bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

1 # Synergy and remarkable specificity of antimicrobial peptides *in*
2 # *vivo* using a systematic knockout approach

3 M.A. Hanson[1*], A. Dostálová[1], C. Ceroni[1], M. Poidevin[2], S. Kondo[3], and B. Lemaitre[1*]

4

5 1 Global Health Institute, School of Life Science, École Polytechnique Fédérale de
6 Lausanne (EPFL), Lausanne, Switzerland.
7 2 Institute for Integrative Biology of the Cell, Université Paris-Saclay, CEA, CNRS,
8 Université Paris Sud, 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette, France
9 3 Invertebrate Genetics Laboratory, Genetic Strains Research Center, National
10 Institute of Genetics, Mishima, Japan
11 * Corresponding authors: M.A. Hanson (mark.hanson@epfl.ch), B. Lemaitre
12 (bruno.lemaitre@epfl.ch)
13
14 ORCID IDs:
15 Hanson: https://orcid.org/0000-0002-6125-3672
16 Kondo : https://orcid.org/0000-0002-4625-8379
17 Lemaitre: https://orcid.org/0000-0001-7970-1667
18

19 # Abstract

20 Antimicrobial peptides (AMPs) are host-encoded antibiotics that combat invading
21 microorganisms. These short, cationic peptides have been implicated in many
22 biological processes, primarily involving innate immunity. *In vitro* studies have
23 shown AMPs kill bacteria and fungi at physiological concentrations, but little
24 validation has been done *in vivo*. We utilised CRISPR gene editing to delete all
25 known immune inducible AMPs of *Drosophila,* namely: 4 Attacins, 4 Cecropins, 2
26 Diptericins, Drosocin, Drosomycin, Metchnikowin and Defensin. Using individual
27 and multiple knockouts, including flies lacking all 14 AMP genes, we characterize
28 the *in vivo* function of individual and groups of AMPs against diverse bacterial and
29 fungal pathogens. We found that *Drosophila* AMPs act primarily against Gram-
30 negative bacteria and fungi, acting either additively or synergistically. We also
31 describe remarkable specificity wherein certain AMPs contribute the bulk of
32 microbicidal activity against specific pathogens, providing functional
33 demonstrations of highly specific AMP-pathogen interactions in an *in vivo* setting.

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

34 # Introduction

35 While innate immune mechanisms were neglected during the decades where
36 adaptive immunity captured most of the attention, they have become central to our
37 understanding of immunology. Recent emphasis on innate immunity has, however,
38 mostly focused on the first two phases of the immune response: microbial
39 recognition and associated downstream signaling pathways. In contrast, how innate
40 immune effectors individually or collectively contribute to host resistance has not
41 been investigated to the same extent. The existence of multiple effectors that
42 redundantly contribute to host resistance has hampered their functional
43 characterization by genetic approaches[1]. The single mutation methodology that still
44 prevails today has obvious limits in the study of immune effectors, which often
45 belong to large gene families. As such, our current understanding of the logic
46 underlying the roles of immune effectors is only poorly defined. As a consequence,
47 the key parameters that influence host survival associated with a successful immune
48 response are not well characterized. In this paper, we harnessed the power of the
49 CRISPR gene editing approach to study the function of *Drosophila* antimicrobial
50 peptides in host defence both individually and collectively.

51 Antimicrobial peptides (AMPs) are small, cationic, usually amphipathic peptides
52 that contribute to innate immune defence in plants and animals [2–4]. They display
53 potent antimicrobial activity *in vitro* by disrupting negatively-charged microbial
54 membranes, but AMPs can also target specific microbial processes[5–7]. Their
55 expression is induced to very high levels upon challenge to provide microbicidal
56 concentrations in the µM range. Numerous studies have revealed unique roles that
57 AMPs may play in host physiology, including anti-tumour activity[8,9], inflammation in
58 aging[10–12], involvement in memory[13,14], mammalian immune signaling[15,16], wound-
59 healing[17,18], regulation of the host microbiota[19,20], tolerance to oxidative stress[21,22],
60 and of course microbicidal activity[1,2,23]. The fact that AMP genes are immune
61 inducible and expressed at high levels has led to the common assumption they play
62 a vital role in the innate immune response[24]. However, little is known in most cases

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

63 about how AMPs individually or collectively contribute to animal host defence. *In*
64 *vivo* functional analysis of AMPs has been hampered by the sheer number and small
65 size of these genes, making them difficult to mutate with traditional genetic tools
66 (but *e.g.* see[25,26]).

67 Since the first animal AMPs were discovered in silk moths[27], insects and particularly
68 *Drosophila melanogaster* have emerged as a powerful model for characterizing their
69 function. There are currently seven known families of inducible AMPs in *D.*
70 *melanogaster*. Their activities have been determined either *in vitro* by using
71 peptides directly purified from flies or produced in heterologous systems, or
72 deduced by comparison with homologous peptides isolated in other insect species:
73 Drosomycin and Metchnikowin show antifungal activity[28,29]; Cecropins (four
74 inducible genes) and Defensin have both antibacterial and some antifungal
75 activities[30–33]; and Drosocin, Attacins (four genes) and Diptericins (two genes)
76 primarily exhibit antibacterial activity[6,34–37]. In *Drosophila*, these AMPs are produced
77 either locally at various surface epithelia in contact with environmental microbes[38–
78 40], or secreted systemically into the hemolymph, the insect blood. During systemic
79 infection, these 14 antimicrobial peptides are strongly induced in the fat body, an
80 organ analogous to the mammalian liver.

81 The systemic production of AMPs is regulated at the transcriptional level by two NF-
82 κB pathways, the Toll and Imd pathways, which are activated by different classes of
83 microbes. The Toll pathway is predominantly responsive to Gram-positive bacteria
84 and fungi, and accordingly plays a major role in defence against these microbes. In
85 contrast, the Imd pathway is activated by Gram-negative bacteria and a subset of
86 Gram-positive bacteria with DAP-type peptidoglycan, and mutations affecting this
87 pathway cause profound susceptibility to Gram-negative bacteria[41,42]. However, the
88 expression pattern of AMP genes is complex as each gene is expressed with different
89 kinetics and can often receive transcriptional input from both pathways[42,43]. This
90 ranges from *Diptericin*, which is tightly regulated by the Imd pathway, to
91 *Drosomycin*, whose expression is mostly regulated by the Toll pathway[41], except at

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

92 surface epithelia where *Drosomycin* is under the control of Imd signaling[44]. While a
93 critical role of AMPs in *Drosophila* host defence is supported by transgenic flies
94 overexpressing a single AMP[33], the specific contributions of each of these AMPs has
95 not been tested. Indeed loss-of-function mutants for most AMP genes were not
96 previously available due to their small size, making them difficult to mutate before
97 the advent of CRISPR/Cas9 technology. Despite this, the great susceptibility to
98 infection of mutants with defective Toll and Imd pathways is commonly attributed
99 to the loss of the AMPs they regulate, though these pathways control hundreds of
100 genes awaiting characterization[42]. Strikingly, Clemmons *et al.*[45] recently reported
101 that flies lacking a set of uncharacterized Toll-responsive peptides (named
102 Bomanins) succumb to infection by Gram-positive bacteria and fungi at rates similar
103 to *Toll*-deficient mutants[45]. This provocatively suggests that Bomanins, and not
104 AMPs, might be the predominant effectors downstream of the Toll pathway; yet
105 synthesized Bomanins do not display antimicrobial activity *in vitro*[46]. Thus, while
106 today the fly represents one of the best-characterized animal immune systems, the
107 contribution of AMPs as immune effectors is poorly defined as we still do not
108 understand why Toll and Imd pathway mutants succumb to infection.

109 In this paper, we took advantage of recent gene editing technologies to delete each
110 of the known immune inducible AMP genes of *Drosophila*. Using single and multiple
111 knockouts, as well as a variety of bacterial and fungal pathogens, we have
112 characterized the *in vivo* function of individual and groups of antimicrobial peptides.
113 We reveal that AMPs can play highly specific roles in defence, being vital for
114 surviving certain infections yet dispensable against others. We highlight key
115 interactions amongst immune effectors and pathogens and reveal to what extent
116 these defence peptides act in concert or alone.

117 Results

118 *Generation and characterization of AMP mutants*

119 We generated null mutants for the fourteen *Drosophila* antimicrobial peptide genes

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

120 that are induced upon systemic infection. These include five single gene mutations
121 affecting *Defensin* (*Def*^{SK3}), *Attacin C* (AttC^{Mi}), *Metchnikowin* (*Mtk*^{R1}), *Attacin D*
122 (*AttD*^{SK1}) and *Drosomycin* (*Drs*^{R1}) respectively, and three small deletions removing
123 both *Diptericins DptA* and *DptB* (*Dpt*^{SK1}), the four *Cecropins CecA1, CecA2, CecB, and*
124 *CecC* (*Cec*^{SK6}) and the gene cluster containing *Drosocin,* and *Attacins AttA* & *AttB*
125 (*Dro-AttAB*^{SK2}). All mutations/deletions were made using the CRISPR editing
126 approach with the exception of *Attacin C*, which was disrupted by insertion of a
127 *Minos* transposable element[47], and the *Drosomycin* and *Metchnikowin* deletions
128 generated by homologous recombination (Fig. 1A). To disentangle the role of
129 *Drosocin* and *AttA/AttB* in the *Dro-AttAB*^{SK2} deletion, we also generated an individual
130 *Drosocin* mutant (*Dro*^{SK4}); for complete information, see Figure S1. We then
131 isogenized these mutations for at least seven generations into the w^{1118} DrosDel
132 isogenic genetic background[48] (*iso* w^{1118}). Then, we recombined these eight
133 independent mutations into a background lacking all 14 inducible AMPs referred to
134 as "*ΔAMPs*." *ΔAMPs* flies were viable and showed no morphological defects. To
135 confirm the absence of AMPs in our *ΔAMPs* background, we performed a MALDI-
136 TOF analysis of hemolymph from both unchallenged and immune-challenged flies
137 infected by a mixture of *Escherichia coli* and *Micrococcus luteus*. This analysis
138 revealed the presence of peaks induced upon challenge corresponding to AMPs in
139 wild-type but not *ΔAMPs* flies. Importantly it also confirmed that induction of most
140 other immune-induced molecules (IMs)[49], was unaffected in *ΔAMPs* flies (Fig. 1B). Of
141 note, we failed to observe two IMs, IM7 and IM21, in our *ΔAMPs* flies, suggesting that
142 these unknown peptides are secondary products of AMP genes. We further
143 confirmed that Toll and Imd NF-κB signaling pathways were intact in *ΔAMPs* flies by
144 measuring the expression of target genes of these pathways (Fig. 1C-D). This
145 demonstrates that *Drosophila* AMPs are not signaling molecules required for Toll or
146 Imd pathway activity. We also assessed the role of AMPs in the melanization
147 response, wound clotting, and hemocyte populations. After clean injury, *ΔAMPs* flies
148 survive as wild-type (Fig. 1 supplement A). We found no defect in melanization (χ^2,
149 p = .34, Fig. 1 supplement B) as both adults and larvae strongly melanize the cuticle
150 following clean injury, (Fig. 1 supplement C). Furthermore, we visualized the

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

151 formation of clot fibers *ex vivo* using the hanging drop assay and PNA staining[50] in
152 hemolymph of both wild-type and *ΔAMPs* larvae (Fig. 1 supplement D). Hemocyte
153 counting (*i.e.* crystal cells, FACS) did not reveal any deficiency in hemocyte
154 populations of *ΔAMPs* larvae (Fig. 1 supplement E, F, and not shown). Altogether,
155 our study suggests that *Drosophila* AMPs are primarily immune effectors, and not
156 regulators of innate immunity.

157 ***AMPs are essential for combating Gram-negative bacterial infection***

158 We used these *ΔAMPs* flies to explore the role that AMPs play in defence against
159 pathogens during systemic infection. We first focused our attention on Gram-
160 negative bacterial infections, which are combatted by Imd pathway-mediated
161 defence in *Drosophila*[1]. We challenged wild-type and *ΔAMPs* flies with six different
162 Gram-negative bacterial species, using inoculation doses (given as OD600) selected
163 such that at least some wild-type flies were killed (Fig. 2). In our survival
164 experiments, we also include *Relish* mutants *(Rel^{E20})* that lack a functional Imd
165 response and are known to be very susceptible to this class of bacteria[51]. Globally,
166 *ΔAMPs* flies were extremely susceptible to all Gram-negative pathogens tested (Fig.
167 2, light blue plots). The susceptibility of AMP-deficient flies to Gram-negative
168 bacteria largely mirrored that of Rel^{E20} flies. For all Gram-negative infections tested,
169 *ΔAMPs* flies show a higher bacterial count at 18 hours post-infection (hpi) indicating
170 that AMPs actively inhibit bacterial growth, as expected of 'antimicrobial peptides'
171 (Fig. 2 supplement A). Use of GFP-expressing bacteria show that bacterial growth in
172 *ΔAMPs* flies radiates from the wound site until it spreads systemically (Fig. 2
173 supplement B,C). Collectively, the use of AMP-deficient flies reveals that AMPs are
174 major players in resistance to Gram-negative bacteria, and likely constitute an
175 essential component of the Imd pathway's contribution for survival against these
176 germs.

177

178

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

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

181 Previous studies have shown that resistance to Gram-positive bacteria and fungi in
182 *Drosophila* is mostly mediated by the Toll pathway, although the Imd pathway also
183 contributes to some extent[41,43,52,53]. Moreover, a deletion removing eight
184 uncharacterized Bomanins (*Bom*$^{\Delta 55C}$) induces a strong susceptibility to both Gram-
185 positive bacteria and fungi[45], suggesting that Bomanins are major players
186 downstream of Toll in the defence against these germs. This prompted us to explore
187 the role of antimicrobial peptides in defence against Gram-positive bacteria and
188 fungi. We first challenged wild-type and *ΔAMPs* flies with two lysine-type (*E.*
189 *faecalis, S. aureus*) and two DAP-type peptidoglycan containing Gram-positive
190 bacterial species (*B. subtilis, L. innocua*). We observed that *ΔAMPs* flies display only
191 weak or no increased susceptibility to infection with these Gram-positive bacterial
192 species, as *ΔAMPs* survival rates were closer to the wild-type than to *spätzle* flies
193 (*spz*^{rm7}) lacking a functional Toll pathway (Fig. 2, orange plots). Meanwhile, *Bom*$^{\Delta 55C}$
194 mutants consistently phenocopied *spz*^{rm7} flies, confirming the important
195 contribution of these peptides in defence against Gram-positive bacteria[45].

196 Next, we monitored the survival of *ΔAMPs* to the yeast *Candida albicans*, the
197 opportunistic fungus *Aspergillus fumigatus* and two entomopathogenic fungi,
198 *Beauveria bassiana,* and *Metarhizium anisopliae.* For the latter two, we used a
199 natural mode of infection by spreading spores on the cuticle[41]. *ΔAMPs* flies were
200 more susceptible to fungal infections with *B. bassiana, A. fumigatus,* and *C. albicans*,
201 but not *M. anisopliae* (Fig. 2, yellow plots). In all instances, *Bom*$^{\Delta 55C}$ mutants were as
202 or more susceptible to fungal infection than *ΔAMPs* flies, approaching *Toll*-deficient
203 mutant levels. Collectively, our data demonstrate that AMPs are major immune
204 effectors in defence against Gram-negative bacteria and have a less essential role in
205 defence against bacteria and fungi.

206

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

207 ***A combinatory approach to explore AMP interactions***

208 The impact of the *ΔAMPs* deletion on survival could be due to the action of certain
209 AMPs having a specific effect, or more likely due to the combinatory action of co-
210 expressed AMPs. Indeed, cooperation of AMPs to potentiate their microbicidal
211 activity has been suggested by numerous *in vitro* approaches[7,54,55], but rarely in an
212 *in vivo* context[56]. Having shown that AMPs as a whole significantly contribute to fly
213 defence, we next explored the contribution of individual peptides to this effect. To
214 tackle this question in a systematic manner, we performed survival analyses using
215 fly lines lacking one or several AMPs, focusing on pathogens with a range of
216 virulence that we previously showed to be sensitive to the action of AMPs. This
217 includes the yeast *C. albicans* and the Gram-negative bacterial species *P.*
218 *burhodogranariea, P. rettgeri, Ecc15,* and *E. cloacae*. Given eight independent AMP
219 mutations, over 250 combinations of mutants are possible, making a systematic
220 analysis of AMP interactions a logistical nightmare. Therefore, we designed an
221 approach that would allow us to characterize their contributions to defence by
222 deleting groups of AMPs. To this end, we generated three groups of combined
223 mutants: flies lacking the primarily antibacterial *Defensin* and *Cecropins* (Group A,
224 mostly regulated by the Imd pathway), flies lacking the antibacterial Proline-rich
225 *Drosocin*, and the antibacterial Glycine-rich *Diptericins* and *Attacins* (Group B,
226 regulated by the Imd pathway), and flies lacking the two antifungal peptide genes
227 *Metchnikowin* and *Drosomycin* (Group C, mostly regulated by the Toll pathway). We
228 then combined these three groups to generate flies lacking AMPs from groups A and
229 B (AB), A and C (AC), or B and C (BC). Finally, flies lacking all three groups are our
230 *ΔAMPs* flies, which are highly susceptible to a number of infections. By screening
231 these seven genotypes as well as individual mutants, we were able to assess
232 potential interactions between AMPs of different groups, as well as decipher the
233 function of individual AMPs.

