Cecropins contribute to *Drosophila* host defence against fungal and Gram-negative bacterial infection

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Cecropins in *Drosophila* innate immunity

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ABSTRACT

Cecropins are small helical secreted peptides with antimicrobial activity that are widely distributed among insects. Genes encoding Cecropins are strongly induced upon infection, pointing to their role in host-defence. In *Drosophila*, four *Cecropin* genes clustered in the genome (*CecA1, CecA2, CecB* and *CecC*) are expressed upon infection downstream of the Toll and Imd pathways. In this study, we generated a short deletion $\Delta Cec^{A\text{-}C}$ removing the whole *Cecropin* locus. Using the $\Delta Cec^{A\text{-}C}$ deficiency alone or in combination with other antimicrobial peptide (AMP) mutations, we addressed the function of Cecropins in the systemic immune response. $\Delta Cec^{A\text{-}C}$ flies were viable and resisted challenge with various microbes as wild-type. However, removing $\Delta Cec^{A\text{-}C}$ in flies already lacking ten other AMP genes revealed a role for Cecropins in defence against Gram-negative bacteria and fungi. Measurements of pathogen loads confirm that Cecropins contribute to the control of certain Gram-negative bacteria, notably *Enterobacter cloacae* and *Providencia heimbachae*. Collectively, our work provides the first genetic demonstration of a role for Cecropins in insect host defence, and confirms their *in vivo* activity primarily against Gram-negative bacteria and fungi. Generation of a fly line (*ΔAMP14*) that lacks fourteen immune inducible AMPs provides a powerful tool to address the function of these immune effectors in host-pathogen interactions and beyond.

INTRODUCTION

In the late nineteen seventies, as immunologists were characterizing the antibody immune response of mammals, pioneering studies revealed that insects could resist infection by fearsome human pathogens despite lacking an adaptive immune system. Eventually a landmark discovery by Hans Boman and colleagues showed that insects

56 produced antimicrobial peptides (AMPs) following infection (Steiner *et al.* 1981),
57 invigorating interest in innate immunity (Ganz *et al.* 1985; Lemaitre 2004). These AMPs
58 differed from other previously identified immune effectors in their small size, cationic
59 charge, and amphipathic structure, which allowed them to directly disrupt the negatively
60 charged membrane of microbes. In contrast to another class of well-known immune
61 effectors, the lysozymes, AMPs lack enzymatic activity and require concentrations into
62 the micromolar range to achieve their microbicidal effects (Imler and Bulet 2005; Seo *et*
63 *al.* 2012; Hanson and Lemaitre 2020). Research has now shown that AMPs are common
64 across the tree of life, with similar molecules contributing to host defence in both plants
65 and animals (Broekaert *et al.* 1995). While they contribute to local defence in barrier
66 epithelia of vertebrates, insect AMPs are most famous for being secreted upon systemic
67 infection from the fat body into the hemolymph, where they reach potent concentrations
68 (Bulet *et al.* 1999). The characterization of a plethora of AMPs with diverse modes of
69 action has enriched our understanding of these immune effectors. However, the
70 functional study of AMPs was limited until recently due to technical challenges in
71 mutating the small AMP genes using traditional genetic approaches. This challenge has
72 now been overcome with the advent of CRISPR/Cas9 technology.

73 Cecropins were the first inducible AMPs to be isolated, found in the hemolymph of
74 infected pupae of the moth *Hyalophora cecropia* (Lepidoptera) (Hultmark *et al.* 1980;
75 Steiner *et al.* 1981). The helical conformation of Cecropins is thought to promote their
76 interaction with negatively charged bacterial membranes, contributing to pore formation
77 and membrane destabilization, and resulting in the bacterial lysis (Steiner *et al.* 1988). *In*
78 *vitro* studies have shown that Cecropins have high efficacy against a large panel of gram-
79 negative bacteria at concentrations below the levels induced in insects upon infection

80 (10μM), as well as against some filamentous fungi (DeLucca *et al.* 1997; Ekengren and
81 Hultmark 1999; Ouyang *et al.* 2015). Heterologous expression of *Cecropin* in transgenic
82 rice has also been shown to confer resistance to the rice blast fungus *Magnaporthe oryzae*
83 (Coca *et al.* 2004). Cecropins *in vitro* do not display antimicrobial activities against Gram-
84 positive bacteria (Smeianov *et al.* 2000). However some studies have reported an activity
85 of Cecropins against tumor cells, bacterial biofilms and viruses (Chiou *et al.* 2002;
86 Suttmann *et al.* 2008; Deslouches and Di 2017; Kalsy *et al.* 2020).

87 AMP regulation and function has been extensively studied in the model insect *Drosophila*
88 *melanogaster*. The *Drosophila* genome encodes four *Cecropin* genes (*CecA1* and *A2, CecB*
89 and *CecC*) and two pseudogenes (*Cec-Ψ1* and *Cec2*) that are clustered at position 99E2 at
90 the tip of the right arm of the third chromosome (Kylsten *et al.* 1990; Samakovlis *et al.*
91 1990; Sackton *et al.* 2007). The *Cecropin* locus is adjacent to another gene named
92 *Andropin,* which encodes a related antibacterial peptide expressed in the ejaculatory duct
93 (Samakovlis *et al.* 1991). CecA1 and CecA2 are identical at the protein level, differing only
94 by a few silent mutations at the nucleotide level, suggesting that they emerged from a
95 recent duplication. The four *Drosophila Cecropin* genes are strongly induced in the fat
96 body and hemocytes upon systemic infection. During systemic infection, *Cecropin* genes
97 are regulated mostly by the Imd pathway while also receiving minor input from the Toll
98 pathway (Lemaitre *et al.* 1996; Hedengren *et al.* 1999). Functional studies analyzing the
99 role of Cecropins *in vivo* are scarce. Overexpression of *CecA* in an otherwise *Imd, Toll*
100 immune-deficient background failed to detect a clear protective effect for *CecA* against a
101 battery of pathogens (Tzou *et al.* 2002). Other studies using overexpression approaches
102 have pointed to a role for *CecA* in the regulation of the gut microbiota (Ryu *et al.* 2008).
103 Transgenic mosquitoes overexpressing both Cecropin and Defensin under the control of

104 the vitellogenin promoter displayed an increased resistance to *Pseudomonas aeruginosa*
105 infection, indicating that these AMPs could act cooperatively against this pathogenic
106 bacterium (Kokoza *et al.* 2010).

107 We have previously generated fly strains with deletions in 10 *Drosophila* AMP genes
108 including: *Defensin*, two *Diptericins* (*DptA* and *B*), *Drosocin*, four *Attacins* (*AttA, B, C,* and
109 *D*), *Metchnikowin*, and *Drosomycin* (Hanson *et al.* 2019a). This study revealed that AMPs
110 play an important role in defence against Gram-negative bacteria and also somewhat in
111 defence against fungi. In contrast, another family of host defence peptides with no overt
112 antimicrobial activity *in vitro*, the Bomanins, plays a major role in the elimination of
113 Gram-positive bacteria and fungi (Clemmons *et al.* 2015; Lindsay *et al.* 2018).
114 Importantly, Hanson et al. (Hanson *et al.* 2019a) revealed evidence for synergy and
115 additivity, but also remarkable specificity in the action of AMPs against certain pathogens.
116 However, this study did not address the function of the four Cecropins due to a failure to
117 generate a proper *Cecropin* locus deletion. In the present study, we have generated fly
118 lines carrying a small deletion that removes the four immune *Cecropin* genes, and by
119 using flies carrying this deletion alone or in combination with other AMP mutations, we
120 address for the first time the role of Cecropins in the systemic immune response.

