1 A constitutively expressed antifungal peptide protects *Tenebrio*
 2 *molitor* during a natural infection by the entomopathogenic
 3 fungus *Beauveria bassiana*.

4 Sevasti Maistrou¹, Véronique Paris², Annette B. Jensen¹, Jens Rolff², Nicolai V.
 5 Meyling^{1*}, Caroline Zanchi^{2*}

6 ¹ University of Copenhagen, Department of Plant and Environmental Sciences,
 7 Thorvaldsensvej 40, 1871 Frederiksberg, Copenhagen

8 ² Free University Berlin, Evolutionary Biology, Königin-Luise-Straße 1-3, 14195
 9 Berlin, Germany

10 * **Correspondence to:**

11 nvm@plen.ku.dk

12 caroline.zanchi@uni-muenster.de

13 Present address : Westfälische Wilhelms Universität, Institute for Evolution &
 14 Biodiversity, Hüfferstraße 1, 48149 Münster, Germany

15

16 Keywords : antifungal peptide, constitutive immune defense, *Beauveria bassiana*,
 17 *Tenebrio molitor*

18 ORCID

19 *Caroline Zanchi* <http://orcid.org/0000-0003-4097-9375>

20 *Jens Rolff* <http://orcid.org/0000-0002-1529-5409>

21 *Nicolai V. Meyling* <http://orcid.org/0000-0003-3025-4370>

22 *Annette B. Jensen* <http://orcid.org/0000-0002-2044-2274>

23 1. Introduction

24 Fungal pathogens infect a great variety of host species including humans, such as
 25 *Candida albicans*, *Naegleria fowleri*, *Aspergillus* sp., or are pests of economically
 26 important crops, such as *Fusarium oxysporum* (Fisher et al., 2012). Fungal pathogens
 27 of insects, called entomopathogenic fungi, share millions of years of coevolution with
 28 their insect hosts (Boomsma et al., 2014; Joop & Vilcinskis, 2016). These fungi play an
 29 important role in controlling insect populations in natural ecosystems by causing
 30 epizootics (Hesketh et al., 2010), thus exerting a strong selection pressure on their
 31 hosts. The relatively short life-cycles of most insects and entomopathogenic fungi, as
 32 well as the knowledge of virulence factors of the fungus (Samuels et al., 1988 ; Hajek
 33 & St. Leger, 1994 ; Valero-Jimenez et al., 2016) and immune reactions of the insect
 34 host (Butt et al., 2016), make these organisms a attractive models for the study of
 35 host-pathogen interactions. Because most entomopathogenic fungi are necrotrophic
 36 parasites (i.e. need to kill their host in order to complete their life cycle), they have
 37 been extensively studied in the context of biocontrol (Lacey et al., 2015).

38 The entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Hypocreales) infects a
 39 broad range of hosts (Ferron, 1978 ; Inglis et al., 2001) such as ants (Broome et al.,
 40 1976), termites (Culliney & Grace, 2000), agricultural pests such as grasshoppers
 41 (Bidochka & Khachatourians, 1991) and arthropod vectors of human diseases (Clark
 42 et al., 1968 ; Kirkland et al., 2004 ; Marti et al., 2005). Once *B. bassiana* conidia land
 43 on a potential host, they attach to the cuticle, germinate and penetrate the cuticular

44 barrier by a combination of mechanical pressure and enzymatic secretions (Ortiz-
 45 Urquiza & Keyhani, 2013). When the fungus reaches the hemolymph, it differentiates
 46 into yeast-like, thin-walled blastospores or hyphal bodies, which divide and exploit
 47 host nutrients. After the death of the host and after catabolizing its last nutrients,
 48 hyphae are produced which breach the cuticle from the inside and form conidia at the
 49 surface of the cadaver, thereby completing the asexual life cycle of the fungus (Ortiz-
 50 Urquiza and Keyhani 2013 ; Pedrini et al. 2013).

51 Once conidia come in contact with the host, the first line of defense is the cuticle that
 52 forms a mechanical barrier which, in combination with defenses such as cuticular
 53 hydrocarbons (Boyle & Cutler, 2012 ; Lopes et al., 2015), and protease inhibitors (Li et
 54 al., 2012), have been well studied for their role in host resistance (Ortiz-Urquiza &
 55 Keyhani, 2013). When the cuticle has been breached, the fungus will face the effectors
 56 of the insect immune system, where immune reactions relying on immune cells and
 57 the phenoloxidase pathway, such as phagocytosis (Gillespie et al., 2000),
 58 encapsulation and nodule formation (Lord et al., 2002), have been shown to play a
 59 central role in the suppression and clearance of *B. bassiana* from the host hemolymph.

60

61 However, insects also possess a battery of antimicrobial peptides (AMPs), small
 62 cationic peptides showing a relatively broad spectrum of antimicrobial activity ranging
 63 from Gram positive and/or negative bacteria, protozoans, viruses, and fungi (Bulet et
 64 al., 1999 ; Hancock, 2000 ; Peschel & Sahl, 2006 ; Mylonakis et al., 2016).

