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Distinct functions of Toll effectors required for *Drosophila* immune defense

A dissertation submitted in partial satisfaction of

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in

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by

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The Dissertation of Samuel Lin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE DISSERTATION

Distinct functions of Toll effectors required for *Drosophila* immune defense

by

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This dissertation explores the overarching question of how the Toll signaling pathway mediates *Drosophila* defense against pathogens. I first discuss my research in collaboration with Scott Lindsay on the Bomanins, a family of Toll-induced secreted peptides required for Toll-mediated defense. We demonstrated that high expression of short-form Bomanins is required for humoral defense against *Candida glabrata* infection and determined that the Bomanins do not affect the presence or processing of two antimicrobial peptides (AMPs).

The second half investigates the function of a previously uncharacterized Toll effector, Bombardier. I demonstrated that Bombardier is required for defense against fungi and Gram-positive bacteria, and that deletion of *bombardier* results in the specific absence of short-form Bomanins from hemolymph. Furthermore, flies lacking Bombardier exhibited a defect in pathogen tolerance linked to aberrant Toll activation, specifically from expression of Bomanins. These

results suggest a model in which the presence of Bombardier enables secretion or intermolecular associations of short-form Bomanins, and the absence of Bombardier disrupts these steps, resulting in defects in both immune resistance and tolerance.

It has been previously thought that AMPs, some of which are conserved throughout eukaryotes, are the primary effectors of innate immunity. However, recent research in *Drosophila* has shown that AMPs play a relatively minor role in Toll-mediated immune defense. Supporting these findings, this dissertation finds that Bomanins and Bombardier, which are *Drosophila*-specific proteins, are vital for Toll-mediated immune defense in an AMP-independent manner, and encourages further exploration into genus-specific proteins.

Chapter 1:

Introduction to *Drosophila* immunity

The immune system plays a vital role in identifying pathogens and mounting a defense against them. In mammals and other vertebrates, immunity is split into two categories: the adaptive and the innate immune systems. Adaptive immunity provides specific defense based on recognition of antigens (Clark and Kupper, 2005). In contrast, innate immunity provides a rapid, general defense (Beutler, 2004). As invertebrates do not have adaptive immunity, they rely on innate immunity to provide defense against pathogens. This allows us to use model organisms like *Drosophila melanogaster*, with its wide range of genetic tools and lack of the adaptive immune system, to investigate innate immune mechanisms.

Innate immunity consists of cellular and humoral immunity. In invertebrates, the cellular response involves three types of hemocytes, comparable to white blood cells in mammals. Plasmatocytes phagocytose pathogens, such as bacteria; lamellocytes encapsulate larger pathogens, such as parasitic wasps; and crystal cells secrete phenoloxidases, which melanize wounds to plug damaged tissues (Meister and Lagueux, 2003; Williams, 2007).

The humoral response will be the focus of this dissertation. Humoral immunity consists of recognition of pathogens, activation of signaling pathways, expression of downstream genes, and secretion of antimicrobial peptides and other effectors into the hemolymph, the invertebrate circulatory fluid. In *Drosophila,* Toll and Imd are the major immune pathways that control humoral immunity (Hoffmann and Reichhart, 2002; Imler, 2014; Lemaitre and Hoffmann, 2007). The Toll pathway (Anderson et al., 1985) is activated by Lys-type peptidoglycan from most Gram-positive bacteria and by ß-1,3 glycans from fungi, as well as by wounding and by virulence factors, such as specific proteases from pathogens (Lindsay and Wasserman, 2014; Valanne et al., 2011). This recognition, signaling, and response system is comparable to the TLR pathway in mammals. The Imd pathway is activated by DAP-type peptidoglycan from Gram-negative bacteria and some Gram-positive bacteria (Kleino and Silverman, 2014; Myllymäki et al., 2014). It shares similarities

in terms of signaling components with the mammalian TNFR and TLR pathways. Activation of either of these NF-κB pathways induces the expression of many immune proteins (De Gregorio et al., 2002; Troha et al., 2018; Uttenweiler-Joseph et al., 1998). Most of these effectors, including antimicrobial peptides (AMPs), are secreted from the fat body, the *Drosophila* immune organ, into the hemolymph. The effectors then travel throughout the animal and protect the fly from pathogens.

AMPs and other immune effectors

Innate antimicrobial effectors have been studied since the 1920s, when Fleming, working with human secretions, first observed antibacterial activity which he called lysozyme (Fleming, 1922). Lysozyme is conserved throughout the animal kingdom (Callewaert and Michiels, 2010) and was first purified in 1936 from egg white (Meyer et al., 1936). In the 1970s, studies identified antibacterial activities in *Drosophila* hemolymph that increase in response to the activation of innate defenses (Boman 1972). This was followed by the purification and identification of three AMPs from insects in 1980 (Hultmark et al., 1980). Experiments to fractionate *Drosophila* hemolymph resulted in the discovery of multiple AMPs (Bulet et al., 1993; Fehlbaum et al., 1994; Levashina et al., 1995); some are homologous to AMPs in other insects and organisms (Ekengren and Hultmark, 1999; Fehlbaum et al., 1994), while others are *Drosophila*-specific (Bulet et al., 1993). AMPs cover a broad range of activity, with examples of antibacterial (Bulet et al., 1993; Hultmark et al., 1983, 1980; Levashina et al., 1995), as well as antifungal peptides (Fehlbaum et al., 1994; Hedengren et al., 1999). Further research showed that ubiquitous forced expression of single AMPs is immunoprotective in flies deficient in both the Toll and Imd pathways (Tzou et al., 2002), demonstrating that AMPs are sufficient to provide protection against infection. Based on their potent *in vitro* activity and induction after infection, AMPs have long been thought to have a primary role in immune defense. Recent research using flies lacking six of the seven classes of

Drosophila AMPs has revealed that AMPs are the primary effectors for Imd-mediated defense (against Gram-negative bacteria). However, they are not the primary effectors *in vivo* against Gram-positive bacteria or fungi, for which Toll provides defense (Hanson et al., 2019).

What effectors provides defense against Gram-positive bacteria and fungi? Other Toll effectors are highly induced after infection. One intriguing group is the Bomanin (Bom) peptides, a family of twelve Toll-induced peptides. $Bom^{\Delta 55C}$ flies, which have a deletion of ten *Bom* genes, succumb to infection with the same extent and severity as Toll-deficient flies, showing that the Boms are necessary for Toll-mediated immune defense (Clemmons et al., 2015). The family is comprised of three groups: the short-form peptides are 16-17 residues long and contain only the Bom motif; the tailed forms contain the Bom motif followed by a C-terminal tail; finally, the bicipital forms consist of two Bom motifs connected by a linker region.

This dissertation investigates the function of the short-form Boms and characterizes the function of another Toll-induced protein, Bombardier.

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Chapter 2:

Short-Form Bomanins Mediate Humoral Immunity in *Drosophila*

Abstract

The Bomanins (Boms) are a family of a dozen secreted peptides that mediate the innate immune response governed by the *Drosophila* Toll receptor. We recently showed that deleting a cluster of ten *Bom* genes blocks Toll-mediated defenses against a range of fungi and Gram-positive bacteria. Here, we characterize the activity of individual Bom family members. We provide evidence that the Boms overlap in function and that a single *Bom* gene encoding a mature peptide of just 16 amino acids can act largely or entirely independent of other family members to provide phenotypic rescue *in vivo*. We further demonstrate that the Boms function in *Drosophila* humoral immunity, mediating killing of the fungal pathogen *Candida glabrata* in an *in vitro* assay of cell-free hemolymph. In addition, we find that the level of antifungal activity both *in vivo* and *in vitro* is linked to the level of *Bom* gene expression. Although Toll dictates expression of the antimicrobial peptides Drosomycin and Metchnikowin, we find no evidence that Boms act by modifying expression of the mature forms of these antifungal AMPs.

Introduction

The innate immune system provides essential defenses against pathogens in both plants and animals and are the sole basis for defense in all nonvertebrates. Substantial insight into such systems grew out of the discovery of the Toll signaling pathway and its conservation from insects to humans (Anderson and Nüsslein-Volhard, 1984; Hoffmann et al., 1999). We now have a fairly comprehensive picture of how pathogen invasion of body tissues is recognized and how an ensuing cascade of signaling triggers immune defense activation (Lemaitre and Hoffmann, 2007; Kawai and Akira, 2011). In many organisms these defenses are demonstrably essential: infection of wild-type hosts by a range of bacteria, fungi, or viruses results in minimal morbidity or mortality, whereas hosts with compromised innate immune function succumb, often rapidly. Nevertheless, a fundamental question remains – what effector activities mediate survival in the absence of a lymphocyte-directed adaptive response?

The study of innate antimicrobial effectors began in the 1920s with the discovery of lysozyme, which Fleming found can lyse bacterial cell walls (Fleming, 1922). In the 1970s, studies on insects identified activities in hemolymph that kill bacteria or fungi and increase in response to the activation of innate defenses (Boman et al., 1972; Boman et al., 1974). This culminated in the identification and isolation of the first three antimicrobial peptides (AMPs) in 1980 (Hultmark et al., 1980). There was a flurry of interest in AMPs as potential therapeutic agents and a general assumption that they represent the primary effector mechanism. However, very few mutations have been reported that link inactivation of one or more AMP genes to an immune deficiency. Moreover, in *Drosophila,* such a link has been established not for an AMP, but rather for a set of novel effectors – the *Bomanin*, or *Bom*, gene family.

Bomanin genes encode three forms of mature peptide – short, tailed, and bicipital – that are secreted into the hemolymph upon Toll pathway activation (Uttenweiler-Joseph et al., 1998; Clemmons et al., 2015). Ten of the twelve defined *Bomanin* genes are tandemly arrayed in a cluster at position 55C on the *Drosophila melanogaster* second chromosome. We have demonstrated that excising this cluster disrupts immune defenses to the same extent and with the same specificity as blocking the entire Toll pathway (Clemmons et al., 2015). This finding raises a number of questions regarding Bom peptides as immune effectors. Are they humoral effectors or do they promote cellular immunity? Do they complex with one another or do they act as individual peptides? Is each Bomanin specific for a different pathogen?

In this paper, we explore the effector role of Boms via a two-pronged approach. We begin by using transgenic studies to define the activity of particular *Bom* genes. We then use microbiological and biophysical assays to define the nature of Bom activity *in vitro*. Together, these two approaches reveal that expression of individual Boms in hemolymph can provide both resistance to infection *in vivo* and pathogen killing *in vitro*.

Materials and Methods

Flies and Transgenic Strains

Flies were raised at 25°C on standard cornmeal molasses food. The w^{1118} strain was used as the wild-type. $Bom^{\Delta 55C}$ flies were described previously (Clemmons et al., 2015).

All transgenic constructs were based on a genomic fragment – hereafter 55C-Right – that encodes the four 55C *Bom* genes (*Bom3, Bom836, Bom065, Bom068*) remaining in the $Bom^{\Delta left}$ second chromosome deficiency (Clemmons et al., 2015). Specifically, genomic DNA encompassing region 2R:18,387,209 to 18,391,496 was amplified and cloned into attB-pNot-CaSpeR (Liu and Posakony, 2014). Next, individual *Bom* ORFs were deleted using PCR SOEing (Ho et al., 1989) in such a way as to generate four constructs, each deleted for three of the four *Bom* genes in 55C-Right.

To generate transgenic constructs expressing a single *Bom* gene under control of the *Bom3* promoter (*pBOM3*), the starting point was the 55C-Right construct with *Bom3* intact and the remaining *Bom* ORFs deleted. The *Bom3* ORF was then swapped out for another ORF using PCR SOEing, placing the introduced ORF directly downstream of *pBOM3*.

All constructs were introduced into the *Drosophila* genome by ΦC31-mediated transgenesis (Bischof et al., 2007) at an *attP* landing site located at 86Fb (BDSC stock #24749). Stable lines established for each construct carry homozygous transgenes on the third chromosome in a $Bom^{\Delta 55C}$ background.

CRISPR deletion of 55C region

Cloning and injections were performed using established protocols (Gratz et al., 2014). A pair of gRNAs designed to delete the region 2R:18,380,931 to 18,391,053 were cloned into pU6-BbsI-chiRNA (Addgene plasmid # 45946). Homology arms (766bp and 840bp left and right,

respectively) were cloned into pDsRed-attP (Addgene plasmid # 51019). pBS-Hsp70-Cas9 (Addgene plasmid # 46294) was used as the Cas9 source. Constructs were injected into w^{1118} flies.

Microbial Culture

Micrococcus luteus was cultured overnight in LB media at 37°C, heat-killed by autoclaving for 20 minutes at 121°C, and then concentrated to OD_{600} = 300 in 20% glycerol. For infection experiments, *Candida glabrata* strain *CBS 138* [ATCC 2001] was cultured overnight in YPD media at 37°C and concentrated to OD_{600} = 100 in fresh PBS containing 0.01% Tween. For antimicrobial assay experiments, *C. glabrata* was grown to mid-log phase in YPD and then diluted 1:1000 in YPD.

***Drosophila* Infection, Survival Assays, and Statistical Analyses**

Septic wounding, survival assays, and statistical analyses using GraphPad Prism were conducted essentially as previously described (Clemmons et al., 2015).

Bomanin Peptide Preparations

The 16-amino acid mature forms of Bom1 (GNVIINGDCRVCNVHG) and Bom3 (GNVIINGDCRVCNVRA) were synthesized by Lifetein and Biomatik, respectively, using a solid support resin with Fmoc and Boc chemistry. Peptides were delivered at 95% purity, confirmed by HPLC as well as mass spectrometry. Based on previously published mass spectrometry analysis (Uttenweiler-Joseph et al., 1998), an intramolecular disulfide bond was introduced into both synthetic peptides and Bom1 was amidated at the carboxyl-terminus. Bom1 was dissolved in sterile water, and Bom3 was dissolved in 50% DMSO. Peptide concentrations were determined

using the Qubit protein assay (Life Technologies), and were measured to be 43 μM (Bom1) and 56 μM (Bom3). These peptide solutions were introduced neat into the in vitro antimicrobial assay.

Hemolymph Preparation and Antimicrobial Assays

Toll pathway activation was achieved by wounding 2-7 day old male flies with heat-killed *M. luteus*. After incubating the flies at 29°C for 24 hours, cell-free hemolymph was collected from groups of seventy flies by a modification of a standard method (Neyen et al., 2014). Flies were loaded into a Zymo-Spin IC column (Zymo Research) previously washed twice with 200 μl sterile water. The flies were covered with 2 mm glass beads (Walter Stern) and centrifuged at 13,500 rpm atop a collection tube for 20 minutes at 4°C, yielding approximately four μl of cell-free hemolymph.

Antimicrobial assays with synthetic peptide or hemolymph were carried out as prescribed for standard antimicrobial peptide assays (Wiegand et al., 2008). Two μl of peptide, hemolymph, or a combination of the two was mixed with an equal volume of log-phase *C. glabrata* suspended at 1:1000 in YPD. Samples were incubated either at room temperature for 1 hour or on ice for 24 hours. Following incubation, samples were diluted to 100 μl with YPD, spread onto YPD plates, and grown overnight at 37°C. Yeast colonies were counted the next day.

Hemolymph Extraction and MALDI-TOF Analysis

After Toll activation as above, hemolymph samples for MALDI-TOF were extracted from 2-7 day old male flies using glass capillaries, and transferred with a Narishige IM-300 Microinjector into two μl 0.1% trifluoroacetic acid (TFA)/ 50% acetonitrile (ACN). The samples were then mixed 1:1 with α-cyano-4-hydroxycinnamic acid (HCCA) matrix (Agilent: 2037A), and

two µl were spotted onto a MALDI plate where sample and matrix cocrystallized *in situ* under a flow of warm air.

MALDI mass spectra were acquired using a Bruker Biflex IV MALDI-TOF mass spectrometer operated in either linear or reflectron mode with positive polarization. Desorption and ionization were achieved using a 337 nm laser. As an external mass calibration for linear mode spectra, equal parts of Peptide Calibration Standard II (Bruker: 8222570) and Protein Calibration Standard I (Bruker: 8206355) were mixed with HCCA matrix and spotted onto the same sample plate. For calibration of reflectron mode spectra, Peptide Calibration Standard II was mixed with HCCA matrix. For each genotype and condition, spectra from at least five individual flies were collected, yielding reproducible results. To identify particular peptides, the m/z values of peaks in our spectra were matched to those of the corresponding peaks in a prior study (Uttenweiler-Joseph et al., 1998). Spectra, for which representative examples are shown, were visualized using R 3.3.2, RStudio 1.0.136 and ggplot2 2.2.1.

Results

A Transgenic Assay Reveals the Effector Activity of a Single Bom Peptide

Ten *Bomanin* genes are clustered on the *D. melanogaster* second chromosome (Figure 1). Our prior studies (Clemmons et al., 2015) defined two deletions affecting this cluster, designated 55C for its position on the polytene map. $Bom^{\Delta 55C}$ flies, which lack the entire 55C *Bom* cluster, fail to mount a successful immune response against pathogens normally counteracted by the Toll pathway. In contrast, flies carrying the smaller $Bom^{\Delta left}$ deletion are susceptible to a number of pathogens, but have wild-type resistance to a relatively weak pathogen, the yeast *Candida glabrata*. Because $Bom^{\Delta left}$ lacks the six genes on the left (proximal) side of the *Bom* cluster, the four remaining *Bom* genes provided a useful starting point for defining the functional unit of Bom activity.

