2021; Troha et al., 2018). Reflecting on the differences between in vitro activity and in vivo relevance (Chapters 2, 4, and 5): and the previous lack of attention paid to the Drosocin and Defensin propeptides: what should one conclude based on direct or inferential evidence of AMP activities in other animals?

Lessons for human AMP processing: the curious case of Cathelin

In this sense, many studies of AMP genes have a peculiar and possibly important blind spot. One example of this is the human *Cathelicidin* gene, which encodes the AMP LL-37 at its C-terminus. LL-37 is an α-helical peptide with broad-spectrum membrane-disrupting activity against various Gram-negative and Gram-positive bacterial species. However, the *Cathelicidin* gene family is not defined by LL-37, but rather its universal N-terminal "Cathelin" domain. To date, no study has demonstrated an antimicrobial role for the Cathelin domain in vitro. In fact, the Cathelin domain is so frequently ignored that the most up-to-date commentary I am aware of is by Margherita Zanetti in 2005 (Zanetti, 2005). Indeed an article search on PubMed yields the following number of results for the search terms Cathelicidin (3311), LL37 (1974), LL-37 (1793), and Cathelin (219) (accessed September 28th 2021). Thus the mere mention of Cathelin occurs at only ~1/15th the frequency of the very gene family it defines, or the human version of its C-terminal peptide. Yet the same Cathelin domain is found in Atlantic Cod upstream of canonical RXRR furin cleavage sites, which distinguish the Cod Cathelin from its mature AMP C-terminus. Similar 4-residue RXRR/RXKR furin cleavage motifs are found in Cathelicidin genes of frogs, fish, chicken, marsupials, horse, and rat (Fig. 6.2 red block arrows). In primates, instead a 5-residue domain rich in lysine and arginine is universally found upstream of LL-37 peptides (Fig. 6.2 black block arrows), which I suspect reflects a cleavage motif adapted for another member of the furin gene family (furin-like enzymes of the PCSK family are reviewed in (Braun and Sauter, 2019)). Thus furin-like processing of Cathelicidins is found across vertebrates, suggesting both domains might be intended as secreted gene products (like Baramicin, Drosocin, and other polypeptide AMPs of flies). At the time of writing, I could find no discussion of Cathelicidin processing by furin or related PCSK family enzymes

querying the PMC database with the following search terms: “Furin AND Cathelicidin NOT virus” or “PCSK AND Cathelicidin NOT virus”. I added “NOT virus” to avoid Sars-CoV-2, Human Papillomavirus, and other virus studies where viral cell entry is mediated by furin cleavage (Day and Schiller, 2009; Hoffmann et al., 2020).

The Cathelicidins thus represent the exact sort of blind spot that AMP research has, and which my work in flies suggests might be problematic. I will highlight three points to consider: **i)** Despite Cathelicidins being united by their Cathelin domain, almost no research is dedicated to understanding what the Cathelin domain actually does. While the Cathelin domain does not display apparent activity in vitro, my 2019 study (Hanson et al., 2019a) and the Bomanins (Clemmons et al., 2015; Lindsay et al., 2018) emphasize that in vitro activity does not always correlate with in vivo importance. **ii)** My survey of the *Cathelicidin* genes in Fig. 6.2 suggests that furin-like enzymes mediate polypeptide cleavage of this AMP family in all vertebrates. Thus both Cathelin and LL-37 peptides are intended as separate immune-regulated products. **iii)** My work in flies, particularly the contributions of Drosocin vs. IM7 to defence (Chapter 5) and IM24 in either immunity and/or neurology (Chapter 3 and (Hanson and Lemaitre, 2021)), demonstrates that any observation on an AMP polypeptide gene should consider each mature peptide’s contribution to defence or disease processes.

In this light the following is a list of already-known fly AMP genes where small propeptides are evident, but which have not received attention previously: *Attacin A, Attacin B, Defensin, Diptericin B,* and *Drosocin*. Chapter 4 in this thesis addresses the Drosocin propeptide IM7, however the possible functions of propeptides in the other AMP genes remains enigmatic.

6.2.2 Putative AMPs awaiting formal description

Mtk-like, IBIN, and edin

Beyond the classical immune-induced molecules, a number of other AMP-like genes (including polypeptide genes) are awaiting characterization (Box 6.1). Recently a paralogue of *Metchnikowin* was discovered through a single cell RNAseq study of hemocytes, and named *Mtk-like (CG43236)* (Tattikota et al., 2020). Two other immune effector genes have had complicated histories, and are known only by monikers related to the fact that they are immune-induced (Induced By INfection = *IBIN*, and Elevated during infection = *edin*). *IBIN* was previously thought to be a non-coding RNA before re-annotation of the *Drosophila* genome corrected it to a protein-coding gene (Ebrahim et al., 2021; Valanne et al., 2019). *edin* is an Attacin family member with clear homology to other Attacins and is regulated by Imd signalling (Vanha-Aho et al., 2012), but previous studies have not confirmed a role for edin in defence against Gram-negative bacteria. Instead, previous work has suggested mild susceptibilities to *Listeria* and *Enterococcus* Gram-positive bacterial infections (Gordon et al., 2008; Vanha-Aho et al., 2012) and wasp infestation (Vanha-Aho et al., 2015). For posterity, I will note that these studies on *edin* have used only overexpression or RNAi tools, which were associated with mortality after eclosion (Gordon et al., 2008), or varied in hemocyte differentation (Vanha-Aho et al., 2015) – an effect that varies wildly depending on genetic background. Vanha-Aho et al. (Vanha-Aho et al., 2012) dismissed *edin* antibacterial activity based on a lack of activity of heterologous *edin* expressed in S2 cells. I will note here that their pMT-edin-V5 tagged ex-

pression construct failed to exhibit furin cleavage (Vanha-Aho et al. (2012 - Fig. 1B), and so the precursor protein was never maturated properly. The discussion here perhaps sheds light on previous *edin* results, and again emphasizes how single gene approaches may be insufficient to reveal major susceptibilities. Combination of *edin* mutation with other *Attacins* may reveal a role for *edin* in defence against Gram-negative bacteria.

Other short peptide genes and the broader Metchnikowin gene family

As previously mentioned, the source gene of IM18 is known (*CG33706*), but to date no study has investigated its role in defence. The microbicidal activities of two other putative AMPs, Listericin (Goto et al., 2010) and GNBP-like3 (Barajas-azpeleta et al., 2018), have also been described through heterologous expression methods in vitro. However no measured in vitro activity at known concentrations has been performed, making it unclear if observed activity is in the realm of physiological relevance.