AMPs have attracted a lot of attention in the context of bacterial infections (Yokoi et al., 2012 ; Johnston et al., 2014) and because of their potential as new therapeutical agents (Zasloff, 2002). Some AMPs have shown antifungal activity either while being also antibacterial (Levashina et al., 1995 ; Hekengren & Hultmark, 1999 ; Lamberty et al., 2001a), or solely antifungal (Ijima et al., 1993 ; Fehlbauum et al., 1994 ; Schuhmann et al., 2003 ; Lamberty et al., 2001b). The antifungal activity of these AMPs has often been deduced from *in vitro* tests against opportunistic fungi (Levashina et al., 1995 ; Souhail et al., 2016 ; Fehlbauum et al., 1994 ; Yuan et al., 2007 ; Gao & Zhu, 2008 ; Tian et al., 2008, Zhang & Zhu 2009 ; Yang et al., 2006 ; Liu et al., 2016 ; Kim et al., 2001), but only a few times against ecologically relevant pathogens of insects (Hekengren & Hultmark, 1999 ; Tzou et al., 2002 ; Lu et al., 2016 ; Lamberty et al., 2001b). Moreover, the *in vitro* spectrum of activity of AMPs is not always transposable *in vivo* (Zanchi et al., 2017). This makes their contribution to insect fitness in case of a natural infection hard to predict, especially regarding peptides whose expression is not elicited by a fungal infection. In the mealworm beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae), it has recently been shown that an infection with *B. bassiana* activated the Toll pathway which lead to the expression of some antimicrobial peptides among such as the defensin Tenecin 1 and the coleopteracin Tenecin 2 (Yang et al., 2017), and that the knock down of this pathway decreased the survival of fungal infected beetles. However, the contribution of the constitutively expressed thaumatin Tenecin 3 (Makarova et al., 2016 ; Johnston et al., 2014 ; Yang et al., 2017) during *B. bassiana* infection in *T. molitor* is unknown.

87 It is commonly assumed that immune defense, including the production of
88 antimicrobial peptides, comes at a cost (Johnston et al., 2014 ; Poulsen et al., 2002),
89 and that its fitness benefits for the host have to outweigh these costs in order to evolve
90 (Schmid-Hempel, 2005). Thus, we can expect the constitutive expression of Tenecin 3
91 to confer a benefit to *T. molitor* in the case of a natural fungal infection. The spectrum
92 of activity of Tenecin 3 has been investigated *in vitro* towards several bacterial species
93 whose growth were not affected, and against two opportunistic fungi, *Candida*
94 *albicans* and *Saccharomyces cerevisiae* against which it was active (Kim et al., 1998 ;
95 Kim et al., 2001).

96 In this study, we used the *Tenebrio molitor* - *Beauveria bassiana* model system to shed
97 light on the contribution of a constitutively expressed antifungal peptide to host
98 defense against a fungal infection. We used a gene knock-down approach by RNA
99 interference on Tenecin 3, and monitored in parallel host fitness in terms of survival,
100 and fungal fitness in terms of dynamics of within-host growth. We also performed an
101 *in vitro* experiment in order to confirm the fungicidal effect of Tenecin 3 on *B.*
102 *bassiana*.

103 Understanding the contribution of specific elements of the insect immune system in
104 the resistance towards *B. bassiana* can help explain the success or failure of biocontrol
105 strategies, as well as improve our understanding of the evolution of constitutive
106 antifungal peptides as a defense mechanism.

107 **2. Materials and Methods**

108 **2.1. Insect rearing**

109 Larvae of the mealworm beetle (*Tenebrio molitor*) were purchased from a supplier and
 110 maintained at 25°C in the dark in plastic boxes (18 x 18 x 8 cm) at a density of 500
 111 larvae in 400 g of wheat bran supplemented with rat chow. Fresh apple and albumin
 112 (from chicken egg white, Sigma) were added to the boxes every third day. Nymphs
 113 were collected every second day and kept apart until emergence. Newly emerged adult
 114 beetles were placed individually into grid boxes with wheat bran and a piece of filter
 115 paper. They received a piece of apple and ~1g of albumin twice a week. Beetles of both
 116 sexes, aged 8-12 days post-hatching were used for the experiments.

117

118 **2.2. Fungal cultures and conidia suspensions**

119 A *Beauveria bassiana* strain (KVL 03-144) isolated from a natural infection of
 120 *Leptopterna dolabrata* (Homoptera: Miridae) from an agroecosystem in Denmark
 121 (Meyling et al., 2009) was kept at -80°C in a culture collection at the University of
 122 Copenhagen before cultivation. Isolates were cultivated on quarter-strength
 123 Sabouraud Dextrose Agar + Yeast 10 % (SDAY) and incubated for 10 days at 23°C to
 124 allow for sporulation. Conidia were collected by scrapping the surface of the culture
 125 with a sterile loop and transferred in 1 ml PBS with 0.05% of Triton-X. The resulting
 126 solution was centrifuged twice at 23°C, 4000 rpm for 3.5 min and the supernatant

discarded in order to remove agar and hyphae. The pellet was resuspended in 1 ml Phosphate Buffered Saline (PBS) + Triton-X 0.05%, and the conidia concentration assessed with a hemocytometer (Neubauer improved). The concentration of the inoculum was adjusted through serial dilutions before exposing the beetles. After each infection bout, the germination rate of the inoculum was assessed by plating 100 μ L of a 10^5 conidia/ml solution on quarter strength SDAY 10% plates. After incubation for 24 h at 23°C the germination of 3 x 100 conidia was counted. We discarded any batch of beetles that turned out to be infected with an inoculum of a germination rate lower than 90%.