We generated a transgenic construct, hereafter 55C-Right, spanning the four intact *Bom* genes to the right of the deletion in $Bom^{\Delta left}$. By generating flies carrying the 55C-Right transgene on each third chromosome and the $Bom^{\Delta 55C}$ deletion on each second chromosome, we could recapitulate the genome of $Bom^{\Delta left}$. Using 55C-Right as a template, we next generated four related constructs, each expressing only a single ORF from the four *Bom* genes originally present. We then introduced two copies of the wild-type or single-gene 55C-Right constructs into the $Bom^{\Delta 55C}$ background and assayed survival after challenge with *C. glabrata* (Figure 2).

The data in Figure 2 demonstrate that a single *Bom* gene – either *Bom3* or *Bom065* – can restore resistance to *C. glabrata* in $Bom^{\Delta 55C}$ flies. We note that both *Bom3* and *Bom065* encode short-form Bom peptides, defined as having a mature length of just 16 amino acids (Clemmons et al., 2015). A third short-form *Bom* gene, *Bom068,* provided lesser but still significant rescue. Intriguingly, resistance strength appeared to correlate with *Bom* transcript level, as measured in

Toll-induced wild-type adults (Table 1). Thus, resistance was higher for *Bom3* and *Bom065* and lower for *Bom068*.

Robust Expression of Short-Form Boms Conveys Resistance to *C. glabrata*

By further modifying the 55C-Right construct, we set out to compare the activity of different *Bom* forms when expressed at comparable levels. In particular, we replaced the *Bom3* coding region with that of other *Bom* genes, i.e., fused different *Bom* ORFs to the strongly Toll responsive *Bom3* promoter (*pBOM3*). In this fashion, we generated 55C-Right constructs that should express a single short, tailed, or bicipital Bom at the high levels normally observed for *Bom3* upon Toll pathway activation. We then assayed survival after challenge with *C. glabrata* (Figure 3).

All three short-form *Bom* genes tested provided full resistance to *C. glabrata* when expressed under control of *pBOM3*. This included *Bom068*, which provided considerably weaker resistance when driven by its own promoter (compare Figures 2 and 3). These data support the hypothesis that it is the level of expression, rather than the sequence composition, of short-form Boms that determines their contribution to survival. Furthermore, we find no evidence that the short-form Bom peptides vary intrinsically in specificity for *C. glabrata*.

We turned next to representative examples of tailed and bicipital Bomanins. A tailed form, Bom836, provided no rescue of $Bom^{\Delta 55C}$ when controlled by its endogenous promoter. Analysis of endogenous transcript levels following Toll activation revealed far less transcript accumulation for *Bom836* than for either *Bom3* or *Bom065* (see Figure 2 and Table 1). However, the *Bom836* ORF failed to generate detectable rescue even when fused to *pBOM3* (Figure 3). Likewise, we did not observe rescue with *pBOM3*-driven expression of a bicipital form, Bom23 (Figure 3). Thus, neither a tailed nor a bicipital Bom exhibited activity against *C. glabrata*. For subsequent studies

we therefore focused on the short-form Boms, for which our survival assay provides a robust readout *in vivo* for an individual gene product.

Short-Form Boms Mediate Cell-Free Killing of *C. glabrata*

Having found that expression of a single short-form Bom could rescue adults otherwise immunodeficient against *C. glabrata* infection, we turned our attention to *in vitro* studies. In initial experiments we assayed synthetic mature short-form Bom peptides under conditions permissive for antimicrobial activity against *C. glabrata*. No such activity was detected with synthetic forms of mature Bom1 or Bom3 (data not shown).

In parallel, we set out to explore the *in vitro* activity of hemolymph, in which many of the Bom peptides are abundantly expressed upon infection. We stimulated the Toll pathway, collected cell-free hemolymph after 24 hours, and mixed it with an equal volume of mid-log *C. glabrata.* After incubation, the mixture was plated and the yeast colonies counted the next day. Two alternative conditions were used for incubation. The first – a one-hour incubation at room temperature – was designed to mimic conditions under which infection would normally occur. The second – a 24-hour incubation at 4°C – was designed to increase assay sensitivity. Wild-type hemolymph was fungicidal under either condition, with no detectable *C. glabrata* colonies formed (Figure 4).

When we assayed hemolymph from $Bom^{\Delta 55C}$ adults, there was a clear and dramatic difference from the wild type: deleting the 55C *Bom* genes eliminated the fungicidal activity of hemolymph. The *Bom* genes thus mediate a humoral antimicrobial activity against *C. glabrata*. Furthermore, the Bom-dependent activity of hemolymph *in vitro* parallels the Bom-dependent resistance observed *in vivo* against the same pathogen.

Hemolymph Activity Correlates with *Bom* Gene Dosage

To assay the effect of gene dosage on the level of Bom-dependent hemolymph activity, we took advantage of genetic backgrounds representing different overall levels of Bom expression. We first assayed hemolymph from $Bom^{\Delta 55C}$*; {55C-Right}* flies. Antimicrobial activity was comparable to the wild type under both conditions (Figure 4). We next assayed hemolymph from $Bom^{\Delta 55C}$; *{Bom065}* flies, in which expression of a single Bom peptide (Bom065) confers *C. glabrata* resistance in the $Bom^{\Delta 55C}$ background (see Figure 2). In this case we found no fungicidal activity by $Bom^{\Delta 55C}$*; {Bom065}* hemolymph at room temperature, but readily detectable and significant killing at 4°C overnight (Figure 4). Thus, the strength of antimicrobial activity *in vitro*, like resistance *in vivo*, appears to correlate with the overall level of short-form Bom expression.

The Levels and Composition of Antifungal AMPs Appears Bom-Independent

Although the Bomanins mediate antimicrobial activity, they could act upstream of known antimicrobial peptides (AMPs) to, for example, bring about accumulation or modification of AMP gene products. In the case of antifungal defenses, there are two AMPs that are robustly induced upon Toll activation and display strong antifungal activity *in vitro*: Drosomycin (Drs) and Metchnikowin (Mtk) (Fehlbaum et al., 1994; Levashina et al., 1995). (A third AMP, Cecropin, is active against a wide range of pathogens (Ekengren and Hultmark, 1999), but is inactive against *C. glabrata* (Lowenberger et al., 1999).) We have previously shown that induction of mRNA expression by Toll does not require Bom function (Clemmons et al., 2015). To determine if Boms act on these AMPs at the protein level, we turned to mass spectrometry.

Hoffmann, Bulet, and colleagues have reported an approach for characterizing the peptide composition of hemolymph from a single *Drosophila* adult (Uttenweiler-Joseph et al., 1998). Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF

MS), they identified singly-charged ions for a set of 24 immune induced molecules (IMs). Adopting their approach, we obtained near identical spectrograms (Figure 5 a,b, compare with Figure 3 in (Uttenweiler-Joseph et al., 1998)), enabling us to identify individual peptides by their m/z ratio. Upon inducing innate immune signaling, the IMs were readily detectable, including Drs (IM19), Mtk (IM17), and three Boms (1, 2 and 3) in the 55C cluster (Figure 5 b,c). The m/z for an additional 55C Bom peptide, Bom23, falls outside the mass range examined.

Next, we carried out parallel experiments with hemolymph from $Bom^{\Delta 55C}$ flies (Figure 5 d,e). As expected, no peaks were found with an m/z ratio matching that of Bom1, Bom2, or Bom3. Peaks were present, however, for both Drs and Mtk. In the case of Drs, the signal remained robust and with the same signal maximum. We note, however, that Drs has a relatively high molecular weight, precluding detection of a minor change in m/z in our study.

For Mtk, we did observe a significant difference in peak maxima, with a shift from 3044 m/z in the w^{1118} control to 3022 m/z in $Bom^{\Delta 55C}$. This shift, however, is not Bom-dependent. Rather, it reflects a difference in Mtk isoform between the two genetic backgrounds. There are two known isoforms of Mtk, encoding either a His or Arg residue at position 3 in the mature peptide. Both isoforms are active (Levashina et al., 1995). The m/z values corresponding to the two species exactly match those we detect here.

To verify that the Bom phenotypes do not reflect a difference in Mtk isoform, we first sequenced the *Mtk* gene in the w^{1118} and $Bom^{\Delta 55C}$ backgrounds. This analysis confirmed the isoform identification by mass spectrometry: Mtk^{R3} in the w^{1118} background and Mtk^{H3} in the $Bom^{\Delta 55C}$ background. Next, we used CRISPR-Cas9 to generate a *Bom* 55C deletion in the w^{1118} background, eliminating the polymorphism in Mtk between the control and experimental samples. Using the newly derived $Bom^{\Delta 55C\text{-}2}$ flies, we obtained survival curves after infection identical to those of the previously published TALEN-generated $Bom^{\Delta 55C}$ flies (Figure 6).

Taken together, these studies reveal that deleting the *Bom* 55C cluster has no readily detectable effect on accumulation or post-translational modification of two major Toll-induced antifungal peptides in hemolymph.

Refined Understanding of the Bom Repertoire

Six of the 55C Boms are not detected even in wild-type hemolymph. Why? They are unlikely to represent pseudogenes, since they are well conserved at the amino acid level and lack any obvious nonsense or missense mutations. Instead, we favor two explanations. First, for a Bom with robust expression at the mRNA level, the peptide is likely present but masked. For example, we note that the calculated mass of the amidated form of Bom065 is 1723.8 m/z. This peptide is likely to fall in the shoulder of the robust peak for IM4 (calculated mass 1721.9 m/z) and therefore not be detected. Second, a number of Boms may have been missed simply because their level of immune induced expression is relatively low. Here, Bom068 provides a useful example. Compared to other short-form Boms, *Bom068* mRNA accumulates to only modest levels upon Toll activation (see Table 1). Consistent with this weak expression, we observed modest resistance to *C. glabrata* infection in flies expressing Bom068 from its endogenous promoter in a $Bom^{\Delta 55C}$ background, and were unable to detect the peptide in MALDI-TOF. However, when the *Bom068* ORF was heterologously expressed under control of the strongly Toll responsive *pBOM3* promoter we observed wild-type resistance to *C. glabrata* infection and, furthermore, ready detection of a peak at 1787 m/z, matching the predicted value in MALDI-TOF for Bom068 that has undergone proteolytic processing but not amidation (Figure 5 f,g).

Discussion

Individual 55C *Bom* Genes Mediate Antimicrobial Defense

Transgenic analyses revealed that a single, short-form 55C *Bom* gene provides resistance to *C. glabrata* in the absence of the remaining nine genes from the cluster. Furthermore, every short-form *Bom* gene tested displayed such activity. The highly similar structure of the short-form Boms thus appears to reflect a shared activity found in each. These data make it unlikely that all ten genes of the cluster form a supramolecular complex or are all part of a common activity cascade.

We did not find transgenic activity for tailed or bicipital Boms. Two explanations appear very reasonable. First, these Bom forms might have specificity for particular pathogens other than *C. glabrata*. Bom activity has been observed not just for yeast, but also for representatives of the hyphal fungi and Gram-positive bacteria (Clemmons et al., 2015). Second, the tailed and bicipital Boms might be active only in combination with other Bom family members.

Boms exist in a cluster across the *Drosophila* genus. Why did such a cluster arise if individual genes are active and interchangeable, at least in certain circumstances? Our results indicate that for particularly virulent pathogens, such as *Enterococcus faecalis*, successful immune defense requires very high levels of Bomanins (Clemmons et al., 2015). We hypothesize that a single transcription unit is insufficient to provide enough Bom peptide in the time frame required. By this model, duplication provided multiple, near-identical genes that could act as a battery for defense. Several lines of evidence support this idea. First, there are multiple, strongly Toll responsive *Bomanin* genes, each immediately downstream of a canonical TATA start site and almost always one or more perfect or near-perfect Toll response elements (Busse et al., 2007). Second, the 55C-Right construct, inserted at 86Fb and lacking more than half the 55C cluster, expresses each of the four remaining *Bom* genes at the same level as in the full-length cluster, suggesting an absence of control elements shared across the ten genes (Table 2).

If duplication was driven by a need for a high overall level of expression, what should we make of the variation we observe in sequence among Bom family members? It could provide sequence specificity for Bom action. It could also provide protection against pathogen countermeasures, varying residues targeted by microbial enzymes or inhibitors. Lastly, variation could also be a simple consequence of duplication and drift.

It is important to note that removing the 55C *Bom* cluster drastically reduces, but does not eliminate, Bom expression. A pair of additional *Bomanins* – *Bom778* (tailed) and *Bom791* (bicipital) – remain intact, as do two additional genes (*IM4*, *IM14*) that are immune induced and encode peptides that bear sequence similarity to the Bomanins. For this reason, caution is required in drawing conclusions with regard to the complete repertoire of the Bomanin family.

Bom Peptides Are Required for the Toll-Directed Humoral Defense

Bomanins are clearly required for the humoral response, as a preparation free from intact cells provides Bomanin-dependent fungicidal activity *in vitro*. Furthermore, this activity acts rapidly, killing *C. glabrata* within an hour under normal growth conditions. An involvement in humoral immunity makes good sense, as the Bomanins are secreted from the fat body into the hemolymph (Uttenweiler-Joseph et al., 1998). Indeed, they are loaded into hemolymph at concentrations sufficiently high as to suggest a stoichiometric role in defense rather than a role as cytokines.

To date, synthetic Boms have not been active in our *in vitro* assays. We have no easy explanation. The problem is not one of concentration, since MALDI-TOF revealed that synthetic peptide concentrations were well matched to those of the endogenously expressed forms. Furthermore, the measured mass to charge (m/z) values were also identical. Nor is the problem one of the exogenous peptides requiring a partner protein or other factor, since the synthetic

Bomanins are also inactive when mixed with induced $Bom^{\Delta 55C}$ hemolymph (unpublished observations). This failure to observe complementation *in vitro* suggests that the synthetic forms lack a conformation or stable interaction that is required for activity and that is normally provided *in vivo* prior to release into hemolymph.

Loss-of-function mutations in multiple family members provide a powerful tool for dissecting effector function. In this study, for example, we were able to define an essential role for the *Bom* family, assess the significance of gene duplication and divergence, and define effector specificity both *in vivo* and *in vitro*. Whereas small genes of overlapping function are a poor target for traditional mutagenesis, generating mutations in a number of such genes is now both straightforward and feasible. More insights and more surprises lie ahead.

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Chapter 2, in full, is a reprint of the material as it appears in Lindsay SA, Lin SJH, Wasserman SA. (2018). Short-form Bomanins Mediate Humoral Immunity in Drosophila. *J Innate Immun*, 10(4):306-314. The dissertation author was a co-author of this material.

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Figures

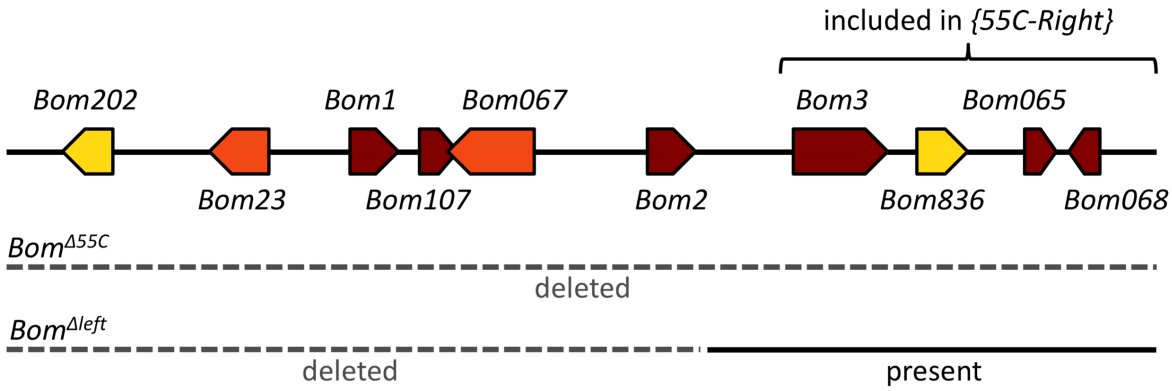


Figure 1. Organization of the *Bom* family cluster at cytogenetic map position 55C
This figure reflects a renaming of the *Bom* family members, with the CG or IM prefixes used previously replaced with the Bom prefix while retaining the gene number for both DIMs (one or two digits) and CGs (last three digits) for continuity with prior literature (De Gregorio et al., 2001; Uttenweiler-Joseph et al., 1998). Accordingly, the two remaining *Bom* genes, located outside the 55C region, are now *Bom778* and *Bom791*. For the ten genes shown in this drawing, arrowhead direction denotes gene orientation. Color indicates form of the encoded Bom peptide: short (red), tailed (yellow), or bicipital (orange). Diagrams below illustrate the extent of the chromosomal deletions in $Bom^{\Delta 55C}$ and $Bom^{\Delta left}$. The bracket at the top of the figure encompasses the *Bom* genes in the 55C-Right genomic construct.