121

122 **MATERIALS AND METHODS**

123 **Fly stocks and genetics**

124 The w^{1118} DrosDel isogenic (iso w^{1118}) wild type was used as the genetic background for
125 mutant isogenization, as described by Ferreira and colleagues (Ferreira *et al.* 2014). The
126 $\Delta Cec^{A\text{-}C}$ mutation was generated using CRISPR with two gRNAs and a homology directed
127 repair vector by cloning 5' and 3' region-homologous arms into the pHD-DsRed vector,

128 and consequently *ΔCec*$^{A-C}$ flies express DsRed in their eyes, ocelli and abdomen. The
129 *ΔCec*$^{A-C}$ mutation was generated by Cas9 mediated injection into the iso *Mtk*^{R1}*; Drs*^{R1}
130 background. Following this, two rounds of backcrossing were performed to replace the
131 1st and 2nd chromosome with the *iso DrosDel* 1st and 2nd chromosome, and to recombine
132 the *ΔCec*$^{A-C}$ mutation away from other mutations. The resulting stock is here called iso
133 *ΔCec*$^{A-C}$. Afterwards, the *ΔCec*$^{A-C}$ mutation was recombined independently with *Drs*^{R1} and
134 *AttD*^{SK1} on chromosome 3, and introgressed alongside the other AMP mutations on
135 chromosome 2 (Hanson *et al.* 2019a) to generate *ΔAMP14* flies lacking the 14 classical
136 AMP genes from the *Defensin, Drosocin, Attacin, Diptericin, Metchnikowin, Drosomycin*,
137 and *Cecropin* gene families. The iso *Bom*$^{\Delta 55C}$ and iso *Relish*^{E20} flies are the same as used in
138 Hanson et al. (Hanson *et al.* 2019a). Finally, the *ΔAMP10* genotype is described in Hanson
139 *et al.* ; however we removed the aberrant *Cecropin* locus (*Cec*^{SK6}) detected in this line to
140 avoid any potential effects this locus could have on Cecropin-mediated resistance to
141 infection (see Hanson et al. correction notice (Hanson *et al.* 2019b)).
142
143 **Microbial culture conditions**
144 Bacteria were grown overnight on a shaking plate at 200 RPM in their respective growth
145 media and at their optimal temperature conditions. They were then pelleted by
146 centrifugation (4000 RPM) at 4°C. The bacterial pellets were diluted to the desired optical
147 density at 600nm (OD_{600}).
148 *Pectobacterium carotovorum carotovorum 15* (*Ecc15*) and *Micrococcus luteus* were grown
149 in LB media at 29°C. *Escherichia coli* strain 1106, *Providencia burhodogranariea*,
150 *Providencia rettgeri* and *Providencia heimbachae* were grown in LB media at 37°C.
151 *Enterococcus faecalis*, *Listeria monocytogenes* and *Enterobacter cloacae* were cultured in

152 BHI media at 37°C. *Streptococcus pneumoniae* was grown as described by Krejčová and
153 colleagues (Krejčová *et al.* 2019). *Candida albicans* strain ATCC 2001 was cultured in YPG
154 media at 37°C. *Aspergillus fumigatus* was grown at 37°C on Malt Agar; spores were
155 collected in sterile PBS, pelleted by centrifugation and resuspended at the desired OD.
156 *Beauveria bassiana* strain R444 and *Metharizium rileyi* strain PHP1705 commercial
157 spores were produced by Andermatt Biocontrol (products: BB-PROTEC and NOMU-
158 PROTEC).
159

160 **Infection experiments and survival**

161 Systemic infections with *P. carotovorum carotovorum 15* (*Ecc15*) (Basset *et al.* 2000), *M.*
162 *luteus*, *E. coli* strain 1106, *P. burhodogranariea*, *P. rettgeri, P. heimbachae* (Galac and
163 Lazzaro 2011), *E. faecalis*, *L. monocytogenes*, *E. cloacae* and *C. albicans* were performed
164 as followed: 3-5 day old adult females were pricked in the thorax with a 100μm thick
165 needle dipped into a concentrated pellet of bacteria at the desired OD_{600}. Infected flies
166 were then maintained at 25 or 29°C and survivals were tracked. Systemic infection with
167 *S. pneumoniae* (Krejčová *et al.* 2019) or *M. rileyi* was performed by injecting 50nL of a
168 concentrated pellet of bacteria or suspension of fungal spores using a nanoinjector and
169 glass capillary needles.
170 Natural infections with *B. bassiana* were performed by shaking anesthetized flies in a vial
171 with 200mg of spores. Flies were flipped into fresh vials one day after fungal inoculation.
172 A minimum of two replicate survival experiments were performed for each infection with
173 20 flies per vial on standard fly medium without yeast. Survival was scored daily.
174

175 **Bacterial load of flies**

176 Flies were infected (systemic infection) with bacteria at the desired OD_{600}. At the
177 indicated time post-infection, flies were anaesthetized using CO_2, surface sterilized by
178 washing briefly in 70% EtOH, and blotted dry. Pools of 5 flies were transferred in 200μL
179 of sterile PBS and macerated using a pestle. The homogenates were centrifuged at 8,000
180 RPM for 3 minutes. The supernatants were serially diluted and 7μL droplets (6
181 replicates) were inoculated on LB agar overnight at 29°C. Colony-forming units (CFUs)
182 were manually counted the following day.
183
184 **Gene expression levels by RT-qPCR**
185 Flies that either were unchallenged or were infected systemically by pricking in the
186 thorax with a needle dipped in a pellet of *Ecc15* or *M. luteus* (OD_{600}=200) were frozen at
187 -20°C 6h or 12h post-infection, respectively. Gene expression measurements were then
188 performed by RT-qPCR as previously described (Hanson *et al.* 2019a). Briefly, 5 whole
189 flies were homogenized and their RNA was extracted using TRIzol reagent and
190 resuspended in RNase-free water. Reverse transcription was carried out using
191 PrimeScript RT (TAKARA) with random hexamers and oligo dTs. Quantitative PCRs were
192 performed on a LightCycler 480 (Roche) using PowerUp SYBR Green Master Mix.
193
194 **Cecropin A injection**
195 Commercially available *Hyalophora cecropia* Cecropin A (Sigma-Aldrich) was diluted in
196 PBS to a concentration of 50μM. Fifty nL of Cecropin was injected into the thorax using a
197 nanoinjector and glass capillary. Flies were left to recover for 2 hours and then pricked
198 with the desired pathogen.
199
200 **MALDI-TOF**

201 Hemolymph samples were collected from either unchallenged flies, or flies pricked with
202 a 1:1 cocktail of *E. coli* and *M. luteus* (OD=200) in 0.1% TFA, as described previously
203 (Hanson *et al.* 2019a). Samples were then added to an acetonitrile universal matrix and
204 subjected to desorption and ionization. Representative spectra are shown. Immune
205 induced peaks were identified based on previous studies (Uttenweiler-Joseph *et al.* 1998;
206 Levy *et al.* 2004) to confirm the absence of AMP-associated peaks, and presence of
207 immune-induced peptides not affected by the various AMP mutations. Spectra were
208 visualized using mMass and figures were additionally prepared using Inkscape v0.92.
209