136

2.3. Gene knock-down by RNA interference

We used RNA interference to knock down the expression of the *tenecin 3* gene. RNAi has been previously shown to be efficient in *Tribolium castaneum* (Coleoptera: Tenebrionidae) and *T. molitor* (Fabrick et al., 2009 ; Miller et al. 2012) and the effect to last at least 14 days (Zanchi et al., 2017). We generated double-stranded RNA (dsRNA) of a *tenecin 3* gene construct (Eurofins, Operon) which consisted in the sequence of the whole *tenecin 3* gene minus the sequence that was amplified by qPCR in order to confirm the efficiency of the knock-down of *tenecin 3* gene expression (Supplemental Information S1 figure S1). As a control, we used the dsRNA of the *Galleria mellonella* lysozyme, which has no homology of sequence with any known gene of *T. molitor* (Johnston & Rolff, 2015). The full sequences of the resulting

148 products as well as the qPCR primers and products are given in the Supplemental
149 Information S1. The template for dsRNA synthesis was amplified from the constructs
150 by PCR (KAPA2G Fast ReadyMix, KAPA Biosystems) using gene-specific primers
151 tailed with the T7 polymerase promoter sequence (Metabion International AG). After
152 checking the length of our amplicon on a 2 % agarose gel and cleanup (PCR DNA
153 Clean-Up Kit, Roboklon), the resulting amplicon was used as a template for RNA
154 synthesis (High Yield MEGAscript T7 kit, Applied Biosystems/Ambion) according to
155 the manufacturer's recommendations. We then purified the RNA with a phenol-
156 chloroform extraction and resuspended the pellet in a nuclease-free insect Ringer
157 solution (128 mM NaCl, 18 mM CaCl₂, 1.3 mM KCl, 2.3 mM NaHCO₃). Before being
158 used, the RNA was annealed by heating it up at 90°C and allowed to slowly cool down,
159 in order to obtain dsRNA. We injected 500 ng of dsRNA per beetle at a concentration
160 of 100 ng/μl in 5μL of insect Ringer solution. To do so, we chilled the insects on ice for
161 10-15 minutes before dsRNA injection, which was performed with a sterile pulled
162 glass capillary inserted into the pleural membrane below the elytra, directly into the
163 hemocoel. Care was taken that the needle was parallel to the anterior/posterior axis of
164 the beetle to avoid injury of the organs. Beetles that received the Tenecin 3 dsRNA
165 will be referred to as “Ten3KD” whereas beetles that received *G. mellonella* dsRNA
166 will be referred to as “control”.

167

168

169 2.4. Fungal exposure and maintenance of infected beetles

170 Exposures to *B. bassiana* were performed by applying a 0.5 µl droplet of conidia/PBS-
 171 TritonX solution prepared as described above on the intersegmental membrane
 172 between the sclerotized parts of the sternum and the abdomen. We performed the
 173 exposures 7 days after the injection of dsRNA. Care was taken so that the whole
 174 droplet was adsorbed on the membrane before placing the beetles individually at 23°C
 175 in medicine cups (Carl Roth GmbH) containing a 2x2cm wet filter paper in order to
 176 keep the humidity level high in the cup and stimulate *B. bassiana* conidia to
 177 germinate. Beetles were provided with food (ca. 5 g sterilized rolled oats) a day later in
 178 order to avoid the transfer of conidia in the medium. The filter papers were replaced
 179 every day, and the medicine cups along with the food were replaced every four days.

180

181 2.5. Mortality bioassays

182 We tested the effect of three different concentrations of *B. bassiana* conidia on beetles
 183 survival: 1×10^5 , 5×10^5 and 1×10^6 /ml, corresponding to 50, 250, 500 conidia applied per
 184 beetle, since previous laboratory experiments showed that these concentrations span
 185 the LC50 (3×10^5 conidia/ml) of the strain KVL 03-144 of *B. bassiana* (S. Maistrou,
 186 unpublished). As procedural control we included a group of beetles that each received
 187 only a 0.5 µl PBS-0.05% Triton-X solution. The beetles were maintained as described
 188 above and their mortality was checked daily for 14 days after exposure. All dead

189 beetles exposed to *B. bassiana* developed mycosis after death. Each treatment group
190 consisted of 20 beetles and the bioassay was repeated twice. The final sample sizes are
191 presented in the Supplemental Information S2 Figure S2.

192

193 **2.6. Recovery of hyphal bodies from the beetles hemolymph**

194 We chose to perform this experiment on beetles exposed to 0.5 µl of 5x10⁵ conidia/ml
195 since this was the concentration which yielded the biggest effect on mortality between
196 Ten3KD and control beetles (Figure 1). Beetles exposed to *B. bassiana* were split into
197 two groups of ~100 beetles/treatment (control or Ten3KD). Since hemolymph collection
198 was an invasive process, it was performed only once per beetle. Therefore, at each time
199 point (1, 2, 3, 4 and 5 days post infection) we collected the hemolymph of ~20 randomly
200 selected beetles of each of the two treatment groups.