234

235

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

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

238 We first applied this AMP-groups approach to infections with the relatively
239 avirulent yeast *C. albicans*. Previous studies have shown that Toll, but not Imd,
240 contributes to defence against this fungus[57,58]. Thus, we suspected that the two
241 antifungal peptides, Drosomycin and Metchnikowin, could play a significant role in
242 the susceptibility of *ΔAMPs* flies to this yeast. Consistent with this, Group C flies
243 lacking *Metchnikowin* and *Drosomycin* were more susceptible to infection ($p < .001$
244 relative to *iso* w^{1118}) with a survival rate similar to *ΔAMPs* flies (Fig. 3A). Curiously,
245 AC deficient flies that also lack *Cecropins* and *Defensin* survived better than Group C
246 deficient flies (Log-Rank $p = .014$). We have no explanation for this interaction, but
247 this could be due to i) a better canalization of the immune response by preventing
248 the induction of ineffective AMPs, ii) complex biochemical interactions amongst the
249 AMPs involved, or iii) differences in genetic background generated by additional
250 recombination. We then investigated the individual contributions of *Metchnikowin*
251 and *Drosomycin* to survival to *C. albicans*. We found that both Mtk^{R1} and Drs^{R1}
252 individual mutants were somewhat susceptible to infection, but notably only *Mtk;*
253 *Drs* compound mutants reached *ΔAMPs* levels of susceptibility (Fig. 3B). This co-
254 occurring loss of resistance appears to be primarily additive (Mutant, Cox Hazard
255 Ratio (HR), p-value: Mtk^{R1}, *HR = +1.17, p = .008;* Drs^{R1}, *HR = +1.85, p < .001; Mtk*Drs,*
256 *HR = -0.80, p =.116*). We observed that Group C deficient flies eventually succumb to
257 uncontrolled *C. albicans* growth by monitoring yeast titre, indicating that these
258 AMPs indeed act by suppressing yeast growth (Fig. 3C).

259 In conclusion, our study provides an *in vivo* validation of the potent antifungal
260 activities of Metchnikowin and Drosomycin[28,29], and highlights a clear example of
261 additive cooperation of AMPs.

262 ***AMPs synergistically contribute to defence against P. burhodogranariea***

263 We next analyzed the contribution of AMPs in resistance to infection with the

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

264 moderately virulent Gram-negative bacterium *P. burhodogranariea*. We found that
265 Group B mutants lacking *Drosocin*, the two *Diptericins*, and the four *Attacins*, were as
266 susceptible to infection as *ΔAMPs* flies (Fig. 4A), while flies lacking the antifungal
267 peptides Drosomycin and Metchnikowin (Toll-regulated, Group C) resisted the
268 infection as wild-type. Flies lacking *Defensin* and the four *Cecropins* (Group A)
269 showed an intermediate susceptibility, but behave as wild-type in the additional
270 absence of Toll Group C peptides (Group AC). Thus, we again observed a better
271 survival rate with the co-occurring loss of Group A and C peptides (see possible
272 explanation above). In this case Group A flies were susceptible while AC flies were
273 not. Flies individually lacking *Defensin* or the four *Cecropins* were weakly susceptible
274 to *P. burhodogranariea* ($p = .022$ and $p = 0.040$ respectively), however the
275 interaction term between *Defensin and* the *Cecropins* was not significant
276 ($Def^{SK3}*Cec^{SK6}$, HR = -0.28, $p = .382$), indicating the susceptibility of Group A flies
277 arises from additive loss of resistance (Figure 4 supplement A).

278 Following the observation that Group B flies were as susceptible as *ΔAMPs* flies, we
279 sought to better decipher the contribution of each Group B AMP to resistance to *P.*
280 *burhodogranariea*. We observed that mutants for *Drosocin* alone (Dro^{SK4}), or the
281 *DiptericinA/B* deficiency were not susceptible to this bacterium (Fig. 4B). We
282 additionally saw no marked susceptibility of *Drosocin-Attacin A/B* deficient flies, nor
283 *Attacin C* or *Attacin D* mutants (not shown). Interestingly, we found that compound
284 mutants lacking *Drosocin* and *Attacins A, B, C,* and *D* (Fig. 4B: '*ΔDro, ΔAtt*'), or
285 *Drosocin* and *Diptericins DptA* and *DptB* ('*ΔDro, ΔDpt*') displayed an intermediate
286 susceptibility. Only the Group B mutants lacking *Drosocin*, all *Attacins*, and both
287 *Diptericins* (*ΔDro, ΔAtt, ΔDpt)* phenocopied *ΔAMPs* flies (Fig. 4B), with synergistic
288 interactions observed upon co-occurring loss of *Attacins* and *Diptericins* (*ΔAtt*ΔDpt*:
289 HR = +1.45, $p < .001$). By 6hpi, bacterial titres of individual flies already showed
290 significant differences in the most susceptible genotypes (Fig. 4C), though these
291 differences were reduced by 18hpi likely owing to the high chronic load *P.*
292 *burhodogranariea* establishes in surviving flies[24]; also see Fig. 2 supplement A.

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

293 Collectively, the use of various compound mutants reveals that several Imd-
294 responsive AMPs, notably Drosocin, Attacins, and Diptericins, jointly contribute to
295 defence against *P. burhodogranariea* infection. A strong susceptibility of Group B
296 flies was also observed upon infection with *Ecc15*, another Gram-negative
297 bacterium commonly used to infect flies[59] (Fig. 4 supplement B).

298 ***Diptericins* alone contribute to defence against *P. rettgeri***

299 We continued our exploration of AMP interactions using our AMP groups approach
300 with the fairly virulent *P. rettgeri* (strain Dmel), a strain isolated from wild-caught
301 *Drosophila* hemolymph[60]. We were especially interested by this bacterium as
302 previous studies[61,62] have shown a correlation between susceptibility to *P. rettgeri*
303 and a polymorphism in the *Diptericin A* gene pointing to a specific AMP-pathogen
304 interaction. Use of compound mutants revealed only loss of Group B AMPs was
305 needed to reach the susceptibility of *ΔAMPs* and Rel^{E20} flies (Fig. 5A). Use of
306 individual mutant lines however revealed a pattern strikingly different from that *P.*
307 *burhodogranariea,* as the sole *Diptericin A/B* deficiency caused susceptibility similar
308 to Group B, *ΔAMPs,* and Rel^{E20} flies (Fig. 5B,C). We further confirmed this
309 susceptibility using a *DptA RNAi* construct (Fig. 5 supplement A, B). Moreover, flies
310 carrying the Dpt^{SK1} mutation over a deficiency (*Df(2R)Exel6067*) were also highly
311 susceptible to *P. rettgeri* (Fig. 5D). Interestingly, flies that were heterozygotes for
312 Dpt^{SK1} or the *Df(2R)Exel6067* that have only one copy of the two *Diptericins* were
313 markedly susceptible to infection with *P. rettgeri* (Fig. 5D). This indicates that a full
314 transcriptional output of *Diptericin* is required over the course of the infection to
315 resist *P. rettgeri* infection (Fig. 5E). Altogether, our results suggest that only the
316 *Diptericin* gene family, amongst the many AMPs regulated by the Imd pathway,
317 provides the full AMP-based contribution to defence against this bacterium. To test
318 this hypothesis, we generated a fly line lacking all the AMPs except *DptA* and *DptB*
319 ($\Delta AMPs^{+Dpt}$). Strikingly, $\Delta AMPs^{+Dpt}$ flies have the same survival rate as wild-type flies,
320 further emphasizing the specificity of this interaction (Fig. 5B). Bacterial counts
321 confirm that the susceptibility of these *Diptericin* mutants arises from an inability of

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

322 the host to suppress bacterial growth (Fig. 5C).

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

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

331 In the course of our exploration of AMP-pathogen interactions, we identified
332 another highly specific interaction between *E. cloacae* and Drosocin. Use of
333 compound mutants revealed that alone, Group B flies were already susceptible to *E.*
334 *cloacae*. Meanwhile, Group AB flies reached *ΔAMPs* levels of susceptibility, while
335 Group A and Group C flies resisted as wild-type (Fig. 6A). The high susceptibility of
336 Group AB flies results from a synergistic interaction amongst Group A and Group B
337 peptides in defence against *E. cloacae* (*A*B*, HR = +2.55, p = .003).

338 We chose to further explore the AMPs deleted in Group B flies, as alone this
339 genotype already displayed a strong susceptibility. Use of individual mutant lines
340 revealed that mutants for *Drosocin* alone (Dro^{SK4}) or the *Drosocin-Attacin A/B*
341 deficiency ($Dro\text{-}AttAB^{SK2}$), but not *AttC, AttD, nor* Dpt^{SK1} (not shown), recapitulate the
342 susceptibility observed in Group B flies (Fig. 6B). At 18hpi, both Dro^{SK4} and *ΔAMPs*
343 flies had significantly higher bacterial loads compared to wild-type flies, while Rel^{E20}
344 mutants were already moribund with much higher bacterial loads (Fig. 6C). Indeed,
345 the deletion of *Drosocin* alone drastically alters the fly's ability to control the
346 otherwise avirulent *E. cloacae* with inoculations at OD=200 (~39,000 bacteria, Fig.
347 6A-C) or even OD=10 (~7,000 bacteria, Fig. 6 supplement A).

348 We confirmed the high susceptibility of *Drosocin* mutant flies to *E. cloacae* in various
349 contexts: transheterozygote flies carrying Dro^{SK4} over a *Drosocin* deficiency

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

350 *(Df(2R)BSC858)* that also lacks flanking genes including *AttA* and *AttB* ((Fig. 6D), the
351 *Dro* ^{SK4} mutations in an alternate genetic background (*yw*, Fig. 6E), and, *Drosocin*
352 *RNAi* (Fig. 6 supplement B,C). Thus, we recovered two highly specific AMP-
353 pathogen interactions: Diptericins are essential to combat *P. rettgeri* infection, while
354 *Drosocin* is paramount to surviving *E. cloacae* infection.

355 # Discussion

356 ***A combinatory approach to study AMPs***

357 Despite the recent emphasis on innate immunity, little is known on how immune
358 effectors contribute individually or collectively to host defence, exemplified by the
359 lack of in depth *in vivo* functional characterization of *Drosophila* AMPs. Taking
360 advantage of new gene editing approaches, we developed a systematic mutation
361 approach to study the function of *Drosophila* AMPs. With eight distinct mutations,
362 we were able to generate a fly line lacking the 14 AMPs that are inducible during the
363 systemic immune response. A striking first finding is that *ΔAMPs* flies were perfectly
364 healthy and have an otherwise wild-type immune response. This indicates that in
365 contrast to mammals[15], *Drosophila* AMPs are not likely to function as signaling
366 molecules. Most flies lacking a single AMP family exhibited a higher susceptibility to
367 certain pathogens consistent with their *in vitro* activity. We found activity of
368 Diptericins against *P. rettgeri*, Drosocin against *E. cloacae*, Drosomycin and
369 Metchnikowin against *C. albicans*, and Defensin and Cecropin against *P.*
370 *burhodogranariea* (Fig. 4 supplement A). In most cases, the susceptibility of single
371 mutants was slight, and the contribution of individual AMPs could be revealed only
372 when combined to other AMP mutations as illustrated by the susceptibility of
373 *Drosocin, Attacin,* and *Diptericin* combined mutants to *P. burhodogranariea*. Thus,
374 the use of compound rather than single mutations provides a better strategy to
375 decipher the contribution of AMPs to host defence.

376

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

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

379 The Toll and Imd pathways provide a paradigm of innate immunity, illustrating how
380 two distinct pathways link pathogen recognition to distinct but overlapping sets of
381 downstream immune effectors[1,63]. However, a method of deciphering the
382 contributions of the different downstream effectors to the specificity of these
383 pathways remained out of reach, as mutations in these immune effectors were
384 lacking. Our study shows that AMPs contribute greatly to resistance to Gram-
385 negative bacteria. Consistent with this, *ΔAMPs* flies are almost as susceptible as Imd-
386 deficient mutants to most Gram-negative bacteria. In contrast, flies lacking AMPs
387 were only slightly more susceptible to Gram-positive bacteria and fungal infections
388 compared to wild-type flies, and this susceptibility rarely approached the
389 susceptibility of *Bomanin* mutants. This may be due to the cell walls of Gram-
390 negative bacteria being thinner and more fluid than the rigid cell walls of Gram-
391 positive bacteria[64], consequently making Gram-negative bacteria more prone to the
392 action of pore-forming cationic peptides. It would be interesting to know if the
393 specificity of AMPs to primarily combatting Gram-negative bacteria is also true in
394 other species.

395 Based on our study and Clemmons et al.[45], we can now explain the susceptibility of
396 Toll and Imd mutants at the level of the effectors, as we show that mutations
397 affecting Imd-pathway responsive antibacterial peptide genes are highly susceptible
398 to Gram-negative bacteria while the Toll-responsive targets Drosomycin,
399 Metchnikowin, and especially the Bomanins, confer resistance to fungi and Gram-
400 positive bacteria. Thus, the susceptibility of these two pathways to different sets of
401 microbes not only reflects specificity at the level of recognition, but can now also be
402 translated to the activities of downstream effectors. It remains to be seen how
403 Bomanins contribute to the microbicidal activity of immune-induced hemolymph, as
404 attempts to synthesize Bomanins have not revealed direct antimicrobial activity[46]. It
405 should also be noted that many putative effectors downstream of Toll and Imd

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

406 remain uncharacterized, and so could also contribute to host defence beyond AMPs
407 and Bomanins.

408 ***AMPs act additively and synergistically to suppress bacterial growth in vivo***

409 In the last few years, numerous *in vitro* studies have focused on the potential for
410 synergistic interactions of AMPs in microbial killing[7,54,56,65–70]. Our collection of AMP
411 mutant fly lines placed us in an ideal position to investigate AMP interactions in an
412 *in vivo* setting. While Toll-responsive AMPs (Group C: Metchnikowin, Drosomycin)
413 additively contributed to defence against the yeast *C. albicans*, we found that certain
414 combinations of AMPs have synergistic contributions to defence against *P.*
415 *burhodogranariea*. Synergistic loss of resistance may arise in two general fashions:
416 first, co-operation of AMPs using similar mechanisms of action may breach a
417 threshold microbicidal activity whereupon pathogens are no longer able to resist.
418 This may be the case for our observations of synergy amongst Diptericins and
419 Attacins against *P. burhodogranariea*, as only co-occurring loss of both these related
420 glycine-rich peptide families[36] led to complete loss of resistance. Alternatively,
421 synergy may arise due to complementary mechanisms of action, whereupon one
422 AMP potentiates the other AMP's ability to act. For instance, the action of the
423 bumblebee AMP Abaecin, which binds to the molecular chaperone DnaK to inhibit
424 bacterial DNA replication, is potentiated by the presence of the pore-forming
425 peptide Hymenoptaecin[71]. *Drosophila* Drosocin is highly similar to Abaecin,
426 including O-glycosylation of a critical threonine residue[2,72], and thus likely acts in a
427 similar fashion. Furthermore, *Drosophila* Attacin C is maturated into both a glycine-
428 rich peptide and a Drosocin-like peptide called MPAC[73]. As such, co-occuring loss of
429 Drosocin, MPAC, and other possible MPAC-like peptides encoded by the
430 Attacin/Diptericin superfamily may be responsible for the synergistic loss of
431 resistance in *Drosocin, Attacin, Diptericin* combined mutants.

432 ***AMPs can act with great specificity against certain pathogens***

433 It is commonly thought that the innate immune response lacks the specificity of the

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

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

443 *Diptericin* has previously been highlighted for its important role in defence against
444 *P. rettgeri*[62], but it was previously unknown whether other AMPs may confer
445 defence in this infection model. Astoundingly, flies mutant for all other inducible
446 AMPs resisted *P. rettgeri* infection as wild-type, while only *Diptericin* mutants
447 succumbed to infection. This means that Diptericins do not co-operate with other
448 AMPs in defence against *P. rettgeri*, and are solely responsible for defence in this
449 specific host-pathogen interaction. Moreover, +/Dpt^{SK1} heterozygote flies were
450 nonetheless extremely susceptible to infection, demonstrating that a full
451 transcriptional output over the course of infection is required to effectively prevent
452 pathogen growth. A previous study has shown that ~7hpi appears to be the critical
453 time point at which *P. rettgeri* either grows unimpeded or the infection is
454 controlled[24]. This time point correlates with the time at which the *Diptericin*
455 transcriptional output is in full-force[41]. Thus, a lag in the transcriptional response in
456 Dpt^{SK1}/+ flies likely prevents the host from reaching a competent Diptericin
457 concentration, indicating that *Diptericin* expression level is a key factor in successful
458 host defence.

459 We also show that *Drosocin* is specifically required for defence against *E. cloacae*.
460 This striking finding validates previous biochemical analyses showing Drosocin *in*
461 *vitro* activity against several Enterobacteriaceae, including *E. cloacae*[37]. As *ΔAMPs*
462 flies are more susceptible than *Drosocin* single mutants, other AMPs also contribute

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

463 to Drosocin-mediated control of *E. cloacae*. As highlighted above, Drosocin is similar
464 to other Proline-rich AMPs (*e.g.* Abaecin, Pyrrhocoricin) that have been shown to
465 target bacterial DnaK[6,7]. Alone, these peptides still penetrate bacteria cell walls
466 through their uptake by bacterial permeases[71,74]. Thus, while Drosocin would
467 benefit from the presence of pore-forming toxins to enter bacterial cells[71], the
468 veritable "stake to the heart" is likely the plunging of Drosocin itself into vital
469 bacterial machinery.

470 ***On the role of AMPs in host defence***

471 It has often been questioned why flies should need so many AMPs[1,4,75]. A common
472 idea, supported by *in vitro* experiments[7,65,70], is that AMPs work as cocktails,
473 wherein multiple effectors are needed to kill invading pathogens. However, we find
474 support for an alternative hypothesis that suggests AMP diversity may be due to
475 highly specific interactions between AMPs and subsets of pathogens that they target.
476 Burgeoning support for this idea also comes from recent evolutionary studies that
477 show *Drosophila* and vertebrate AMPs experience positive selection[62,72,75–81], a
478 hallmark of host-pathogen evolutionary conflict . Our functional demonstrations of
479 AMP-pathogen specificity, using naturally relevant pathogens[60,82], suggest that such
480 specificity is fairly common, and that certain AMPs can act as the arbiters of life or
481 death upon infection by certain pathogens. This stands in contrast to the classical
482 view that the AMP response contains such redundancy that single peptides should
483 have little effect on organism-level immunity[4,61,75,83]. Nevertheless, it seems these
484 immune effectors play non-redundant roles in defence.