There are also many more short immune effector genes encoding AMP-like peptides that have received little attention. For instance, a two gene cluster of 1.2kb (*CG16978* and *CG9928*) encodes short peptide genes that others have found were immune-induced (Duneau et al., 2017; Ramírez-Camejo and Bayman, 2020), but no validation or focused investigation has been performed. Schlamp et al. (Schlamp et al., 2021) recently highlighted a number of highly upregulated effector genes after Imd pathway stimulation. Their list includes some of the previously discussed genes, but also CG45045 and CG43920. Upon closer inspection, I realized that many of these putative AMP genes are in fact members of a larger Metchnikowin gene family (Fig. 6.3). This includes *Mtk, Mtk-like, IBIN, CG45045*, and *CG43920*. There is also an additional short effector gene neighbouring *CG43920 (CG42649)* that shares the signal peptide + N-terminal HRH motif of Metchnikowin family members, but now encodes a highly divergent mature peptide product. Neither *CG43920* nor *CG42649* have robust expression in databases like FlyAtlas (Leader et al., 2018), despite *CG43920* being clearly inducible (Schlamp et al., 2021).

CG33493: an ancient Attacin of Diptera

Finally, I will note two additional AMP-like genes of special interest, as both have implications well beyond *Drosophila.*

First : *CG33493* encodes a clear Attacin homologue that clusters with outgroup Attacins of other flies. Intriguingly, this *Attacin* gene encodes two isoforms that differ in their C-terminal sequence. One isoform bears homology to conserved Attacins of other flies (Diptera), while the other terminates the peptide with a sequence reminiscent of Diptericin, including an aspartate (D) at the residue homologous to the *Diptericin A* polymorphism site in a conserved DXRXG motif (Fig. 6.4); Aspartate is common in outgroup Diptericins, and encoded in a DXRXG consensus motif (Hanson et al., 2019b). What is particularly striking is that this dual isoform structure is conserved in all other flies including mosquitoes (Fig. 6.4D and personal communication Rob Waterhouse). Thus the *D. melanogaster Attacin CG33493* represents an ancient Attacin structure encoding both Attacin- and Diptericin-like C-termini that predates the derivation of the Diptericin gene family (Hanson et al., 2019b).

CG4269: a cysteine-bridge stabilized immune peptide of Arthropods

Second : CG4269 encodes a short (77 residues), immune-induced (Troha et al., 2018), Cysteine-rich peptide that is predicted to fold into 4 alpha helices (Jumper et al., 2021) likely stabilized by Cysteine bridges (Fig. 6.5). What is striking about this gene is that it is universally conserved in flies, beetles, and moths, but also water fleas, millipedes, arachnids, and shrimp (personal observation). This evolutionary conservation equates to the clade Pancrustacea, whose common ancestor extends back >500ma to the Cambrian explosion. In almost all cases, a clear dipeptidyl peptidase motif (XP/XA) is present directly after the predicted signal peptide. Investigating CG4269 therefore has clear implications for arthropod immunity, with a highly conserved gene structure reminiscent of cysteine-bridge mediated Defensins.

6.3 AMPs in non-canonical roles

There has been a surge of interest in how the immune system and immune effectors might mediate disease processes. For instance, the Alzheimer's disease peptide Amyloid-beta has now been shown to have antibacterial activity in vitro (Gosztyla et al., 2018), and longstanding correlational evidence between gingivitis and Alzheimer's disease risk appears to be mediated by eventual invasion of gingival bacteria into the nervous system (Dominy et al., 2019). The brain-gut axis protein alpha-synuclein is also a major determinant of Parkinson's disease, and alpha-synuclein encodes a protein that is now understood to have antimicrobial properties (Park et al., 2016; Pineda and Burré, 2017). In nematodes, an immune-induced AMP is bound by a G-protein coupled receptor in motor neurons that triggers autophagy signalling and motor neuron degeneration (Lezi et al., 2018). These studies suggest a toxicity model where aberrant immune responses lead to disease progression, and these effects could be pinpointed to specific immune effectors.

Supporting ideas for this model come from AMP-cancer studies that have shown AMPs can suppress tumors. Typically it is thought that AMPs target non-self microbes by virtue of more negatively charged membranes and cell walls of microbes. However one change common to tumors and neurons is the exposure of negatively charged cell surface molecules like phosphatidylserine. We proposed a model where these transient effects on membrane charge might make neurons or tumors specifically susceptible to AMP perturbation (Hanson and Lemaitre, 2020). This thinking was motivated by recent studies in the fly where AMPs like Defensin were shown to inhibit tumor growth through both mutation and overexpression approaches, which can be regulated by modulating phosphatidylserine exposure (Araki et al., 2019; Parvy et al., 2019). Human AMPs such as the Cathelicidin LL-37 are a topic of significant research as an endogenous anti-cancer molecule that might avoid aberrant toxicity by acting on tumours directly while also triggering endogenous defences (Piktel et al., 2016).

AMP dynamics in vivo are likely far more complex than dynamics in vitro. This is especially true in vertebrates, where AMPs are typically thought of primarily as cytokines implicated in disease, and not especially for their antimicrobial roles in defence (Van Wetering et al., 2002). It is therefore interesting that the human Cathelicidin LL-37 can bind amyloid beta peptide, inhibiting fibril assembly (De Lorenzi et al., 2017). Genetic models of Alzheimer's have failed to reach a consensus on the importance of amyloid beta in Alzheimer's disease progression, as amyloid beta plaque levels are only poorly correlate with disease severity (Moir et al., 2018). Immune-neuronal interactions have received little attention to date. This is somewhat ex-

pected, particularly considering functional evidence of an Alzheimer's infectious process was published only recently (Dominy et al., 2019), and remains controversial in terms of importance (Abbott, 2020). However AMP heterodimer interactions like LL-37 and amyloid beta could be more commonplace than we appreciate. An elegant recent study showed that human alpha Defensin and LL-37 peptides form heterodimers (Drab and Sugihara, 2020). What is striking about their findings is that the pore size formed by these heterodimers differs from the pore size of the same peptides as homodimers. Moreover, Defensin-LL-37 heterodimers are more disruptive to bacterial membranes than homodimers, and also less disruptive to eukaryotic membranes (Drab and Sugihara, 2020). Thus Defensin-LL37 heterodimers display synergy both in killing microbes and avoiding host autotoxicity. Such synergies are missed in single-molecule studies focused on developing AMPs as therapeutics, despite a clearly important biological implication for their specificity and potential toxicity.

Studies on AMP interactions in the nervous system are also increasingly suggesting commonalities between neuropeptides and AMPs (Brogden, 2005). For instance, a neuropeptide regulating sleep in flies also improves defence against infection and has antimicrobial properties in vitro (Toda et al., 2019), and antibacterial/neuropeptides of *Hydra* jellyfish coordinate microbiome colonization (Augustin et al., 2017). As previously mentioned, motor neuron degeneration in nematodes is determined by an AMP-autophagy axis (Lezi et al., 2018), and it has now also been shown that the same AMP-receptor complex communicates with RIS-type sleep interneurons to promote lethargy in nematodes (Sinner et al., 2021). In fruit flies, *Diptericin B* and *GNBP-like3* deletions are associated with failure to form long-term memories (Barajas-azpeleta et al., 2018). Intriguingly, this effect is mediated by intraneuronal expression of *GNBP-like3*, but fat body-mediated expression of *Diptericin B*. We also showed an erect wing behavioural phenotype upon Toll pathway stimulation in male *BaraA* mutants (Hanson et al., 2021), alongside expression patterns of *BaraA* both in the fat body and nervous system. The *BaraA* gene family further adapts its sequence to primarily encode the IM24 domain in genes adapted to nervous system specific expression (Hanson and Lemaitre, 2021), suggesting different elements of the Baramicin polypeptide precursor might have either host (IM24) or microbial (IM10-like) targets. Lastly, a recent study of sexual behavioural changes upon wasp sighting honed in on the Mtk-like gene *IBIN* as mediating the behavioural response to wasp presence. *IBIN* is upregulated in the optic lobes upon wasp sighting, and *IBIN* mutants fail to exhibit an expected shift in mating behaviour (Ebrahim et al., 2021). Here I provide evidence that *IBIN* is in fact an Mtk-like gene, which is an AMP family known for its antifungal role ((Levashina et al., 1995) and Fig. 6.3). It is interesting in this light that *Mtk* mutants apparently suffer reduced neurodegeneration following traumatic brain injury (Swanson et al., 2020).