210 **Statistical analysis**

211 Survival analyses were performed using a Cox proportional hazards (CoxPH) multiple
212 comparison model, with Benjamini-Hochberg corrections for p-values, in R 3.6.3. Survival
213 curves included at least one survival experiment with 2 cohorts of 20 flies per treatment.
214 Statistics were represented using a Compact Letter Display (CLD) graphical technique:
215 groups were assigned the same letter if they were not significantly different ($p>.05$).
216 Quantitative PCR data were compared by one-way ANOVA with Holm-
217 Šídák multiple test correction in Prism R7. Bacterial load values were transformed as
218 $\log_{10}$(value+1) to allow graphical representation of the absence (0) of CFUs. Bacterial load
219 data were compared by one-way ANOVA with Holm- Šídák multiple test correction in
220 Prism 7. Statistics were represented using a CLD graphical technique.
221

222 **RESULTS**

223 **Generation and characterization of *Cecropin* mutants**

224 We generated a fly line lacking the four *Cecropin* genes, *CecA1, CecA2, CecB*, and *CecC,*
225 which are clustered at 99E and are inducible during the systemic response. For this, we
226 used the CRISPR/Cas9 editing method to generate a 6kb deletion (referred as $\Delta Cec^{A\text{-}C}$)
227 that removes the four inducible *Cecropins* but leaves the neighboring *Andropin* gene
228 intact (Fig. 1A). The $\Delta Cec^{A\text{-}C}$ mutation was generated by Cas9 mediated injection in the *w,*
229 *Drosdel* (referred to as w^{1118}) background. The background of the $\Delta Cec^{A\text{-}C}$ mutation was
230 then cleaned by 2 successive crosses to the w^{1118} *iso* background to remove potential off-
231 target alterations. To confirm the absence of *Cecropin* genes in $\Delta Cec^{A\text{-}C}$ flies, we performed
232 RT-qPCR of *CecA*1 and *CecC* genes, which are located at the two extremities of the
233 *Cecropin* locus. Expression of *CecA1* or *CecC*, readily observed in the wild type, was not
234 detected in $\Delta Cec^{A\text{-}C}$ flies (Fig. 1B-C).

235 We previously generated a fly line in the w^{1118} *iso* background here referred to as
236 "*ΔAMP10*", harbouring six mutations that remove ten antimicrobial peptide genes:
237 *Defensin*, *Metchnikowin*, the four Attacins (*A/B/C/D*), *Drosomycin*, two Diptericins (*A/B*),
238 and *Drosocin* (Hanson et al., 2019). We recombined the iso $\Delta Cec^{A\text{-}C}$ mutation with the iso
239 *ΔAMP10* mutations to generate an iso fly line lacking all 14 'classical' antimicrobial
240 peptides (referred to as "*ΔAMP14"*). MALDI-TOF and RT-qPCR analysis confirms the
241 absence of these 14 antimicrobial peptides in *ΔAMP10* and *ΔAMP14* flies (Fig. 1B-C and
242 Fig. S1). The $\Delta Cec^{A\text{-}C}$, *ΔAMP10* and *ΔAMP14* flies were healthy and showed no
243 morphological defects. We also confirmed that the two central NF-κB signaling pathways,
244 Toll and Imd, were functional, as demonstrated by measuring expression of genes
245 characteristic of each of these pathways (Fig. 1D-F). Furthermore, MALDI-TOF proteomic

246 analysis of hemolymph from infected flies 24 hours post infection (hpi) reveals a wild
247 type-like induction of peaks associated with other NF-κB effectors (e.g., Bomanins,
248 Daishos, and Baramicin A) (Fig. S1). Collectively, our study indicates that we have
249 generated a fly line lacking all the Drosophila 'classical' AMPs, and that deleting these
250 AMPs does not impact the production of other NF-κB effectors.
251

252 **Cecropins contribute to survival against certain Gram-negative bacterial**
253 **infections**

254 We used wild-type, *ΔCec*$^{A-C}$, *ΔAMP10* and *ΔAMP14* flies to explore the role that Cecropins
255 play in defence against pathogens during systemic infection. By performing survival
256 analyses with wild-type and *ΔCec*$^{A-C}$ flies, we assessed if the absence of the four Cecropins
257 is sufficient to cause an immune deficiency. Likewise, any difference in survival rates
258 between *ΔAMP10* and *ΔAMP14* flies would suggest a contribution of Cecropins that is only
259 apparent in the absence of other AMPs. We first focused our attention on Gram-negative
260 bacterial infections, as cecropins were initially identified for their activity against this
261 class of bacteria, and the four *Cecropin* genes are mostly regulated by the Imd pathway
262 that responds to Gram-negative bacterial infection (Lemaitre and Hoffmann 2007). We
263 challenged wild-type, *ΔCec*$^{A-C}$, *ΔAMP10* and *ΔAMP14* flies with six different Gram-negative
264 bacterial species, using inoculation doses (given as OD_{600}) selected such that Imd
265 deficient, iso *Rel*^{E20} mutant control flies were killed. Our survival experiments did not
266 reveal an overt contribution of Cecropins to resistance against the Gram-negative
267 bacteria *Escherichia coli, Pectobacterium carotovorum carotovorum (Ecc15)*, *Providencia*
268 *rettgeri* or *Providencia burhodogranariea* (Fig 2A-D). In all cases, *ΔCec*$^{A-C}$ flies survived as
269 well as wild-type flies, while *ΔAMP10* flies were as susceptible as *ΔAMP14*. One exception

270 was found for *P. burhodogranariea* infection: death of *ΔAMP10* flies was delayed by one
271 day compared to *ΔAMP14* flies, suggesting a contribution of Cecropins in combatting this
272 bacterium early in infection.
273 Interestingly, we did identify a prominent role for Cecropins against two Gram-negative
274 bacterial strains: *Enterobacter cloacae and Providencia heimbachae*. Although ΔCec^{A-C} flies
275 survived *E. cloacae* infection like wild-type flies and *ΔAMP10* flies had only a slight
276 reduction in survival, *ΔAMP14* flies infected with *E. cloacae* instead behaved like Rel^{E20}
277 mutants lacking Imd signaling entirely (Fig. 3A). This result suggests that the presence of
278 the four *Cecropin* genes confers a mild protective effect against this bacterium in flies that
279 lack ten other AMP genes. A significant difference in CFUs between *ΔAMP10* and *ΔAMP14*
280 flies at 8h post-infection (hpi) confirmed a role of Cecropins in limiting the growth of *E.*
281 *cloacae* (Fig. 3B). We also observed a consistently higher bacterial load in ΔCec^{A-C} flies
282 compared to wild-type controls, though this was not significant ($p = .18$). Moreover,
283 *ΔAMP14* and Rel^{E20} fly CFUs were similar, consistent with survival data showing complete
284 mortality of these genotypes within 24 hours. These results indicate that the knock-out
285 of the 'classical' AMPs in the *ΔAMP14* line fully explains the susceptibility of Imd pathway
286 mutants to *E. cloacae* infection. Next, we attempted to rescue the susceptibility of *ΔAMP10*
287 and *ΔAMP14* flies using commercially available Cecropin A. We injected 50 nL of 50μM
288 *Hyalophora cecropia* Cecropin A (Sigma-Aldrich) or PBS (control) two hours before
289 challenging flies with *E. cloacae*. Interestingly, when we injected Cecropin A two hours
290 prior to *E. cloacae* infection, *ΔAMP10* flies survived as well as wild-type flies previously
291 injected with PBS (Fig. 3C). This result suggests that priming the fly defence by increasing
292 circulating levels of Cecropin is sufficient to combat *E. cloacae* infection, even in flies
293 lacking a broad range of other AMPs. However we did not succeed in rescuing the