485 By providing a long-awaited *in vivo* functional validation for the role of AMPs in host
486 defence, we also pave the way for a better understanding of the functions of immune
487 effectors. Our approach of using multiple compound mutants, now possible with the
488 development of new genome editing approaches, was especially effective to
489 decipher the logic of immune effectors. Understanding the role of AMPs in innate
490 immunity holds great promise for the development of novel antibiotics[18,84,85],
491 insight into autoimmune diseases[86–89], and given their potential for remarkably

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

492 specific interactions, perhaps in predicting key parameters that predispose
493 individuals or populations to certain kinds of infections[61,75,76]. Finally, our set of
494 isogenized *AMP* mutant lines provides long-awaited tools to decipher the role of
495 AMPs not only in immunity, but also in the various roles that AMPs may play in
496 aging, neurodegeneration, anti-tumour activity, regulation of the microbiota and
497 more, where disparate evidence has pointed to their involvement.

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

498 # Figure Captions
499

500 **Figure 1:** Description of *AMP* mutants. A) Chromosomal location of *AMP* genes that
501 were deleted. Each mutation is color-coded with the mutagenic agent: black, a *Minos*
502 insertion or homologous recombination, red, CRISPR-CAS9 mediated deletion, and
503 blue CRISPR CAS9 mediated indel causing a nonsense peptide. B) A representative
504 MALDI-TOF analysis of hemolymph samples from immune-challenged (1:1 *E. coli*
505 and *M. luteus* at OD600 = 200) *iso* w^{1118} and *ΔAMPs* flies as described in
506 Üttenweiller-Joseph et al.[49]. No AMP-derived products were detected in the
507 hemolymph samples of *ΔAMPs* flies. No signals for IM7, nor IM21 were observed in
508 the hemolymph samples of *ΔAMPs* mutants suggesting that these uncharacterized
509 immune-induced molecules are the products of AMP genes. The Imd pathway (C)
510 and Toll pathway (D) are active and respond to immune challenge in *ΔAMPs* flies.
511 We used alternate readouts to monitor the Toll and Imd pathways: *pirk* and *PGRP-*
512 *LB* for Imd pathway and *CG5791 (Bomanin)* and *IMPPP* for Toll signaling[42,72]. UC =
513 unchallenged, Inf = infected. hpi = hours post-infection. Expression normalized with
514 *iso* w^{1118}*-UC* set to a value of 1.

515 **Figure 1 supplement:** *ΔAMPs* flies have otherwise wild-type immune reactions. A)
516 *ΔAMPs* flies survive clean injury like wild-type flies, while *ΔPPO* mutants deficient
517 for melanization have reduced survival over time. B) *ΔAMPs* flies melanize the
518 cuticle similar to wild-type flies following pricking (χ^2 = 2.14, p = .34). Melanization
519 categories (None, Weak, Normal) were as described in Dudzic et al.[90]. Sample sizes
520 (n) are included in each bar. C) Melanization in *iso* w^{1118}, *ΔAMPs*, and *ΔPPO* flies of
521 the cuticle in adults (*i*, yellow arrowheads), larvae (*ii*, melanized wounds), and larval
522 hemolymph (*iii*). D) To investigate clotting ability, we used the hanging drop assay[50]
523 with *ΔAMPs* larval hemolymph and visualized clot fibers with PNA staining (green).
524 Both *iso* w^{1118} and *ΔAMPs* hemolymph produced visible clot fibres measured after 20
525 minutes. Hemocyte populations are normal in *ΔAMPs* flies, including crystal cell
526 distribution (E) and number (F).

527 **Figure 2:** Survival of *ΔAMPs* flies to diverse microbial challenges. Control lines for
528 survival experiments included two wild-types (*w;Drosdel* (*iso* w^{1118}) and Oregon R
529 (*OR-R*) as an alternate wild-type), mutants for the Imd response (*Rel*^{E20}), mutants for
530 Toll signaling (*spz*^{rm7}), and mutants for Bomanins (*Bom*$^{\Delta 55C}$). *ΔAMPs* flies are
531 extremely susceptible to infection with Gram-negative bacteria (blue backgrounds).
532 Unexpectedly, *ΔAMPs* flies were not markedly susceptible to infection with Gram-
533 positive bacteria (orange backgrounds), while *Bom*$^{\Delta 55C}$ flies were extremely

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

534 susceptible, often mirroring *spz*^{rm7} mutants. This pattern of *Bom*$^{\Delta55C}$ susceptibility
535 held true for fungal infections (yellow backgrounds). *ΔAMPs* flies are somewhat
536 susceptible to fungal infections, but the severity shifts with different fungi. Pellet
537 densities are reported for all systemic infections in OD at 600nm. P-values are given
538 for *ΔAMPs* flies compared to *iso w*^{1118} using a Cox-proportional hazards model.

539 **Figure 2 supplement:** *ΔAMPs* flies fail to suppress Gram-negative bacterial growth.
540 Colony counts were performed on pooled samples (5 flies) for bacteria amenable to
541 LB agar, a medium that avoids overnight growth of the host microbiota. A) For
542 Gram-negative bacterial infections, *ΔAMPs* flies have significantly higher bacterial
543 loads than *iso w*^{1118} at 18 hours post-infection (hpi) (*i*). This is not true for any of the
544 Gram-positive bacteria tested (*ii*), while *spz*^{rm7} mutants carried higher bacterial
545 loads, significantly so in *E. faecalis* infections. Gram-negative (B) and Gram-positive
546 (C) infections with GFP-labelled bacteria spread from the wound site systemically in
547 all genotypes tested. Thus *ΔAMPs* fly mortality is likely not due to tissue-specific
548 colonization by invading bacteria, but rather a failure to suppress bacterial growth
549 first locally, and then systemically. One-way ANOVA: not significant = *ns*, $p < .05$ = *,
550 $p < .01$ = **, and $p < .001$ = *** relative to *iso w*^{1118}.

551 **Figure 3:** Identification of AMPs involved in the susceptibility of *ΔAMPs* flies to *C.*
552 *albicans*. A) Survival of mutants for groups of AMPs reveals that loss of only Toll-
553 responsive Group C peptides (Metchnikowin and Drosomycin) is required to
554 recapitulate the susceptibility of *ΔAMPs* flies. Co-occurring loss of groups A and C
555 has a net protective effect (*A*C*: HR = -1.71, p = .002). B) Further dissection of Group
556 C mutations reveals that both Metchnikowin and Drosomycin contribute to resist *C.*
557 *albicans* survival (p = .008 and $p < .001$ respectively). The interaction of
558 Metchnikowin and Drosomycin was not different from the sum of their individual
559 effects (*Mtk*Drs*: HR = -0.80, p = .116). Fungal loads of individual flies at 18hpi. At
560 this time point, *Bom*$^{\Delta55C}$ mutants and *spz*^{rm7} flies have already failed to constrain *C.*
561 *albicans* growth (C'). Fungal titres at 36hpi (C''), a time point closer to mortality for
562 many AMP mutants, show that some AMP mutants fail to control fungal load, while
563 wild-type flies consistently controlled fungal titre. One-way ANOVA: not significant
564 = *ns*, $p < .05$ = *, $p < .01$ = **, and $p < .001$ = *** relative to *iso w*^{1118}.

565 **Figure 4:** Identification of AMPs involved in the susceptibility of *ΔAMPs* flies to *P.*
566 *burhodogranariea*. A) Survival of mutants for groups of AMPs reveals that loss of
567 Imd-responsive Group B peptides (Drosocin, Attacins, and Diptericins) recapitulates
568 the susceptibility of *ΔAMPs* flies. Loss of Group A peptides also resulted in strong
569 susceptibility ($p< .001$) due to additive effects of Defensin and Cecropins (Fig. 4

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

570 supplement). B) Further dissection of AMPs deleted in Group B reveals that only the
571 loss of all Drosocin, Attacin, and Diptericin gene families leads to susceptibility
572 similar to *ΔAMPs* flies. Simultaneous loss of *Attacins* and *Diptericins* results in a
573 synergistic loss of resistance (*ΔAtt*ΔDpt*: HR = +1.45, p < .001). C) Bacterial loads of
574 individual flies at 6hpi (C'). At this time point, most AMP mutants had significantly
575 higher bacterial loads compared to wild-type flies. At 18hpi (C''), differences in
576 bacterial load are reduced, likely owing to the high chronic load *P. burhodogranariea*
577 establishes even in surviving flies[24]. Meanwhile Rel^{E20} flies succumb ~18 hours
578 earlier than *ΔAMPs* flies in survival experiments, and already have significantly
579 higher loads. One-way ANOVA: not significant = *ns*, p < .05 = *, p < .01 = **, and p <
580 .001 = *** relative to *iso* w^{1118}.

581 **Figure 4 supplement:** A) Dissection of the susceptibility of Group A flies lacking
582 *Defensin* and *Cecropins* reveals that combined mutants have an additive loss of
583 resistance (*Def*Cec*, HR = +0.36, p = .342). B) Upon infection with the Gram-negative
584 *Ecc15*, Group B peptides (Drosocin, Attacins and Diptericins) explain the bulk of
585 mortality, but additional loss of other peptides in *ΔAMPs* flies leads to increased
586 mortality (Log-Rank p = .013).

587 **Figure 5:** Identification of AMPs involved in the susceptibility of *ΔAMPs* flies to *P.*
588 *rettgeri*. A) Survival of mutants for groups of AMPs reveals that only loss of Imd-
589 responsive Group B peptides (Drosocin, Attacins, and Diptericins) recapitulates the
590 susceptibility of *ΔAMPs* flies. B) Further dissection of the mutations affected in
591 Group B reveals that only the loss of Diptericins (Dpt^{SK1}) leads to susceptibility
592 similar to *ΔAMPs* flies. Remarkably, flies lacking all other AMPs ($ΔAMPs^{+Dpt}$) resist as
593 wild-type. C) Bacterial loads of individual flies are similar at 6hpi (C'), but by 18hpi
594 (C''), *Dpt* mutants and Rel^{E20} flies have all failed to control *P. rettgeri* growth. D)
595 Heterozygote flies for Dpt^{SK1} and a deficiency including the *Diptericins* and flanking
596 genes *(Df(2R)Exel6067)* recapitulates the susceptibility of *Diptericin* mutants.
597 Intriguingly, heterozygotes with one functional copy of the Diptericins ($+/Dpt^{SK1}$ or
598 *+/ Df(2R)Exel6067*) are nonetheless highly susceptible to infection. E) *Diptericin A*
599 transcriptional output is strongly reduced in heterozygotes 6hpi compared to wild-
600 type flies. One-way ANOVA: not significant = *ns*, p < .05 = *, p < .01 = **, and p < .001
601 = *** relative to *iso* w^{1118}.

602 **Figure 5 supplement:** A) Silencing of *Diptericin by RNAi* leads to higher
603 susceptibility to *P. rettgeri* infection (p < .001). B) Validation of the *Diptericin* RNAi
604 construct 6hpi. C) Mutants lacking multiple peptides (Attacins, Drosocin, and
605 Metchnikowin) succumb to *P. stuartii* infection as wild-type ('*ΔAtt, ΔDro,* Mtk^{R1}'),

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

606 while Diptericin mutation alone (*Dpt*^{SK1}) or combined (*'ΔAtt, ΔDro, Mtk*^{R1}*, ΔDpt'*)
607 leads to a susceptibility similar to *Rel*^{E20} mutants. This pattern of survival was
608 similar to the pattern observed with *P. rettgeri*. One-way ANOVA: p < .001 = ***.

609 **Figure 6:** Identification of AMPs involved in the susceptibility of *ΔAMPs* flies to *E.*
610 *cloacae*. A) Survival of mutants for groups of AMPs reveals that loss of Imd-
611 responsive Group B peptides (Drosocin, Attacins, and Diptericins) results in a strong
612 susceptibility to infection (p < .001), while loss of Group A or C peptides alone
613 resists as wild-type (p > 0.1 each). Group AB flies were as susceptible as *ΔAMPs* flies,
614 and we observed a synergistic interaction between Group A and B mutations (A*B:
615 HR = +2.55, p = .003). B) Further dissection of the mutations in Group B revealed
616 that loss of *Drosocin* alone (*Dro*^{SK4}), or a deficiency lacking both *Drosocin* and
617 *Attacins AttA* and *AttB* (*Dro-AttAB*^{SK2}) recapitulates the susceptibility of Group B
618 flies. C) By 18hpi, bacterial loads in individual *Drosocin* mutants or *Rel*^{E20} flies are
619 significantly higher than wild-type. D) Heterozygote flies for *Dro*^{SK4} and
620 *Df(2R)BSC858* (a deficiency removing *Drosocin, Attacins AttA* and *AttB*, and other
621 genes) are strongly susceptible to *E. cloacae* infection. E) *Drosocin* mutants in an
622 alternate genetic background (*yw*) are susceptible to *E. cloacae*. One-way ANOVA:
623 not significant = *ns*, and p < .001 = *** relative to *iso w*^{1118}.

624 **Figure 6 supplement:** A) *Drosocin* mutant susceptibility remains even at a lower
625 dose (OD=10, ~7000 bacteria/fly), while *Rel*^{E20} flies succumb rapidly regardless of
626 initial dose. B) Silencing of *Drosocin by RNAi* leads to significant mortality from *E.*
627 *cloacae* infection (p < .001). C) Validation of the *Drosocin* RNAi construct 6hpi.

628 **Figure S1:** Genetic description of mutations generated in this study. *Mtk*^{R1} and *Drs*^{R1}
629 mutations entirely replaced the CDS with an insert from the piHR vector. Non-
630 synonymous nucleotides in mutants are given in red.

631 **Table S1:** Primers used in this study to track AMP mutations or measure gene
632 expression.

633

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

634 # Materials and Methods

635 *Drosophila genetics and mutant generation*

636 The DrosDel[48] isogenic w^{1118} (*iso* w^{1118}) wild type was used as a genetic background
637 for mutant isogenization. Alternate wild-types used throughout include Oregon R
638 (*OR-R*), w^{1118} from the Vienna Drosophila Resource Centre, and the Canton-S
639 isogenic line Exelexis w^{1118}, which was kindly provided by Brian McCabe. $Bom^{\Delta 55C}$
640 mutants were generously provided by Steven Wasserman, and $Bom^{\Delta 55C}$ was
641 isogenized into the *iso* w^{1118} background. Rel^{E20} and spz^{rm7} *iso* w^{1118} flies were
642 provided by Luis Teixeira[51,91]. Prophenoloxidase mutants (*ΔPPO*) are described in
643 Dudzic et al.[92]. P-element mediated homologous recombination according to Baena-
644 Lopez et al.[93] was used to generate mutants for *Mtk* (Mtk^{R1}) and *Drs* (Drs^{R1}).
645 Plasmids were provided by Mickael Poidevin. *Attacin C* mutants ($AttC^{Mi}$, #25598),
646 the *Diptericin* deficiency (*Df(2R)Exel6067*, #7549), the *Drosocin* deficiency
647 (*Df(2R)BSC858*, #27928), *UAS-Diptericin RNAi* (Dpt^{RNAi}, #53923), *UAS-Drosocin RNAi*
648 (Dro^{RNAi}, #67223), and *Actin5C-Gal4* (*ActGal4*, #4414) were ordered from the
649 Bloomington stock centre (stock #s included). CRISPR mutations were performed
650 by Shu Kondo according to Kondo and Ueda[94], and full descriptions are given in
651 Figure S1. In brief, flies deficient for *Drosocin, Attacin A,* and *Attacin B* (*Dro-*
652 $AttAB^{SK2}$), *Diptericin A* and *Diptericin B* (Dpt^{SK1}), and *Cecropins CecA1, CecA2, CecB,*
653 *CecC* (Cec^{SK6}) were all produced by gene region deletion specific to those AMPs
654 without affecting other genes. Single mutants for *Defensin* (Def^{SK3}), *Drosocin* (Dro^{SK4}),
655 and *Attacin D* ($AttD^{SK1}$) are small indels resulting in the production of short (80-107
656 residues) nonsense peptides. Mutations were isogenized for a minimum of seven
657 generations into the *iso* w^{1118} background prior to subsequent recombination.
658

659 *Microbial culture conditions*

660 Bacteria were grown overnight on a shaking plate at 200rpm in their respective
661 growth media and temperature conditions, and then pelleted by centrifugation at
662 4°C. These bacterial pellets were diluted to the desired optical density at 600nm
663 (OD) as indicated. The following bacteria were grown at 37°C in LB media:
664 *Escherichia coli strain 1106, Salmonella typhimurium, Enterobacter cloacae β12,*
665 *Providencia rettgeri strain Dmel, Providencia burhodogranariea strain B, Providencia*
666 *stuartii strain DSM 4539, Providencia sneebia strain Dmel, Providencia alcalifaciens*
667 *strain Dmel, Providencia vermicola strain DSM 17385, Bacillus subtilis,* and
668 *Staphylococcus aureus. Erwinia carotovora carotovora (Ecc15)* and *Micrococcus*
669 *luteus* were grown overnight in LB at 29°C. *Enterococcus faecalis* and *Listeria*

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

670 *innocua* were cultured in BHI medium at 37°C. *Candida albicans* strain ATCC 2001
671 was cultured in YPG medium at 37°C. *Aspergillus fumigatus* was grown at room
672 temperature on Malt Agar, and spores were collected in sterile PBS rinses, pelleted
673 by centrifugation, and then resuspended to the desired OD in PBS. The
674 entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* were
675 grown on Malt Agar at room temperature until sporulation.