These observations collectively suggest AMPs and neuropeptides can play dual roles in both immune and nervous system contexts. The mechanisms behind functions of AMPs in either immunity or neurology appears to be a clear avenue for future study. As suggested by studies in flies and nematodes, AMP dysregulation could impact neurological processes in unexpected fashions.

6.4 Conclusion

Since the 1980s, the innate immune response has commanded far more attention than in the decades prior. Isolation of antimicrobial peptides from immune-induced blood samples provided an intuitive mechanism to explain the microbicidal host defence of insects. The idea of specific interactions between single host effector genes and microbes was largely ignored assuming redundancy governed the success of the innate immune response. This assumption was also supported by evolutionary patterns of AMP gain/loss, collectively motivating a focus on understanding the upstream signalling pathways that regulated innate immune molecules. It is only in the last few years that challenges to the redundancy model of innate immune defence have emerged. The work in this thesis supports the notion that broad redundant mechanisms of defence may exist, but these alone do not dictate a successful defence response. We are now on the cusp of a paradigm shift in how we view the body's molecular defences against infection: the recognition of broad microbial patterns like peptidoglycan reflects a need for a consistent signal to detect, allowing as rapid a response as possible upon infection. However by using such generic molecules to activate immune signalling, information is lost between the recognition and effector stages of the response. Thus the broad panel of inducible effectors reflects an antimicrobial cocktail strategy given limited information. The innate immune response deploys every possible armament that could be relevant for defence, even though many are expected to be irrelevant; when the trade-off is life or death, throwing everything plus the kitchen sink at a pathogen is a small price to pay for a better guarantee of survival. However it should be noted that the incredible specificity of Diptericin A, Drosocin, Buletin, and Diptericin B in defence against *Providencia* species, *Enterobacter*, or *Acetobacter*, remains to be demonstrated as a general biological phenomenon in other model organisms. At least in the fly, the work in this thesis reveals that such interactions are not one-off events. The fact that AMP copy number variation and presence of AMP polymorphisms is common in animals has major implications on the logic behind the innate immune response more generally. Could AMP polymorphisms have important predictive power in understanding host-pathogen interactions in human disease contexts? AMP polymorphisms could affect not only disease progression, but also explain underlying risk factors important for preventing infection in the first place. The idea that AMP variation could contribute significantly to these processes offers an intuitive and testable model to better understand risk factors behind enigmatic diseases.

In the CRISPR age of genetic manipulation, we now have the necessary tools to disrupt whatever gene is of interest no matter how large or small. With modern transcriptomics, it is now common practice to highlight signalling pathways and regulatory networks that shift upon infection, highlighting hundreds of differentially expressed genes. However it is important to remember that most differential expression is a consequence of disease, and should not be confused with a focused defence strategy (Porcu et al., 2021). Understanding signalling networks at the effector level is often presumed to be so inordinately complex that no single protein could explain the logic behind a given pathway's activation. This thesis provides a powerful demonstration defying this assumption. In the fly, we are beginning to understand how broadly relevant immune pathways confer defence against specific infections at the effector level. So far, investigations have narrowed down that single AMP genes can be the key to explaining the susceptibility of flies lacking an entire NF-κB signalling pathway. Could this speci-

ficity really only be true of flies? Or are such highly specific AMP-pathogen interactions common in innate immunity?

Infection is complex, but defence can be simple.

~Mark Austin Hanson

6.5 References

Abbott A. 2020. Are infections seeding some cases of Alzheimer's disease? *Nature* **587**:22–25. doi:10.1038/d41586-020-03084-9

Araki M, Kurihara M, Kinoshita S, Awane R, Sato T, Ohkawa Y, Inoue Y. 2019. Anti-tumour effects of antimicrobial peptides, components of the innate immune system, against haematopoietic tumours in Drosophila mxc mutants. *Disease Models & Mechanisms* **12**. doi:doi: 10.1242/dmm.037721

Augustin R, Schröder K, Murillo Rincón AP, Fraune S, Anton-Erxleben F, Herbst E-M, Wittlieb J, Schwentner M, Grötzinger J, Wassenaar TM, Bosch TCG. 2017. A secreted antibacterial neuropeptide shapes the microbiome of Hydra. *Nat Commun* **8**:698. doi:10.1038/s41467-017-00625-1

Barajas-azpeleta R, Wu J, Gill J, Welte R. 2018. Antimicrobial peptides modulate long-term memory. *PLoS Genetics* 1–26. doi:10.1371/journal.pgen.1007440

Bellen HJ, Levis RW, He Y, Carlson JW, Evans-Holm M, Bae E, Kim J, Metaxakis A, Savakis C, Schulze KL, Hoskins RA, Spradling AC. 2011. The Drosophila gene disruption project: Progress using transposons with distinctive site specificities. *Genetics* **188**:731–743. doi:10.1534/genetics.111.126995

Braun E, Sauter D. 2019. Furin-mediated protein processing in infectious diseases and cancer. *Clin Transl Immunol* **8**. doi:10.1002/cti2.1073

Brogden KA. 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? doi:10.1038/nrmicro1098

Carton Y. 2019. Innate immunity: from Louis Pasteur to Jules Hoffmann.

Clemmons AW, Lindsay SA, Wasserman SA. 2015. An Effector Peptide Family Required for Drosophila Toll-Mediated Immunity. *PLoS Pathogens* **11**. doi:10.1371/journal.ppat.1004876

Cociancich S, Ghazi A, Hetru C, Hoffmann JA, Letellier L. 1993. Insect defensin, an inducible antibacterial peptide, forms voltage- dependent channels in Micrococcus luteus. *Journal of Biological Chemistry* **268**:19239–19245.