294 susceptibility of *ΔAMP14* flies using the same approach. This suggests the rescue effect
295 we observed using *ΔAMP10* flies relies on the total Cecropin levels, which includes both
296 endogenously produced Cecropin and the supplemental Cecropin A we injected.
297 Collectively, our *in vivo* analysis is consistent with previous *in vitro* studies that showed
298 commercial Cecropin A from *Hyalophora cecropia* has activity against *E. cloacae* (Brey
299 and Ashida 1993).
300 Similarly, we observed a contribution of Cecropins against the Gram-negative bacterium
301 *P. heimbachae* in flies lacking other AMPs (fig. 3D-F). While *ΔAMP10* flies were able to
302 survive this infection as wild type at OD_{600}=50, *ΔAMP14* flies again behaved like Rel^{E20}
303 mutants and suffered complete mortality (Fig. 3D); the ΔCec^{A-C} mutation alone did not
304 increase susceptibility. Bacterial load measurement performed on flies collected 24 hpi
305 revealed a contribution of Cecropins both in the presence and absence of other AMPs (Fig.
306 3E). We again injected commercial *H. cecropia* Cecropin A in an attempt to rescue the
307 susceptibility of *ΔAMP10* and *ΔAMP14* flies to *P. heimbachae* (Fig. 3F). Using this bacterial
308 infection model, previous injection of PBS increased the susceptibility of wild-type flies
309 to *P. heimbachae*. Strikingly, however, injection of Cecropin A prior to infection rescued
310 survival of *ΔAMP10* flies to a level comparable to uninjured wild-type flies. Moreover,
311 injection of *H. cecropia* Cecropin A improved the survival of *ΔAMP14* flies against *P.*
312 *heimbachae*, extending *ΔAMP14* lifespan compared to PBS controls.
313 In summary, our results reveal that Cecropins contribute to *Drosophila* host defence
314 against a subset of Gram-negative bacteria, and that this contribution is more readily
315 apparent when other AMPs are also lacking.
316

317 **Cecropins are not involved in the resistance to Gram-positive bacteria**

318 Previous work with the *ΔAMP10* flies did not reveal a role of *Drosophila* AMPs against
319 Gram-positive bacteria, indicating instead that other immune effectors – notably the
320 bomanins – play a predominant role against this class of microbes (Hanson *et al.* 2019a;
321 Lin *et al.* 2020). Therefore, we were curious if the added loss of Cecropins would reveal a
322 cryptic contribution of *Drosophila* AMPs to defence against Gram-positive bacteria. For
323 this, we challenged wild-type, ΔCec^{A-C}, *ΔAMP10* and *ΔAMP14* flies with three Gram-
324 positive bacteria: *E. faecalis*, *S. pneumoniae* and *L. monocytogenes* (Fig. 4A-C). *E. faecalis*
325 and *S. pneumoniae* contain Lysine-type peptidoglycan that is known to predominantly
326 activate the Toll pathway while *L. monocytogenes* has DAP-type peptidoglycan, and is
327 known to activate both the Toll and Imd pathways (Leulier *et al.* 2003). In these
328 experiments, we included iso $Bom^{\Delta 55C}$ control flies, which lack ten *Bomanin* genes and are
329 known to be susceptible to Gram-positive bacterial and fungal infections (Clemmons *et*
330 *al.* 2015). Our survival experiments did not reveal a major role of Cecropins individually
331 or alongside other AMPs in combating these Gram-positive bacterial species, but
332 confirmed the importance of bomanins (Fig. 4A-C).

333

334 **Cecropins can contribute to antifungal defence**

335 While Cecropins were initially identified as antibacterial peptides, further *in vitro* studies
336 have suggested an antifungal activity as well (Ekengren and Hultmark 1999; Andrä *et al.*
337 2001). We therefore investigated the contribution of Cecropins to resistance upon septic
338 injury with any of four fungal species: the entomopathogenic fungi *Metarhizium rileyi* and
339 *Beauvaria bassiana* R444, the opportunistic mould *Aspergillus fumigatus,* and the yeast
340 *Candida albicans*. Survival analysis did not reveal a prominent role for AMPs or Cecropins
341 against *M. rileyi*, *A. fumigatus* or *C. albicans,* as ΔCec^{A-C}, *ΔAMP10* and *ΔAMP14* flies

342 generally survived as the wild-type (Fig. 5A-C). However, *ΔAMP14* flies suffered greater
343 mortality to *B. bassiana* using a natural infection mode, compared to wild type flies (Fig.
344 5D). We additionally introduced *B. bassiana* spores directly into the hemolymph by septic
345 injury for more controlled fungal infection kinetics, and measured fungal load at 48h hpi
346 by RT-qPCR. Monitoring pathogen load revealed that in *ΔAMP14* flies, *B. bassiana* loads
347 were higher than levels found in wild type (p=.07) and *ΔAMP10* flies (Fig. 5E). Taken
348 together, these results show a contribution of Cecropins to defence against the
349 entomopathogenic fungus *B. bassiana*.

350

351

352 **DISCUSSION**

353

354 In this study, we generated flies lacking the four-immune inducible *Cecropin* genes to
355 address their function alone or in combination with other *AMP* gene mutations. $\Delta Cec^{A\text{-}C}$
356 and *ΔAMP14* flies were viable, fertile and did not show any morphological defect.
357 Moreover, they display normal activation of the Imd and Toll pathways, suggesting that
358 the classical *Drosophila* AMPs do not contribute to immune signaling, in contrast to
359 mammalian AMPs (Mookherjee *et al.* 2020).

360 Our survival analyses reveal a role of Cecropins in the defence against certain Gram-
361 negative bacterial species. However, we could not identify a bacterial species or context
362 for which flies mutant for *Cecropin* genes alone succumb faster than wild-type. Studies of
363 other AMPs have revealed that certain AMPs exhibit a high degree of specificity in
364 determining host-pathogen interactions, as illustrated by the requirement of *Diptericin*
365 in defence against *P. rettgeri*, *Drosocin* in defence against *E. cloacae*, and the recently-
366 described *Daisho* and *Baramicin A* genes in defence against *Fusarium oxysporum* and

367 *Beauveria bassiana* fungi, respectively (Unckless *et al.* 2016; Hanson *et al.* 2019a, 2021;
368 Cohen *et al.* 2020). Further studies may reveal bacteria for which the presence of
369 Cecropins is essential for survival.

370 The most striking phenotype in the present study was that loss of Cecropins has a
371 marked effect on *E. cloacae* and *P. heimbachae* infection in flies also lacking other AMP
372 genes. As such, we reveal an important but cryptic contribution of Cecropins in defence
373 against these bacteria. Generation of flies lacking refined subsets of AMPs might narrow
374 down the specific groups of peptides key to defence against *E. cloacae* and *P. heimbachae*.
375 The enhanced growth of *E. cloacae* in AMP mutants that also lack Cecropins is a
376 particularly striking demonstration of their importance. In this infection model, the
377 presence of Cecropins dictates whether AMP mutant flies initially suppress bacterial
378 growth, or phenocopy Rel^{E20} flies deficient for Imd signaling. Cecropins are induced with
379 faster kinetics than most other AMPs, with a peak expression as early as 3hpi (Lemaitre
380 *et al.* 1997; De Gregorio *et al.* 2002). As *Cecropins* encode simple helical peptides that do
381 not require extensive post-translational modification, it is tempting to speculate that they
382 become functional more rapidly, and play an important role in combatting bacteria
383 specifically at this early phase of infection.