676 *Systemic infections and survival*

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

692 *Quantification of microbial load*

693 The native *Drosophila* microbiota does not readily grow overnight on LB, allowing
694 for a simple assay to estimate bacterial load. Flies were infected with bacteria at the
695 indicated OD as described, and allowed to recover. At the indicated time post-
696 infection, flies were anaesthetized using CO_2 and surface sterilized by washing them
697 in 70% ethanol. Ethanol was removed, and then flies were homogenized using a
698 Precellys™ bead beater at 6500rpm for 30 seconds in LB broth, with 300ul for
699 individual samples, or 500uL for pools of 5-7 flies. These homogenates were serially
700 diluted and 150uL was plated on LB agar. Bacterial plates were incubated overnight,
701 and colony-forming units (CFUs) were counted manually. Statistical analyses were
702 performed using One-way ANOVA with Sidak's correction. P-values are reported as
703 < 0.05 = *, < 0.01 = **, and < 0.001 = ***. For *C. albicans*, BiGGY agar was used instead
704 to select for *Candida* colonies from fly homogenates.

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

705 *Gene expression by qPCR*

706 Flies were infected by pricking flies with a needle dipped in a pellet of either *E. coli*
707 or *M. luteus* (OD600 = 200), and frozen at -20°C 6h and 24h post-infection
708 respectively. Total RNA was then extracted from pooled samples of five flies each
709 using TRIzol reagent, and re-suspended in MilliQ dH_2O. Reverse transcription was
710 performed using 0.5 micrograms total RNA in 10 µl reactions using PrimeScript RT
711 (TAKARA) with random hexamer and oligo dT primers. Quantitative PCR was
712 performed on a LightCycler 480 (Roche) in 96-well plates using Applied
713 Biosystems™ SYBR™ Select Master Mix. Values represent the mean from three
714 replicate experiments. Error bars represent one standard deviation from the mean.
715 Primers used in this study can be found in Table S1. Statistical analyses were
716 performed using one-way ANOVA with Tukey post-hoc comparisons. P-values are
717 reported as not significant = ns, < 0.05 = *, < 0.01 = **, and < 0.001 = ***. qPCR
718 primers and sources[11,72,95] are included in Table S1.

719 *MALDI-TOF peptide analysis*

720 Two methods were used to collect hemolymph from adult flies: in the first method,
721 pools of five adult females were pricked twice in the thorax and once in the
722 abdomen. Wounded flies were then spun down with 15µL of 0.1% trifluoroacetic
723 acid (TFA) at 21000 RCF at 4°C in a mini-column fitted with a 10µm pore to prevent
724 contamination by circulating hemocytes. These samples were frozen at -20°C until
725 analysis, and three biological replicates were performed with 4 technical replicates.
726 In the second method, approximately 20nL of fresh hemolymph was extracted from
727 individual adult males using a Nanoject, and immediately added to 1µL of 1% TFA,
728 and the matrix was added after drying. Peptide expression was visualized as
729 described in Üttenweiller-Joseph et al.[49]. Both methods produced similar results,
730 and representative expression profiles are given.

731 *Melanization and hemocyte characterization, image acquisition*

732 Melanization assays[90] and peanut agglutinin (PNA) clot staining[50] was performed as
733 previously described. In brief, flies or L3 larvae were pricked, and the level of
734 melanization was assessed at the wound site. We used FACS sorting to count
735 circulating hemocytes. For sessile crystal cell visualization, L3 larvae were cooked in
736 dH_2O at 70°C for 20 minutes, and crystal cells were visualized on a Leica DFC300FX
737 camera using Leica Application Suite and counted manually.

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

738 # Literature Cited

739 1. Lemaitre B, Hoffmann J. The Host Defense of. *Annu Rev Immunol*.
740 2007;25:697-743. doi:10.1146/annurev.immunol.25.022106.141615.

741 2. Imler J-L, Bulet P. Antimicrobial peptides in Drosophila: structures, activities
742 and gene regulation. *Chem Immunol Allergy*. 2005;86(1):1-21.
743 doi:10.1159/000086648.

744 3. Guaní-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM. Antimicrobial
745 peptides: General overview and clinical implications in human health and
746 disease. *Clin Immunol*. 2010;135(1):1-11. doi:10.1016/j.clim.2009.12.004.

747 4. Rolff J, Schmid-Hempel P. Perspectives on the evolutionary ecology of
748 arthropod antimicrobial peptides. *Philos Trans R Soc Lond B Biol Sci*.
749 2016;371(1695):20150297-. doi:10.1098/rstb.2015.0297.

750 5. Park CB, Kim HS, Kim SC. Mechanism of action of the antimicrobial peptide
751 buforin II: Buforin II kills microorganisms by penetrating the cell membrane
752 and inhibiting cellular functions. *Biochem Biophys Res Commun*.
753 1998;244(1):253-257. doi:10.1006/bbrc.1998.8159.

754 6. Kragol G, Lovas S, Varadi G, Condie BA, Hoffmann R, Otvos L. The antibacterial
755 peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents
756 chaperone-assisted protein folding. *Biochemistry*. 2001;40(10):3016-3026.
757 doi:10.1021/bi002656a.

758 7. Rahnamaeian M, Cytry ska M, Zdybicka-Barabas A, et al. Insect antimicrobial
759 peptides show potentiating functional interactions against Gram-negative
760 bacteria. *Proc R Soc B Biol Sci*. 2015;282(1806):20150293-20150293.
761 doi:10.1098/rspb.2015.0293.

762 8. Suttmann H, Retz M, Paulsen F, et al. Antimicrobial peptides of the Cecropin-
763 family show potent antitumor activity against bladder cancer cells. *BMC Urol*.
764 2008;8:5. doi:10.1186/1471-2490-8-5.

765 9. Kuroda K, Okumura K, Isogai H, Isogai E. The Human Cathelicidin
766 Antimicrobial Peptide LL-37 and Mimics are Potential Anticancer Drugs. *Front*
767 *Oncol*. 2015;5. doi:10.3389/fonc.2015.00144.

768 10. Cao Y, Chtarbanova S, Petersen AJ, Ganetzky B. Dnr1 mutations cause
769 neurodegeneration in Drosophila by activating the innate immune response
770 in the brain. *Proc Natl Acad Sci*. 2013;110(19):E1752-E1760.
771 doi:10.1073/pnas.1306220110.

772 11. Kounatidis I, Chtarbanova S, Cao Y, et al. NF-κB Immunity in the Brain
773 Determines Fly Lifespan in Healthy Aging and Age-Related

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

774 Neurodegeneration. *Cell Rep*. 2017;19(4):836-848.
775 doi:10.1016/j.celrep.2017.04.007.

776 12. Lezi E, Zhou T, Koh S, et al. An Antimicrobial Peptide and Its Neuronal
777 Receptor Regulate Dendrite Degeneration in Aging and Infection. *Neuron*.
778 2018;97(1):125-138.e5. doi:10.1016/j.neuron.2017.12.001.

779 13. Bozler J, Kacsoh BZ, Chen H, Theurkauf WE, Weng Z, Bosco G. A systems level
780 approach to temporal expression dynamics in Drosophila reveals clusters of
781 long term memory genes. *PLoS Genet*. 2017;13(10).
782 doi:10.1371/journal.pgen.1007054.

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

786 15. Van Wetering S, Mannesse-Lazeroms SPG, Van Sterkenburg MAJA, Hiemstra
787 PS. Neutrophil defensins stimulate the release of cytokines by airway
788 epithelial cells: Modulation by dexamethasone. In: *Inflammation Research*. Vol
789 51. ; 2002:8-15. doi:10.1007/PL00000282.

790 16. Tjabringa GS, Aarbiou J, Ninaber DK, et al. The Antimicrobial Peptide LL-37
791 Activates Innate Immunity at the Airway Epithelial Surface by Transactivation
792 of the Epidermal Growth Factor Receptor. *J Immunol*. 2003;171(12):6690-
793 6696. doi:10.4049/jimmunol.171.12.6690.

794 17. Tokumaru S, Sayama K, Shirakata Y, et al. Induction of Keratinocyte Migration
795 via Transactivation of the Epidermal Growth Factor Receptor by the
796 Antimicrobial Peptide LL-37. *J Immunol*. 2005;175(7):4662-4668.
797 doi:10.4049/jimmunol.175.7.4662.

798 18. Chung EMC, Dean SN, Propst CN, Bishop BM, Van Hoek ML. Komodo dragon-
799 inspired synthetic peptide DRGN-1 promotes wound-healing of a mixed-
800 biofilm infected wound. *npj Biofilms Microbiomes*. 2017;3(1).
801 doi:10.1038/s41522-017-0017-2.

802 19. Login FH, Balmand S, Vallier A, et al. Antimicrobial peptides keep insect
803 endosymbionts under control. *Science (80-)*. 2011;334(6054):362-365.
804 doi:10.1126/science.1209728.

805 20. Mergaert P, Kikuchi Y, Shigenobu S, Nowack ECM. Metabolic Integration of
806 Bacterial Endosymbionts through Antimicrobial Peptides. *Trends Microbiol*.
807 2017. doi:10.1016/j.tim.2017.04.007.

808 21. Zhao HW, Zhou D, Haddad GG. Antimicrobial peptides increase tolerance to
809 oxidant stress in Drosophila melanogaster. *J Biol Chem*. 2011;286(8):6211-
810 6218. doi:10.1074/jbc.M110.181206.

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

811 22. Zheng Y, Niyonsaba F, Ushio H, et al. Cathelicidin LL-37 induces the generation
812 of reactive oxygen species and release of human alpha-defensins from
813 neutrophils. *Br J Dermatol*. 2007;157(6):1124-1131. doi:10.1111/j.1365-
814 2133.2007.08196.x.

815 23. Wimley WC. Describing the mechanism of antimicrobial peptide action with
816 the interfacial activity model. *ACS Chem Biol*. 2010;5(10):905-917.
817 doi:10.1021/cb1001558.

818 24. Duneau D, Ferdy JB, Revah J, et al. Stochastic variation in the initial phase of
819 bacterial infection predicts the probability of survival in D. melanogaster.
820 *Elife*. 2017;6. doi:10.7554/eLife.28298.

821 25. Hoeckendorf A, Stanisak M, Leippe M. The saposin-like protein SPP-12 is an
822 antimicrobial polypeptide in the pharyngeal neurons of *Caenorhabditis*
823 *elegans* and participates in defence against a natural bacterial pathogen.
824 *Biochem J*. 2012;445(2):205-212. doi:10.1042/BJ20112102.

825 26. Nakatsuji T, Chen TH, Two AM, et al. Staphylococcus aureus Exploits
826 Epidermal Barrier Defects in Atopic Dermatitis to Trigger Cytokine
827 Expression. *J Invest Dermatol*. 2016;136(11):2192-2200.
828 doi:10.1016/j.jid.2016.05.127.

829 27. Steiner H, Hultmark D, Engström Å, Bennich H, Boman HG. Sequence and
830 specificity of two antibacterial proteins involved in insect immunity. *Nature*.
831 1981;292(5820):246-248. doi:10.1038/292246a0.

832 28. Fehlbaum P, Bulet P, Michaut L, et al. Insect immunity: Septic injury of
833 drosophila induces the synthesis of a potent antifungal peptide with sequence
834 homology to plant antifungal peptides. *J Biol Chem*. 1994;269(52):33159-
835 33163.

836 29. Levashina EA, Ohresser S, Bulet P, Reichhart J -M, Hetru C, Hoffmann JA.
837 Metchnikowin, a Novel Immune-Inducible Proline-Rich Peptide from
838 Drosophila with Antibacterial and Antifungal Properties. *Eur J Biochem*.
839 1995;233(2):694-700. doi:10.1111/j.1432-1033.1995.694_2.x.

840 30. Hultmark D, Steiner H, Rasmuson T, Boman HG. Insect Immunity. Purification
841 and Properties of Three Inducible Bactericidal Proteins from Hemolymph of
842 Immunized Pupae of Hyalophora cecropia. *Eur J Biochem*. 1980;106(1):7-16.
843 doi:10.1111/j.1432-1033.1980.tb05991.x.

844 31. Ekengren S, Hultmark D. Drosophila cecropin as an antifungal agent. *Insect*
845 *Biochem Mol Biol*. 1999;29(11):965-972. doi:10.1016/S0965-
846 1748(99)00071-5.

847 32. Cociancich S, Ghazi A, Hetru C, Hoffmann JA, Letellier L. Insect defensin, an

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

848 inducible antibacterial peptide, forms voltage- dependent channels in
849 Micrococcus luteus. *J Biol Chem*. 1993;268(26):19239-19245.

850 33. Tzou P, Reichhart J-M, Lemaitre B. Constitutive expression of a single
851 antimicrobial peptide can restore wild-type resistance to infection in
852 immunodeficient Drosophila mutants. *Proc Natl Acad Sci U S A*.
853 2002;99(4):2152-2157. doi:10.1073/pnas.042411999.

854 34. Åsling B, Dushay MS, Hultmark D. Identification of early genes in the
855 Drosophila immune response by PCR-based differential display: the Attacin A
856 gene and the evolution of attacin-like proteins. *Insect Biochem Mol Biol*.
857 1995;25(4):511-518. doi:10.1016/0965-1748(94)00091-C.

858 35. Cudic M, Bulet P, Hoffmann R, Craik DJ, Otvos L. Chemical synthesis,
859 antibacterial activity and conformation of diptericin, an 82-mer peptide
860 originally isolated from insects. *Eur J Biochem*. 1999;266(2):549-558.
861 doi:10.1046/j.1432-1327.1999.00894.x.

862 36. Hedengren M, Borge K, Hultmark D. Expression and evolution of the
863 Drosophila attacin/diptericin gene family. *Biochem Biophys Res Commun*.
864 2000;279(2):574-581. doi:10.1006/bbrc.2000.3988.

865 37. Bulet P, Urge L, Ohresser S, Hetru C, Otvos L. Enlarged scale chemical
866 synthesis and range of activity of drosocin, an O-glycosylated antibacterial
867 peptide of Drosophila. *Eur J Biochem*. 1996;238(1):64-69. doi:10.1111/j.1432-
868 1033.1996.0064q.x.

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

873 39. Zaidman-Rémy A, Hervé M, Poidevin M, et al. The Drosophila Amidase PGRP-
874 LB Modulates the Immune Response to Bacterial Infection. *Immunity*.
875 2006;24(4):463-473. doi:10.1016/j.immuni.2006.02.012.

876 40. Gendrin M, Welchman DP, Poidevin M, Hervé M, Lemaitre B. Long-range
877 activation of systemic immunity through peptidoglycan diffusion in
878 Drosophila. *PLoS Pathog*. 2009;5(12). doi:10.1371/journal.ppat.1000694.

879 41. Lemaitre B, Reichhart JM, Hoffmann JA. Drosophila host defense: differential
880 induction of antimicrobial peptide genes after infection by various classes of
881 microorganisms. *Proc Natl Acad Sci U S A*. 1997;94(26):14614-14619.
882 doi:10.1073/pnas.94.26.14614.

883 42. De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. The Toll and Imd
884 pathways are the major regulators of the immune response in Drosophila.

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

885 *EMBO J*. 2002;21(11):2568-2579. doi:10.1093/emboj/21.11.2568.

886 43. Leulier F, Rodriguez A, Khush RS, Abrams JM, Lemaitre B. The Drosophila
887 caspase Dredd is required to resist Gram-negative bacterial infection. *EMBO*
888 *Rep*. 2000;1(4):353-358. doi:10.1093/embo-reports/kvd073.

889 44. Ferrandon D, Jung AC, Criqui MC, et al. A drosomycin-GFP reporter transgene
890 reveals a local immune response in Drosophila that is not dependent on the
891 Toll pathway. *EMBO J*. 1998;17(5):1217-1227. doi:10.1093/emboj/17.5.1217.

892 45. Clemmons AW, Lindsay SA, Wasserman SA. An Effector Peptide Family
893 Required for Drosophila Toll-Mediated Immunity. *PLoS Pathog*. 2015;11(4).
894 doi:10.1371/journal.ppat.1004876.

895 46. Lindsay SA, Lin SJH, Wasserman SA. Short-Form Bomanins Mediate Humoral
896 Immunity in Drosophila. 2018. doi:10.1159/000489831.

897 47. Bellen HJ, Levis RW, He Y, et al. The Drosophila gene disruption project:
898 Progress using transposons with distinctive site specificities. *Genetics*.
899 2011;188(3):731-743. doi:10.1534/genetics.111.126995.

900 48. Ryder E, Blows F, Ashburner M, et al. The DrosDel collection: A set of P-
901 element insertions for generating custom chromosomal aberrations in
902 Drosophila melanogaster. *Genetics*. 2004;167(2):797-813.
903 doi:10.1534/genetics.104.026658.

904 49. Uttenweiler-Joseph S, Moniatte M, Lagueux M, Van Dorsselaer a, Hoffmann J a,
905 Bulet P. Differential display of peptides induced during the immune response
906 of Drosophila: a matrix-assisted laser desorption ionization time-of-flight
907 mass spectrometry study. *Proc Natl Acad Sci U S A*. 1998;95(19):11342-
908 11347. doi:10.1073/pnas.95.19.11342.

909 50. Scherfer C, Karlsson C, Loseva O, et al. Isolation and characterization of
910 hemolymph clotting factors in Drosophila melanogaster by a pullout method.
911 *Curr Biol*. 2004;14(7):625-629. doi:10.1016/j.cub.2004.03.030.

912 51. Hedengren M, Åsling B, Dushay MS, et al. Relish, a central factor in the control
913 of humoral but not cellular immunity in Drosophila. *Mol Cell*. 1999;4(5):827-
914 837. doi:10.1016/S1097-2765(00)80392-5.

915 52. Rutschmann S, Jung AC, Hetru C, Reichhart JM, Hoffmann JA, Ferrandon D. The
916 Rel protein DIF mediates the antifungal but not the antibacterial host defense
917 in Drosophila. *Immunity*. 2000;12(5):569-580. doi:10.1016/S1074-
918 7613(00)80208-3.

919 53. Tanji T, Hu X, Weber ANR, Ip YT. Toll and IMD Pathways Synergistically
920 Activate an Innate Immune Response in Drosophila melanogaster. *Mol Cell*

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

921 *Biol*. 2007;27(12):4578-4588. doi:10.1128/MCB.01814-06.