Day PM, Schiller JT. 2009. The role of furin in papillomavirus infection. *Future Microbiol* **4**:1255–1262. doi:10.2217/fmb.09.86

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. 2002. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. *EMBO Journal* **21**:2568–2579. doi:10.1093/emboj/21.11.2568

De Lorenzi E, Chiari M, Colombo R, Cretich M, Sola L, Vanna R, Gagni P, Bisceglia F, Morasso C, Lin JS, Lee M, Mcgeer PL, Barron AE. 2017. Evidence that the human innate immune peptide LL-37 may be a binding partner of amyloid-β and inhibitor of fibril assembly. *Journal of Alzheimer's Disease* **59**:1213–1226. doi:10.3233/JAD-170223

Dietzl G, Chen D, Schnorrer F, Su K-C, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ. 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* **448**:151–156. doi:10.1038/nature05954

Dimarcq J -L, Hoffmann D, Meister M, Bulet P, Lanot R, Reichhart J -M, Hoffmann JA. 1994. Characterization and transcriptional profiles of a Drosophila gene encoding an insect defensin: A study in insect immunity. *European Journal of Biochemistry* **221**:201–209. doi:10.1111/j.1432-1033.1994.tb18730.x

Dominy SS, Lynch C, Ermini F, Benedyk M, Marczyk A, Konradi A, Nguyen M, Haditsch U, Raha D, Griffin C, Holsinger LJ, Arastu-Kapur S, Kaba S, Lee A, Ryder MI, Potempa B, Mydel P, Hellvard A, Adamowicz K, Hasturk H, Walker GD, Reynolds EC, Faull RLM, Curtis MA, Dragunow M, Potempa J. 2019. Porphyromonas gingivalis in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. *Science Advances* **5**. doi:10.1126/sciadv.aau3333

Drab E, Sugihara K. 2020. Cooperative Function of LL-37 and HNP1 Protects Mammalian Cell Membranes from Lysis. *Biophys J* **119**:2440–2450. doi:10.1016/j.bpj.2020.10.031

Duneau DF, Kondolf HC, Im JH, Ortiz GA, Chow C, Fox MA, Eugénio AT, Revah J, Buchon N, Lazzaro BP. 2017. The Toll pathway underlies host sexual dimorphism in resistance to both Gram-negative and Gram-positive bacteria in mated Drosophila. *BMC Biology* **15**. doi:10.1186/s12915-017-0466-3

Ebrahim SAM, Talross GJS, Carlson JR. 2021. Sight of parasitoid wasps accelerates sexual behavior and upregulates a micropeptide gene in Drosophila. *Nat Commun* **12**:2453. doi:10.1038/s41467-021-22712-0

Faye I, Lindberg BG. 2016. Towards a paradigm shift in innate immunity—seminal work by Hans G. Boman and co-workers. *Philosophical Transactions of the Royal Society B: Biological Sciences*. doi:10.1098/rstb.2015.0303

Gerdol M, Schmitt P, Venier P, Rocha G, Rosa RD, Destoumieux-Garzón D. 2020. Functional Insights From the Evolutionary Diversification of Big Defensins. *Front Immunol* **11**:758. doi:10.3389/fimmu.2020.00758

Gordon MD, Ayres JS, Schneider DS, Nusse R. 2008. Pathogenesis of listeria-infected Drosophila wntD mutants is associated with elevated levels of the novel immunity gene edin. *PLoS Pathog* **4**:e1000111. doi:10.1371/journal.ppat.1000111

Gosztyla ML, Brothers HM, Robinson SR. 2018. Alzheimer's Amyloid-β is an Antimicrobial Peptide: A Review of the Evidence. doi:10.3233/JAD-171133

Goto A, Yano T, Terashima J, Iwashita S, Oshima Y, Kurata S. 2010. Cooperative regulation of the induction of the novel antibacterial Listericin by peptidoglycan recognition protein LE and the JAK-STAT pathway. *J Biol Chem* **285**:15731–15738. doi:10.1074/jbc.M109.082115

Halldórsdóttir K, Árnason E. 2015. Trans-species polymorphism at antimicrobial innate immunity cathelicidin genes of Atlantic cod and related species. *PeerJ* **3**:e976. doi:10.7717/peerj.976

Hanson MA, Cohen LB, Marra A, Iatsenko I, Wasserman SA, Lemaitre B. 2021. The Drosophila Baramicin polypeptide gene protects against fungal infection. *PLoS Pathog* **17**:e1009846. doi:10.1371/journal.ppat.1009846

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

Hanson MA, Hamilton PT, Perlman SJ. 2016. Immune genes and divergent antimicrobial peptides in flies of the subgenus Drosophila. *BMC evolutionary biology* **16**:228. doi:10.1186/s12862-016-0805-y

Hanson MA, Lemaitre B. 2021. Repeated subfunctionalization of a modular antimicrobial peptide gene for neural function (preprint). bioRxiv. doi:10.1101/2021.02.24.432738

Hanson MA, Lemaitre B. 2020. New insights on Drosophila antimicrobial peptide function in host defense and beyond. *Curr Opin Immunol* **62**:22–30. doi:10.1016/j.coi.2019.11.008

Hanson MA, Lemaitre B, Unckless RL. 2019b. Dynamic Evolution of Antimicrobial Peptides Underscores Trade-Offs Between Immunity and Ecological Fitness. *Frontiers in Immunology* **10**:2620. doi:doi: 10.3389/fimmu.2019.02620

Hoffmann M, Kleine-Weber H, Pöhlmann S. 2020. A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Molecular Cell* **78**:779-784.e5. doi:10.1016/j.molcel.2020.04.022

Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, Bridgland A, Meyer C, Kohl SAA, Ballard AJ, Cowie A, Romera-Paredes B, Nikolov S, Jain R, Adler J, Back T, Petersen S, Reiman D, Clancy E, Zielinski M, Steinegger M, Pacholska M, Berghammer T, Bodenstein S, Silver D, Vinyals O, Senior AW, Kavukcuoglu K, Kohli P, Hassabis D. 2021. Highly accurate protein structure prediction with AlphaFold. *Nature*. doi:10.1038/s41586-021-03819-2

Lazzaro BP, Zasloff M, Rolff J. 2020. Antimicrobial peptides: Application informed by evolution. *Science* **368**. doi:10.1126/science.aau5480

Leader DP, Krause SA, Pandit A, Davies SA, Dow JAT. 2018. FlyAtlas 2: A new version of the Drosophila melanogaster expression atlas with RNA-Seq, miRNA-Seq and sex-specific data. *Nucleic Acids Research* **46**:D809–D815. doi:10.1093/nar/gkx976

Levashina EA, Ohresser S, Bulet P, Reichhart J -M, Hetru C, Hoffmann JA. 1995. Metchnikowin, a Novel Immune-Inducible Proline-Rich Peptide from Drosophila with Antibacterial and Antifungal Properties. *European Journal of Biochemistry* **233**:694–700. doi:10.1111/j.1432-1033.1995.694_2.x

Lezi E, Zhou T, Koh S, Chuang M, Sharma R, Pujol N, Chisholm AD, Eroglu C, Matsunami H, Yan D. 2018. An Antimicrobial Peptide and Its Neuronal Receptor Regulate Dendrite Degeneration in Aging and Infection. *Neuron* **97**:125-138.e5. doi:10.1016/j.neuron.2017.12.001

Lin SJH, Cohen LB, Wasserman SA. 2020. Effector specificity and function in Drosophila innate immunity: Getting AMPed and dropping Boms. *PLoS Pathog* **16**:e1008480. doi:10.1371/journal.ppat.1008480