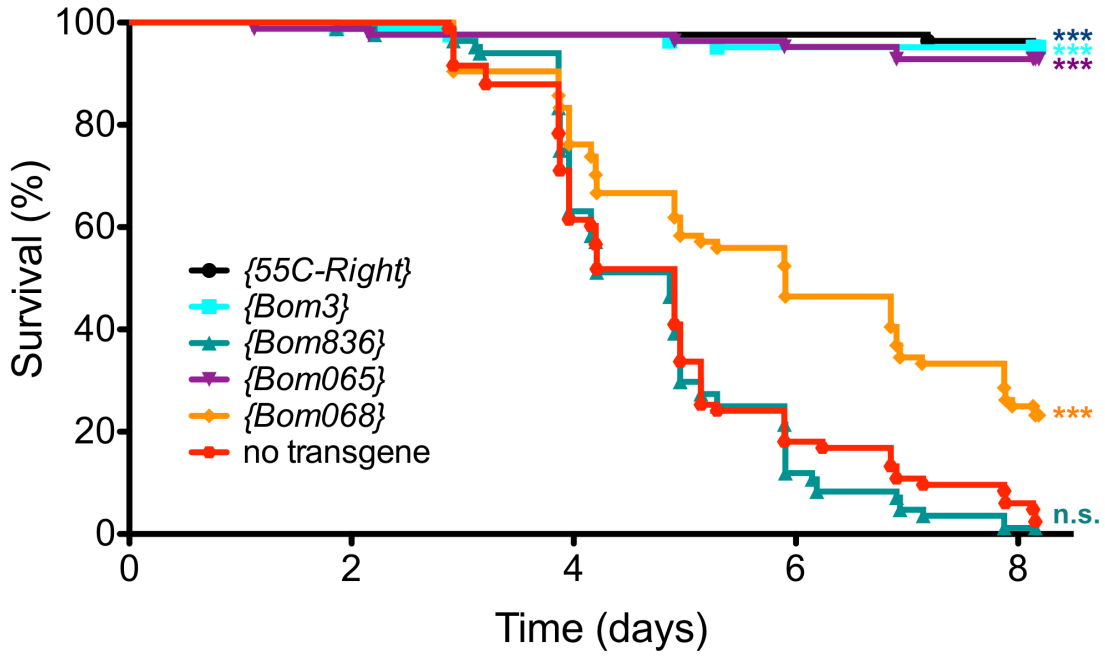


Figure 2. A single *Bom* gene rescues the *Bom*$^{\Delta 55C}$ immunodeficiency toward *C. glabrata*
This graph illustrates survival of transgenic adults at indicated intervals post-infection with *C. glabrata*. Each transgene encodes either the four Bom peptides of 55C-Right or a single one of those peptides, as indicated. All transgenes were present in two copies in a *Bom*$^{\Delta 55C}$ background. Each curve represents the pooled results of three independent experiments involving 25 or more flies per genotype. Survival curves were compared using the Gehan-Breslow-Wilcoxon test. Significance is shown relative to the *no transgene* control (*Bom*$^{\Delta 55C}$) and adjusted for multiple comparisons (*** = $p<0.0001$; n.s. = not significant, $p>0.05$).

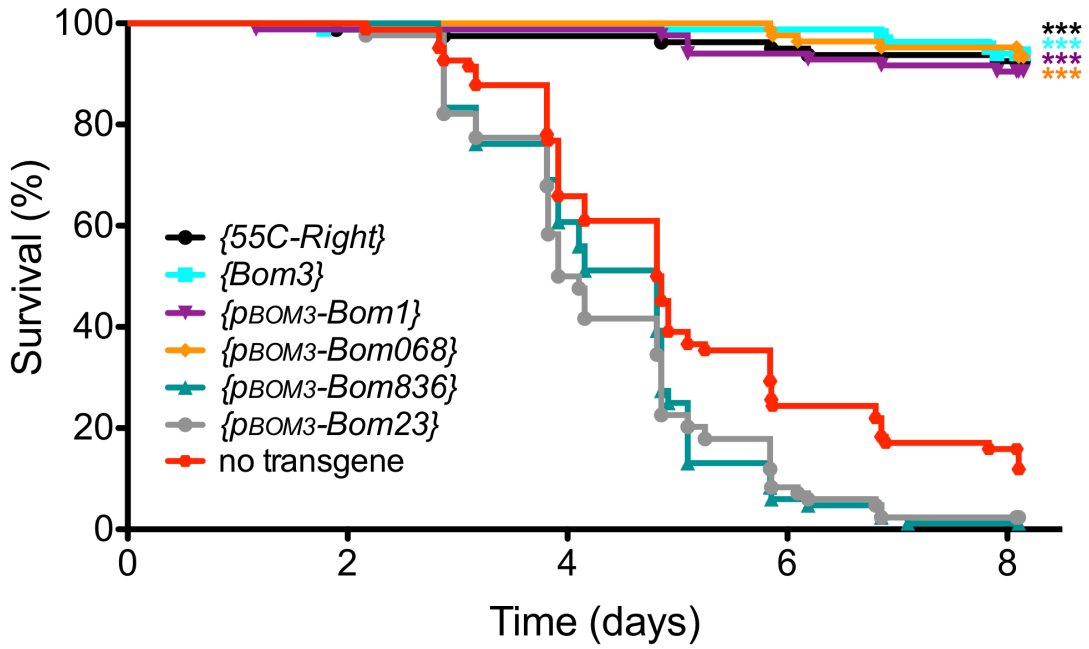


Figure 3. Short-form Bom peptides mediate survival to *C. glabrata* infection
As in Figure 2, this graph plots survival of adults at indicated intervals post-infection with *C. glabrata*. *Bom* transgenes were expressed under control of either their endogenous promoter or *pBOM3*. All transgenes were present in two copies in a $Bom^{\Delta 55C}$ background. Data were captured and analyzed as in Figure 2.

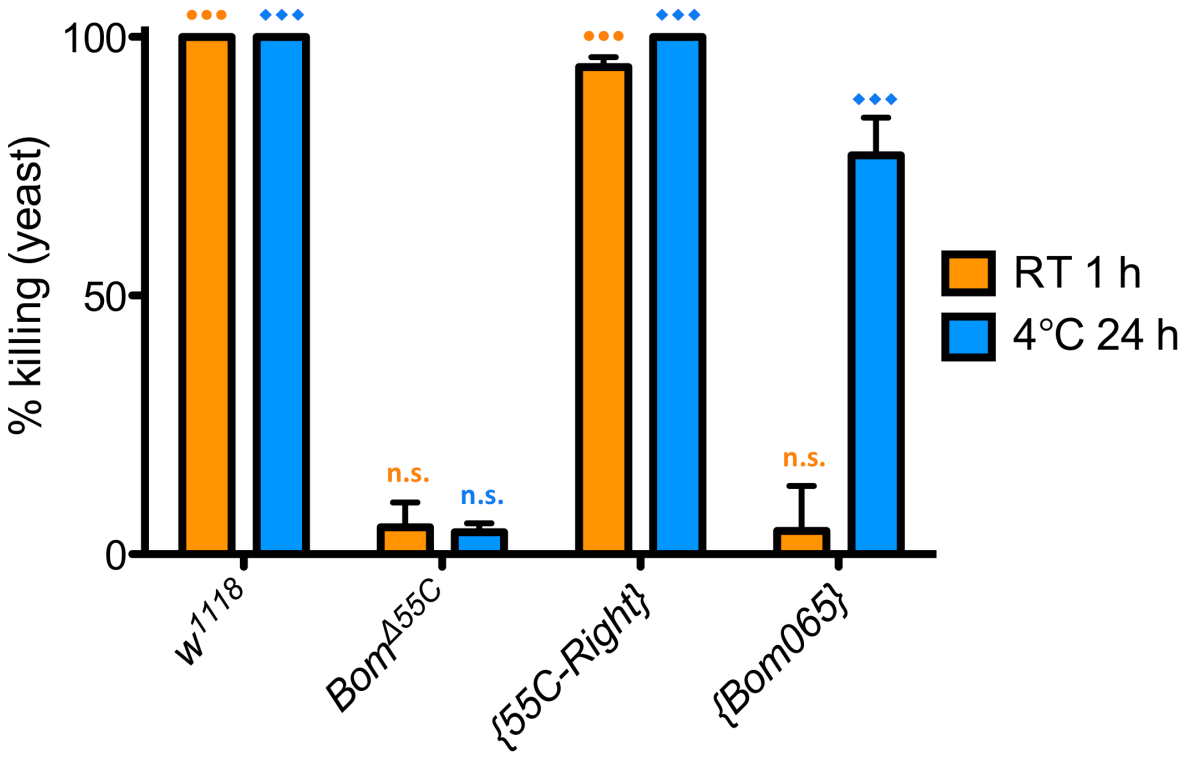
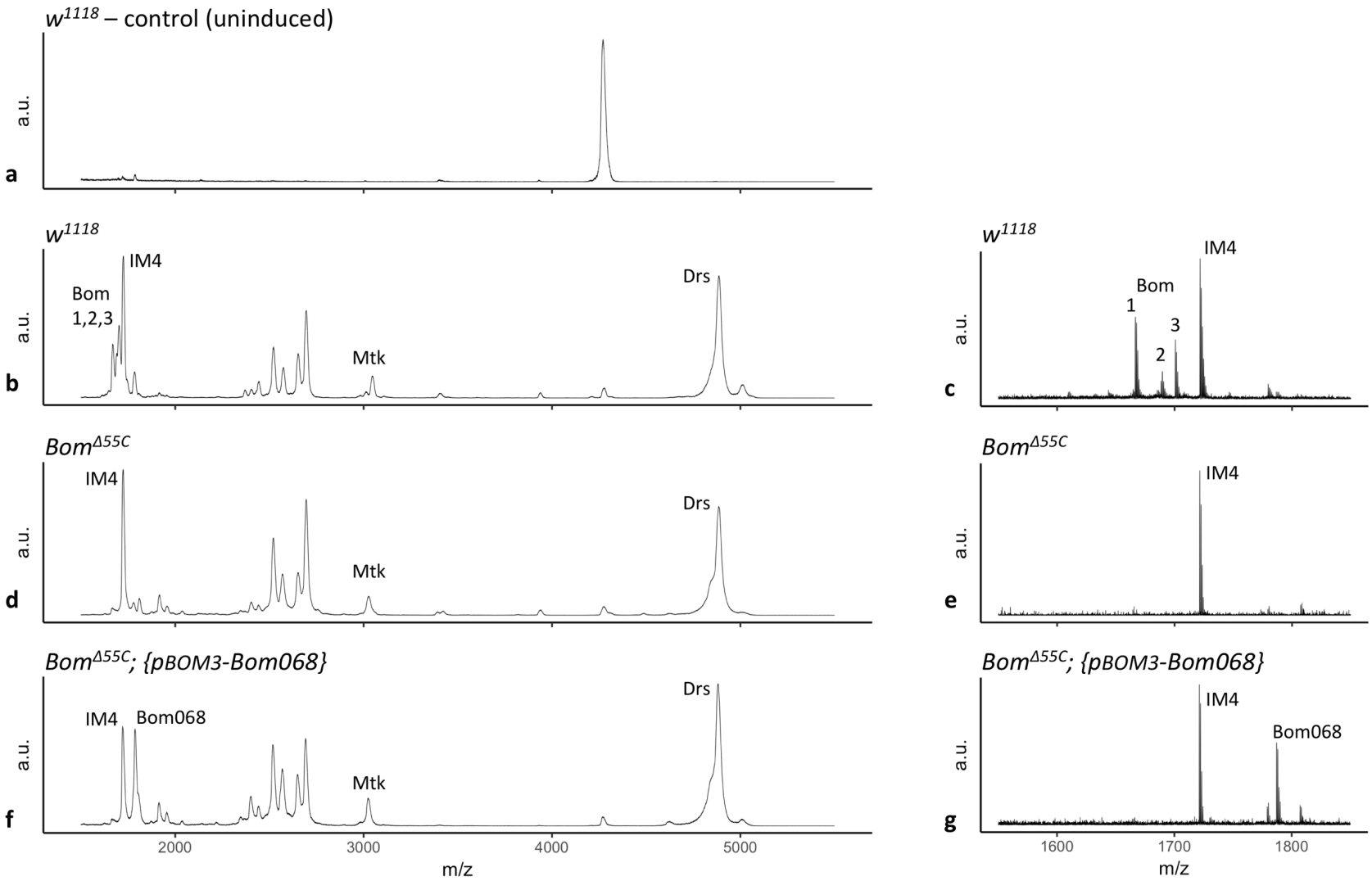


Figure 4. Fungicidal activity of hemolymph is Bom-dependent
Hemolymph activity was assayed for four sets of flies: w^{1118} (wild-type), $Bom^{\Delta 55C}$, and $Bom^{\Delta 55C}$ carrying either a 55C-Right (four-gene) or *Bom065* (single-gene) construct. All flies had been pricked one day earlier with heat-killed *M. luteus* to induce Toll-mediated gene expression. Fungicidal activity was assayed by incubating cell-free hemolymph with *C. glabrata* for 1 hour at room temperature (RT) or 24 hours at 4°C before spreading the mixture onto a yeast (YPD) plate. Percent killing was calculated by comparing the colony count for each sample to that for yeast mixed with a w^{1118} uninduced hemolymph control. Experiments were performed in triplicate; error bars represent standard error of the mean. One-way ANOVA followed by Tukey's test was performed for each incubation condition. Significance is shown relative to the w^{1118} uninduced control (••• and ◆◆◆ = $p<0.0001$; n.s. = not significant, $p>0.05$).

Figure 5. Analysis of hemolymph from adult *Drosophila* by MALDI-TOF MS
Hemolymph (50 nl) was collected from a single fly from each genotype and analyzed by MALDI-TOF MS using linear (**a**, **b, d**, **f**) or reflectron mode (**c**, **e**, **g**) (see Materials and Methods). Representative MALDI spectra from five or more such samples are shown for hemolymph from a w^{1118} fly either uninduced (control) (**a**), or induced 24 h earlier by Toll pathway stimulation with heat-killed *M. luteus* (**b, c**), and induced flies of the following genotypes: $Bom^{\Delta 55C}$ (**d, e**) and $Bom^{\Delta 55C}$*; {pBOM3-Bom068}* (**f, g**). a.u. = arbitrary units. The y-axis was scaled to the tallest peak within the window examined. Numbering and naming of induced peptides corresponds to published conventions (Uttenweiler-Joseph et al., 1998). As observed previously (Uttenweiler-Joseph et al., 1998), a prominent peak appears in control spectra that becomes insignificant in the context of Toll induction.


w1118 – control (uninduced)
a.u.
a
w1118
IM4
Bom
1,2,3
Drs
Mtk
b
w1118
IM4
Bom
1
3
2
c
BomΔ55C
IM4
Drs
Mtk
d
BomΔ55C
IM4
e
BomΔ55C; {pBOM3-Bom068}
IM4
Bom068
Drs
Mtk
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1600
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m/z

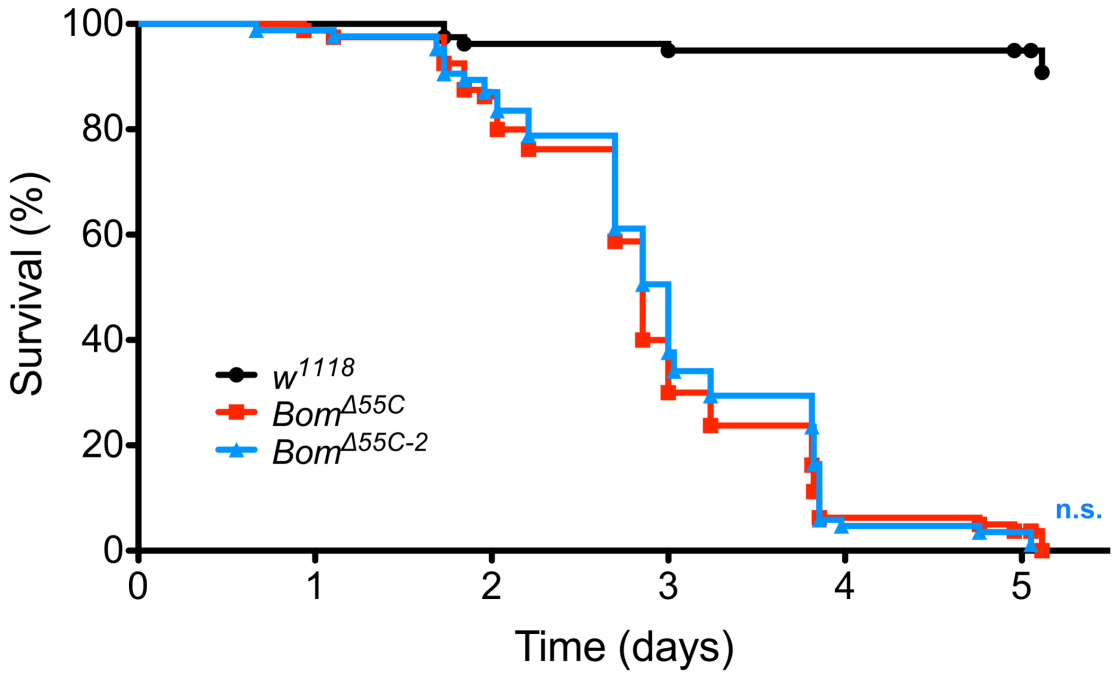

Figure 6. *Bom*$^{\Delta 55C\text{-}2}$ phenocopies *Bom*$^{\Delta 55C}$
Survival of flies after *C. glabrata* infection was monitored for wild type (w^{1118}), TALEN-generated Δ*Bom*55C (*Bom*$^{\Delta 55C}$), and CRISPR-generated Δ*Bom*55C (*Bom*$^{55C\text{-}2}$). Data were captured and analyzed as in Figure 2. Significance is shown relative to *Bom*$^{\Delta 55C}$ (n.s. = not significant, p>0.05).