922 54. Yu G, Baeder DY, Regoes RR, Rolff J. The More The Better? Combination Effects
923 of Antimicrobial Peptides. *Antimicrob Agents Chemother*.
924 2016;(January):AAC.02434-15. doi:10.1128/AAC.02434-15.

925 55. Mohan KVK, Rao SS, Gao Y, Atreya CD. Enhanced antimicrobial activity of
926 peptide-cocktails against common bacterial contaminants of ex vivo stored
927 platelets. *Clin Microbiol Infect*. 2014;20(1). doi:10.1111/1469-0691.12326.

928 56. Zanchi C, Johnston PR, Rolff J. Evolution of defence cocktails: Antimicrobial
929 peptide combinations reduce mortality and persistent infection. *Mol Ecol*.
930 2017;26(19):5334-5343. doi:10.1111/mec.14267.

931 57. Gottar M, Gobert V, Matskevich AA, et al. Dual Detection of Fungal Infections
932 in Drosophila via Recognition of Glucans and Sensing of Virulence Factors.
933 *Cell*. 2006;127(7):1425-1437. doi:10.1016/j.cell.2006.10.046.

934 58. Glittenberg MT, Silas, SukritGlittenberg, M. T., Silas, S., MacCallum, D. M., Gow,
935 N. a R., & Ligoxygakis P. Wild-type Drosophila melanogaster as an alternative
936 model system for investigating the pathogenicity of Candida albicans. *Dis*
937 *Model Mech*. 2011;4(4):504-514. doi:10.1242/dmm.006619.

938 59. Neyen C, Bretscher AJ, Binggeli O, Lemaitre B. Methods to study Drosophila
939 immunity. *Methods*. 2014;68(1):116-128. doi:10.1016/j.ymeth.2014.02.023.

940 60. Juneja P, Lazzaro BP. Providencia sneebia sp. nov. and Providencia
941 burhodogranariea sp. nov., isolated from wild Drosophila melanogaster. *Int J*
942 *Syst Evol Microbiol*. 2009;59(5):1108-1111. doi:10.1099/ijs.0.000117-0.

943 61. Unckless RL, Rottschaefer SM, Lazzaro BP. The Complex Contributions of
944 Genetics and Nutrition to Immunity in Drosophila melanogaster. *PLoS Genet*.
945 2015;11(3). doi:10.1371/journal.pgen.1005030.

946 62. Unckless RL, Howick VM, Lazzaro BP. Convergent Balancing Selection on an
947 Antimicrobial Peptide in Drosophila. *Curr Biol*. 2016;26(2):257-262.
948 doi:10.1016/j.cub.2015.11.063.

949 63. Buchon N, Silverman N, Cherry S. Immunity in Drosophila melanogaster -
950 from microbial recognition to whole-organism physiology. *Nat Rev Immunol*.
951 2014;14(12). doi:10.1038/nri3763.

952 64. Fayaz AM, Balaji K, Girilal M, Yadav R, Kalaichelvan PT, Venketesan R. 15
953 Biogenic synthesis of silver nanoparticles and their synergistic effect with
954 antibiotics: a study against gram-positive and gram-negative bacteria.
955 *Nanomedicine*. 2010;6(1):103-109. doi:10.1016/j.nano.2009.04.006.

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

956 65. Yan H, Hancock REW. Synergistic interactions between mammalian
957 antimicrobial defense peptides. *Antimicrob Agents Chemother*.
958 2001;45(5):1558-1560. doi:10.1128/AAC.45.5.1558-1560.2001.

959 66. Nuding S, Frasch T, Schaller M, Stange EF, Zabel LT. Synergistic effects of
960 antimicrobial peptides and antibiotics against clostridium difficile. *Antimicrob*
961 *Agents Chemother*. 2014;58(10):5719-5725. doi:10.1128/AAC.02542-14.

962 67. Zerweck J, Strandberg E, Kukharenko O, et al. Molecular mechanism of
963 synergy between the antimicrobial peptides PGLa and magainin 2. *Sci Rep*.
964 2017;7(1). doi:10.1038/s41598-017-12599-7.

965 68. Chen X, Niyonsaba F, Ushio H, et al. Synergistic effect of antibacterial agents
966 human β-defensins, cathelicidin LL-37 and lysozyme against Staphylococcus
967 aureus and Escherichia coli. *J Dermatol Sci*. 2005;40(2):123-132.
968 doi:10.1016/j.jdermsci.2005.03.014.

969 69. Stewart SE, Kondos SC, Matthews AY, et al. The perforin pore facilitates the
970 delivery of cationic cargos. *J Biol Chem*. 2014;289(13):9172-9181.
971 doi:10.1074/jbc.M113.544890.

972 70. Zdybicka-Barabas A, Mak P, Klys A, et al. Synergistic action of *Galleria*
973 *mellonella* anionic peptide 2 and lysozyme against Gram-negative bacteria.
974 *Biochim Biophys Acta - Biomembr*. 2012;1818(11):2623-2635.
975 doi:10.1016/j.bbamem.2012.06.008.

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

980 72. Hanson MA, Hamilton PT, Perlman SJ. Immune genes and divergent
981 antimicrobial peptides in flies of the subgenus Drosophila. *BMC Evol Biol*.
982 2016;16(1):228. doi:10.1186/s12862-016-0805-y.

983 73. Rabel D, Charlet M, Ehret-Sabatier L, et al. Primary structure and in vitro
984 antibacterial properties of the Drosophila melanogaster attacin C Pro-domain.
985 *J Biol Chem*. 2004;279(15):14853-14859. doi:10.1074/jbc.M313608200.

986 74. Narayanan S, Modak JK, Ryan CS, Garcia-Bustos J, Davies JK, Roujeinikova A.
987 Mechanism of escherichia coli resistance to pyrrhocoricin. *Antimicrob Agents*
988 *Chemother*. 2014;58(5):2754-2762. doi:10.1128/AAC.02565-13.

989 75. Unckless RL, Lazzaro BP. The potential for adaptive maintenance of diversity
990 in insect antimicrobial peptides. *Philos Trans R Soc Lond B Biol Sci*.
991 2016;371(1695):20150291-. doi:10.1098/rstb.2015.0291.

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

992 76. Chapman JR, Hill T, Unckless RL. Balancing selection drives maintenance of
993 genetic variation in Drosophila antimicrobial peptides. *bioRxiv*. 2018:298893.
994 doi:10.1101/298893.

995 77. Sackton TB, Lazzaro BP, Schlenke T a, Evans JD, Hultmark D, Clark AG.
996 Dynamic evolution of the innate immune system in Drosophila. *Nat Genet*.
997 2007;39(12):1461-1468. doi:10.1038/ng.2007.60.

998 78. Hellgren O, Sheldon BC. Locus-specific protocol for nine different innate
999 immune genes (antimicrobial peptides: β-defensins) across passerine bird
1000 species reveals within-species coding variation and a case of trans-species
1001 polymorphisms. *Mol Ecol Resour*. 2011;11(4):686-692. doi:10.1111/j.1755-
1002 0998.2011.02995.x.

1003 79. Halldórsdóttir K, Árnason E. Trans-species polymorphism at antimicrobial
1004 innate immunity cathelicidin genes of Atlantic cod and related species. *PeerJ*.
1005 2015;3:e976. doi:10.7717/peerj.976.

1006 80. Jiggins FM, Kim KW. A screen for immunity genes evolving under positive
1007 selection in Drosophila. *J Evol Biol*. 2007;20(3):965-970. doi:10.1111/j.1420-
1008 9101.2007.01305.x.

1009 81. Tennessen JA, Blouin MS. Balancing selection at a frog antimicrobial peptide
1010 locus: Fluctuating immune effector alleles? *Mol Biol Evol*. 2008;25(12):2669-
1011 2680. doi:10.1093/molbev/msn208.

1012 82. Cox CR, Gilmore MS. Native microbial colonization of Drosophila
1013 melanogaster and its use as a model of Enterococcus faecalis pathogenesis.
1014 *Infect Immun*. 2007;75(4):1565-1576. doi:10.1128/IAI.01496-06.

1015 83. Tzou P, Ohresser S, Ferrandon D, et al. Tissue-specific inducible expression of
1016 antimicrobial peptide genes in Drosophila surface epithelia. *Immunity*.
1017 2000;13(5):737-748. doi:10.1016/S1074-7613(00)00072-8.

1018 84. Mylonakis E, Podsiadlowski L, Muhammed M, Vilcinskas A. Diversity,
1019 evolution and medical applications of insect antimicrobial peptides. *Philos
1020 Trans R Soc Lond B Biol Sci*. 2016;371(1695):169-184.
1021 doi:10.1098/rstb.2015.0290.

1022 85. Mahlapuu M, Håkansson J, Ringstad L, Björn C. Antimicrobial Peptides: An
1023 Emerging Category of Therapeutic Agents. *Front Cell Infect Microbiol*. 2016;6.
1024 doi:10.3389/fcimb.2016.00194.

1025 86. Schluesener HJ, Radermacher S, Melms A, Jung S. Leukocytic antimicrobial
1026 peptides kill autoimmune T cells. *J Neuroimmunol*. 1993;47(2):199-202.
1027 doi:10.1016/0165-5728(93)90030-3.

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

1028 87. Gilliet M, Lande R. Antimicrobial peptides and self-DNA in autoimmune skin
1029 inflammation. *Curr Opin Immunol*. 2008;20(4):401-407.
1030 doi:10.1016/j.coi.2008.06.008.

1031 88. Sun J, Furio L, Mecheri R, et al. Pancreatic β-Cells Limit Autoimmune Diabetes
1032 via an Immunoregulatory Antimicrobial Peptide Expressed under the
1033 Influence of the Gut Microbiota. *Immunity*. 2015;43(2):304-317.
1034 doi:10.1016/j.immuni.2015.07.013.

1035 89. Kumar DKV, Choi SH, Washicosky KJ, et al. Amyloid-β peptide protects against
1036 microbial infection in mouse and worm models of Alzheimer's disease. *Sci*
1037 *Transl Med*. 2016;8(340):340ra72. doi:10.1126/scitranslmed.aaf1059.

1038 90. Dudzic JP, Hanson MA, Iatsenko I, Kondo S, Lemaitre B. More than black and
1039 white: complex relationships involving serine proteases regulate the Toll
1040 pathway and the melanization response in Drosophila. *bioRxiv*. January 2018.
1041 http://biorxiv.org/content/early/2018/08/02/383257.abstract.

1042 91. Ferreira ÁG, Naylor H, Esteves SS, Pais IS, Martins NE, Teixeira L. The Toll-
1043 Dorsal Pathway Is Required for Resistance to Viral Oral Infection in
1044 Drosophila. *PLoS Pathog*. 2014;10(12). doi:10.1371/journal.ppat.1004507.

1045 92. Dudzic JP, Kondo S, Ueda R, Bergman CM, Lemaitre B. Drosophila innate
1046 immunity: regional and functional specialization of prophenoloxidases. *BMC*
1047 *Biol*. 2015;13(1):81-97. doi:10.1186/s12915-015-0193-6.

1048 93. Baena-Lopez LA, Alexandre C, Mitchell A, Pasakarnis L, Vincent J-P.
1049 Accelerated homologous recombination and subsequent genome modification
1050 in Drosophila. *Development*. 2013;140(23):4818-4825.
1051 doi:10.1242/dev.100933.

1052 94. Kondo S, Ueda R. Highly Improved gene targeting by germline-specific Cas9
1053 expression in Drosophila. *Genetics*. 2013;195(3):715-721.
1054 doi:10.1534/genetics.113.156737.

1055 95. Iatsenko I, Kondo S, Mengin-Lecreulx D, Lemaitre B. PGRP-SD, an Extracellular
1056 Pattern-Recognition Receptor, Enhances Peptidoglycan-Mediated Activation
1057 of the Drosophila Imd Pathway. *Immunity*. 2016;45(5):1013-1023.
1058 doi:10.1016/j.immuni.2016.10.029.

1059

1060 **Author contributions:**

1061

1062 MAH, AD and BL designed the study. MAH and AD performed DrosDel isogenization
1063 and recombination. MP and SK supplied critical reagents. MAH performed the

bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

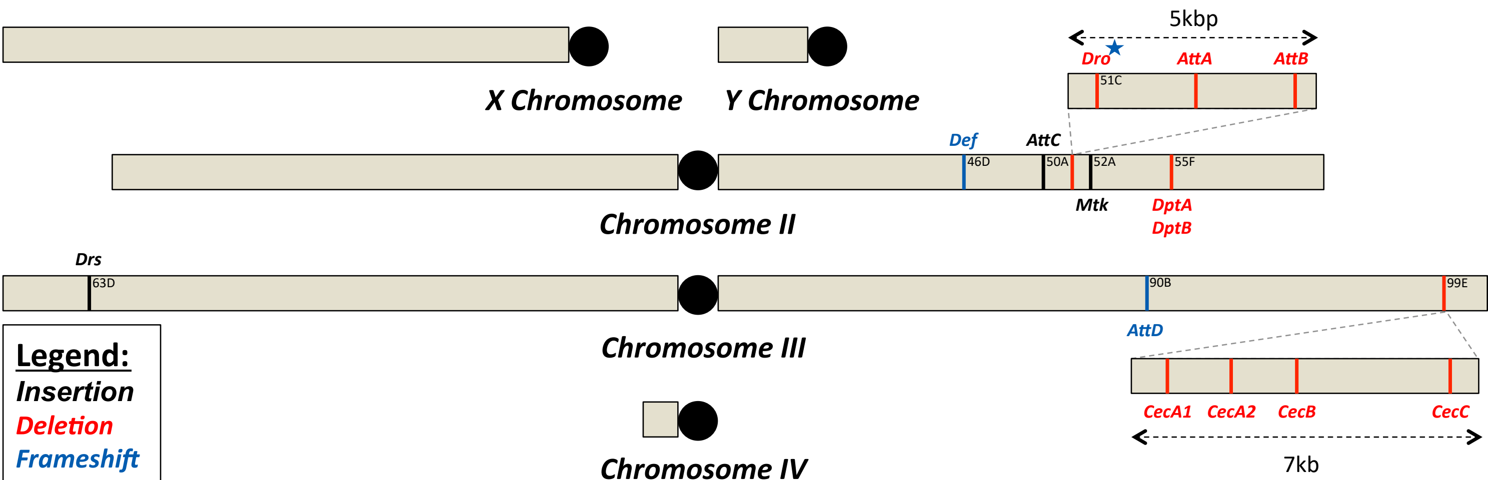
1064 experiments, and CC provided experimental support. MAH and BL analyzed the data
1065 and wrote the manuscript. All authors read and approved the final manuscript.
1066

1067 **Acknowledgements:**

1068 We would like to thank Marc Moniatte for assistance with MALDI-TOF analysis,
1069 Claudia Melcarne for assistance with hemocyte characterization, and Igor Iatsenko
1070 for help in preparation of critical reagents. Brian Lazzaro generously provided
1071 *Providencia* species used in this study. We would like to thank Hannah Westlake for
1072 useful comments on the manuscript. MAH would like to extend special thanks to Jan
1073 Dudzic for many illuminating discussions had over coffee.

Figure 1

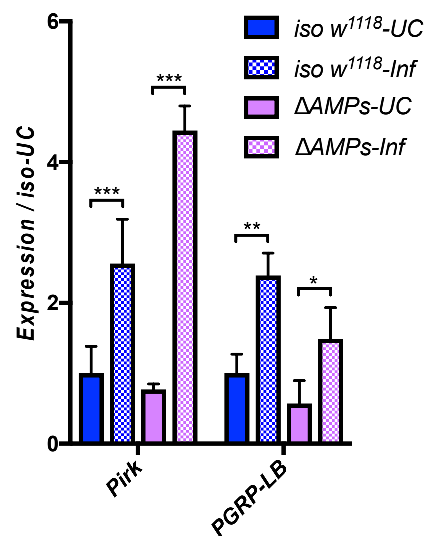
A



B

IM7
Dro-MS
MPAC
Mtk
CecA-C
Def
Drs
IM21
DptA
DptB
iso w^{1118}
IM8
IM16
IMs1-4
IM5
IM6
IM10
IM12
IM13
IM14
IM15
IM18
IM20
IM22
IM23
IM24
ΔAMPs
Present in both iso w^{1118} and ΔAMPs
Absent in ΔAMPs (expected)
Absent in ΔAMPs (unexpected)
Not detected in both iso w^{1118} and ΔAMPs
Signal Intensity
6000
3000
0
3000
6000
1000 2000 3000 4000 5000 6000 7000 8000 9000 10000 11000 12000
m/z