Lindsay SA, Lin SJH, Wasserman SA. 2018. Short-Form Bomanins Mediate Humoral Immunity in Drosophila. *J Innate Immun* **10**:306–314. doi:10.1159/000489831

Moir RD, Lathe R, Tanzi RE. 2018. The antimicrobial protection hypothesis of Alzheimer's disease. *Alzheimer's & Dementia* **14**:1602–1614. doi:10.1016/j.jalz.2018.06.3040

Park SC, Moon JC, Shin SY, Son H, Jung YJ, Kim NH, Kim YM, Jang MK, Lee JR. 2016. Functional characterization of alpha-synuclein protein with antimicrobial activity. *Biochemical and Biophysical Research Communications* **478**:924–928. doi:10.1016/j.bbrc.2016.08.052

Parvy J-P, Yu Y, Dostalova A, Kondo S, Kurjan A, Bulet P, Lemaitre B, Vidal M, Cordero J. 2019. The antimicrobial peptide defensin cooperates with tumour necrosis factor to drive tumour cell death in Drosophila. *eLife* eLife 2019;8:e45061. doi:10.7554/eLife.45061

Piktel E, Niemirowicz K, Wnorowska U, Wątek M, Wollny T, Głuszek K, Góźdź S, Levental I, Bucki R. 2016. The Role of Cathelicidin LL-37 in Cancer Development. *Arch Immunol Ther Exp (Warsz)* **64**:33–46. doi:10.1007/s00005-015-0359-5

Pineda A, Burré J. 2017. Modulating membrane binding of α-synuclein as a therapeutic strategy. *Proceedings of the National Academy of Sciences* **114**:1223–1225. doi:10.1073/pnas.1620159114

Porcu E, Sadler MC, Lepik K, Auwerx C, Wood AR, Weihs A, Sleiman MSB, Ribeiro DM, Bandinelli S, Tanaka T, Nauck M, Völker U, Delaneau O, Metspalu A, Teumer A, Frayling T, Santoni FA, Reymond A, Kutalik Z. 2021. Differentially expressed genes reflect disease-induced rather than disease-causing changes in the transcriptome. *Nat Commun* **12**:5647. doi:10.1038/s41467-021-25805-y

Ramírez-Camejo LA, Bayman P. 2020. Gene expression on the fly: A transcriptome-level view of Drosophila's immune response to the opportunistic fungal pathogen Aspergillus flavus. *Infection, Genetics and Evolution* **82**:104308. doi:10.1016/j.meegid.2020.104308

Robinson SW, Herzyk P, Dow JAT, Leader DP. 2013. FlyAtlas: database of gene expression in the tissues of Drosophila melanogaster. *Nucleic Acids Research* **41**:D744–D750. doi:10.1093/nar/gks1141

Schlamp F, Delbare SYN, Early AM, Wells MT, Basu S, Clark AG. 2021. Dense time-course gene expression profiling of the Drosophila melanogaster innate immune response. *BMC Genomics* **22**:304. doi:10.1186/s12864-021-07593-3

Sinner MP, Masurat F, Ewbank JJ, Pujol N, Bringmann H. 2021. Innate Immunity Promotes Sleep through Epidermal Antimicrobial Peptides. *Current Biology* **31**:564-577.e12. doi:10.1016/j.cub.2020.10.076

Swanson LC, Rimkus SA, Ganetzky B, Wassarman DA. 2020. Loss of the Antimicrobial Peptide Metchnikowin Protects Against Traumatic Brain Injury Outcomes in Drosophila melanogaster. *G3 (Bethesda)* **10**:3109–3119. doi:10.1534/g3.120.401377

Tattikota SG, Cho B, Liu Y, Hu Y, Barrera V, Steinbaugh MJ, Yoon S-H, Comjean A, Li F, Dervis F, Hung R-J, Nam J-W, Ho Sui S, Shim J, Perrimon N. 2020. A single-cell survey of

Drosophila blood. *eLife* **9**:e54818. doi:10.7554/eLife.54818

Toda H, Williams JA, Gulledge M. 2019. A sleep-inducing gene, nemuri , links sleep and immune function in Drosophila **515**:509–515.

Troha K, Im JH, Revah J, Lazzaro BP, Buchon N. 2018. Comparative transcriptomics reveals CrebA as a novel regulator of infection tolerance in D. melanogaster. *PLoS Pathog* **14**:e1006847. doi:10.1371/journal.ppat.1006847

Unckless RL, Howick VM, Lazzaro BP. 2016. Convergent Balancing Selection on an Antimicrobial Peptide in Drosophila. *Current Biology* **26**:257–262. doi:10.1016/j.cub.2015.11.063

Uttenweiler-Joseph S, Moniatte M, Lagueux M, Van Dorsselaer a, Hoffmann J a, Bulet P. 1998. Differential display of peptides induced during the immune response of Drosophila: a matrix-assisted laser desorption ionization time-of-flight mass spectrometry study. *Proceedings of the National Academy of Sciences of the United States of America* **95**:11342–11347. doi:10.1073/pnas.95.19.11342

Valanne S, Salminen TS, Järvelä-Stölting M, Vesala L, Rämet M. 2019. Immune-inducible non-coding RNA molecule lincRNA-IBIN connects immunity and metabolism in Drosophila melanogaster. *PLoS Pathog* **15**:e1007504. doi:10.1371/journal.ppat.1007504

Van Wetering S, Mannesse-Lazeroms SPG, Van Sterkenburg MAJA, Hiemstra PS. 2002. Neutrophil defensins stimulate the release of cytokines by airway epithelial cells: Modulation by dexamethasoneInflammation Research. pp. 8–15. doi:10.1007/PL00000282

Vanha-Aho L-M, Anderl I, Vesala L, Hultmark D, Valanne S, Rämet M. 2015. Edin Expression in the Fat Body Is Required in the Defense Against Parasitic Wasps in Drosophila melanogaster. *PLoS Pathog* **11**:e1004895. doi:10.1371/journal.ppat.1004895

Vanha-Aho L-M, Kleino A, Kaustio M, Ulvila J, Wilke B, Hultmark D, Valanne S, Rämet M. 2012. Functional characterization of the infection-inducible peptide Edin in Drosophila melanogaster. *PLoS One* **7**:e37153. doi:10.1371/journal.pone.0037153

Zanetti M. 2005. The role of cathelicidins in the innate host defenses of mammals. *Curr Issues Mol Biol* **7**:179–196.