201 Before collection, beetles were chilled on ice (10-15 minutes) in a glass tube containing
202 a piece of cotton soaked in ethyl-acetate and briefly dipped in 70% ethanol to remove
203 any contamination from the cuticle. An incision was made above their genitals before
204 inserting a hypodermic needle of a syringe containing 0.5 ml PBS which was
205 subsequently perfused. The resulting haemolymph/PBS extract was collected into a
206 microcentrifuge tube kept on ice. 100 µL of this suspension was plated on SDA + 5
207 mg/ml tetracyclin using sterile glass beads, and incubated at 23°C for 36 h (we
208 established beforehand that tetracycline did not affect the growth of blastospores in

terms of speed of germination and density, data not shown). The number of colony forming units (CFU) on each plate was counted and used as a proxy to assess the concentration of *B. bassiana* hyphal bodies in the hemolymph of each individual beetle at a given time point. Few beetles died before the end of the experiment, which was compensated in our sample size by the fact that we injected slightly more than 100 beetles at the start. Some cadavers developed mycosis as expected (i.e. succumbed to *B. bassiana* infection) ; there were 1 beetle in both the control and the Ten3KD while 2 beetles in the control and 3 in the Ten3 KD treatment died without showing mycosis symptoms. The final sample sizes are presented in the Supplemental Information S2 Figure S3.

219

2.7. *In vitro* effect of Tenecin 3 on *Beauveria bassiana*

2.7.1. Growth and collection of blastospores

500 µl of conidial suspension (10^7 conidia/ml) was added into a flask containing 100 ml of Sabouraud Dextrose broth + 10% yeast extract. The solution was incubated for 72-96 h at 23 °C with agitation at 150 rpm, in order for the conidia to germinate. After incubation, the solution was filtered through a sterile filter paper (595 grade, Schleicher & Schuell) in order to recover the blastospores and discard the mycelium. The resulting solution was washed by centrifugation three times at 10 000 g and 25 °C for 10 minutes, and the pellet resuspended in 1ml PBS. The concentration of

229 blastospores in this solution was assessed in a hemocytometer, and adjusted to 5.10^6
230 blastospores/ml.

231

232 **2.7.2. Survival of blastospores *in vitro* against a recombinant Tenecin 3**

233 We first established a growth curve of the *B. bassiana* strain KVL 03-144 in our
234 experimental conditions in order to focus on a time point which was in the exponential
235 phase for the rest of the experiments. The protocol and the growth curve are presented
236 in the Supplemental Information S3.

237 We chose to carry out the rest of the next experiment on the 6 hour time point,
238 corresponding to the beginning of the exponential growth phase. We inoculated 19.5 μ l
239 of Sabouraud Dextrose broth with 0.5 μ l of blastospore solution (10^3 blastospores) in
240 the wells of a 96 well plate (Sarstedt). We added 5 μ l of a solution of water and
241 recombinant Tenecin 3 resulting in a final concentration of 0.05, 0.1, 0.2, and 0.4 ng/ μ l
242 of Tenecin 3. After incubation at 25 °C and agitation at 220 rpm for 6 h, we suspended
243 the content of the wells in PBS up to 100 μ l and plated 100 μ l of serial dilutions by a
244 factor of 10 and 100 on SDA. We incubated the Petri dishes at 23°C in the dark for 48
245 hours after which we counted the number of CFU. As a control, we replaced Tenecin 3
246 with bovine Serum Albumin (BSA) at the same concentrations. The process was
247 repeated 5 times, with conidia originating from different culture plates.

248

2.8. Data analysis

All statistical analyses were performed using the R software (R Core Team, 2016). The survival of the beetles after *B. bassiana* exposure was analyzed with the “survreg” function of the 'survival' package (Therneau, 1999), since the hazards were not proportional between treatment (checked with the “coxph” function). We checked whether survival of the beetles was affected by the concentration in conidia of *B. bassiana* used for exposure and their knock-down treatment, as well as the interaction between them and the replicate as random factor, and with an exponential distribution. Both sham infected treatments showed a very low mortality and impaired the fit of the model, therefore we decided to exclude them during the analyses, but show them on the figure.

The concentration of hyphal bodies present in the hemolymph of the beetles was analyzed with a Generalized Linear Model fitted with a negative binomial distribution with the package 'MASS' (Venables & Ripley, 2002). We tested whether the concentration of hyphal bodies was explained by the knock down treatment and the time as well as their interaction, in order to highlight the dynamics of the progression of the infection.

The *in vitro* growth of blastospores in the presence of Tenecin 3, BSA or alone was compared using a Generalized Linear Mixed Model with a Poisson distribution corrected for overdispersion with the package 'lme4' (Bates, 2015), including the treatment (protein added, either BSA or Tenecin 3) as well as the protein

270 concentration as explanatory variables as well as the interactions between them, and
271 the replicate as a random factor.