C



D

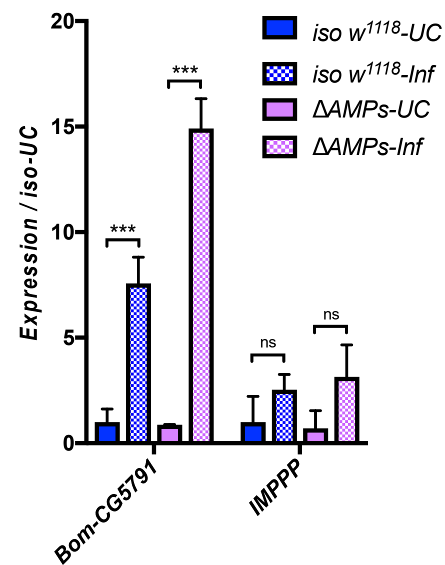
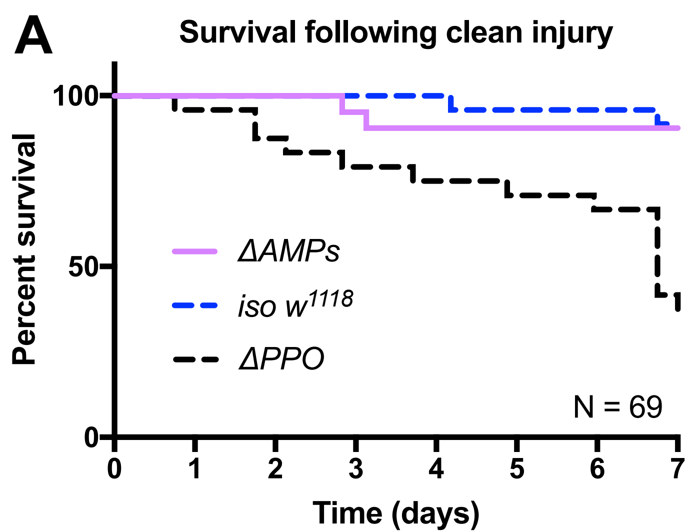
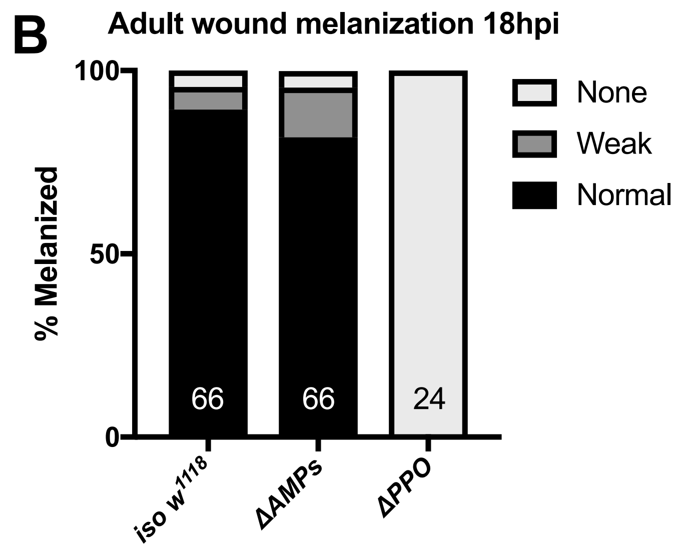
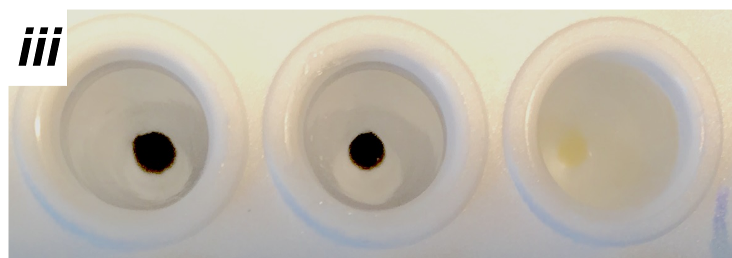
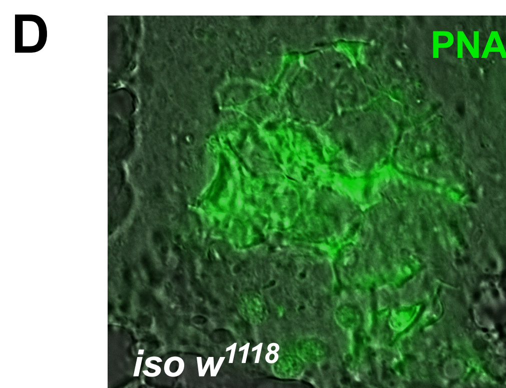


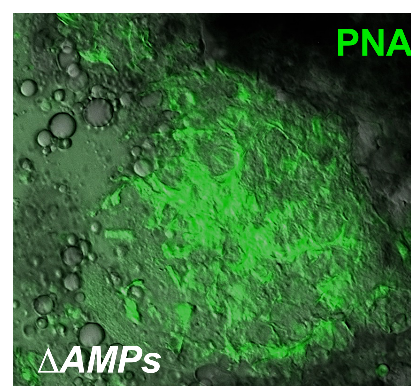
Figure 1 supplement

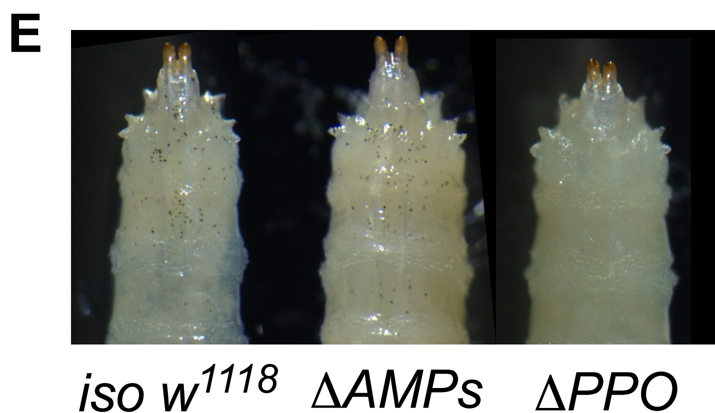












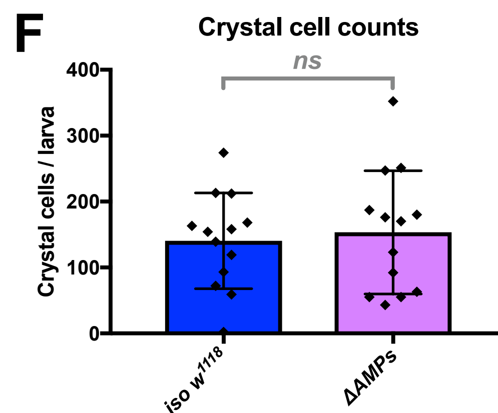
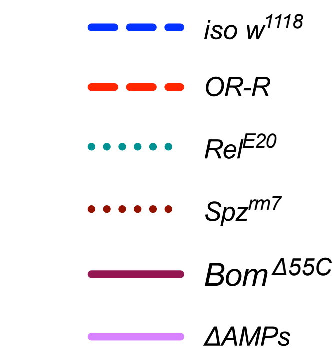
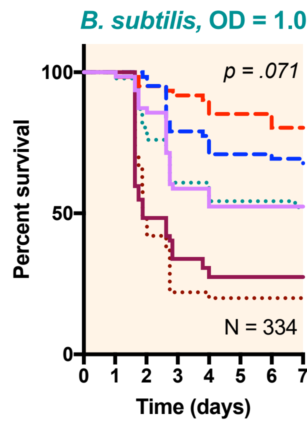
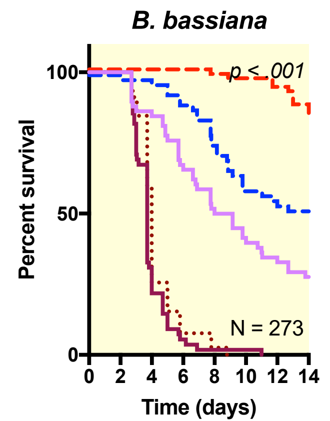
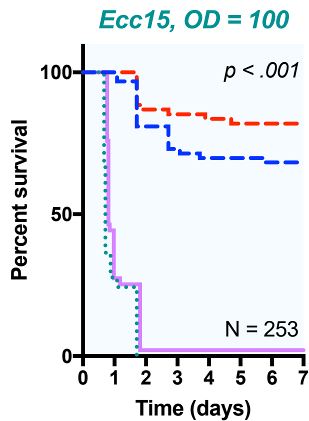


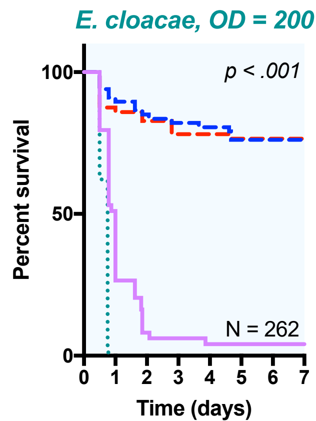
Figure 2

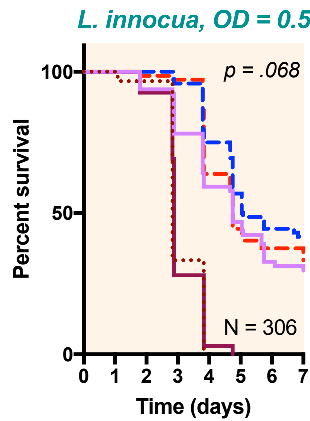


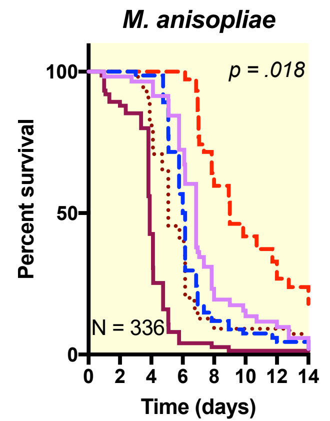


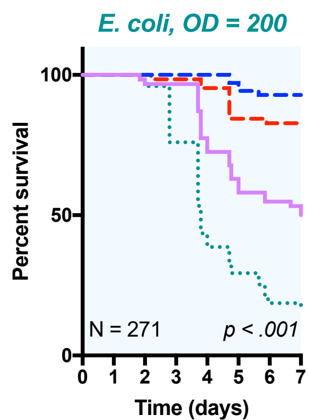


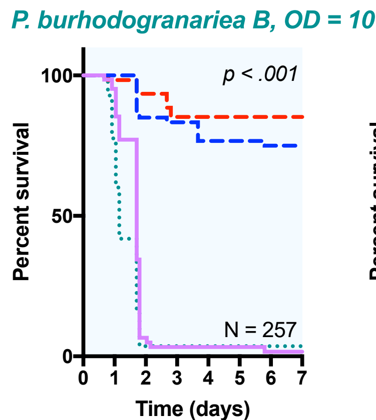


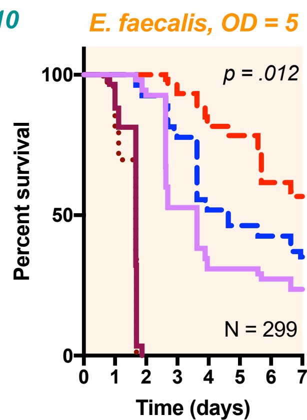


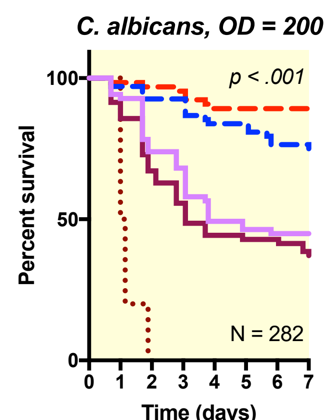


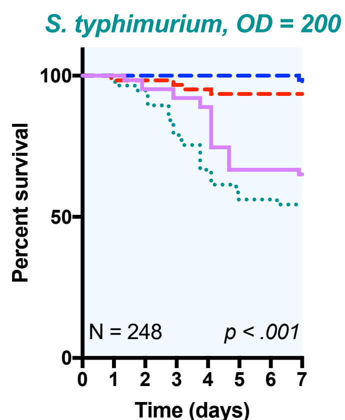


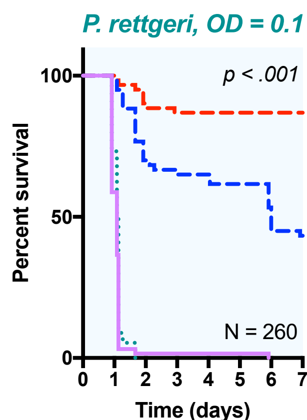


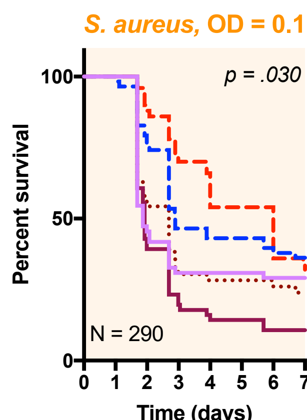












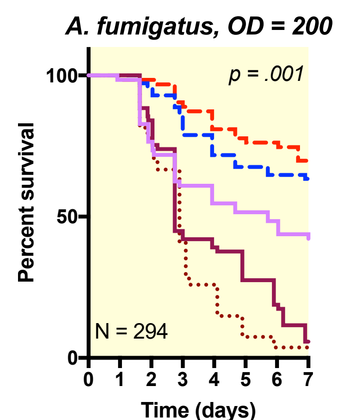
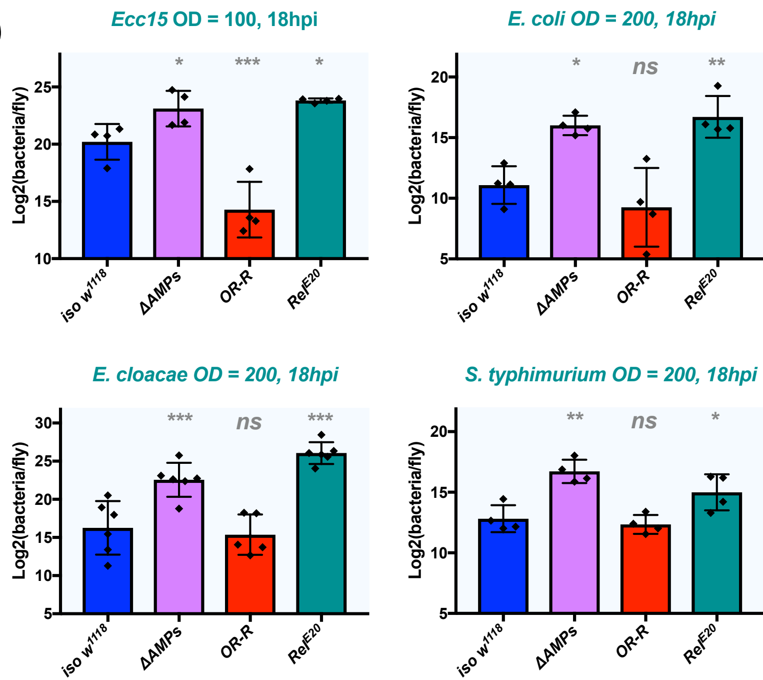
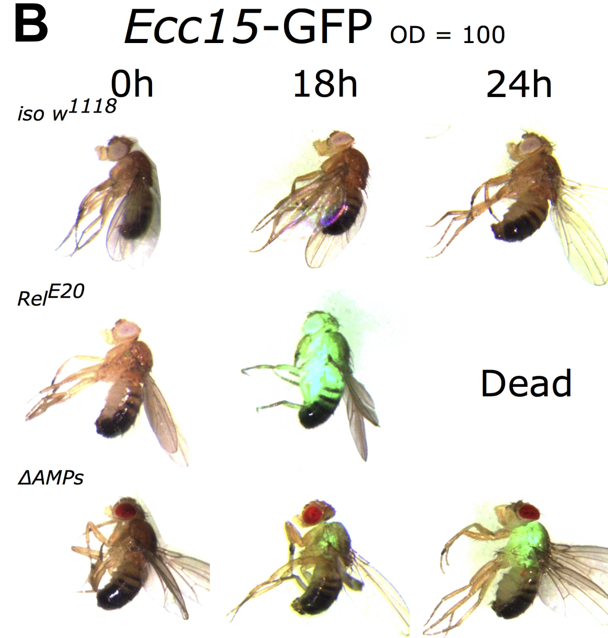
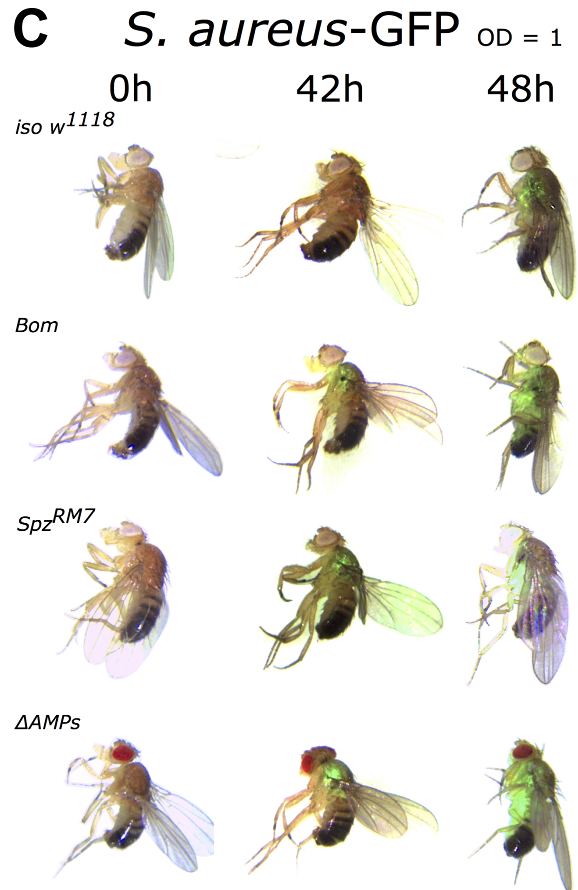