6.6 Figures and Tables chapter 6

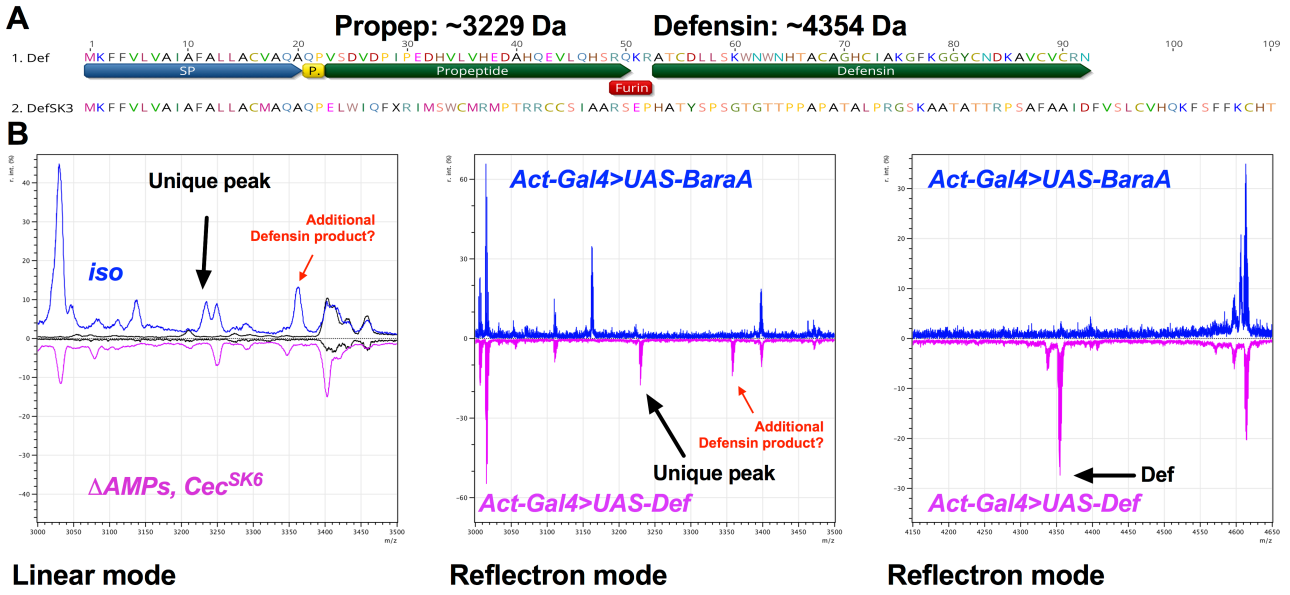

Figure 6.1: The Defensin propeptide is a prominent molecule present in immune-induced hemolymph.

A) Alignment of wild-type Defensin and the Def^{SK3} frameshift mutation present in *ΔAMP, Cec^{SK6}* flies (spectra generated by (Hanson et al., 2019a)). The signal peptide (SP, blue), Dipeptidyl peptidase motif (P, yellow), Def propeptide (green), RXKR Furin site (red), and mature Defensin peptide (green) are annotated by block arrows. Presuming Dipeptidyl peptidase activity and furin cleavage, the Defensin propeptide is predicted to have a mass of ~3229.5 Da. B) A unique immune-induced peak of ~3229 Da is observed in wild-type flies but not AMP mutants. This peak is also specific to Defensin overexpression, but not overexpression of Baramicin A. The 4354 Da peak of mature Defensin is also shown. An additional peptide product is uniquely absent in *ΔAMP, Cec^{SK6}* flies and present in the Defensin overexpression condition, suggesting this peak corresponds to a Defensin peptide product with some form of post-translational modification, or that the production of this molecule relies on the presence of Defensin (e.g. acting as a cytokine).

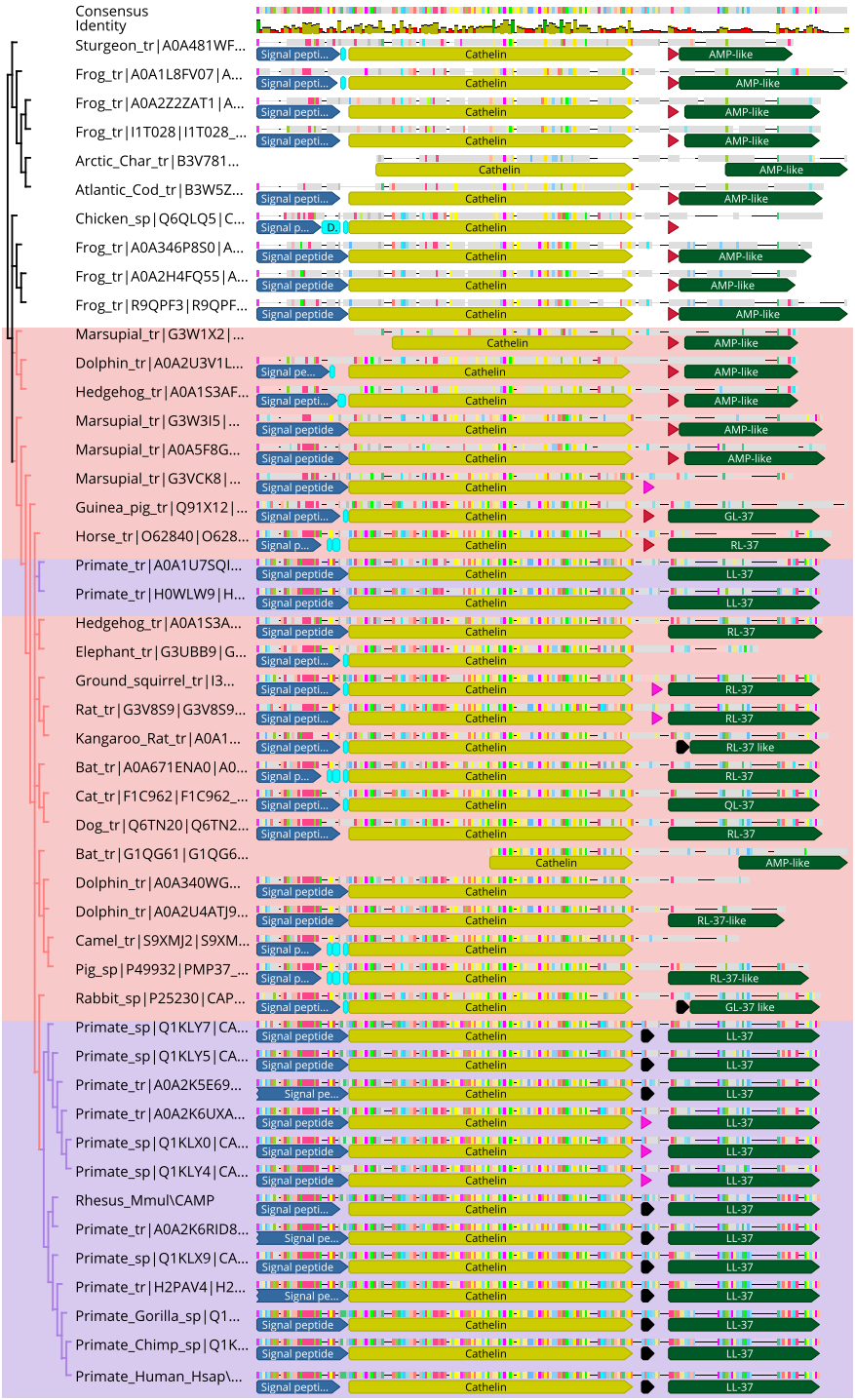

Figure 6.2: the vertebrate Cathelicidin gene family ancestrally encodes AMP-like peptides after canonical furin motifs.