384 Our study also reveals that endogenous Cecropins can play a role in defence against
385 fungi, but not against Gram-positive bacteria. Thus, our *in vivo* study corroborates the
386 antifungal and antibacterial activities of Cecropins previously observed with *in vitro*
387 studies (Samakovlis *et al.* 1990; DeLucca *et al.* 1997; Ekengren and Hultmark 1999).
388 While Imd is crucial for the expression of the four Cecropins, the Cecropin response to
389 infection relies in part on Toll signaling (De Gregorio *et al.* 2002). As such, the
390 contribution of Cecropins to defence against fungi could help explain the regulation of the
391 *Cecropin* locus by both Toll and Imd pathways.

392 The observations that AMP genes are induced to an incredible extent, reach high
393 peptide concentrations in the hemolymph, and display *in vitro* microbicidal activity are
394 all consistent with their role as immune effectors. Use of both *ΔAMP10* and *ΔAMP14* flies
395 has confirmed the important contribution of AMPs to host defence against Gram-negative
396 bacteria and fungi, but not against Gram-positive bacteria. *Drosophila* AMPs also regulate
397 the gut microbiota downstream of the Imd pathway, a function consistent with their
398 bactericidal activity (Marra *et al.* 2021). However, recent studies have suggested that
399 AMP-like genes may play more subtle roles in other processes like memory formation
400 (Barajas-Azpeleta *et al.* 2018), an erect wing response upon infection (Hanson *et al.*
401 2021), tumor control (Parvy *et al.* 2019; Araki *et al.* 2019), or regulation of JNK signaling
402 in the salivary gland (Krautz *et al.* 2020). While we confirm a primary importance for
403 Cecropins and other AMPs in the systemic immune response, exploring the functions of
404 AMPs including Cecropins in non-canonical roles is an exciting future direction of
405 research.

406 Our study and others contribute to the rapid progress made towards understanding
407 the roles of *Drosophila* immune effectors. Research on the effector response has
408 stagnated for over a decade, but recent functional characterizations by loss of function of
409 key effectors (*Cecropins, Defensin, Attacins, Diptericins, Drosocin, Drosomycin,*
410 *Metchnikowin, Bomanins, Daishos,* and *Baramicin*) has greatly advanced our
411 understanding of the roles of these effectors (Lindsay *et al.* 2018; Hanson *et al.* 2019a;
412 Cohen *et al.* 2020; Huang *et al.* 2020). Most importantly, these studies correct the
413 assumptions of the previous "cocktail" model for AMP-pathogen interactions (Yan and
414 Hancock 2001; Lazzaro 2008; Zdybicka-Barabas *et al.* 2012; Rahnamaeian *et al.* 2016),
415 revealing some AMPs to be general effectors against most pathogens, while others act as
416 "silver bullets" specifically required for defence against certain pathogens. The

417 susceptibility of Toll and Imd pathway mutants to specific pathogens can now be directly
418 linked to the susceptibility of mutants for immune effectors regulated by these pathways
419 (Hanson and Lemaitre 2020). As new genetic techniques allow greater characterization
420 of the roles of known immune effectors, many of them yet remain to be characterized,
421 notably a number of short peptide genes highlighted by old and recent transcriptomic
422 studies (Gregorio *et al.* 2001; Troha *et al.* 2018; Tattikota *et al.* 2020; Schlamp *et al.* 2021).
423 However, we are likely still exploring inside the box when assuming a unique immune
424 role for these peptides.

425 Our study also highlights the power of multiple mutation analysis, as the role of
426 C*ecropins* would not have been uncovered *in vivo* by mutating individual genes. While we
427 have begun exploring the combinatorial potential of AMPs in defence against infection,
428 future studies will benefit from probing the interaction of immune effectors like AMPs
429 with other mechanisms of host defence such as phagocytosis or melanization. With the
430 advent of CRISPR/Cas9 technology and many recently described mutants, the
431 interactions of AMPs in defence are just the tip of the iceberg in developing a global
432 framework to understand the *Drosophila* immune response.

433

434

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441

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640

641 # Figure legends

642

643 **Figure 1 : Description and validation of *ΔCecA-C*, *ΔAMP10* and *ΔAMP14* mutants.**
644 (**A**) Schema of the *Cecropin* locus chromosomal deletion removing *CecA1* and *A2, CecB* and
645 *CecC,* plus 2 pseudo genes, *Cec2* and *Cec- Ψ 1* clustered at position 99E2 (III) (**B,C**) RT-
646 qPCR of *CecA* (B) and *CecC* (C) expression in w^{1118}, $\Delta Cec^{A\text{-}C}$, *ΔAMP10* and *ΔAMP14* flies 6
647 hours post *Ecc15* infection. (**D-F**) The Imd (D-E) and Toll (F) pathways are functional in

648 *ΔCec^{A-C}*, *ΔAMP10* and *ΔAMP14* flies after challenge as revealed by expression of target
649 genes upon septic injury with *Ecc15* or *M. luteus. PGRP-LB* and *Pirk* were used as readouts
650 for Imd pathway and *Bomanin (BomBc3)* for Toll pathway. Expression was normalized
651 with *w^{1118}* UC to a value of 1. Statistical significance levels were represented as: * = P≤0.05
652 ; ** = P≤0.01 ; *** = P≤0.001 ; **** = P≤0.0001.
653
654
655 **Figure 2 : Cecropins are not involved in resistance to a broad spectrum of Gram-**
656 **negative bacteria.**
657 *w^{1118}* were used as wild-type flies and *Rel^{E20}* as susceptible flies lacking the Imd pathway
658 for all survival experiments to Gram-negative bacterial infection. Female *w^{1118}*, *ΔCec^{A-C}*,
659 *ΔAMP10, ΔAMP14* and *Rel^{E20}* flies were pricked in the thorax with an inoculum of (**A**) *P.*
660 *rettgeri,* (**B**) *Ecc15,* (**C**) *E. coli* or (**D**) *P. burhodogranariea.* Cecropins were not critically
661 involved in combating infection with any bacteria presented here as *ΔCec^{A-C}* survived as
662 well as *w^{1118}* flies, while *ΔAMP10* and *ΔAMP14* mutant flies died as fast as *Rel^{E20}* mutants.
663 Bacterial concentrations are indicated in the figure.
664
665
666 **Figure 3 : Cecropins are essential in the absence of other AMPs to resist *E. cloacae***
667 **and *P. heimbachae* infection.**
668 (**A**) Survival experiments upon infection with *E. cloacae* reveal that AMP deficient flies
669 having Cecropins (*ΔAMP10*) are significantly more resistant than those without
670 Cecropins (*ΔAMP14*). (**B**) Bacterial loads (CFU counts) of *w^{1118}*, *ΔCec^{A-C}*, *ΔAMP10, ΔAMP14*
671 and *Rel^{E20}* flies 8 hours post infection reveal a significant role for Cecropins in clearing
672 and controlling *E. cloacae.* (**C**) Commercial Cecropin A injection (50 nL at 50 μM) 2 hours
673 prior to *E. cloacae* infection increases the resistance of *ΔAMP10* mutant flies. However,
674 CecA injection did not rescue the susceptibility of *ΔAMP14* flies to *E. cloacae*. (**D-F**)
675 Survival analysis (D) bacterial load measurements 24 hours post infection (E) and
676 Cecropin supplementation experiments (F) in *w^{1118}*, *ΔCec^{A-C}*, *ΔAMP10, ΔAMP14* and *Rel^{E20}*
677 flies upon infection with *P. heimbachae* (as described for A-C).
678
679 **Figure 4 : Cecropins are not involved in resistance to Gram-positive bacteria**