Tables

Table 1. Survival after infection with *C. glabrata* correlated with total *Bom* transcript level

Transgene in $Bom^{\Delta 55C}$	Gene: Present (+) or Absent (-)				Survival at 8 days (see Figure 2)	Total transcript level from 55C *Bom* genes[§]
	Bom3	*Bom836*	*Bom065*	*Bom068*		
{55C-Right}	+	+	+	+	96%	59
{Bom3}	+	-	-	-	95%	18
{Bom836}	-	+	-	-	1%	3
{Bom065}	-	-	+	-	93%	24
{Bom068}	-	-	-	+	25%	9
no transgene	-	-	-	-	6%	none

[§] *Bom* transcript levels from adult males (2-7 days old), quantified by real-time PCR and shown relative to *rp49*, were measured 24 h after Toll induction with heat-killed *M. luteus* (see Table 2).

Table 2. Induced *Bom* transcript levels measured in wild-type and transgenic flies

Genotype	Induced *Bom* transcript level†			
	Bom3	*Bom836*	*Bom065*	*Bom068*
w^{1118}	18 ± 1.10	2.8 ± 1.26	25 ± 4.00	3.2 ± 0.047
$Bom^{\Delta 55C}$; *{55C-Right}*	19 ± 1.78	3.5 ± 1.36	30 ± 4.80	6.5 ± 0.074
$Bom^{\Delta 55C}$; *{Bom3}*	18 ± 1.84	na	na	na
$Bom^{\Delta 55C}$; *{Bom836}*	na	3.0 ± 1.34	na	na
$Bom^{\Delta 55C}$; *{Bom065}*	na	na	24 ± 1.20	na
$Bom^{\Delta 55C}$; *{Bom068}*	na	na	na	8.9 ± 0.480

†Total RNA was extracted, using Trizol, from three separate groups of flies (2-7 day old adult males, n =6) for each genotype 24 hours after Toll induction with heat-killed *M. luteus*. RNA was reverse-transcribed (SuperScript II, Invitrogen) and real-time PCR was carried out using iQ SYBR Green Supermix (Bio-Rad) with an iCycler iQ instrument. Transcript levels are shown relative to *rp49*, quantified using the $2^{-\Delta\Delta Ct}$ method, and corrected for primer amplification efficiencies. Standard error of the mean is indicated. "na" = not applicable.

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Chapter 3:

Bombardier enables delivery of short-form Bomanins in the *Drosophila* Toll response

Abstract

Toll mediates a robust and effective innate immune response across vertebrates and invertebrates. In *Drosophila melanogaster*, activation of Toll by systemic infection drives the accumulation of a rich repertoire of immune effectors in hemolymph, including the recently characterized Bomanins, as well as the classical antimicrobial peptides (AMPs). Here we report the functional characterization of a Toll-induced hemolymph protein encoded by the *bombardier* (*CG18067*) gene. Using the CRISPR/Cas9 system to generate a precise deletion of the *bombardier* transcriptional unit, we found that Bombardier is required for Toll-mediated defense against fungi and Gram-positive bacteria. Assaying cell-free hemolymph, we found that the Bomanin-dependent candidacidal activity is also dependent on Bombardier, but is independent of the antifungal AMPs Drosomycin and Metchnikowin. Using mass spectrometry, we demonstrated that deletion of *bombardier* results in the specific absence of short-form Bomanins from hemolymph. In addition, flies lacking Bombardier exhibited a defect in pathogen tolerance that we link to an aberrant Toll activation, specifically from overexpression of Bomanins. These results lead us to a model in which the presence of Bombardier in wild-type flies enables the proper folding, secretion, or intermolecular associations of short-form Bomanins, and the absence of Bombardier disrupts one or more of these steps, resulting in defects in both immune resistance and tolerance.

Introduction

Innate immune pathways are found in plants, fungi, and animals and provide a rapid defense against a broad range of pathogens (Beutler, 2004; Boller and He, 2009; Kombrink et al., 2019). In the fruit fly *Drosophila melanogaster*, the two major innate immune pathways are Toll and Imd (Hoffmann and Reichhart, 2002; Lemaitre and Hoffmann, 2007; Imler, 2014). The Toll pathway is activated by Gram-positive bacteria with Lys-type peptidoglycan and by fungi, and is required for defense against these microbes (Rutschmann et al., 2002; Gottar et al., 2006; Valanne et al., 2011; Lindsay and Wasserman, 2014). Conversely, the Imd pathway is activated by and plays a major role in survival against Gram-negative bacteria and Gram-positive bacteria with DAP-type peptidoglycan (Kleino and Silverman, 2014; Myllymäki et al., 2014). These pathways, which are both mediated by NF-κB transcription factors, are broadly conserved as initiators of innate immune responses. Activation of either pathway induces robust production of an array of immune molecules, including antimicrobial peptides (AMPs) (Uttenweiler-Joseph et al., 1998; De Gregorio et al., 2002; Levy et al., 2004; Verleyen et al., 2006; Troha et al., 2018).

AMPs are found in all kingdoms of life (Hultmark et al., 1980; Imler and Bulet, 2005; Radek and Gallo, 2007; Maróti Gergely et al., 2011; Bahar and Ren, 2013). These peptides have long been thought to play the principal effector role in innate immune defense due to their demonstrated *in vitro* antimicrobial activity and their marked upregulation after infection. However, recent research in *D. melanogaster* suggests that AMPs play a major role in Imd-mediated defense, but a relatively minor role in Toll-mediated immunity (Hanson et al., 2019).

In contrast to the AMPs, the *Drosophila*-specific Bomanin peptides (Boms), which are highly induced after infection, are indispensable for resistance against pathogens controlled by the Toll pathway (Clemmons et al., 2015). $Bom^{\Delta 55C}$ flies, which lack ten of the twelve *Bom* genes, succumb to fungal and Gram-positive bacterial infections at rates indistinguishable from Toll-

deficient flies (Clemmons et al., 2015; Hanson et al., 2019), suggesting that Boms rather than AMPs are the primary Toll effectors.

Bom peptides, like AMPs, are secreted from the fat body, the *Drosophila* immune organ, into the hemolymph, the *Drosophila* circulatory fluid. The family is comprised of three groups. The short-form peptides are 16-17 residues long and contain only the Bom motif. The tailed forms contain the Bom motif followed by a C-terminal tail. Finally, the bicipital forms consist of two Bom motifs connected by a linker region (Clemmons et al., 2015). $Bom^{\Delta 55C}$ flies lack all six of the short-form Boms, two of the three tailed Boms, and two of the three bicipital Boms. High-level expression of short-form Boms is sufficient to rescue the sensitivity of $Bom^{\Delta 55C}$ flies to *C. glabrata* infection (Lindsay et al., 2018). Furthermore, the absence of Toll-induced candidacidal activity in $Bom^{\Delta 55C}$ hemolymph can be rescued by high-level expression of a short-form Bom (Lindsay et al., 2018). However, no *in vitro* antimicrobial activity has been observed with Bom peptides alone (Lindsay et al., 2018), suggesting that the Bomanins act in coordination with additional humoral effectors.

In this study, we demonstrate an essential role in Toll-mediated humoral defense for a previously uncharacterized hemolymph protein, Bombardier (one that deploys Boms).

Materials and Methods

CRISPR/Cas9 deletion of *bombardier* locus

The *bombardier* gene (CG18067) was deleted using CRISPR/Cas9 technology according to established protocols (Gratz et al., 2014). Briefly, a pair of gRNAs designed to delete the region 2R: 20,534,248 to 20,536,154 were cloned into pU6-BbsI-chiRNA (Addgene plasmid # 45946). Homology arms (1017 bp left and 1022 bp right) were cloned into pDsRed-attP (Addgene plasmid #51019). The plasmid pBS-Hsp70-Cas9 (Addgene plasmid #46294) was used as the Cas9 source. Constructs were injected into w^{1118} embryos. F1 progeny were screened for DsRed eyes and homozygous lines were established. See Table 3 for gRNA and homology arm primer sequences.

Toll activation, *Drosophila* infection, and survival analysis

Flies were raised at 25°C on standard cornmeal molasses agar media. The w^{1118} strain was used as the wild type. Microbial isolates, culture conditions, and conditions for infection for *Enterococcus faecalis, Enterobacter cloacae, Fusarium oxysporum,* and *Candida glabrata* were as described previously (Clemmons et al., 2015), except that *C. glabrata* was concentrated to OD_{600} = 100. Flies were incubated at 25°C after live bacterial infection and at 29°C after fungal infection. For heat-killed challenge, bacterial cultures were autoclaved and resuspended in 20% glycerol to OD_{600} = 10 for *E. faecalis* and OD_{600} = 300 for *M. luteus*. For both survival assays and hemolymph preparation, flies challenged with heat-killed bacteria were incubated at 29°C.

Hemolymph antimicrobial assays

Candidacidal activity of hemolymph was assayed as described previously (Lindsay et al., 2018), except that hemolymph was prepared from groups of 30 flies and all activity assays were

carried out for 1 h at room temperature. The number of colonies representing zero percent killing was set as the value obtained by assaying uninduced w^{1118} hemolymph.

ELP-intein construct generation

The plasmid pET/ELP-I-CAT was a gift from David Wood (Addgene # 71461). The plasmid was digested with restriction enzymes BsrGI and HindIII (New England Biolabs) to remove the CAT sequence, and the vector was gel extracted. Primers were designed as described (Shi et al. 2017) to place the target genes in frame with the intein and incorporate BsrGI and HindIII cut sites into the insert. The coding sequences of the secreted forms of Bombardier and Defensin were amplified from w^{1118} genomic DNA, such that the purified recombinant protein would be identical to that found in the hemolymph. The insert was digested with BsrGI and HindIII, followed by ligation into pET/ELP-I and transformation into DH5α cells. Insertion of *bombardier* required two rounds of transformation due to a BsrGI site in the coding sequence of *bombardier*. The pET/ELP-I-Bombardier and pET/ELP-I-Defensin plasmids were then extracted, the sequences confirmed, and the plasmids transformed into BLR(DE3) competent cells (Sigma-Aldrich) for protein expression. See Table 3 for ELP-intein primer sequences.

ELP-intein protein purification

Protein purification was as described (Shi et al., 2017) with slight modifications, owing to the use of the ELP-intein method in place of the ELP-split intein method. A single colony of BLR cells transformed with pET/ELP-I-Bombardier or pET/ELP-I-Defensin was grown overnight in 3ml LB with ampicillin (100 μg/ml) at 37°C. One ml overnight culture was added into 100 mL Terrific Broth plus ampicillin (100 μg/ml) in a 500 ml baffle flask and grown at 37°C until reaching

an OD_{600} of ~0.8. IPTG (100 µl at 0.8 M) was then added to induce protein expression at room temperature overnight.

The protein purification protocol was as previously described until the cleaving step. ELP fusion protein was then resuspended in 1.5ml cleaving buffer and incubated at room temperature to allow for intein cleavage overnight. One half ml of 1.6 M $(NH_4)_2SO_4$ was added and the solution incubated at room temperature for 5 minutes to precipitate the ELP-intein. The solution was spun down and the supernatant, which contains the purified target protein, withdrawn. Protein concentration was determined using a BCA assay.

MALDI-TOF analysis of hemolymph

The Toll pathway was activated in flies using heat-killed *M. luteus,* then incubated at 29°C for 24 h. Hemolymph was extracted as in (Lindsay et al., 2018), with slight modifications. Hemolymph extracted with glass capillaries from five male flies was pooled and transferred into 0.1% trifluoroacetic acid (TFA)/50% acetonitrile (ACN). One µl of each mixture was spotted on a Bruker MSP 96 ground steel plate, mixed 1:1 with a saturated solution of Universal MALDI matrix (Sigma-Aldrich) in 0.1% TFA/78% ACN, and air-dried. MALDI-TOF spectra were acquired using a Bruker Autoflex mass spectrometer. Data were collected from 1,500 to 10,000 m/z in positive linear mode, and 1,000 to 5,000 m/z in positive reflectron mode. Peptide calibration standard II (Bruker) was mixed with Universal MALDI matrix and used as an external calibration standard. At least ten independent samples were collected for each genotype. For peptide identification, peaks were matched to those of corresponding peaks in prior studies (Lindsay et al., 2018; Uttenweiler-Joseph et al., 1998). Representative spectra were visualized using R 3.3.2 and ggplot2 2.2.1 (R Core Development Team, 2013; Wickham, 2016).

Gene expression quantitation

The Toll pathway was activated with heat-killed *M. luteus*. Using TRIzol (Ambion), total RNA was extracted 18 h after Toll activation from four to six adult flies (2-5 days old). Next, cDNA was synthesized from 500 ng total RNA using the SuperScript II Reverse Transcriptase kit (Invitrogen). Quantitative RT-PCR was performed on an iQ5 cycler (BioRad) using iQ SYBR Green Supermix (BioRad). Quantification of mRNA levels was calculated relative to levels of the ribosomal protein gene *rp49* using the Pfaffl method (Pfaffl, 2001). Three independent replicates were completed. See Table 3 for qPCR primer sequences.

Hemolymph LC-MS

Flies were challenged with heat-killed *M. luteus* to activate the Toll pathway. Hemolymph was extracted from 100 to 110 each of w^{1118}, Δ*bbd*, and $Bom^{\Delta 55C}$ flies using the same method as in the hemolymph antimicrobial assays, with 50-60 flies processed per Zymo-Spin IC column (Zymo Research) and yielding a total of ~10 µl hemolymph per genotype. Three independent biological replicates were processed for Δ*bbd* and $Bom^{\Delta 55C}$, and two independent biological replicates were processed for w^{1118}. Extracted hemolymph was mixed 1:1 (vol/vol) with denaturing buffer (8 M Urea, 50 mM Tris, pH 7.8, 150 mM NaCl, protease and phosphatase inhibitors) and protein concentration was determined using a BCA assay. For each sample, 40 µg of hemolymph was diluted to 1 M urea using 50 mM ammonium bicarbonate and digested overnight with trypsin (Promega, V511A) at a 1:100 (trypsin:protein) ratio. After digestion, peptides were reduced with 1 mM dithiothreitol at room temperature for 30 min and then alkylated with 5 mM iodoacetamide at room temperature in the dark for 30 min. Formic acid was added to a 0.1% final concentration and peptides were desalted using the C18-Stage-Tip method and then vacuum dried. The dried peptides were reconstituted in 5% formic acid/5% acetonitrile and 1 µg of total peptide for each

sample was loaded for MS analysis. Samples were run in technical triplicates on a Q-Exactive mass spectrometer with instrument and chromatography settings as described previously (Markmiller et al., 2018), except for the following modifications: the RAW files were analyzed using Andromeda/MaxQuant (version 1.6.7.0) (Cox and Mann, 2008) with default settings except the match between the run and LFQ quantitation settings was enabled for label free quantification. Data were searched against a concatenated target-decoy database comprised of forward and reversed sequences from the unreviewed UniprotKB/Swiss-Prot FASTA *Drosophila* database (2019). A mass accuracy of 20 ppm was assigned for the first search and 4.5 ppm for the main search. The statistical analysis was calculated using the DEP analysis R-package (Zhang et al., 2018).

Bacterial load quantification

Bacterial load upon death (BLUD) was obtained as in (Duneau et al., 2017), with slight modifications. Briefly, flies were infected with *E. faecalis* and vials were monitored every 30 min for newly dead flies. These flies were then individually homogenized with a pestle in 400 µl LB media. Homogenates were also prepared from individual live w^{1118} flies 120 hours post infection (hpi). Homogenates were diluted serially in LB and spread on LB agar plates for incubation at 37°C overnight. Colonies were counted manually and the number of viable bacteria per fly was calculated. Data were obtained from three independent experiments.

Data analysis

GraphPad Prism 5 was used for statistical tests. Survival data were plotted as Kaplan-Meier curves and were analyzed using the Gehan-Breslow-Wilcoxon test to determine statistical significance. Statistical differences in candidacidal activity were calculated using one-way

ANOVA followed by Tukey's test. Multiple Mann-Whitney U tests were used to calculate differences between BLUD samples ($p= 0.0085$ after Šidák correction for multiple comparisons, $\alpha=0.05$, $k=6$).