Block arrows annotate protein domains: Signal peptide (blue), Dipeptidyl peptidase sites (light blue), Cathelin domain (yellow), 4-residue furin cleavage site (red, RXRR/RXKR), 4-residue furin-like cleavage site (pink, e.g. RXKK, KXKR), 5-residue polybasic cleavage cleavage site (black, commonly KXXKR), AMP-like C-terminus (green). Amino acid similarity is highlighted by coloured bars, which indicate alignment to Hsap/CAMP as the reference protein (bottom sequence). The red background indicates mammal genes, and the purple background indicates primate genes. Protein sequences were obtained from https://www.uniprot.org. Annotations were manually curated, and major peptide product start sites were cross-checked with reference annotations from https://www.uniprot.org. The cladogram represents a Neighbour Joining tree (1000bs) used primarily for simple lineage sorting.

Box 6.1: Summary of AMP-like genes awaiting formal characterization.

peptides ≥ 10AA and predicted Size (AA) assumes furin cleavage and dipeptidyl peptidase activity. Genomic location is given as "chromosome: recombination map (cytogenetic map)". When cytogenetic map was unavailable, an approximate position was inferred from recombination maps. Characteristics are labelled as Furin-cleaved (Furin), Dipeptidyl peptidase maturated (DPase), or whether a specific amino acid is enriched (common parlance in describing AMPs). Confirmed induction is given for genes after infection by Gram-negative bacteria (G-), or in the case of specific hypothesis testing, Imd, Toll or JAK-STAT regulation when confirmed. References are as follows: De Gregorio et al. (2002) [1], Troha et al. (2018) (FlySick) [2], Schlamp et al. (2021) [3], Goto et al. (2010) [4]. Conservation levels are annotated as: Drosophila, Diptera, Insecta, or Arthropoda.

AMP family	Gene	# peptides ≥ 10AA	Location	Size (AA)	Characteristics	Confirmed Expression	Conservation
Metchnikowin	*Mtk-like (CG43236)*	1	2R: 2-72 (~51)	25	P-rich	G- [2][3]	Diptera
	IBIN (CG44404)	1	2R: 2-83 (~53)	23	P-rich	G- [2][3]	Diptera
	CG43920	1	2R: 2-83 (~53)	29	P-rich, DPase	G- [2][3]	Drosophila
	CG42649	1	2R: 2-83 (~53)	52	P-rich, divergent	G- [2][3]	Drosophila
	CG45045	1	3R: 3-61 (~90)	24	P-rich	G- [2][3]	Diptera
Attacin	*Edin (CG32185)*	2	3L: 3-45 (74D)	25 and 64	Furin, DPase, P-rich, G-rich	G- [2][3]	Insecta
	CG33493	1	3L: 3-33 (67E)	76	Furin, G-rich	Constitutive [2][3]	Insecta
IM18	*CG33706*	1	2R: 2-104 (~60)	45	DPase, P-rich	Unknown	Diptera
Listericin	*Lstn*	1, possibly 2	3R: 2-64 (47E)	99, or 25 and 72	Furin?, DPase, G-rich	Imd [1], JAK-STAT [4]	Drosophila
GNBP-like3	*GNBP-like3 (CG13422)*	1	2R: 2-91 (57A)	129		Toll [1][2]	Drosophila
CG4269	*CG4269*	1	2R: 2-100 (58F)	77	DPase, C-rich	Imd [1][2] Toll [1]	Arthropoda

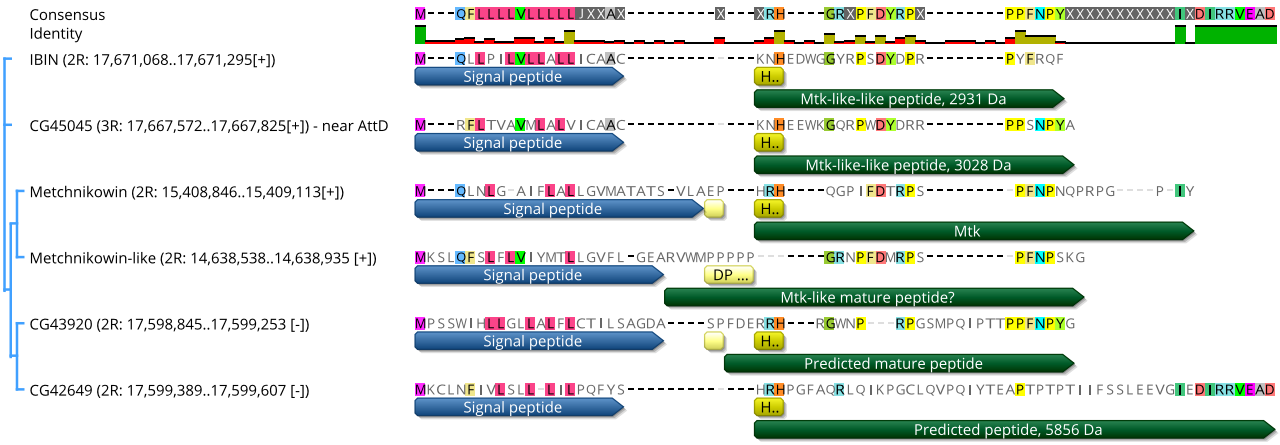

Figure 6.3: the *Metchnikowin* gene family.

Four additional genes with sequence homology to Mtk (Mtk-like, IBIN, CGG45045, CG43920), and also CG42649, which is found in tandem with CG43920 in the genome (genomic locations included). Most of these Mtk family relatives encode a polybasic motif following the signal peptide or dipeptidyl peptidase sites ending in a Histidine (e.g. KNH or HRH). Most family members also encode something resembling the consensus motif GXXPXDXRP and/or PFNP at the C-terminus. *CG42649* is not especially homologous to other Mtk family relatives, but is found within 1kb of *CG43920*, and encodes an Mtk-like HRH motif following its signal peptide. The predicted mature peptide is however highly divergent, and no longer resembles Mtk-like sequence (but bears some similarity to its sister gene CG43920: e.g. PQIXTXXP). This situation is reminiscent of the *Drosocin* gene being found directly adjacent to *Attacin A* in the genome, encoding a peptide similar to *Attacin* propeptides but a highly diverged C-terminus. Should *CG42649* encode an AMP, it may deserve its own designation independent of the Metchnikowin family to avoid implying related protein function despite likely sharing a common ancestor gene. The cladogram represents a Maximum Likelihood tree (PhyML, 100bs) used primarily for simple lineage sorting.