680 w^{1118} were used as wild-type flies and $Bom^{\Delta 55C}$ as susceptible flies lacking 10 *Bomanin*
681 genes for all survival experiments to Gram-positive bacterial infection. w^{1118}, $\Delta Cec^{A\text{-}C}$,
682 *ΔAMP10, ΔAMP14* and $Bom^{\Delta 55C}$ flies were pricked in the thorax with an inoculum of (**A**)
683 *E. faecalis,* (**B**) *S. pneumoniae* and (**C)** *L. monocytogenes*. Cecropins were not involved in
684 combating infection of 3 bacterial species: $\Delta Cec^{A\text{-}C}$, *ΔAMP10* and *ΔAMP14* flies survived as
685 well as w^{1118} flies. Bacterial concentrations are indicated in the figure.
686
687 **Figure 5: Cecropins contribute to antifungal defence against *B. bassiana***
688 (**A-C**) w^{1118} were used as wild-type flies and Bom^{D55C} as susceptible flies lacking 10
689 *Bomanin* genes for all survival experiments to fungal infections. Cecropins were not
690 involved in combating infection of (**A**) *M. rileyi,* (**B**) *A. fumigatus* and (**C**) *C. albicans*, as
691 w^{1118}, $\Delta Cec^{A\text{-}C}$, *ΔAMP10* and *ΔAMP14* flies survived as well as w^{1118} flies. (**D**) Survival upon
692 (**D**) natural infection with *B. bassiana* reveals a significant increase in resistance of
693 *ΔAMP10* flies compared to *ΔAMP14* flies, suggesting an important role for cecropins in
694 combating *B. bassiana*. (**E**) *B. bassiana* load (measured by *B. bassiana* 18S expression
695 related to *D. melanogaster RpL32*) is increased (p = .07) in *ΔAMP14* flies compared to
696 w^{1118}, $\Delta Cec^{A\text{-}C}$ and *ΔAMP10* flies 48 hours post septic infection. Fungal concentrations are
697 indicated in the figure.
698
699 **Figure S1: *ΔAMP14* flies retain the induction of non-AMP Immune Molecules (IMs)**
700 **upon systemic infection.** Pools of ~40 mixed male and female flies were pricked with a
701 1:1 mix of OD_{600}=200 *E. coli* and *M. luteus* bacteria, and ~40nL of total hemolymph was
702 extracted by nanoject 24hpi, and directly placed in 5uL of 0.1% TFA. Peptide samples
703 were run on an acetonitrile matrix as described previously (Hanson *et al.* 2019a).
704 Hemolymph from infected w^{1118} wild-type in blue (top) and *ΔAMP14* flies in pink
705 (bottom) are shown. Peaks corresponding to AMP products in the wild-type are labelled
706 in pink, including IM7 whose sequence is unknown, but is absent in *ΔAMP14* flies. Of note,
707 a novel immune-induced peak was observed at ~3027 Da in *AMP* mutants. This peak
708 corresponds to an unknown peptide that is masked by Metchnikowin (~3026 Da) in the
709 wild-type. Reflectron analysis confirms a different spectrum profile for this peak.
710 *Drosophila* IMs are described in (Levy *et al.* 2004) and (Uttenweiler-Joseph *et al.* 1998)
711

Figure 1

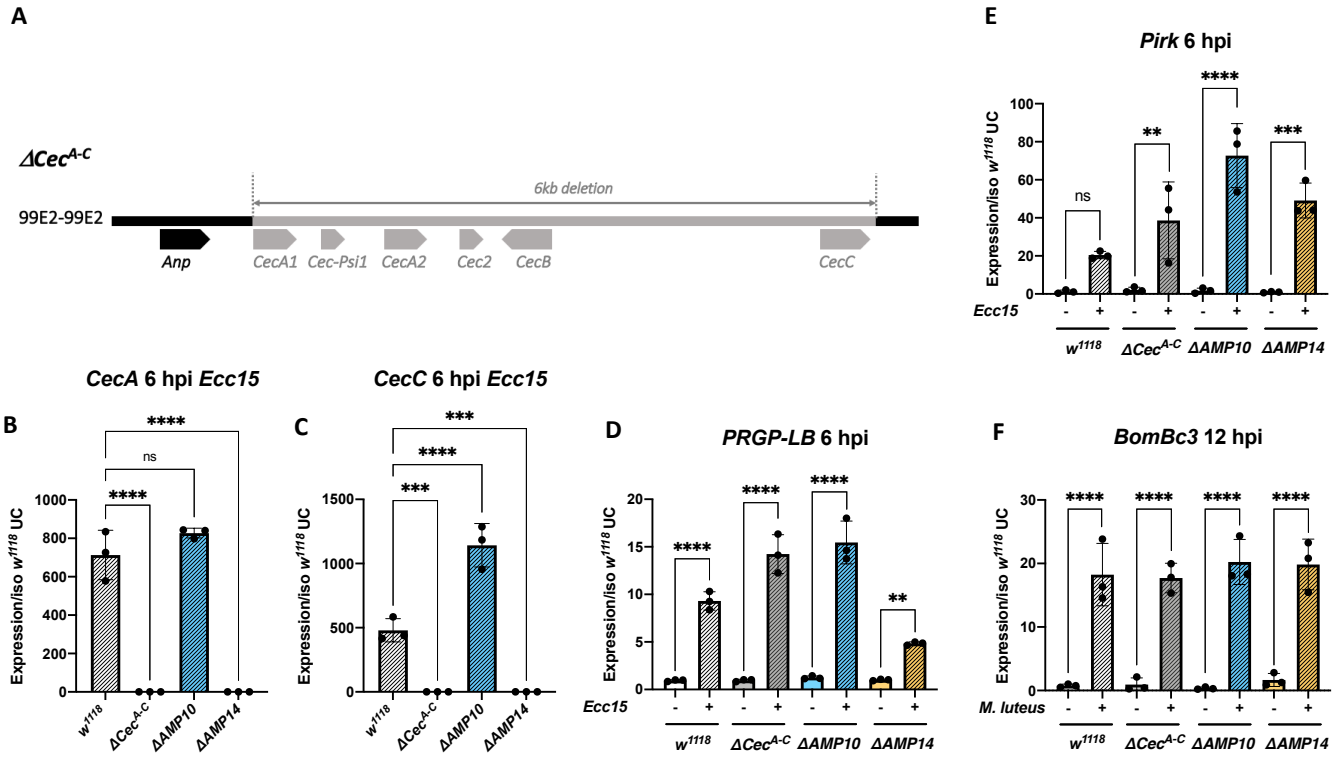

A
ΔCecA-C
6kb deletion
99E2-99E2
Anp
CecA1
Cec-Psi1
CecA2
Cec2
CecB
CecC
B
CecA 6 hpi Ecc15
Expression/iso w1118 UC
ns
****
w1118
ΔCecA-C
ΔAMP10
ΔAMP14
C
CecC 6 hpi Ecc15
***
****
D
PRGP-LB 6 hpi
**
Ecc15
E
Pirk 6 hpi
F
BomBc3 12 hpi
M. luteus