272 For all the analyses, we chose which distribution to use by checking the distribution of
273 the residuals and comparing the estimates of the models with our data with the
274 package 'visreg' (Breheny & Burchett, 2017). We then selected the best model by
275 comparing the Akaike's Information Criterion (AIC) of the full models including
276 interactions to all the nested models and the null model. We kept as the best models
277 the ones with the lowest AICs (Akaike, 1976). Post hoc comparisons when relevant can
278 be performed by comparing the 95% confidence intervals around the estimates of the
279 models on the figures provided in the Supplemental Information S4. A difference
280 between two treatments is deemed significant when the confidence intervals do not
281 overlap on more than half of their length, as advised by Cumming (2009). The figures
282 in the main body of the manuscript were made using the ggplot2 package (Wickham,
283 2009), and the figures of the Supplemental Information were obtained with the
284 package 'effects' (Fox & Hong, 2009).

285

286 **3. Results**

287 **3.1. Survival of the beetles after *B. bassiana* exposure**

288 There was no effect of the concentration of conidia applied on the beetles for infection
289 either in interaction with the treatment (concentration*treatment: $X^2_{6,243} = 9.12$; $p =$

290 0.17) or alone ($X^2_{3,246} = 0.85$; $p = 0.84$) across the range of concentrations we checked.

291 The knock-down of Tenecin 3 significantly reduced survival of the beetles after *B.*

292 *bassiana* exposure ($X^2_{2,247} = 8.13$; $p = 0.017$; LT50 control = 7.5 days ; LT50 Ten3KD =

293 5.5 days).

294 We then decided to keep investigating the effect of the knock-down treatment in

295 beetles exposed to 0.5 µl of 5×10^5 conidial/ml (~ 250 conidia) on the *in vivo* progression

296 of the infection over 5 days, after which the mortality of the beetles would have biased

297 our sampling between treatments.

298

Figure 1 : Tenecin 3 improves the survival of *Tenebrio molitor* to *Beauveria bassiana*. Kaplan-Meier curve showing the survival over 14 days of the control (dashed lines) and Tenecin 3 knock-down (solid lines) beetles either sham (in grey) or exposed to *Beauveria bassiana* (in blue, lighter to darker shades indicating lower to higher concentrations : light blue = 10⁵ conidia/mL ; medium blue = 5.10⁵ conidia/mL ; dark blue = 10⁶ conidia/mL). Only the knock-down treatment had a significant effect on survival of the beetles (dashed vs. solid lines, $\chi^2_{2,247} = 8.13$; $p = 0.017$).

309 **3.2. Progression of the infection in the beetle host.**

310 Very few hyphal bodies could be recovered from the early time points (less than 10 and
 311 less than 50 on the first and second day post infection respectively, see Figure 2). Both
 312 control and Ten3KD treatments showed similar dynamics of the progression of the
 313 infection, i.e. there was no interaction between time and treatment on the number of
 314 hyphal bodies recovered from the beetles (time*treatment : $X^2_{3,202} = 1.28$; $p = 0.257$).
 315 However, both the treatment and time affected the concentration of hyphal bodies
 316 present in the beetles as simple effects. Ten3KD beetles contained overall more hyphal
 317 bodies than control beetles (treatment : $X^2_{1,203} = 7.61$; $p = 0.00578$). As expected, the
 318 concentration of hyphal bodies increased over time (time : $X^2_{1,203} = 211.205$; $p <$
 319 0.0001 , see Supplemental Information S4 Figure S5 for post hoc comparisons).

320

Figure 2 : Tenecin 3 decreases the fungal load of *Beauveria bassiana* in the hemolymph of *Tenebrio molitor*. Box-dotplot representing the number of CFU of *B. bassiana* recovered from the hemolymph of infected beetles according to the time after infection. Each dot represents a data point of control (grey) and Tenecin 3 knock-down (in blue) beetles. The boxplots represent the first to the third quartiles around the median (horizontal grey line) , and the vertical bars the 1.5 interquartile of the lower and upper quartiles. Both time and treatment affect the fungal load in the hemolymph of the beetles, see Supplemental Information S4 Figure S5 for post-hoc comparisons.

bimodal distribution (Figure 2), which is very obvious at 5 days post infection. By setting a cut-off at 600 CFU at this time point we can determine that in the control, 39.1% of the data points are above this threshold, versus 50.0% for the Ten3KD. By looking at the survival curves of the two treatments in the corresponding concentration (5.10^5 conidia/mL, Figure 1 in medium blue) it appears that they diverge at 6 days post-infection and remain relatively parallel after this time point. The proportions of dead beetles at this point are 45.0 and 59.0% for control and Ten3KD respectively (Figure 1). While the 24 h time frame of our sampling does not allow us to capture the concentration of hyphal bodies present in the hemolymph of the beetles immediately prior to death, we can observe that these values are not too different from the proportion of the beetles who carried more than 600 CFU at day 5 (Figure 2).

3.3. In vitro killing of *B. bassiana* blastospores by recombinant Tenecin 3

We investigated whether the lower concentration in hyphal bodies in the hemolymph of control beetles compared to Ten3KD beetles could be due to a direct effect of Tenecin 3 on *B. bassiana* hyphal bodies. We exposed blastospores (hyphal bodies generated *in vitro*) to a recombinant Tenecin 3 or to BSA as a control at different concentrations for 6 hours, and compared the concentration of blastospores recovered after incubation.