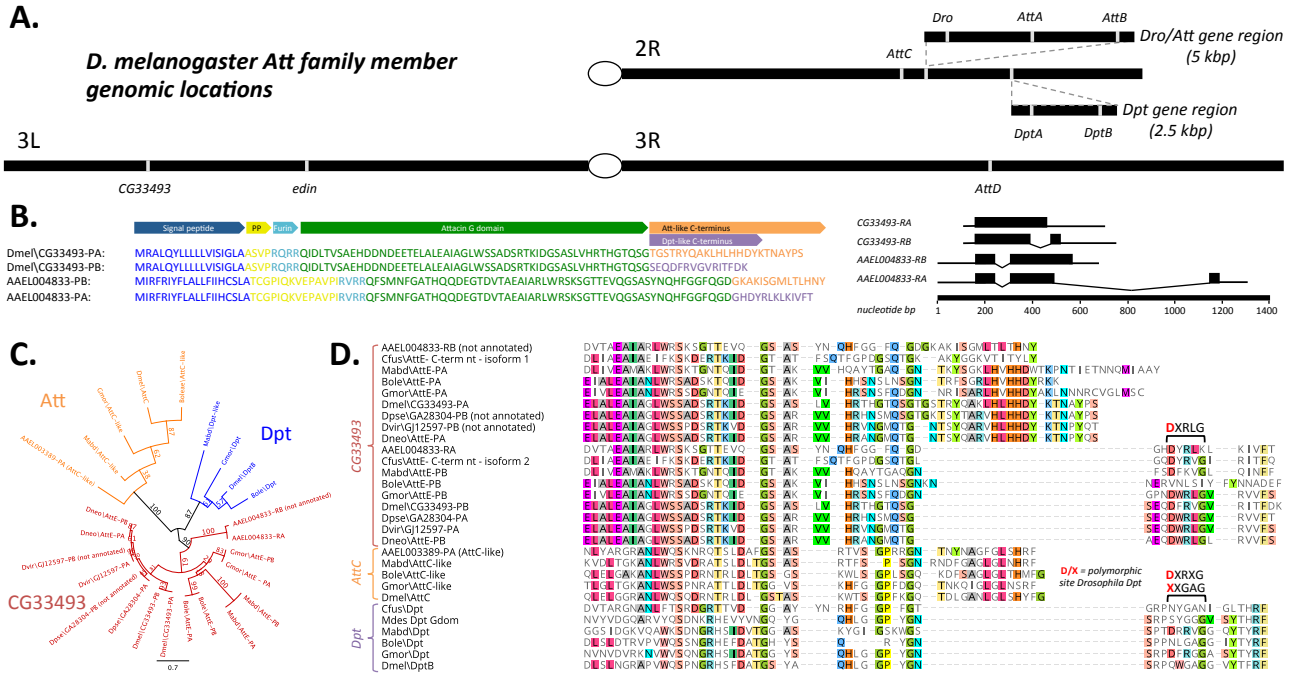

Figure 6.4: *CG33493* is an ancient *Attacin* gene.

A) Genomic locations of the Attacin superfamily genes (*Attacin, Diptericin, Drosocin* (Hedengren et al., 2000) including *CG33493* and *edin*. B) Protein sequences and transcript structures of dual isoforms of *D. melanogaster* and *Aedes aegypti CG33493* orthologues. C) Maximum Likelihood phylogeny (PhyML, 100bs) of *CG33493*-like genes of diverse flies (red branches) compared to Attacins (Att, yellow branches) and Diptericins (Dpt, blue branches). D) Conservation of dual isoforms of *CG33493* across Diptera. The *Diptericin*-like isoform encoding the 5-residue motif DXRXG is conserved across fly *CG33493* orthologues. This motif evolves rapidly in fly Diptericins (Hanson et al., 2019, 2016; Unckless et al., 2016), but remains DXRXG in *Glossina morsitans* and *Megaselia abdita* Diptericins.

Figure 6.5: *CG4269* is an ancient immune effector of arthropods.

Alignment of CG4269 mature peptides from diverse arthropod species with a simple 1000bs neighbour-Joining tree for phylogenetic sorting. Green background species are Diptera, red background species are Lepidoptera, brown background species are Coleoptera, and blue background species are outgroup arthropods including a millipede, water flea, spider, tick, and shrimp. The cysteine bridge motif and many residues within this region are universally conserved. All genes included here have a signal peptide region directly upstream (not shown to save space in alignment).

Curriculum Vitae

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Employment history

- Research Assistant, Perlman lab - University of Victoria, Canada 2015-2016
- Research Assistant, Hintz lab - University of Victoria, Canada 2016-2017

Education

- BSc. Biology - University of Victoria, Canada 2008-2013
- MSc. Biology, focus on molecular evolution - University of Victoria, Canada 2013-2015
- PhD Innate Immunity - École Polytechnique Fèdèrale de Lausanne, Switzerland 2017-present

Awards

- Best graduate student oral presentation – Entomological Society of BC (2014), Vancouver, Canada
- Best graduate student oral presentation – Society for Invertebrate Pathology (2015)

Publications

- Hanson, M.A., Hamilton, P.T. & Perlman, S.J. 2016. Immune genes and divergent antimicrobial peptides in flies of the subgenus Drosophila. *BMC Evol Biol* **16,** 228. https://doi.org/10.1186/s12862-016-0805-y
- Keais, G.L., Hanson, M.A., Gowen, B.E. and Perlman, S.J. 2017. X chromosome drive in a widespread Palearctic woodland fly, *Drosophila testacea*. J. Evol. Biol., 30: 1185-1194. https://doi.org/10.1111/jeb.13089
- Hanson MA, Dostálová A, Ceroni C, Poidevin M, Kondo S, Lemaitre B. 2019. Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. *eLife* **8**:e44341. http://doi.org/10.7554/eLife.44341
- Hanson MA, Lemaitre B, Unckless RL. 2019. Dynamic evolution of antimicrobial peptides underscores trade-offs between immunity and ecological fitness. Front. Immunol., **10**:2620. https://doi.org/10.3389/fimmu.2019.02620
- Dudzic JP, Hanson MA, Iatsenko I, Kondo S, Lemaitre B. 2019. More than black or white: Melanization and Toll share regulatory serine proteases in Drosophila. Cell Reports, **27**;4: 1050-1061.e3. https://doi.org/10.1016/j.celrep.2019.03.101
- Hanson MA and Lemaitre B. 2020. New insights on Drosophila antimicrobial peptide function in host defense and beyond. Curr Op Imm **62**: 22-30. https://doi.org/10.1016/j.coi.2019.11.008
- Hanson MA and Lemaitre B. 2021. Repeated subfunctionalization of a modular antimicrobial peptide gene for neural function. bioRxiv (preprint). https://doi.org/10.1101/2021.02.24.432738
- Marra A, Hanson MA, Kondo S, Erkosar B, Lemaitre B. 2021. *Drosophila* antimicrobial peptides and lysozymes regulate gut microbiota composition and abundance. mBio **12**;4: e00824-21. https://doi.org/10.1128/mBio.00824-21
- A. Carboni, M.A. Hanson, S.A. Lindsay, S.A. Wasserman, B. Lemaitre. 2021. Cecropins contribute to *Drosophila* host defence against fungal and Gram-negative bacterial infection. Genetics, iyab188. https://doi.org/10.1093/genetics/iyab188
- Hanson MA, Cohen LB, Marra A, Iatsenko I, Wasserman SA, Lemaitre B. 2021. The Drosophila Baramicin polypeptide gene protects against fungal infection. *PLoS Pathog* **17**:e1009846. https://doi.org/10.1371/journal.ppat.1009846

Professional memberships

- Genetics Society of America, USA
- Society for Molecular Biology and Evolution, UK