Results

The *bombardier* gene is specifically required for Toll-mediated defense

The *bombardier* (*bbd*) gene contains a consensus Toll-responsive NF-κB binding site within its promoter region and is strongly expressed upon Toll activation by Gram-positive bacterial infection or other inducers (De Gregorio et al., 2002; Busse et al., 2007; Troha et al., 2018; Valanne et al., 2019). The encoded protein is predicted to be secreted and to generate a mature protein of 222 amino acids with a coiled coil near its C-terminus (Lupas et al., 1991; Almagro Armenteros et al., 2019). Orthologs of Bombardier are found across the *Drosophila* genus, but in no other genera (Johnson et al., 2008).

We began our analysis of the *bombardier* gene by generating a null mutant, using CRISPR/Cas9 to delete 1,906 bp encompassing the annotated transcriptional unit. Flies homozygous for this deletion (hereafter Δ*bbd*) were viable and morphologically wild-type. Given that *bombardier* is Toll-inducible, we assayed Δ*bbd* flies for a potential loss-of-function phenotype in Toll-mediated immunity. Specifically, we infected adult Δ*bbd* flies with various pathogens and then monitored survival. Two additional genotypes were used as controls: w^{1118} flies, which served as the wild type, and $Bom^{\Delta 55C}$ flies, which lack Toll-mediated humoral defenses due to deletion of the ten of the twelve *Bom* genes (Clemmons et al., 2015).

As shown in Figure 7, we observed a marked immunodeficiency when Δ*bbd* flies were challenged with representative species for the three classes of microbes against which Toll provides defense. With the yeast *Candida glabrata*, more than 90% of w^{1118}, but no Δ*bbd* flies, survived five days after infection (Figure 7A). In the case of the filamentous fungus *Fusarium oxysporum*, 70% of w^{1118} adults, but fewer than 20% of Δ*bbd* adults, were alive five days post infection (Figure 7B). Finally, with *Enterococcus faecalis*, a Gram-positive bacterium, 50% of wild-type flies, but no Δ*bbd* flies, were alive five days after infection (Figure 7C).

The impairment of Toll-mediated defenses by deletion of *bombardier* was significant for all three pathogens ($p<0.0001$). In the case of *C. glabrata*, the immunodeficiency of Δ*bbd* phenocopied that observed for $Bom^{\Delta 55C}$ flies. In contrast, with either *F. oxysporum* or *E. faecalis,* the rate of death was greater for $Bom^{\Delta 55C}$ than for Δ*bbd* ($p<0.0001$ for both infections). The Δ*bbd* mutant thus displays a substantial, but not complete, loss of Toll-mediated defense.

The expression of *bombardier* is strongly induced by Toll, but not Imd activation (De Gregorio et al., 2002). We therefore hypothesized that Imd-mediated defenses would not require *bombardier* function. To test this prediction, we infected Δ*bbd* flies with *Enterobacter cloacae,* a Gram-negative bacterium. In this experiment, Δ*bbd* flies are as immunocompetent as w^{1118} flies: more than 90% of both genotypes survived at least five days post infection (Figure 7D). In contrast, 100% of Rel^{E20} flies, which are deficient in Imd signaling (Hedengren et al., 1999), succumbed to infection within one day. Thus, *bombardier* functions in defense against a range of pathogens for which Toll mediates defense –yeast, filamentous fungi, and Lys-type Gram-positive bacteria– but not against Gram-negative bacteria, against which the Imd pathway is active.

The candidacidal activity of hemolymph requires Bombardier, but neither Drosomycin nor Metchnikowin

Next, we investigated the potential humoral role of Bombardier by preparing and assaying cell-free hemolymph. We have previously shown that hemolymph from wild-type flies exhibits a Toll-dependent and Bomanin-dependent candidacidal activity (Lindsay et al., 2018). However, we were also curious as to the identity of the active antifungal component. In particular, we considered the potential role of Metchnikowin (Mtk) and Drosomycin (Drs), two antimicrobial peptides (AMPs) that have documented antifungal activity *in vitro* and are strongly Toll-induced *in vivo* (De Gregorio et al., 2002; Fehlbaum et al., 1994; Levashina et al., 1995). We therefore took

advantage of the recently described Δ*AMPs* strain, which is deficient for Mtk and Drs, as well as all other induced AMPs other than the Cecropins (Hanson et al., 2019). Extracting and assaying Toll-induced hemolymph, we found that hemolymph from Δ*AMPs* flies had a killing activity against *C. glabrata* comparable to that of wild-type hemolymph (Figure 8). In contrast, we failed to detect any killing of *C. glabrata* by Δ*bbd* hemolymph. We conclude that Boms and Bombardier, but neither Mtk nor Drs, are required for humoral defense against *C. glabrata*.

To determine whether Bombardier alone was antimicrobial, recombinant Bombardier protein was generated and purified using the ELP-intein protein purification system (Figure 9). When assayed against *M. luteus*, Bombardier did not display antimicrobial activity, even at 10 μM (data not shown). In contrast, *Drosophila* Defensin, an AMP with activity against Gram-positive bacteria also generated using the ELP-intein method, inhibited *M. luteus* with an MIC of 0.2 μM, comparable to previously reported values (Levashina et al., 1995). Against *C. glabrata*, neither Bombardier nor Defensin showed antimicrobial activity.

Short-form Bom peptides are specifically absent from Δ*bbd* hemolymph

MALDI-TOF provides a robust tool for characterizing small (<5,000 MW) peptides present in hemolymph after Toll activation. As shown in Figure 10A,B, such a readout includes the aforementioned AMPs (Mtk and Drs), several short-form Boms (BomS1, S2, S3, and S6; see Table 4 for updated Bomanin nomenclature), and other induced peptides (e.g., IM4). We have previously shown that deleting the 55C Bom gene cluster removes the peaks attributable to the short-form Boms, while leaving the remaining signals unaffected (Lindsay et al., 2018). Remarkably, analysis of Δ*bbd* hemolymph yielded a similar pattern. As shown in Figure 10C,D, the short-form Boms that were readily detectable in the wild type – S1, S2, S3, and S6 – were

absent in Δ*bbd* hemolymph, whereas the remaining peptides, including Mtk, Drs, and IM4, displayed a wild-type profile.

Although Δ*bbd* disrupts the accumulation of short-form Bom peptides in hemolymph, this effect does not reflect a disruption in transcription or stability of the corresponding *Bom* mRNAs: robust induction of Toll-regulated genes, including genes of short-form Boms, was readily detectable with qRT-PCR (Figure 11).

Because proteins such as Bombardier and bicipital Boms are too large to be detected by our MALDI-TOF protocol, we used LC-MS to further characterize the relationship between Bombardier and the Boms in hemolymph. For these studies, we prepared Toll-induced hemolymph from three genotypes: w^{1118}, Δ*bbd*, and $Bom^{\Delta 55C}$. In wild-type hemolymph, we readily detected Bombardier protein (Figure 12), consistent with the presence of a canonical secretion signal sequence in the Bombardier coding sequence. Bombardier, like the Boms, is thus secreted into hemolymph upon Toll induction. We also detected all three bicipital Boms – BomBc1, BomBc2, and BomBc3. The LC-MS studies thus complemented the MALDI-TOF studies, with bicipital Boms detected by the former and short-form Boms by the latter (tailed Boms are not detected by either protocol). Next, we assayed Δ*bbd* hemolymph. As expected, Bombardier was not detected. However, the three bicipital Boms were present at comparable levels in both wild-type and Δ*bbd* hemolymph (see Figure 12). Combined with the MALDI-TOF studies, these results demonstrate that Δ*bbd* blocks accumulation in hemolymph of short-form, but not bicipital, Boms. Lastly, we analyzed hemolymph from $Bom^{\Delta 55C}$ flies, which lack ten of the twelve *Bom* genes. As expected, the products of the two deleted bicipital genes (*BomBc1* and *BomBc2*) were absent, whereas the product of the remaining bicipital gene (*BomBc3*) was present at wild-type levels (see Figure 12). Turning our attention to the Bombardier protein, we observed no effect of the *55C* Bom deletion

on protein presence. Thus, Bombardier is required for the presence of short-form Boms in hemolymph, but the 55C Boms are not required for the presence of Bombardier.

Bombardier mediates both infection resistance and tolerance

The Δ*bbd* survival phenotype could be due to an inability to control pathogen growth – a defect in resistance – or an inability to endure infection – a defect in tolerance. Because flies lacking Bombardier demonstrate an increased susceptibility to infection and decreased levels of known resistance factors, the short-form Boms, it seemed likely that Δ*bbd* flies, like $Bom^{\Delta 55C}$ flies, have a defect in infection resistance. In exploring this hypothesis, we found that the model recently developed by Buchon, Lazzaro, and colleagues provided a useful framework (Duneau et al., 2017). Following infection of an individual fly, there are two stereotypic outcomes: either the pathogen replicates, reaches a lethal burden, and the fly dies; or the pathogen is controlled at a level below the lethal burden and the fly survives with a persistent infection. Variation in survival curves for different pathogens and fly genotypes reflects variation in both the time required to reach lethal burden and in the fraction of flies that are able to control the infection before it reaches such a threshold. In cases where a fraction of flies control infection, group survival typically drops after infection and then reaches a plateau (Hanson et al., 2019).

The survival curve for Δ*bbd* flies infected with *E. faecalis* does not plateau (see Figure 7C). Instead, it exhibits a profile that we hypothesize reflects two phases of death. In the first phase, extending roughly two days post infection, some Δ*bbd* flies reach a lethal burden of *E. faecalis* and die, as reflected in a sharp decline in survival; the remainder control the infection. In the second phase, from 2.5 days onward, those flies with a persistent infection die at a reduced but steady rate, due to a defect in tolerance. If this hypothesis is correct, flies dying in the first phase should have a bacterial load upon death (BLUD) comparable to that of wild-type flies dying from infection.

Furthermore, those dying in the second phase should have a much lower pathogen burden, comparable to that of wild-type survivors with a persistent infection.

To test our predictions regarding pathogen burden, we measured the BLUD of individual flies after infection with live *E. faecalis* and divided the data into two time intervals (Figure 13). For the earlier interval (dead flies obtained between 17 and 51.5 hpi), both $Bom^{\Delta 55C}$ and Δ*bbd* bacterial loads upon death were not significantly different from w^{1118} (Figure 13, red, $p>0.05$). For the later time interval (flies obtained between 68 and 120.5 hpi), Δ*bbd* flies perished at significantly lower bacterial loads compared to that of Δ*bbd* flies which died earlier (Figure 13, Δ*bbd* early compared to Δ*bbd* late, $p<0.0001$), indicating that these two groups die from distinct causes. Importantly, late-death Δ*bbd* flies perished at significantly lower bacterial loads than those of w^{1118} suffering early deaths ($p<0.0001$), demonstrating that Δ*bbd* flies have a defect in tolerance.

Together, the survival curve and BLUD data offer strong support for our two-phase-model: Δ*bbd* flies died early in infection with high bacterial loads, due to a defect in resistance, and died later with lower bacterial loads, reflecting a deficiency in tolerance. However, we note that the bacterial loads of Δ*bbd* flies dying in the later phase were still significantly greater than those of w^{1118} flies alive 120 hpi (Figure 13, Δ*bbd* late compared to live w^{1118}, $p<0.0001$). This indicates that the later-death Δ*bbd* group has not completely controlled infection compared to the live w^{1118} flies, and suggests that both resistance and tolerance contribute to the later Δ*bbd* fly deaths.

Immune activation, specifically *Bom* expression, is deleterious in the absence of Bombardier

What is the nature of the tolerance defect we observed in Δ*bbd* flies? More specifically, do Δ*bbd* flies have a greater sensitivity to the deleterious effects of active infection, is their health impaired by an excessive or toxic immune response, or is death due to another class of impaired

tolerance (Ayres and Schneider, 2012)? To distinguish among these explanations, we assayed the effect of activating the immune response in Δ*bbd* flies in the absence of infection.

When Δ*bbd* flies were challenged with heat-killed *E. faecalis*, we observed a decrease in survival that first was apparent three days post challenge followed by a steady decline in the number of live flies in the following days (Figure 14A), consistent with the timing of the late-phase deaths (see Figure 13). Overall, the death rate was slower than that of live infection, but the extent of killing was similar between heat-killed and live *E. faecalis*: fewer than 20% of flies survived (compare Figures 7C and 14A). In contrast, no effect on survival was observed upon challenge of either w^{1118} or $Bom^{\Delta 55C}$ flies with heat-killed *E. faecalis*: greater than 95% flies survived seven or more days post challenge.

Studies of *Drosophila* immunity have demonstrated that under some circumstances, specific immune stimulants can produce specific primed responses (Pham et al., 2007). Could the effect of heat-killed *E. faecalis* on Δ*bbd* survival be pathogen-specific? We repeated the challenge experiments with heat-killed *Micrococcus luteus*, which activates the Toll response (see Figure 11, as well as (Lemaitre et al., 1997; Lindsay et al., 2018)), and found that heat-killed *M. luteus* also had a marked effect on Δ*bbd* survival: five days after challenge, fewer than 5% of Δ*bbd* flies were alive, compared to survival of greater than 95% of w^{1118} and 85% of $Bom^{\Delta 55C}$ flies over the same period of time (Figure 14B). We therefore see no evidence of pathogen-specific effects in the mortality of Δ*bbd* flies subject to immune stimulation.

As both *M. luteus* and *E. faecalis* induce the Toll pathway, Toll activation could be the key factor in Δ*bbd* mortality. To address this hypothesis, Δ*bbd* flies were crossed with $MyD88^{kra1}$ (Toll-deficient) flies to generate the $MyD88^{kra1}$, Δ*bbd* double mutant, and the resulting flies were challenged with Toll activators. Unlike Δ*bbd* flies, $MyD88^{kra1}$, Δ*bbd* flies survive challenge with Toll activators (Figure 14C,D). Because blocking the Toll pathway with $MyD88^{kra1}$ rescues the

Δ*bbd* phenotype against heat-killed bacteria ($p<0.0001$ compared to Δ*bbd*, $p>0.05$ compared to $MyD88^{kra1}$ for both heat-killed bacteria), we conclude that Toll activation underlies the death of Δ*bbd* flies in the absence of infection.

As described above, *Bom* genes are transcribed in Δ*bbd* flies (Figure 11), but the short-form Bom peptides are not detected in hemolymph (Figure 10). This suggests a mislocalization of these peptides, perhaps in an unprocessed or misfolded state. Given that short-form *Bom* genes are among the most abundantly transcribed genes after infection (Troha et al., 2018; Valanne et al., 2019), such mislocalized or misfolded Boms could rapidly accumulate to high levels in Δ*bbd* flies. Could this explain the death of Δ*bbd* flies upon immune stimulation? To address this question, we generated $Bom^{\Delta 55C}$, Δ*bbd* double mutants and assayed the effect of immune induction alongside both $Bom^{\Delta 55C}$ and Δ*bbd* flies (Figure 14C,D). Introducing $Bom^{\Delta 55C}$, which deletes all of the short-form Boms, eliminated the effect of Δ*bbd* on survival following immune stimulation ($p<0.0001$ compared to Δ*bbd*, $p>0.05$ compared to $Bom^{\Delta 55C}$), similar to the effect observed in $MyD88^{kra1}$, Δ*bbd* flies. The fact that $Bom^{\Delta 55C}$ is epistatic to Δ*bbd* demonstrates that Toll-driven expression of *Bom* genes is specifically responsible for the death of immune stimulated Δ*bbd* flies.

Discussion

The results presented in this study identify a key factor that regulates humoral and Bom-mediated defense in *Drosophila*. We demonstrate that Δ*bbd* flies are defective in resistance to pathogens controlled by the Toll pathway. The results support the hypothesis that this defect results from the absence of short-form Boms in Δ*bbd* hemolymph. Absence of Boms is sufficient to cause a defect in resistance (Clemmons et al., 2015) and Δ*bbd* hemolymph appears to be lacking the short-form Boms but no other component, save Bombardier itself. Furthermore, Δ*bbd* phenocopies $Bom^{\Delta 55C}$ with regard to survival after *C. glabrata* infection, and resistance to *C. glabrata* can be restored in $Bom^{\Delta 55C}$ flies by expression of short-form Boms (Lindsay et al., 2018). Finally, Δ*bbd* hemolymph lacks candidacidal activity, which is dependent on short-form Bom peptides (Lindsay et al., 2018) and which we show here does not require Drs or Mtk.

For pathogens other than *C. glabrata*, the effect of deleting Bombardier is less severe than that of deleting the ten *Bom* genes clustered at 55C. Our mass spectrometry data suggest an explanation. Whereas short-form Boms are absent from Δ*bbd* hemolymph, bicipital Boms are present. (Tailed Boms were not detected with either mass spectrometry method.) Therefore, we postulate that the bicipital Boms, which are not required for resistance to *C. glabrata* (Lindsay et al., 2018), are functional against other pathogens. This would explain why Δ*bbd* flies are more resistant than $Bom^{\Delta 55C}$ flies upon infection with *E. faecalis* or *F. oxysporum* (Figure 1). In this regard, we note that Bombardier and all three forms of Bom proteins – short, tailed, and bicipital – are found across the *Drosophila* genus, supporting the notion that all three classes of Boms are immunoprotective and therefore maintained across the *Drosophila* genus.