Figure 2 supplement







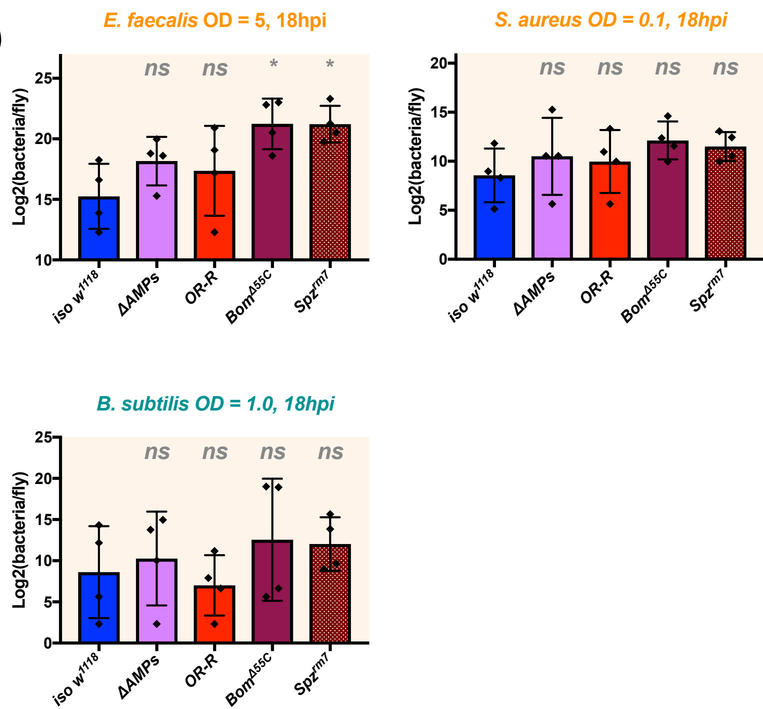
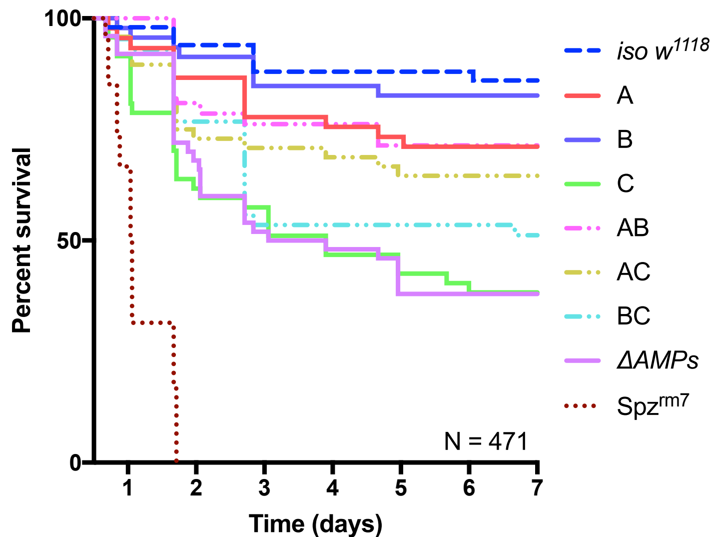
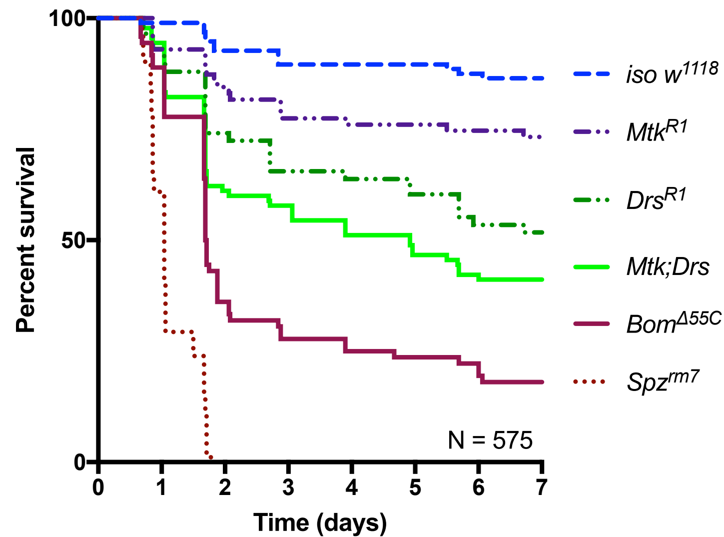
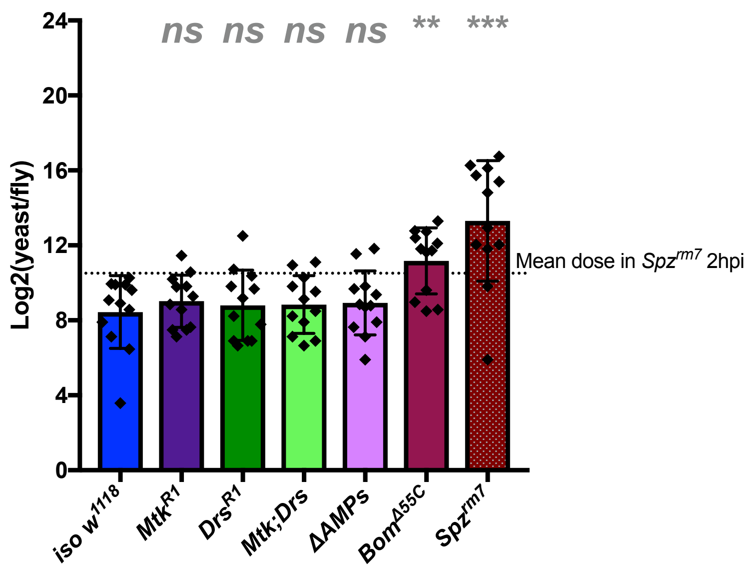


Figure 3: *C. albicans*







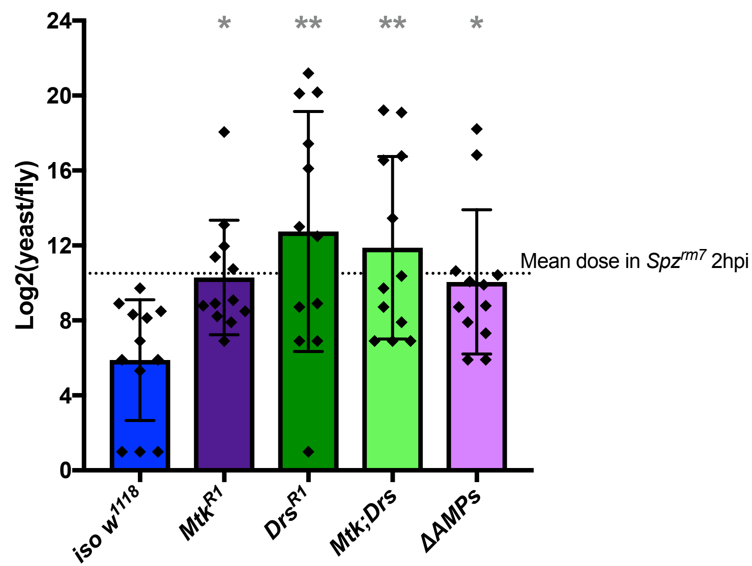
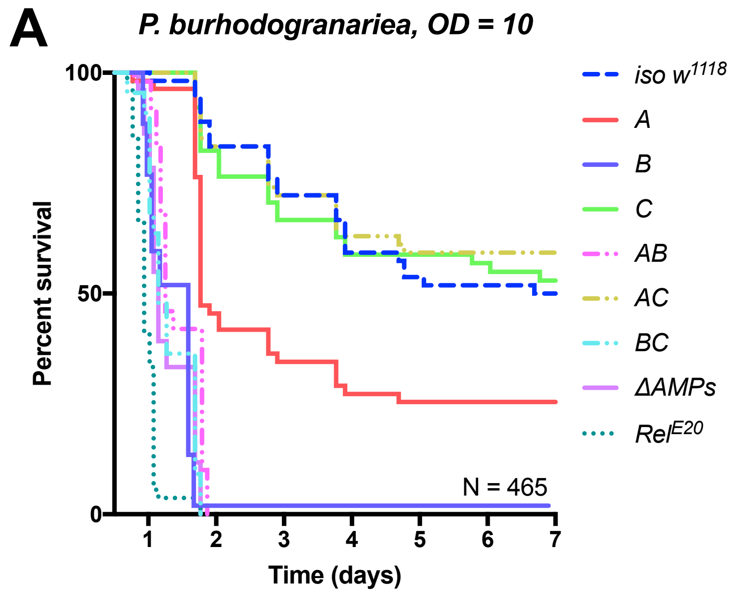
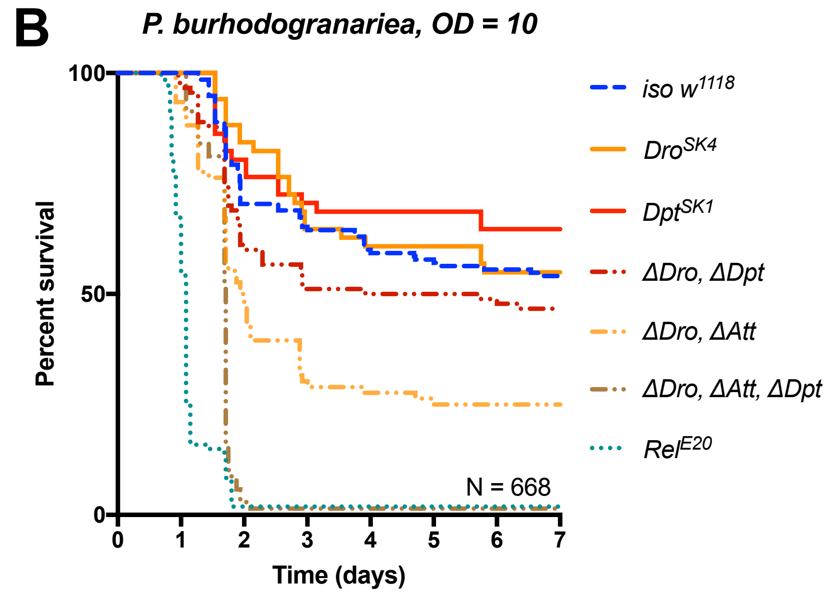
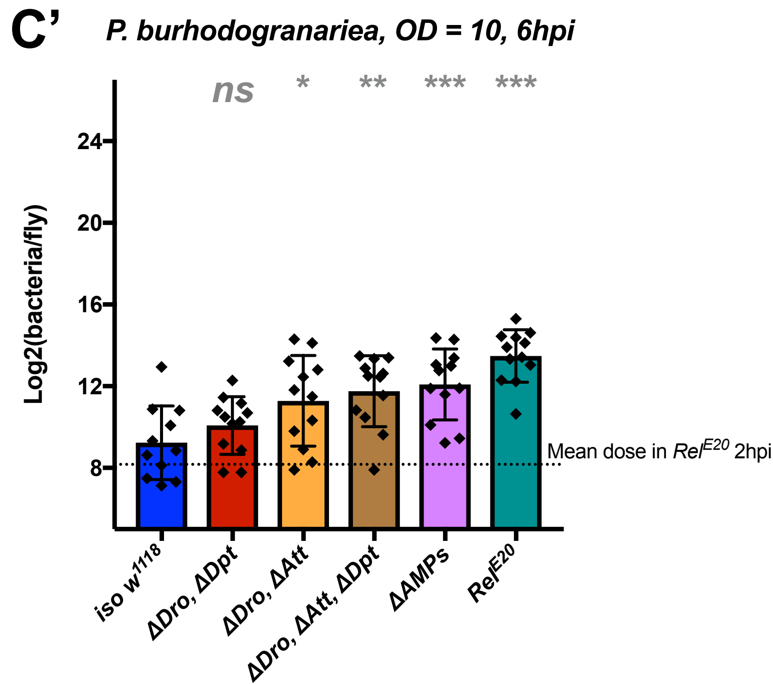


Figure 4: *P. burhodogranariea*







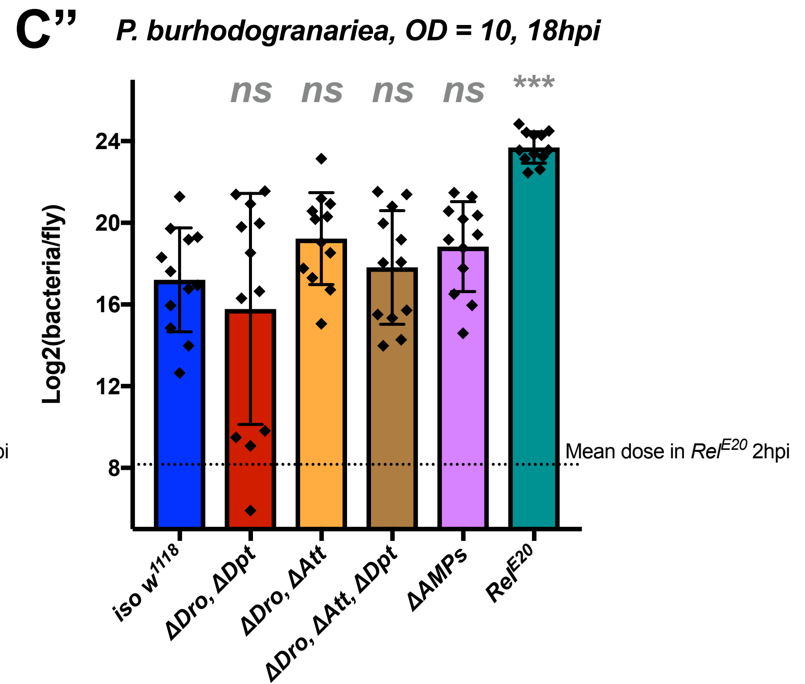
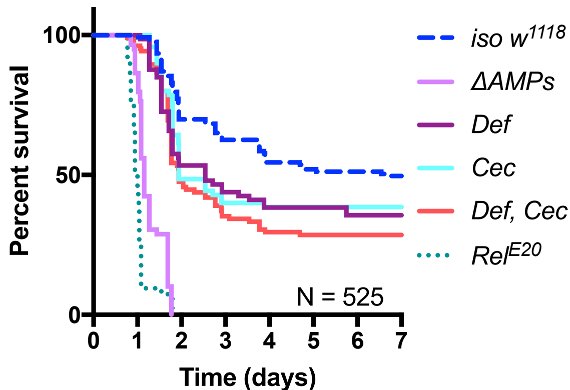


Figure 4 supplement



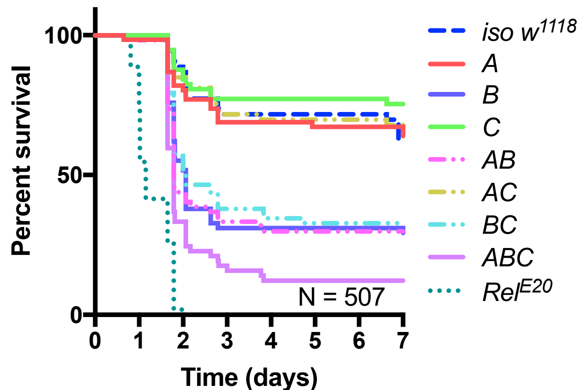
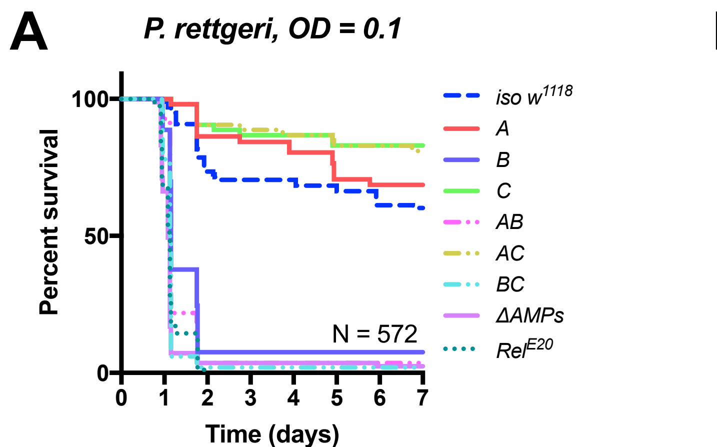
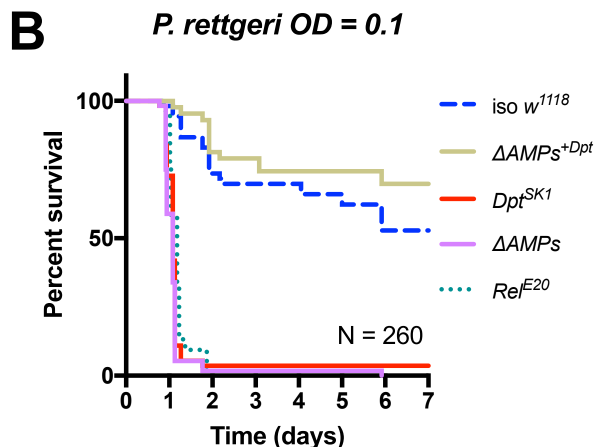
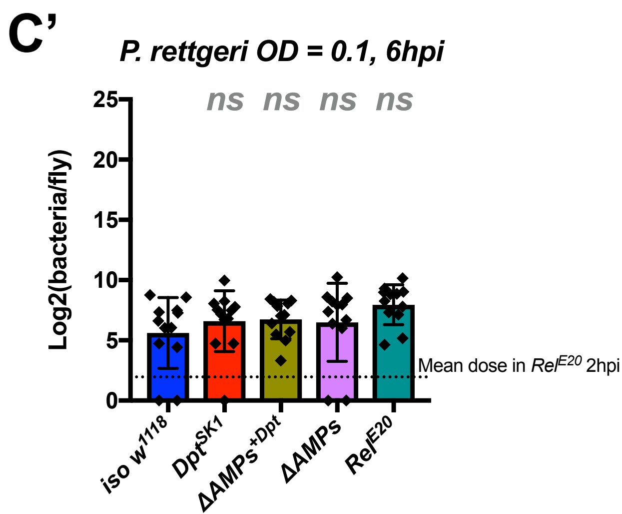
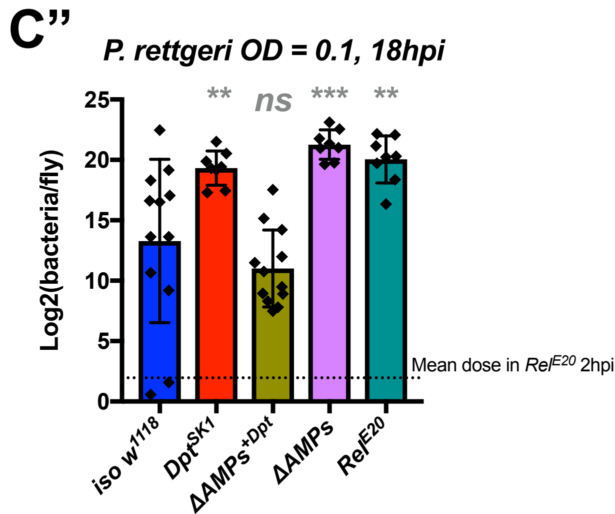