352 Tenecin 3 affected the number of blastospores in the medium differently than BSA at
 353 higher concentrations (0.2 and 0.4 ng/μl : concentration * treatment : $X^2_{8,34} = 552.56$; p
 354 < 0.001, see Supplemental Information 4 Figure S6 for post-hoc comparisons). The
 355 number of blastospores was lower when incubated at both 0.2 and 0.4 ng/μl of Tenecin
 356 3 compared to the inoculum, suggesting a fungicidal effect of Tenecin 3 on *B. bassiana*
 357 (Figure 3).

365 **Figure 3 : Recombinant Tenecin 3 has an antifungal activity against the**
 366 **blastospores of *Beauveria bassiana* *in vitro*.** Dotplot representing the number of
 367 CFU retrieved after *in vitro* incubation of blastospores for 6 hours with either Bovine
 368 Serum Albumin (BSA, in pink) or recombinant Tenecin 3 (in blue). Five replicates
 369 were performed, each dot represents a data point and the thicker dot represents the
 370 mean. The blastospore solution (around 10^3 conidia) was plated after inoculation and

371 is represented in grey as “0(t0)”, meaning a concentration of 0 and time point 0.
 372 Starting from 0.2ng/μL, Tenecin 3 has a fungicidal effect on the blastospores of *B.*
 373 *bassiana*, see Supplemental Information S4 Figure S6 for post hoc comparisons.

374

375 4. Discussion

376 We found that the constitutively expressed antifungal peptide Tenecin 3 increases
 377 beetle survival to a *B. bassiana* infection. This is mirrored by a clear antifungal effect
 378 of Tenecin 3 on *B. bassiana* blastospores *in vitro*.

379 Antimicrobial peptides of insects are well studied in the context of bacterial infections,
 380 in which case they are mostly inducible (Bulet & Stöcklin, 2005). By comparison, *in*
 381 *vivo* studies on antifungal peptides in the context of fungal infections established
 382 through the natural route are scarce. The constitutive expression of Drosomycin, an
 383 otherwise inducible antifungal peptide, in adult *Drosophila melanogaster* (Diptera:
 384 Drosophilidae) mutants deficient for both the Toll and Imd pathways, restored the
 385 wild-type survival to opportunistic or human pathogenic fungi injected in the flies but
 386 did not improve survival to a natural infection with *B. bassiana* (Tzou et al., 2002).
 387 Termicin, an antifungal peptide constitutively expressed in the salivary glands of
 388 some species of termites has been shown to increase the survival of *Reticulitermes*
 389 *flavipes* to a *Metarhizium anisopliae* infection (Hamilton & Bulmer, 2012). The first
 390 result can seem counterintuitive considering that Drosomycin is elicited following *B.*

391 *bassiana* infection (Lemaître et al. 1997). In the second case, it is likely that the
392 selection pressure exerted by fungal pathogens is constant in the environment of these
393 species of termites, making the evolution of an external constitutive antifungal
394 defense relevant (Bulmer & Crozier, 2004 ; Adler & Karban, 1994). In *T. molitor*, by
395 performing knock-downs of the Toll pathway, Yang et al. (2017) highlighted the
396 importance of inducible immune defenses in the survival to *B. bassiana*, although the
397 authors did not directly knock-down the inducible AMPs suspected to be involved,
398 whereas the role of the constitutive Tenecin 3 was not investigated. Even though the
399 pressure exerted by *B. bassiana* on *T. molitor* in its natural habitat is unknown, *B.*
400 *bassiana* is widespread in most ecosystems investigated (e.g. Meyling et al. 2011 ;
401 Hajek & Meyling 2018 ; Eilenberg et al. 2015) and we could expect that similarly to
402 what is observed in the aforementioned termite species, the fact that a constitutively
403 expressed antifungal peptide improves the survival of *T. molitor* during a *B. bassiana*
404 infection indicates that Tenecin 3 might have evolved as a way to fight off fungal a
405 constant selection pressure exerted by fungal pathogens in the environment. If this is
406 the case, the changes of its expression patterns during development (Lee et al., 1996)
407 might even indicate that this pressure is stage-dependent. Since other AMPs are
408 induced in *T. molitor* following *B. bassiana* infection through the Toll pathway (Yang
409 et al., 2017), a future lead to follow could be to perform knock-down on these peptides
410 simultaneously, which would shed light on the interplay of constitutive and inducible
411 immune defenses.