Bombardier function and structure

What is the function of Bombardier? Deleting the gene results in the absence of short-form Boms from hemolymph, an effect we find is at the level of protein. Other mature immune peptides

are present at normal levels in the hemolymph, and there is thus no general defect in translation, secretion, or processing. Based on these findings, we propose that Bombardier normally functions either to chaperone short Boms as they are secreted from the fat body into the hemolymph or, alternatively, to protect the Boms from misfolding or aggregation while in the hemolymph. We further hypothesize that it is the ectopic localization or aberrant form of short-form Boms in Δ*bbd* flies that generates morbidity upon Toll pathway activation. In support of this idea, we showed that *Bom* expression underlies the lethality observed in Δ*bbd* flies (Figure 14). Whether the short-form Boms physically interact with Bombardier, perhaps in the context of a larger antimicrobial complex, is currently unknown.

Activation of Toll-like receptor (TLR) signaling is important for innate immunity, but induction of the pathway can lead to autoimmune disorders and chronic inflammatory disease (Björkbacka et al., 2004; Jiang et al., 2005; Kim et al., 2009; Wu et al., 2010). Here we report an aberrant Bom activity driven by Toll pathway induction in flies lacking a downstream pathway component, Bombardier. To what extent this parallel can be exploited in the context of understanding autoimmune disorders promises to be a significant focus for future investigation.

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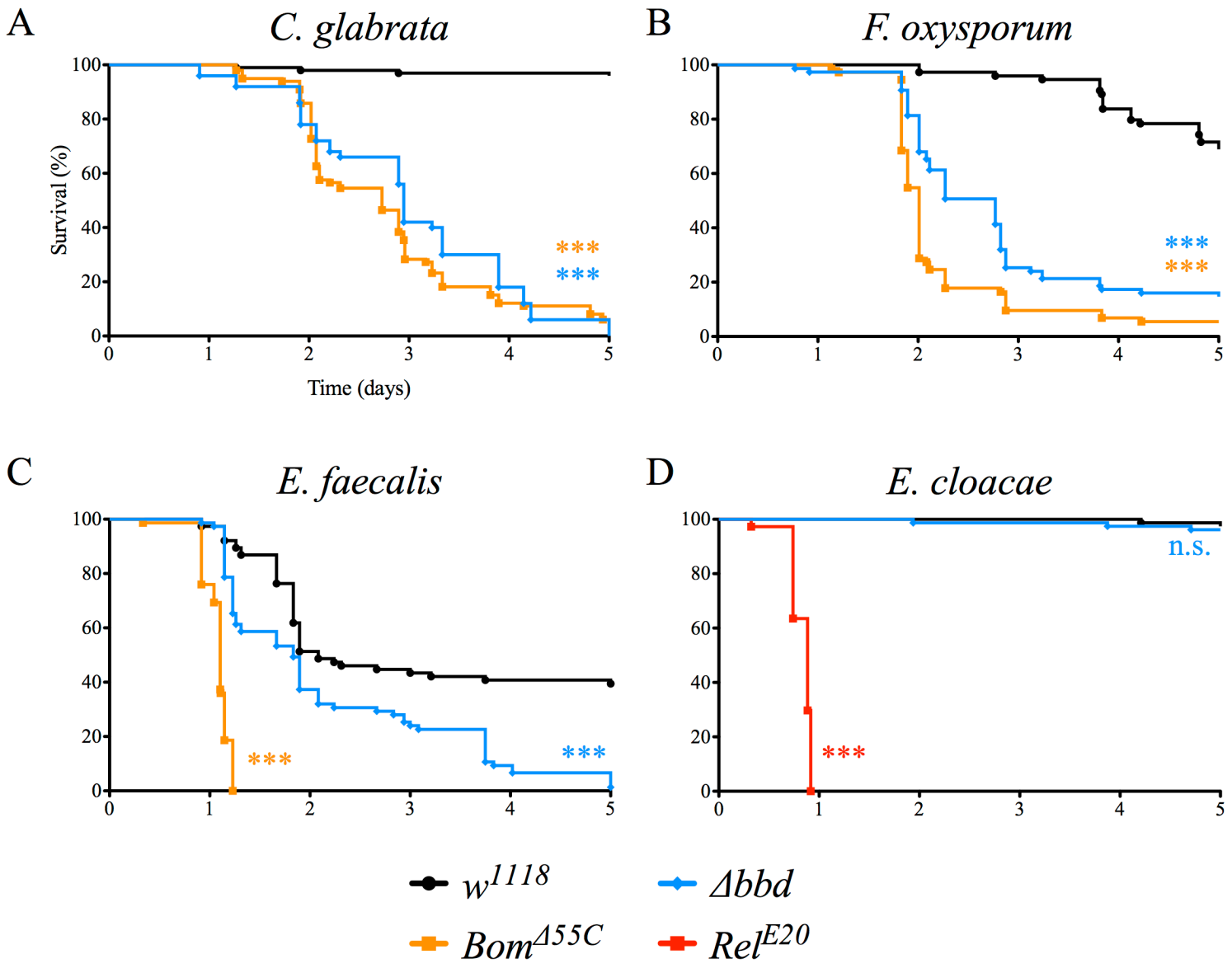
Chapter 3, in large part, has been submitted for publication as it may appear in Lin SJH, Fulzele A, Cohen LB, Bennett EJ, Wasserman SA. 2019. Bombardier enables delivery of short-form Bomanins in the *Drosophila* Toll response. The dissertation author was the primary investigator and author of this material.

Data Availability Statement

The datasets generated for this study have been uploaded to the MassIVE data repository.

Figures

Figure 7. The *bombardier* gene is specifically required for Toll-mediated defense
(A-D) Survival curves of flies infected as indicated. The w^{1118} strain was the wild-type control; $Bom^{\Delta 55C}$ and Rel^{E20} were the susceptible controls (Clemmons et al., 2015; Hedengren et al., 1999). Experiments were completed in triplicate with at least 25 flies per genotype in each replicate. Statistical significance was determined using the Gehan-Breslow-Wilcoxon test and is shown relative to w^{1118} (*** $p<0.0001$; n.s. = not significant, $p>0.05$).


A
C. glabrata
B
F. oxysporum
C
E. faecalis
D
E. cloacae
Survival (%)
Time (days)
***
***
n.s.
w1118
Bom^Δ55C
Δbbd
Rel^E20

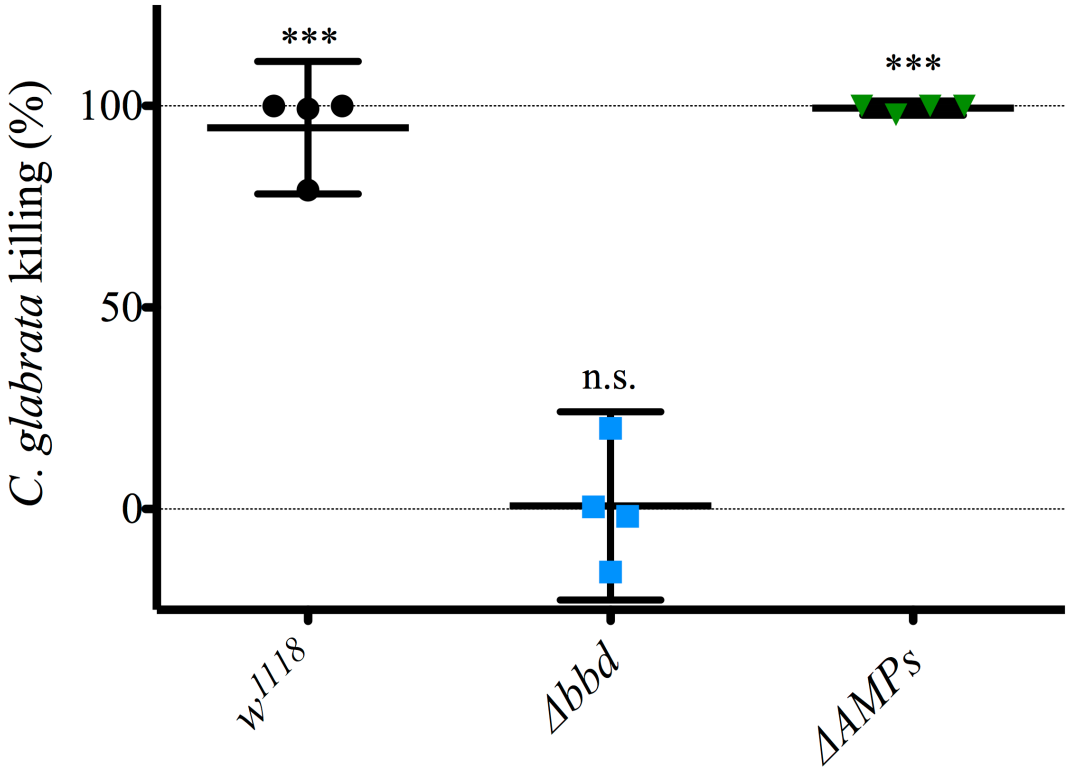


Figure 8. The Toll-induced candidacidal activity of hemolymph requires Bombardier, but neither Drosomycin nor Metchnikowin

Hemolymph was extracted from flies, mixed with *C. glabrata* and incubated for 1 h to allow for killing. The surviving yeast cells were plated, and colonies were counted to determine the level of candidacidal activity in the extracted hemolymph. Colony counts from uninduced w^{1118} hemolymph were used as the control for no (0%) killing. Experiments were completed four times, with each point representing one replicate. One-way ANOVA was calculated followed by Tukey's test. Significance is shown relative to the null hypothesis of 0% killing (*** $p<0.0001$; n.s. = not significant, $p>0.05$). Error bars represent the 95% confidence interval.

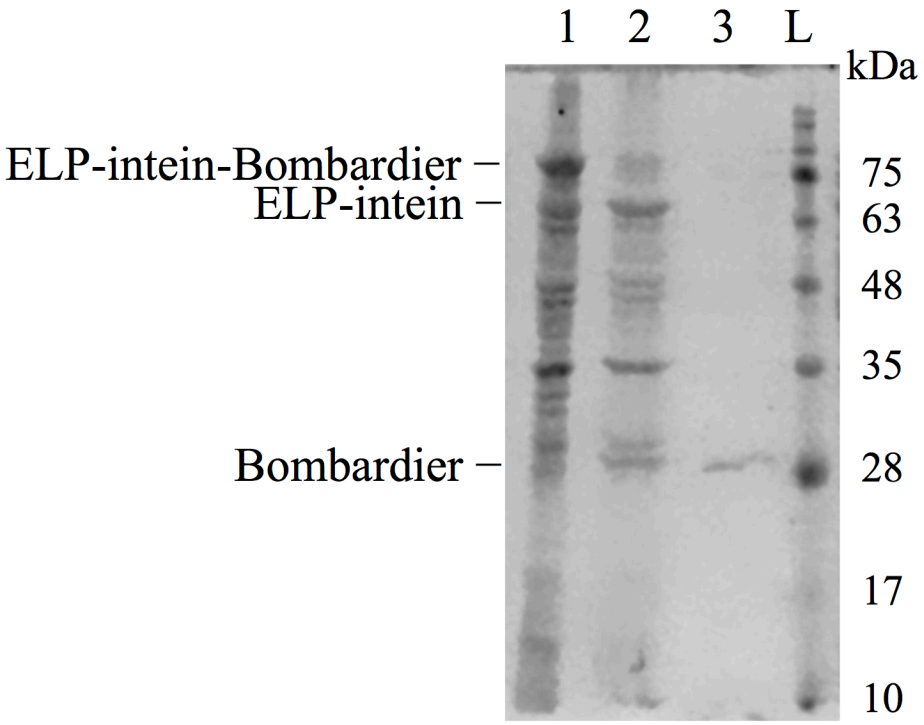
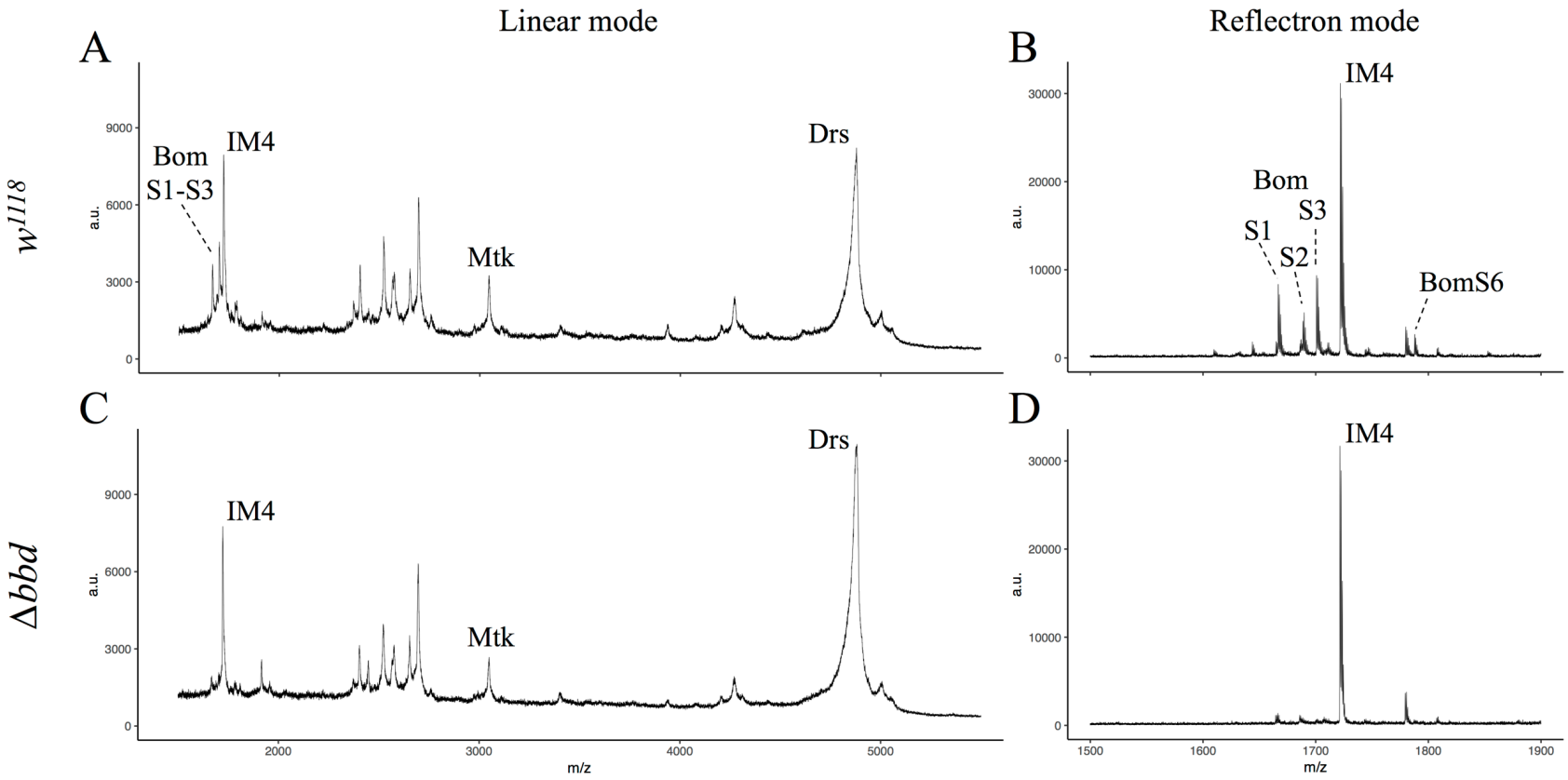


Figure 9. Purification of Bombardier using ELP-intein
Ten µl of each protein sample were mixed with SDS-PAGE loading buffer, samples were run on a 12% SDS-PAGE gel, and stained with Coomassie. 1: ELP-intein-Bombardier protein sample in cleaving buffer, 2: protein sample after 18h incubation, 3: target protein (Bombardier) after precipitation of ELP-intein, L: protein ladder.