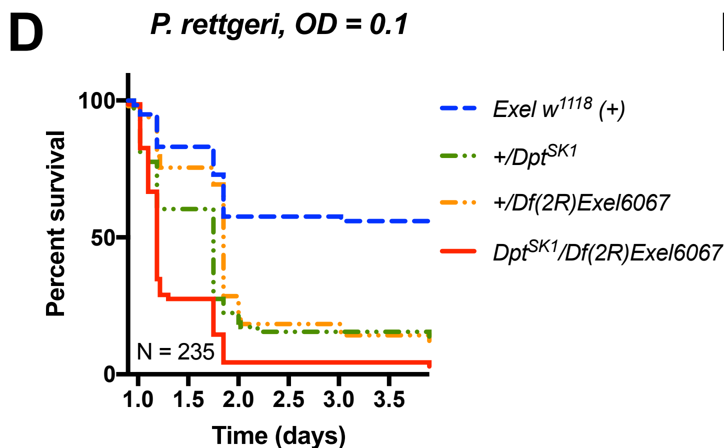
Fig. 5: *P. rettgeri*











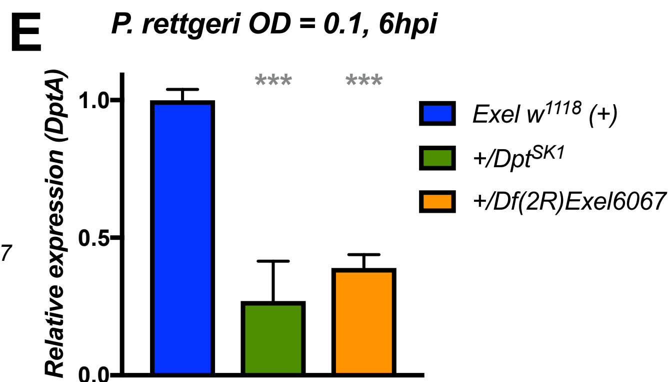
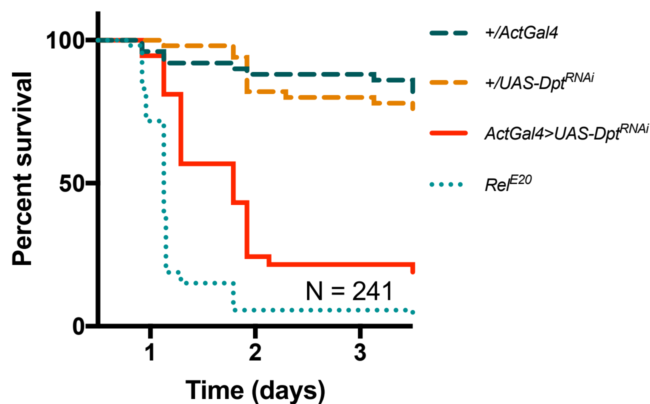
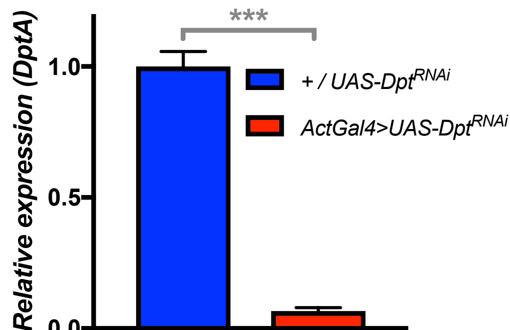


Figure 5 supplement





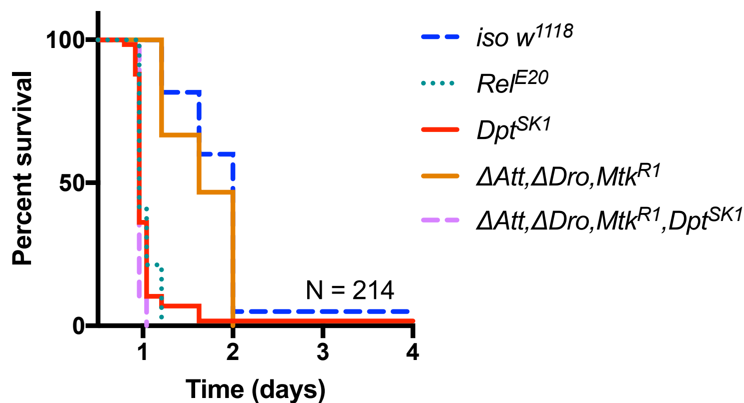
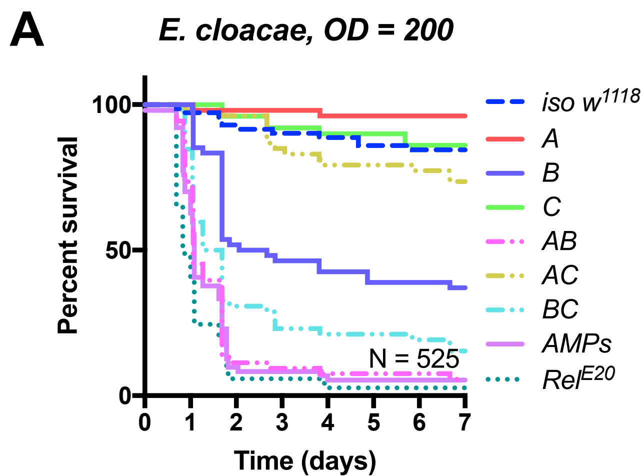
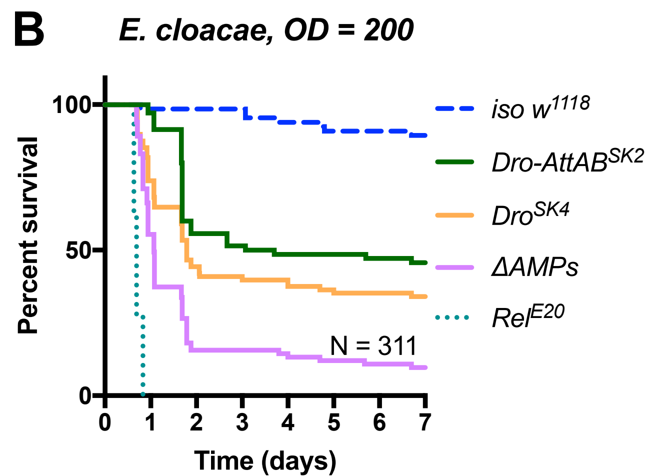
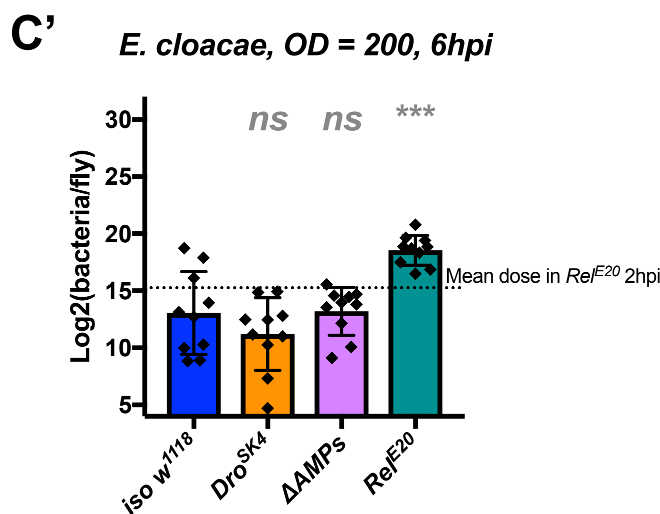
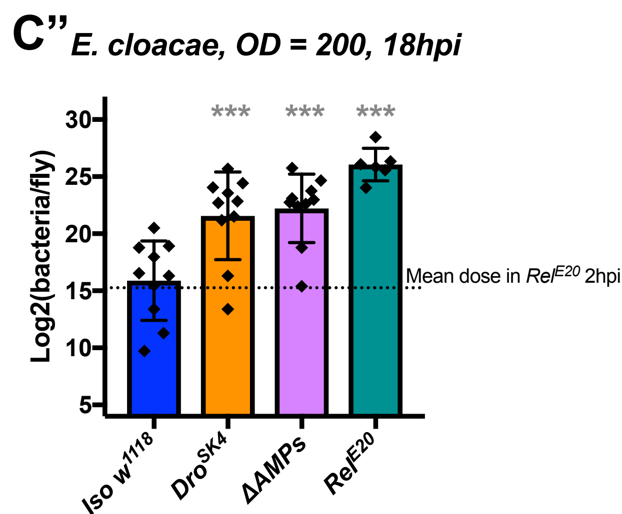


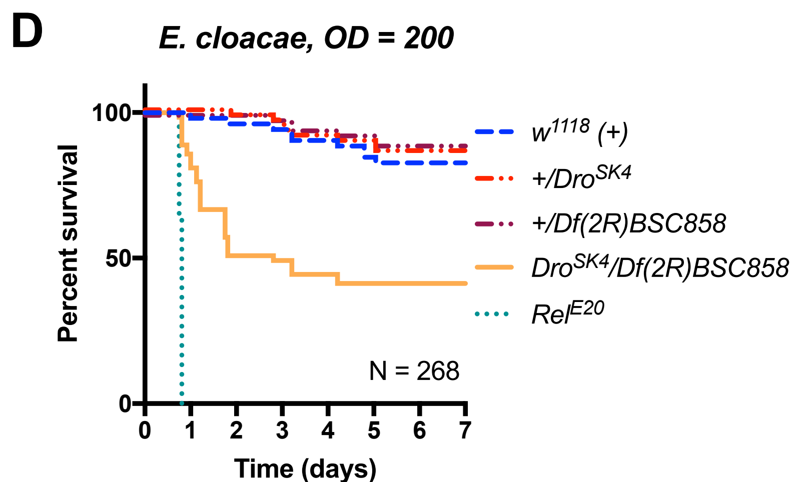
Fig. 6: *E. cloacae*











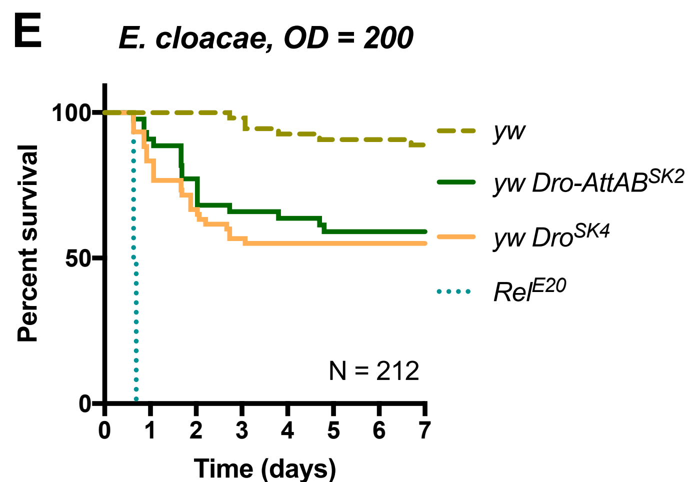
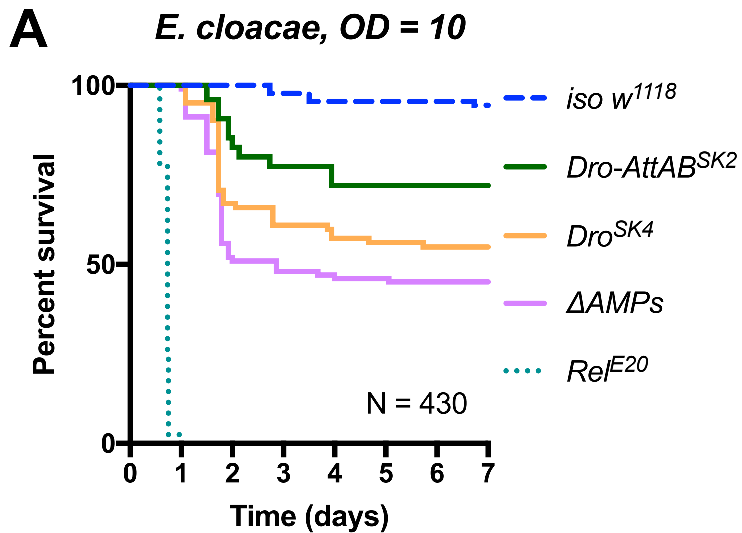
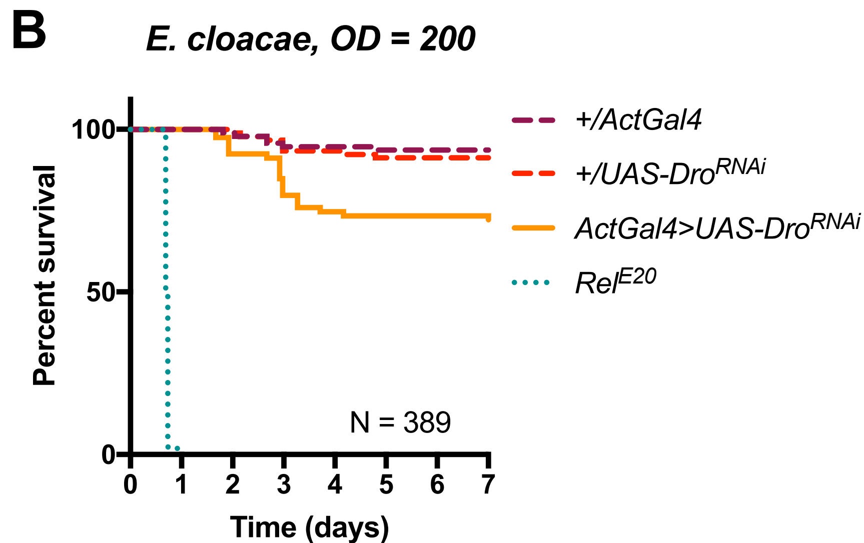


Figure 6 supplement





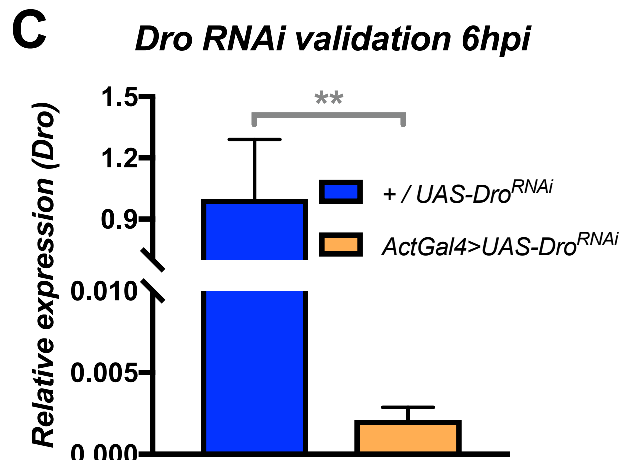
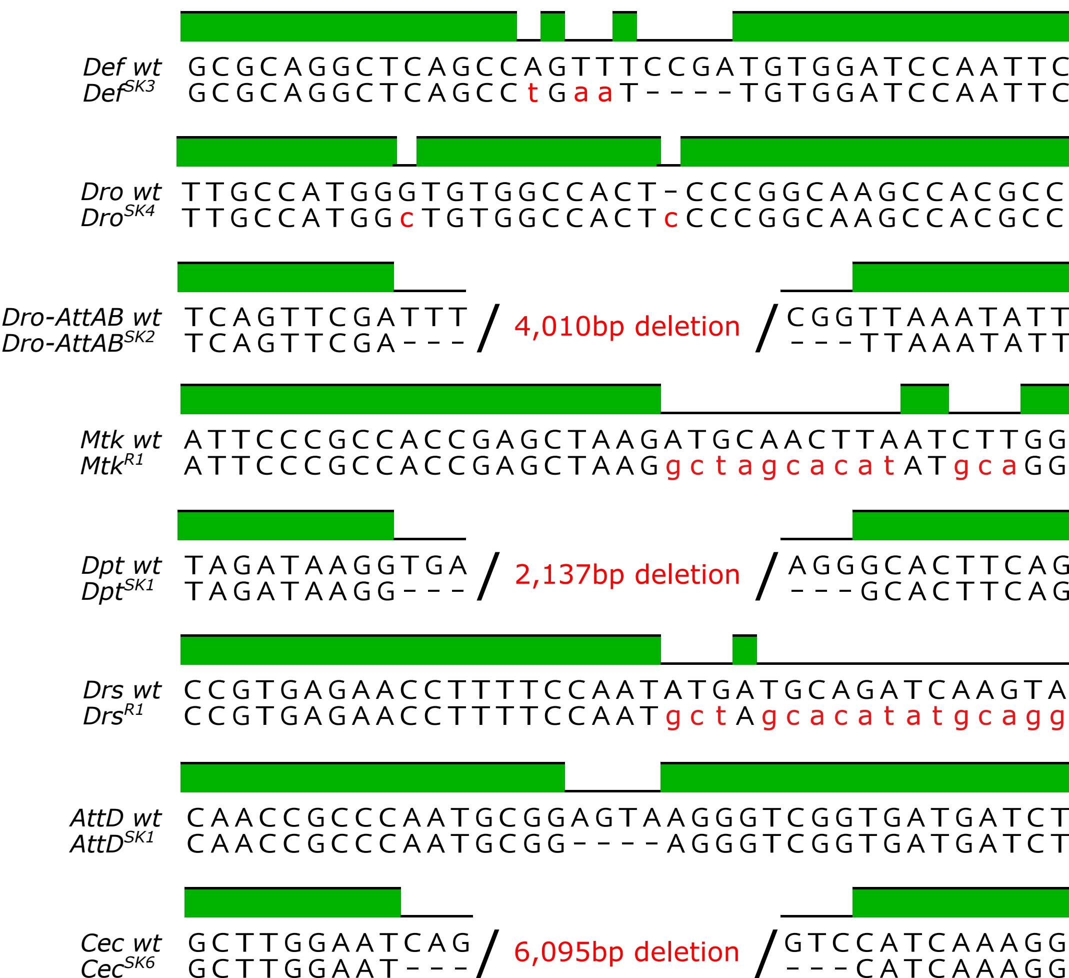


Figure S1



bioRxiv preprint doi: https://doi.org/10.1101/493817; this version posted December 13, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

Table S1

Primers used for tracking mutations

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

Primers used for qPCR

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