412 Our study also adds support to previous observations made in *D. melanogaster* that
 413 the evolution of the pathogen load inside the host over time can show a bimodal
 414 outcome, where one category of hosts carry a low level infection while the other
 415 category will die of high infection levels (Clemmons et al., 2015 ; see Duneau et al.,
 416 2017 for data & review). Similarly to Duneau et al. (2017), the quantity of hyphal
 417 bodies present in the beetles at a certain time point seems to be responsible for their
 418 mortality, and suggests that there is a threshold pathogenic load which needs to be
 419 reached by the fungus in order to kill the host. However the overall pattern of
 420 progression of the infection differs compared to these previous studies : while after
 421 injection of bacteria in both *D. melanogaster* and *T. molitor* there is an immediate
 422 dramatic decrease of the pathogen load in the host, our present infections seems more
 423 to build-up from a low load in the hosts hemolymph. In these previous studies a high
 424 concentration of bacteria was directly injected into the hemolymph, bypassing the
 425 cuticular barrier. With this protocol, Zanchi et al. (2017) did not find an effect of the
 426 knock down of various AMPs on the early stages of the infection, but instead an
 427 increased bacterial load and dispersion of the bacterial counts in later time points
 428 after infection. Our present study therefore confirms this result in the case of a
 429 natural infection, which can be considered like an ecologically relevant setting.

430

431 To conclude, we show that the direct antifungal activity of Tenecin 3 on *B. bassiana*
 432 protects *T. molitor* against the internal progression of infection by this fungus. While

433 many current studies focus on insect defenses which prevent the infection from being
 434 initiated, the role of the immune system of the hemolymph in host resistance to
 435 entomopathogenic fungi should not be underestimated. Understanding the
 436 contribution of specific elements of the insect immune system in the resistance
 437 towards *B. bassiana* can help explain the success or failure of biocontrol strategies, as
 438 well as improve our understanding of the evolution of constitutive antifungal peptides
 439 as a defense mechanism.

440

441 **5. Acknowledgements**

442 We thank P. R. Johnston, O. Makarova & A. Rodriguez Rojas for useful insight and
 443 comments on our experiments, and N. Demandt for useful advice on statistical
 444 analyses.

445

446 **Funding :** This research was funded by the ERC (Grant n° 260986) and the
 447 University of Copenhagen.

448 6. References

- 449 Akaike, H., 1976. Canonical correlation analysis of time series and the use of an
450 information criterion. Academic Press Inc.
- 451 Adler, F.R., Karbn, R., 1994. Defended Fortresses or Moving Targets? Another Model
452 of Inducible Defenses Inspired by Military Metaphors. *Am. Nat.* 144:813:832.
- 453 Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects
454 Models Using lme4. *Journal of Statistical Software*, 67(1), 1-48.
455 doi:10.18637/jss.v067.i01.
- 456 Bidochka, M.J., Kachaturians, G.G., 1991. The implications of metabolic acids
457 produced by *Beauveria bassiana* in pathogenesis of the migratory grasshopper,
458 *Melanopus sanguinipes*. *J. Invertebr. Pathol.* 85:106-117.
- 459 Boomsma, J.J., Jensen, A.B., Meyling, N.V., Eilenberg, J., 2014. Evolutionary
460 interactions networks of insect pathogenic fungi. *Annu. Rev. Entomol.* 59:467–85.
- 461 Boyle, D., Cutler, C.G., 2012. Effects of insect activity, soil, and cuticular factors on
462 virulence of *Beauveria bassiana* toward *Blissus leucopterus hirtus*. *J. Pest Sci.* 85:505-
463 512.
- 464 Breheny P., Burchett W., 2017. Visualization of Regression Models Using visreg. *The*
465 *R Journal*, 9: 56-71.

466 Broome, J.R., Sikorowski, P.P., Norment, A., 1976. A mechanism of pathogenicity of
467 *Beauveria bassiana* on larvae of the imported fire ant, *Solenopsis richteri*. J. Invertebr.
468 Pathol. 28:87-91.

469 Bulet, P., Hetru, C., Dimarcqu, J.L., Hoffmann, D., 1999. Antimicrobial peptides in
470 insects; structure and function. Dev. Comp. Immunol. 23:329-344.

471 Bulet, P., Stöcklin, R., 2005. Insect antimicrobial peptides: structures, properties and
472 gene regulation. Protein Pept. Lett. 12:3-11.

473 Bulmer, M.S., Crozier, R.H., 2004. Duplication and diversifying selection among
474 termite antifungal peptides. Mol. Evol. Biol. 21:2256-2264.

475 Butt, T.M., Coates, C.J., Dubovskiy, I.M., Ratcliffe, N.A., 2016. Entomopathogenic
476 fungi: new insights into host-pathogen interactions. Adv. Genet. 94:37-364.

477 Clark, T.B., Kellen, W.R., Fukuda, T. Lindegren, J.E., 1968. Field and laboratory
478 studies on the pathogenicity of the fungus *Beauveria bassiana* to three genera of
479 mosquitoes. J. Invertebr. Pathol. 11:1-7.

480 Clemmons, A.W., Lindsay, S.A., Wasserman, S.A., 2015. An effector peptide family
481 required for *Drosophila* Toll-mediated immunity. PloS Pathog. 11:e1004876.

482 Culliney, T.W., Grace, J.K., 2000. Prospects for the biological control of subterranean
483 termites (Isoptera: rhinotermitidae), with special reference to *Coptotermes*
484 *formosanus*. Bull. Entomol. Res. 90:9-21.

485 Cumming, G., 2009. Inference by eye: reading the overlap of independent confidence
486 intervals. *Stat. Med.* 28:205-220.