Figure 10. Short-form Bom peptides are specifically absent in hemolymph from Δ*bbd* flies
MALDI-TOF mass spectra of w^{1118} **(A,B)** and Δ*bbd* **(C,D)** hemolymph samples were collected in linear (**A**,**C**) and reflectron mode (**B**,**D**). For peptide identification, peaks were matched to those of corresponding peaks in prior studies (Lindsay et al., 2018; Uttenweiler-Joseph et al., 1998). Spectra were obtained from at least ten independent biological replicates and representative spectra are shown. (a.u.: arbitrary units, m/z: mass/charge)


Linear mode
Reflectron mode
A
B
C
D
w^{1118}
Δbbd
Bom
S1-S3
IM4
Mtk
Drs
S1
S2
S3
BomS6
a.u.
m/z

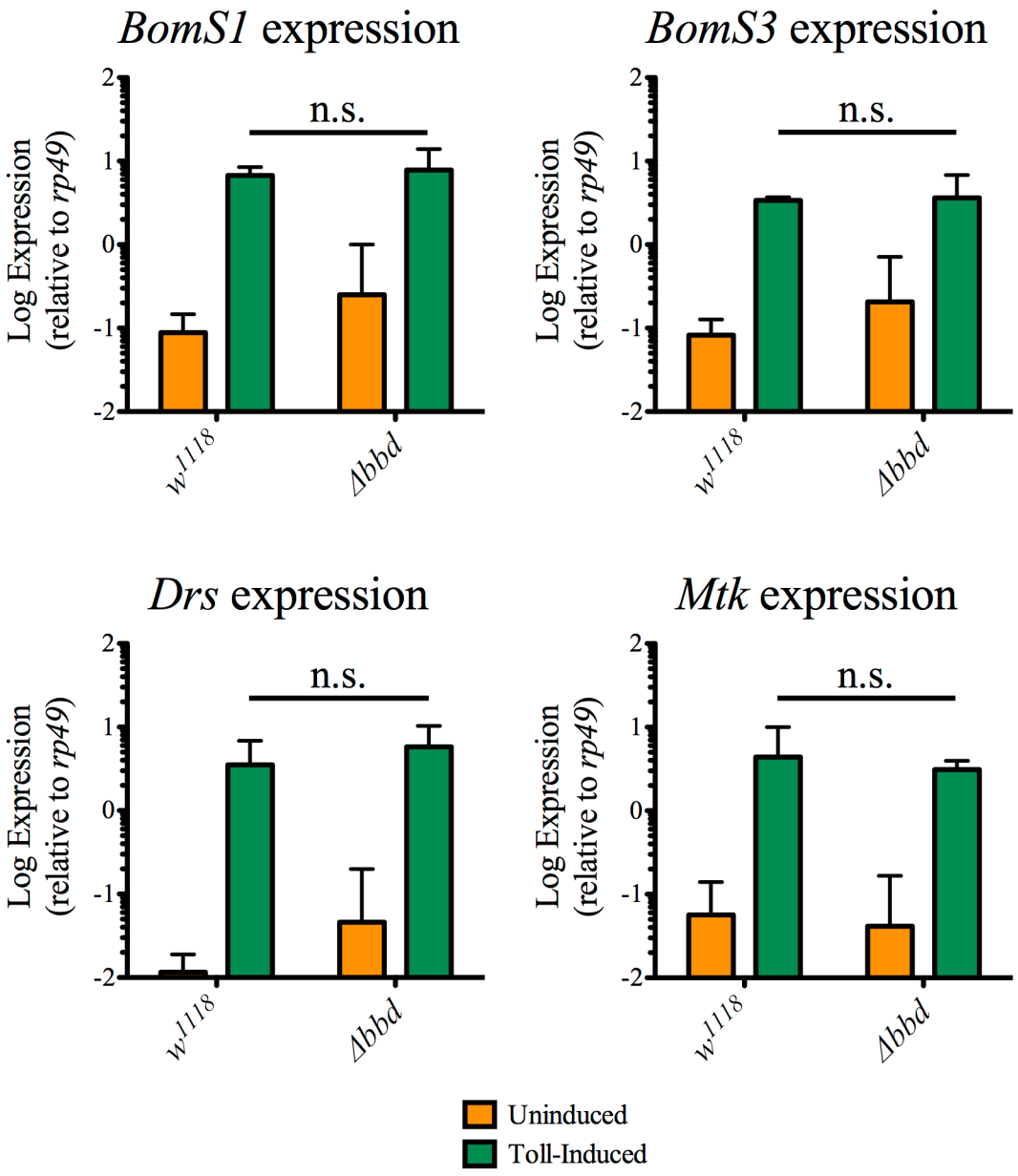


Figure 11. Δ*bbd* deletion does not affect Toll signaling
qRT-PCR analysis of uninjured flies and flies 18 hours after challenge with heat-killed *M. luteus*. Gene mRNA levels were calculated relative to levels of *rp49*, a ribosomal gene. Experiments were completed with independent biological triplicates. Two-way ANOVA was calculated followed by Tukey's test, indicating that Toll-induction is a significant variable for differences in mRNA levels, but fly genotype is not a significant variable. Error bars show 95% confidence interval. (n.s. = not significant, p>0.05)

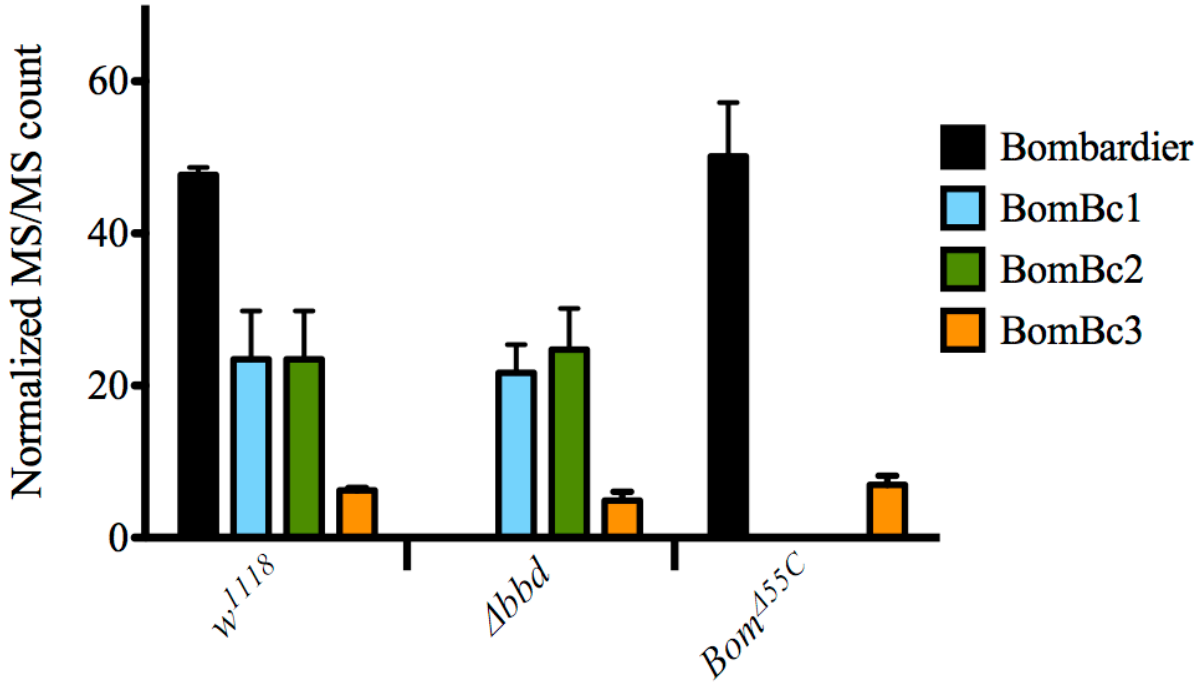


Figure 12. The presence of bicipital Bomanins in hemolymph is unaffected by loss of Bombardier

MS/MS counts for the indicated proteins as determined by Andromeda/MaxQuant were normalized to total MS/MS counts in each run. Error bars represent standard deviation for biological replicates (n=3 for Δ*bbd* and $Bom^{\Delta 55C}$, n=2 for w^{1118}).

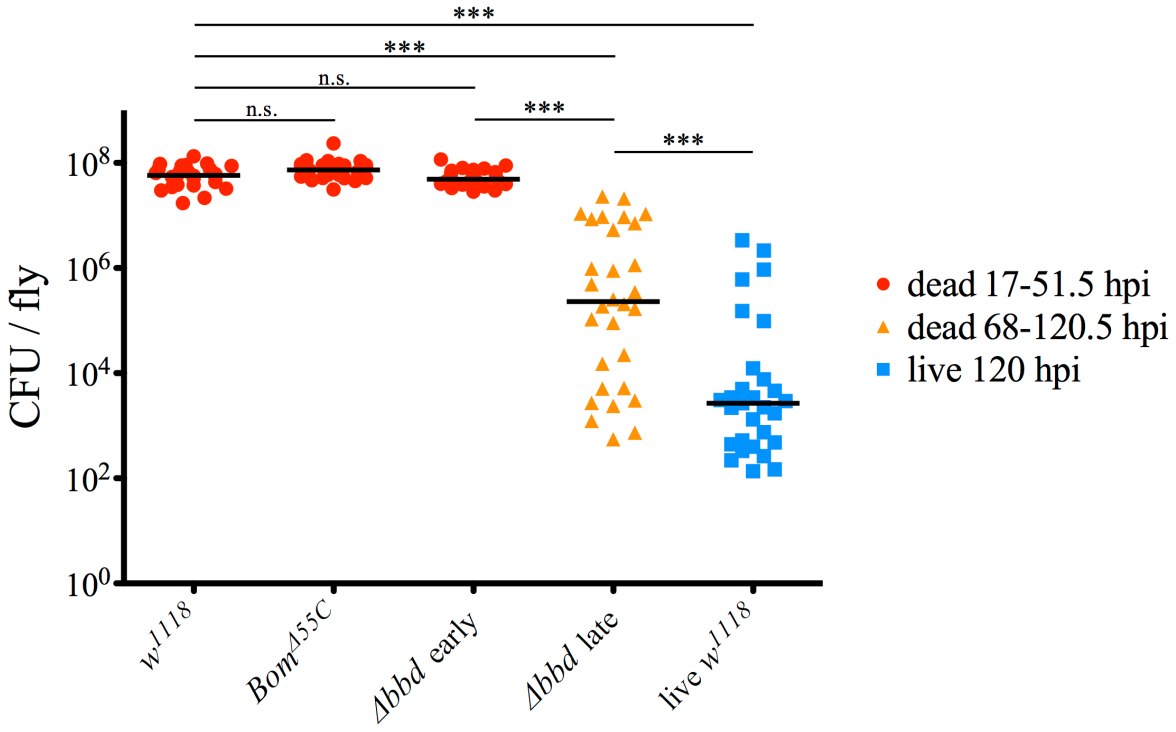
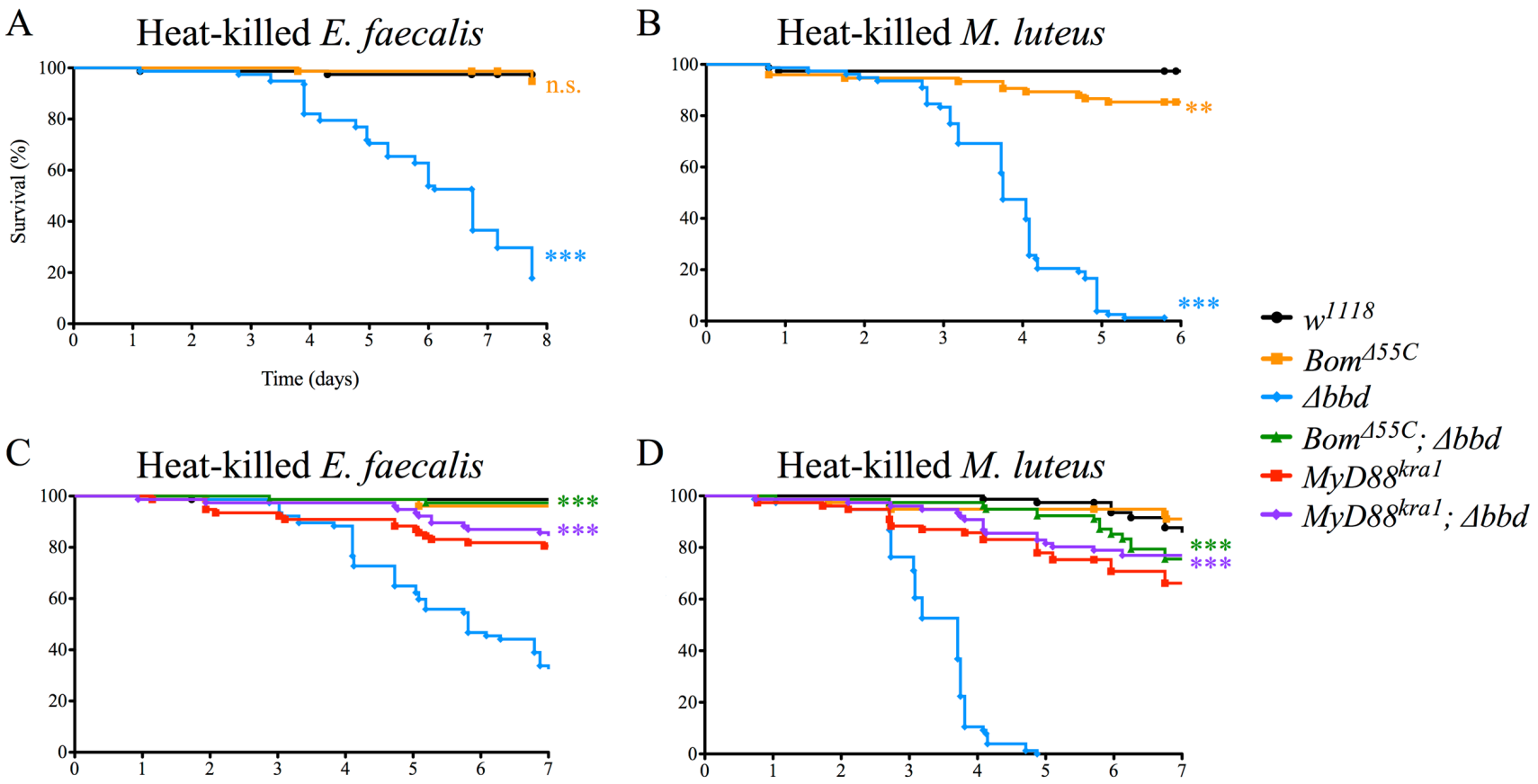


Figure 13. Bombardier mediates both infection resistance and tolerance
Bacterial load upon death (BLUD) of w^{1118}, $Bom^{\Delta 55C}$, and Δ*bbd* flies, plotted by early (17-51.5 hpi, red) or late (68-120.5 hpi, orange) time of death post infection, as well as bacterial load of live w^{1118} flies 120 hpi (blue). Data was obtained and combined from three independent experiments totaling n=26 for w^{1118}, n=30 for $Bom^{\Delta 55C}$, n=33 for Δ*bbd* red, n=30 for Δ*bbd* orange, and n=29 for live w^{1118}. Black bars indicate median values. Statistics were calculated using multiple Mann-Whitney U tests. For significance, p= 0.0085 after Šidák correction for multiple comparisons (α=0.05, k=6). The pathogen loads of early deaths for $Bom^{\Delta 55C}$ and Δ*bbd* were not significantly different from w^{1118} (p>0.05). The pathogen load of late Δ*bbd* fly deaths is significantly different from that of the early-death Δ*bbd* and w^{1118} groups (*** p<0.0001) and also significantly different from that of live w^{1118} flies 120 hpi (*** p<0.0001). Finally, the early-death w^{1118} pathogen load was significantly different from that of live w^{1118} flies 120 hpi (*** p<0.0001). (hpi: hours post infection)

Figure 14. Toll activation, specifically *Bom* expression, is deleterious in absence of Bombardier

Fly survival after introduction of **(A)** heat-killed *E. faecalis* and **(B)** heat-killed *M. luteus*. Experiments were completed in triplicate with at least 25 flies per genotype in each replicate. Statistics were determined using the Gehan-Breslow-Wilcoxon test. Significance is shown relative to w^{1118} (*** $p<0.0001$; n.s. = not significant, $p>0.05$). For survival of double mutant flies challenged with **(C)** heat-killed *E. faecalis* and **(D)** heat-killed *M. luteus*, significance is shown relative to Δ*bbd* (*** $p<0.0001$).


A
Heat-killed E. faecalis
Survival (%)
Time (days)
n.s.
***
B
Heat-killed M. luteus
**
***
C
Heat-killed E. faecalis
***
***
D
Heat-killed M. luteus
***
***
w1118
Bom Δ55C
Δbbd
Bom Δ55C; Δbbd
MyD88 kra1
MyD88 kra1; Δbbd

Tables

Table 3. Sequences of primers used for CRISPR/Cas9, ELP-intein, and qRT-PCR

Primer Name	Function	Sequence
CG18067 HA1 F	CRISPR homology arm 1	ATATCACCTGCATATTCGCAGAATGCCACTAAGAGAGGG
CG18067 HA1 R	CRISPR homology arm 1	ATATCACCTGCATATCTACTGGGGTGAACTGTGTCAAAT
CG18067 HA2 F	CRISPR homology arm 2	GCATGCTCTTCATATAGAGTGATCGTTCAGAATGT
CG18067 HA2 R	CRISPR homology arm 2	ATATGCTCTTCAGACATTTCTTGGGCATAACTCCG
CG18067 gRNA1 F	gRNA1 sense	CTTCGTAGACGGATCGAGGGAGTGG
CG18067 gRNA1 R	gRNA1 antisense	AAACCCACTCCCTCGATCCGTCTAC
CG18067 gRNA2 F	gRNA2 sense	CTTCGGCGGCTTGTCGGCGTAAGA
CG18067 gRNA2 R	gRNA2 antisense	AAACTCTTACGCCGACAAGCCGCC
intein-CG18067-F	ELP-intein-CG18067	AATGTACACAACGCGAATATACAGCGAAATGAGGACCAG
HindIII-CG18067-R	ELP-intein-CG18067	AATAAGCTTCTAATAGAAAATATTTCCCAGGGAATTCTGAAG
intein-Def-F	ELP-intein-Defensin	AATGTACACAACGCCACATGCGACCTACTCTCC
HindIII-Def-R	ELP-intein-Defensin	AATAAGCTTTCAATTGCGGCAAACGC
rp49 qpcr F	rp49 qRT-PCR	CAAGGGTATCGACAACAG
rp49 qpcr R	rp49 qRT-PCR	CTTGTTCGATCCGTAACC
BomS1 qpcr F	BomS1 qRT-PCR	TGAAATTCTTCTCAGTCGTC
BomS1 qpcr R	BomS1 qRT-PCR	TTGAAACTTCCTACTTGCC
BomS3 qpcr F	BomS3 qRT-PCR	TGAAATTCCTATCACTCGCC
BomS3 qpcr R	BomS3 qRT-PCR	TGACATTGCCAGGATTCAG
Drs qpcr F	Drs qRT-PCR	CCGGAAGATACAAGGGTC
Drs qpcr R	Drs qRT-PCR	TTTAGCATCCTTCGCACC
Mtk qpcr F	Mtk qRT-PCR	GCCCTTCAATCCTAACCA
Mtk qpcr R	Mtk qRT-PCR	GTGTTAACGACATCAGCAG

Table 4. Updated Bomanin nomenclature
In consultation with FlyBase (https://flybase.org) and members of the research community, Bomanin nomenclature was updated to assign names that indicate membership in the Bomanin family and in the three structural classes - Short, Tailed, and Bicipital. Bomanin S (BomS) refers to a short-form Bomanin, Bomanin T (BomT) refers to a tailed-form Bomanin, and Bomanin Bc (BomBc) refers to a bicipital-form Bomanin. IMs were numbered first: IM1, 2, and 3 correspond to BomS1, S2, and S3, and IM23 corresponds to BomBc1. The remaining 55C-cluster Boms were then numbered before the non-55C cluster Boms.