Figure 2

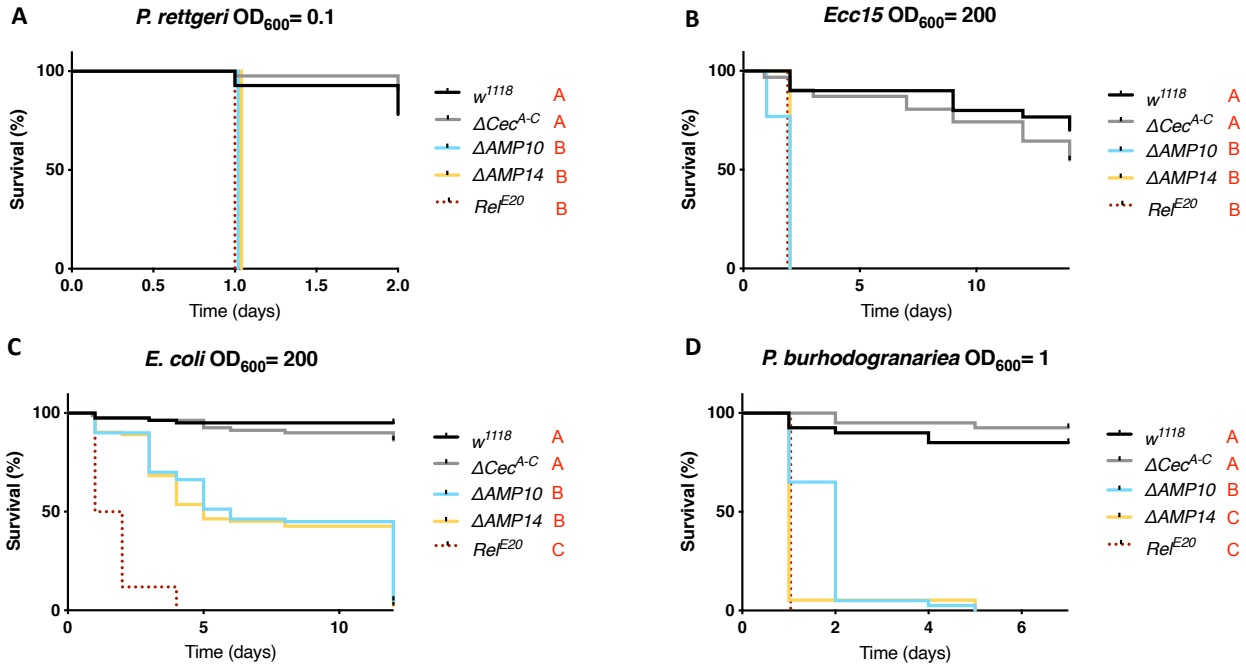

A
P. rettgeri OD600= 0.1
Survival (%)
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 B
ΔAMP14 B
RelE20 B
B
Ecc15 OD600= 200
Survival (%)
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 B
ΔAMP14 B
RelE20 B
C
E. coli OD600= 200
Survival (%)
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 B
ΔAMP14 B
RelE20 C
D
P. burhodogranariea OD600= 1
Survival (%)
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 B
ΔAMP14 C
RelE20 C

Figure 3

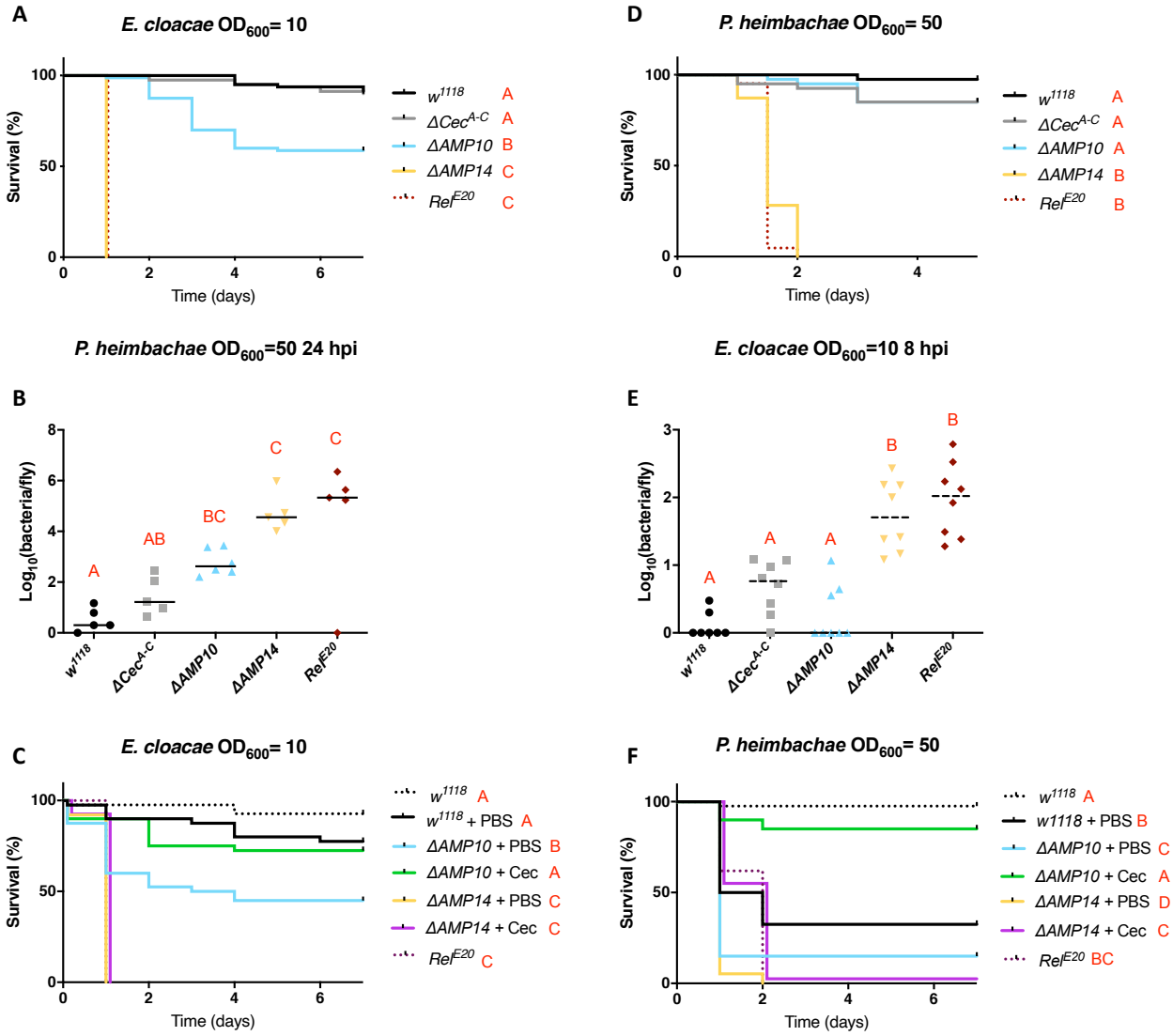

A
E. cloacae OD600= 10
Survival (%)
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 B
ΔAMP14 C
RelE20 C
D
P. heimbachae OD600= 50
Survival (%)
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 A
ΔAMP14 B
RelE20 B
P. heimbachae OD600=50 24 hpi
B
Log10(bacteria/fly)
A
AB
BC
C
C
w1118
ΔCecA-C
ΔAMP10
ΔAMP14
RelE20
E. cloacae OD600=10 8 hpi
E
Log10(bacteria/fly)
A
A
A
B
B
w1118
ΔCecA-C
ΔAMP10
ΔAMP14
RelE20
C
E. cloacae OD600= 10
Survival (%)
Time (days)
w1118 A
w1118 + PBS A
ΔAMP10 + PBS B
ΔAMP10 + Cec A
ΔAMP14 + PBS C
ΔAMP14 + Cec C
RelE20 C
F
P. heimbachae OD600= 50
Survival (%)
Time (days)
w1118 A
w1118 + PBS B
ΔAMP10 + PBS C
ΔAMP10 + Cec A
ΔAMP14 + PBS D
ΔAMP14 + Cec C
RelE20 BC