	IM/CG name	New name; abbreviation
Short-form Bomanin (BomS)	IM1 (CG18108)	Bomanin S1; BomS1
	IM2 (CG18106)	Bomanin S2; BomS2
	IM3 (CG16844)	Bomanin S3; BomS3
	CG18107	Bomanin S4; BomS4
	CG15065	Bomanin S5, BomS5
	CG15068	Bomanin S6, BomS6
Tailed-form Bomanin (BomT)	CG43202	Bomanin T1; BomT1
	CG16836	Bomanin T2; BomT2
	CG5778	Bomanin T3; BomT3
Bicipital-form Bomanin (BomBc)	IM23 (CG15066)	Bomanin Bc1; BomBc1
	CG15067	Bomanin Bc2; BomBc2
	CG5791	Bomanin Bc3; BomBc3

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Chapter 4:

Discussion and Future Directions

The research presented in my dissertation shows the importance of several *Drosophila*-specific immune genes on Toll-mediated immune defense. Investigating the Boms, we found that the short forms are required for defense against *C. glabrata*, and that the classical AMPs are not. With Bombardier, I discovered that this immune protein is required for the presence and activity of short Boms in hemolymph. Additionally, without Bombardier, flies suffer from an autoimmune defect due to Toll-induced *Bom* expression. In this chapter, I identify future directions that can expand on the data shown in this dissertation and build our understanding of Toll-mediated immunity in *Drosophila.*

Bombardier

With regard to Bombardier structure, we note that portions of the amino acid sequence are similar to small portions of other *Drosophila* immune peptides (Figure 15). Some portions of Bombardier are similar to the Bom consensus sequence. These similarities may reflect the shared role I observe and thus suggest a shared functional motif. However, there are no cysteine residues in Bombardier and cysteines are vital to the function of Boms (Scott Lindsay, unpublished data), all of which have one or more disulfide bonds. An 11-amino-acid portion of Bombardier has substantial similarity with a section of the Attacins, a family of antimicrobial peptides active against Gram-negative bacteria (Hultmark et al., 1983), and has some similarity as well to IM4, IM14, and IMPPP, other *Drosophila* immune peptides, so these alignments are not specific to the Bomanins. These alignments were generated from comparisons between the sequences of Bombardier and other immune proteins. Until structure-function analyses are tested, these sequence similarities are just observations that may be due to chance.

What is the function of Bombardier?

As proposed in the discussion section of Chapter 3, my preferred model for Bombardier is that of a chaperone for the Boms, whether that be for Bom peptide secretion, processing, or folding. To show this, I would first investigate whether there is binding, either directly or indirectly, between the two proteins. Preliminary experiments with co-immunoprecipitation of HA-tagged Bombardier were not successful, but this most likely reflects technical issues related to the limited amounts of starting material.

Determining Bom localization in the absence of Bombardier is also vital to addressing Bombardier's role as a chaperone. If Boms were trapped in the fat body in Δ*bbd* flies, it would suggest a role where Bombardier aids in secretion or transport of Boms into the hemolymph. If Boms were aggregated in Δ*bbd* hemolymph, it would suggest a role where Bombardier affects Bom folding and stability. Scott Lindsay has generated flies expressing FLAG-tagged Boms and I have crossed them into the Δ*bbd* background. To date, I have detected the FLAG-tagged bicipital Bom in both control and Δ*bbd* flies, and observed no effect of the *bombardier* deletion on the presence of the tagged form in hemolymph. Using either immunoblot or MALDI-TOF, I have not detected FLAG-tagged short Bom in either the control or Δ*bbd* flies. To address this key question, more reagents will need to be generated.

To further investigate the aberrant condition I observe in Δ*bbd* flies challenged with heat-killed pathogen, one question to consider is how Δ*bbd* flies are dying after challenge. The fat body is not only the immune organ of the fly. It is also essential for energy metabolism, detoxification, and lipid storage (Arrese and Soulages, 2010; Li et al., 2019). If Boms are indeed trapped in the fat body in Δ*bbd* flies, the Boms could be damaging the fat body in such a way that harms metabolism or lipid storage. As such, examining the protein and metabolite levels in hemolymph

and fat body tissue three or four days after induction, when Δ*bbd* flies begin to die after activation of the Toll pathway, would be exciting to pursue.

I observe some defects in the fecundity of the Δ*bbd* stock, consistent with reports based on transcriptomic and proteomic observations that Bombardier is present in *Drosophila* reproductive organs (Allen and Spradling, 2008; Sirot et al., 2014). I do not observe defects in fertility or fecundity in $Bom^{\Delta 55C}$ flies, which suggests that Bombardier could have some Bom-independent functions. Additionally, Bombardier has been linked to cold tolerance (Vermeulen et al., 2013), so investigating how various stresses affect the *bombardier* mutant can be an interesting path to explore Bombardier's function.

Bomanins

What is the mechanism of action of the Boms?

The initial model for mechanism of action of the Boms was that of an AMP, directly killing or inhibiting growth of pathogens. However, in Chapter 2, we noted that we have not observed antimicrobial activity with synthetic Bom peptides. This can be explained in numerous ways. This could mean that they are not antimicrobial. Alternatively, it could also be that the synthetic peptides, while identical in mass to wild-type Bom peptides extracted from flies, are misfolded or aggregated so that no activity is detected. I have attempted a number of experiments varying temperature, salt concentration, buffer concentration, as well as attempting complementation with extracted $Bom^{\Delta 55C}$ hemolymph, and nevertheless have not detected antimicrobial activity against *C. glabrata*. In brief, our data has been negative, which does not eliminate the possibility that Boms could be directly antimicrobial, but in my opinion makes it unlikely.

Another model I investigated is whether Boms affect processing of other immune proteins, like the classical AMPs. The MALDI-TOF spectra (Figure 5) show that at least for the immune

peptides, only the Boms are affected in *Bom*$^{\Delta 55C}$ hemolymph. Drs, Mtk, IMPPP, IM4, IM14, and other detectable peptides did not have a shift in mass, which would have suggested a defect in processing.

One further model is that the Boms form a complex with another immune protein. The requirement for Bombardier suggests that other proteins may also be required for antimicrobial activity. As mentioned earlier, I have tried co-immunoprecipitation of HA-tagged Bombardier. Although I could immunoprecipitate HA-tagged Bombardier, I was unable pull down other proteins. I believe this is a technical issue that can be resolved with further attempts and is definitely worth further investigation. Not only would I expect the short Boms to be pulled down if there is a complex, the unknown proteins could be pulled down as well and assayed for candidacidal activity.

Having an inducible candidacidal activity from hemolymph suggests an alternative approach to co-immunoprecipitation. Could we instead fractionate the activity, then identify candidates with mass spectrometry? Scott Lindsay has attempted to fractionate hemolymph and assay for activity. Again, the problem is technical, in that we are limited by the amount of hemolymph we can extract from flies. Large quantities of hemolymph (from 2,000-50,000 flies) were needed to identify the *Drosophila* AMPs active against bacteria and filamentous fungi (Bulet et al., 1993; Fehlbaum et al., 1994; Levashina et al., 1995). Furthermore, if a protein complex is indeed necessary for activity, it may be difficult to detect after fractionation. Thus, the co-immunoprecipitation would be the preferred approach to the problem.

What is the function of tailed and bicipital Boms?

In Chapter 3, I suggest that the reason why the effect of deleting Bombardier is less severe than that of deleting the ten 55C *Bom* genes is because Δ*bbd* only affects the short-form Boms.

Implicit in this argument is the conclusion that the tailed and bicipital Boms provide some defense. Δ*bbd* flies, which lack the short-form Boms, have at most six Boms present in hemolymph: three tailed and three bicipitals. As a comparison to Δ*bbd* flies, $Bom^{\Delta left}$ flies, which have six of the ten 55C Boms removed (Clemmons et al., 2015), also have six Boms present in hemolymph (four 55C Boms and the two Boms on the 3rd chromosome, totaling to three short, two tailed, and one bicipital) When comparing survival of $Bom^{\Delta left}$ and Δ*bbd* flies after infection with *F. oxysporum*, partial susceptibility for both $Bom^{\Delta left}$ and Δ*bbd* flies was observed (Table 5). However, $Bom^{\Delta left}$ survival against *E. faecalis* phenocopies $Bom^{\Delta 55c}$ survival, while Δ*bbd* flies are more resistant, so the difference in susceptibility is interesting. Could the difference be due to differences in levels of expression of these Boms? Short-form Boms are generally more highly-induced compared to tailed and bicipital Boms, so $Bom^{\Delta left}$ flies should have more Bom molecules present compared to in Δ*bbd* flies. Thus, the expression levels do not explain the difference in susceptibility.

Could the difference in susceptibility between $Bom^{\Delta left}$ and Δ*bbd* be due to tailed and bicipital Boms having specificity to certain pathogens? Examples of AMP specificity have been reported, e.g., Diptericin alone is necessary for survival against *Providencia rettgeri* (Unckless et al., 2016; Hanson et al., 2019). We have shown specificity in that short-form Boms are necessary for survival against *C. glabrata* (Chapter 2), and this is also observed with the difference in susceptibility of $Bom^{\Delta left}$ and Δ*bbd* flies against *C. glabrata.* Infected $Bom^{\Delta left}$ hemolymph has short-form Boms present and the flies can survive against *C. glabrata*, while Δ*bbd* hemolymph does not have short-form Boms, so the flies succumb to *C. glabrata* infection (Table 5). If tailed and bicipital Boms indeed have specificity to certain pathogens, this suggests that the three Bom peptides present in Δ*bbd* and not in $Bom^{\Delta left}$ – BomT1, BomBc1, and BomBc2 – can provide some resistance to *E. faecalis*. Because tailed or bicipital Boms were not required for survival against *C.*

glabrata infection, and we have not currently observed activity for the tailed and bicipital Boms, this is a promising starting point to investigate their function.

Why are *Drosophila*-specific genes required for immunity?

Mechanisms for recognition of pathogens and the signaling pathways that are activated in response are generally conserved, and many AMPs are conserved as well. Why are genus-specific immune genes, *Bomanins* and *bombardier*, required for defense? One possible explanation is that certain pathogens in the fly's environment may be resistant to the conserved AMPs present in *Drosophila*. A conserved AMP that kills the pathogens could have been lost or not expressed in the hemolymph. As an example, lysozymes are present in *Drosophila* and expressed in the gut, but not in the hemolymph (Daffre et al., 1994), so it is unlikely that they play a role in systemic infections. The lack of a conserved AMP that protects against the pathogen leads to selection for flies with the Boms that are active against the pathogens the AMPs were unable to kill. In this scenario, we would observe flies with the *Bom* deletion having susceptibility to only certain pathogens. Instead, we observe susceptibility to a broad spectrum of pathogens in the $Bom^{\Delta 55C}$ mutant, though we show pathogen specificity in that short Boms alone mediate survival against *C. glabrata*. We have only tested the partial *Bom* mutants ($Bom^{\Delta left}$ and Δ*bbd*) against a limited set of five to seven species that induce Toll, and have only tested the single *Bom* gene rescue mutants (Chapter 2) against three species. To obtain more evidence of specificity, these single *Bom* gene and partial *Bom* mutants we have generated should be infected with a wider variety of pathogens. Generating deletions of each Bom structural form (Δ*BomS*, Δ*BomT*, or Δ*BomBc*) would also be useful to address the function of each different form.

In conclusion, this dissertation investigate how Toll effectors affect *Drosophila* immune defense. In the case of Bombardier and the Bomanins, there is the potential for development as

antimicrobials. More broadly, while species-specific and genus-specific genes are often ignored in research to focus on genes conserved in mammals, this dissertation shows the importance of these genes to *Drosophila* immunity and encourages further investigation into highly induced genes, regardless of conservation.

Figures

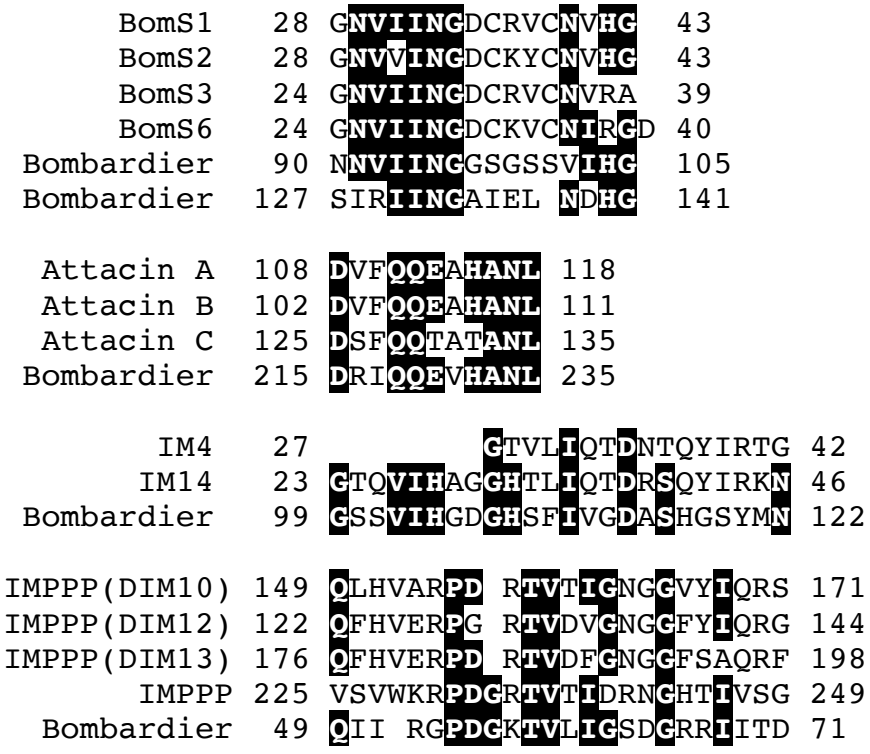

Figure 15. Immune peptide sequence comparison.
The complete mature peptide sequences of the short-form Boms (BomS1, S2, S3, and S6 are shown as examples), IM4, IM14, DIM10, DIM12, and DIM13 are shown, as well as portions of the Attacins and a separate portion of IMPPP, compared to portions of Bombardier. Bold highlighting indicates amino acids identical between a given immune peptide and Bombardier. Numbers before and after each sequence correspond to the protein's amino acid position in its full unprocessed form.

Tables

Table 5. Pathogen resistance of *Bom* and *bombardier* mutants
Wild-type (w^{1118}) flies were categorized as resistant because they are used as the resistant control, even if they eventually succumb to infection (*E. faecalis*). $Bom^{\Delta 55C}$ flies are the susceptible control, and succumb to infection much faster than the wild type. $Bom^{\Delta left}$ and Δ*bbd* flies were labeled as "susceptible" if they phenocopy $Bom^{\Delta 55C}$, "resistant" if they phenocopy w^{1118}, and "partially resistant" if there was an intermediate phenotype between w^{1118} and $Bom^{\Delta 55C}$. $Bom^{\Delta left}$ phenotypes were obtained from Clemmons et al., 2015. See Figure 7 for Δ*bbd* phenotypes.

Fly Genotype	***E. faecalis***	***F. oxysporum***	***C. glabrata***
w^{1118}	Resistant	Resistant	Resistant
$Bom^{\Delta 55C}$	Susceptible	Susceptible	Susceptible
$Bom^{\Delta left}$	Susceptible	Partially resistant	Resistant
Δ***bbd***	Partially resistant	Partially resistant	Susceptible

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