Figure 4

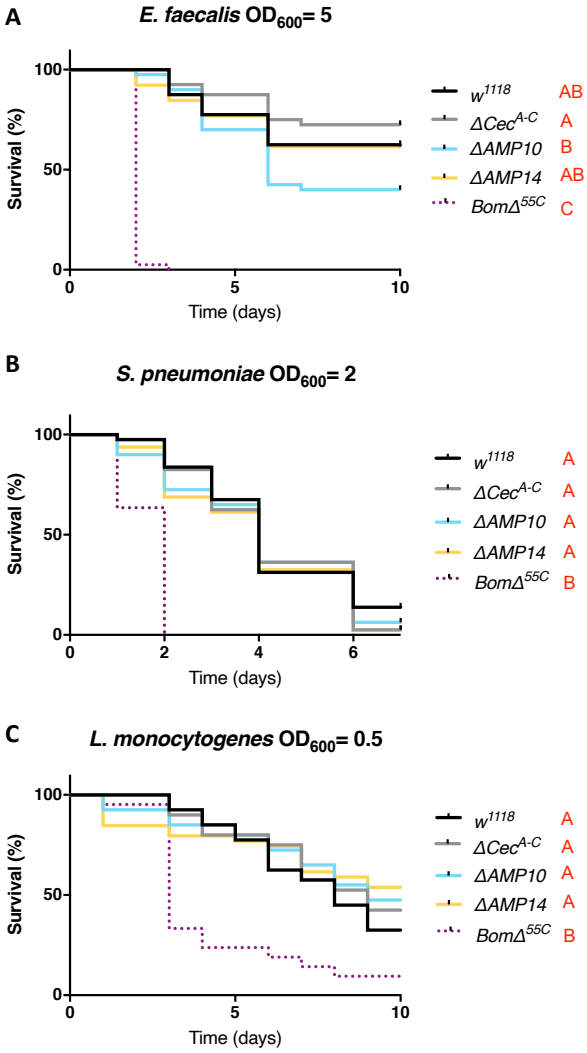
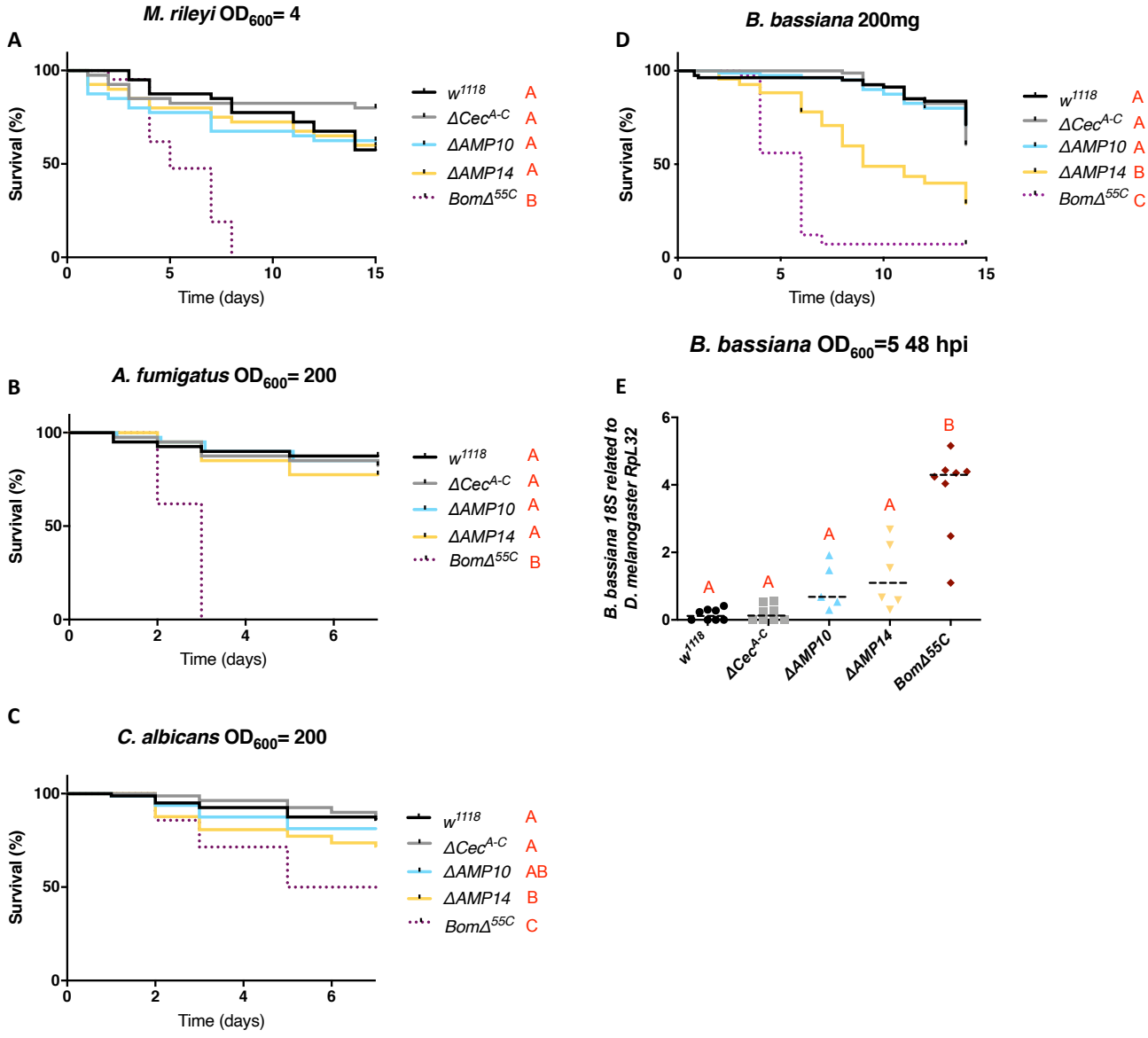

A
E. faecalis OD600= 5
Survival (%)
100
50
0
0
5
10
Time (days)
w1118 AB
ΔCecA-C A
ΔAMP10 B
ΔAMP14 AB
BomΔ55C C
B
S. pneumoniae OD600= 2
Survival (%)
100
50
0
0
2
4
6
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 A
ΔAMP14 A
BomΔ55C B
C
L. monocytogenes OD600= 0.5
Survival (%)
100
50
0
0
5
10
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 A
ΔAMP14 A
BomΔ55C B

Figure 5


A
M. rileyi OD600= 4
Survival (%)
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 A
ΔAMP14 A
BomΔ55C B
B
A. fumigatus OD600= 200
Survival (%)
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 A
ΔAMP14 A
BomΔ55C B
C
C. albicans OD600= 200
Survival (%)
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 AB
ΔAMP14 B
BomΔ55C C
D
B. bassiana 200mg
Survival (%)
Time (days)
w1118 A
ΔCecA-C A
ΔAMP10 A
ΔAMP14 B
BomΔ55C C
E
B. bassiana OD600=5 48 hpi
B. bassiana 18S related to D. melanogaster RpL32
w1118
ΔCecA-C
ΔAMP10
ΔAMP14
BomΔ55C