487 Duneau, D., Ferdy, J.B., Revah, J., Kondolf, H., Ortiz, G.A., Lazzaro, B.P., Buchon, N.,
488 2017. Stochastic variation in the initial phase of bacterial infection predicts the
489 probability of survival in *Drosophila melanogaster*. *Elife* 6. pii: e28298. doi:
490 10.7554/eLife.28298.

491 Eilenberg, J., Vlak, J.M., Nielsen-LeRoux, C., Cappellozza, S., Jensen, A.B., 2015.
492 Diseases in insects produced for food and feed. *Journal of Insects as Food and Feed*
493 1:87-102.

494 Ekengren, S., Hultmark, D., 1999. *Drosophila* cecropin as an antifungal agent. *Insect*
495 *Biochem. Mol. Biol.* 29:965-972.

496 Fabrick, J., Oppert, C., Lorenzen, M.D., Morris, K., Oppert, B., Jurat-Fuentes, J.L.,
497 2009. A novel *Tenebrio molitor* cadherin is a functional receptor for *Bacillus*
498 *thuringiensis* Cry3Aa toxin. *J. Biol. Chem.* 284:18401-18410.

499 Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert, W.F., Hetru, C.,
500 Hoffmann, J.A., 1994. Insect immunity. Septic injury of *Drosophila* induces the
501 synthesis of a potent antifungal peptide with sequence homology to plant antifungal
502 peptides. *J. Biol. Chem.* 269:33159-33163.

503 Ferron, P. 1978., Biological control of insect pests by entomogenous fungi. *Annu. Rev.*
504 *Entomol.* 23:409-442.

505 Fischer, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L.,
506 Gurr, S.J., 2012. Emerging fungal threats to animal, plant and ecosystem health.
507 Nature 484:186–194.

508 Fox, J., Hong, J., 2009. Effect Displays in R for Multinomial and Proportional-Odds
509 Logit Models: Extensions to the effects Package. Journal of Statistical Software, 32(1),
510 1-24. URL <http://www.jstatsoft.org/v32/i01/>.

511 Gao B., Zhu S.Y., 2008. Differential potency of drosomycin to *Neurospora crassa* and
512 its mutant: implications for evolutionary relationship between defensins from insects
513 and plants. Insect Mol. Biol. 17:405–411.

514 Gillespie, J.P., Bailey, A.M., Cobb, B., Vilcinskas, A., 2000. Fungi as elicitors of insect
515 immune responses. Arch. Insect Biochem. Physiol. 44:49-68.

516 Hajek, A.E., St. Leger R.J., 1994. Interactions between fungal pathogens and insect
517 hosts. Annu. Rev. Entomol. 39:293-322.

518 Hajek, A.E., Meyling, N.V., 2018. Ecology of invertebrate pathogens: Fungi. In:
519 Ecology of Invertebrate Diseases (Eds. Hajek AE & Shapiro-Ilan D) John Wiley &
520 Sons Ltd. ISBN: 978-1-119-25607-6.

521 Hamilton, C., Bulmer, M.S., 2012. Molecular antifungal defenses in subterranean
522 termites: RNA interference reveals in vivo roles of termicins and GNBPs against a
523 naturally encountered pathogen. Dev. Comp. Immunol. 36:372-377. doi:
524 10.1016/j.dci.2011.07.008.

- 525 Hancock, R.E., Diamond, G., 2000. The role of cationic antimicrobial peptides in innate
526 host defences. *Trends Microbiol.* 8:402-410.
- 527 Hesketh, H., Roy, H.E., Eilenberg, J., Pell, J.K., Hails, R.S., 2010. Challenges in
528 modelling complexity of fungal entomopathogens in semi-natural populations of
529 insects. *BioControl* 55:55-73.
- 530 Iijima, R., Kurata, S., Natori, S., 1993. Purification, characterization, and cDNA
531 cloning of an antifungal protein from the hemolymph of *Sarcophaga peregrina* (flesh
532 fly) larvae. *J. Biol. Chem.* 268:12055-12061.
- 533 Inglis, G.D., Goettel, M.S., Butt, T.M., Strasser, H., 2001. Use of Hyphomycetous fungi
534 for managing insect pests. In: Butt T.M., Jackson C., Magan N. (Eds.) *Fungi as*
535 *biological control agents: progress, problems and potential*. CABI Publishing, Oxon, pp
536 23–69.
- 537 Johnston, P.R., Makarova, O., Rolff, J., 2014. Inducible defenses stay up late: temporal
538 patterns of immune gene expression in *Tenebrio molitor*. *G3* 4:947-955.
- 539 Johnston, P.R., Rolff, J., 2015. Host and Symbiont Jointly Control Gut Microbiota
540 during Complete Metamorphosis. *PloS Pathog.* 11:e1005246. Doi:
541 10.1371/journal.ppat.1005246.
- 542 Joop, G., Vilcinskas, A., 2016. Coevolution of parasitic fungi and insect hosts. *Zoology*
543 119:350-358.

544 Kim, D.H., Lee, Y.J., Lee, Y.J., Chung, J.H., Lee, B.L., Choi, B.S., Lee, Y., 1998.
545 Bacterial expression of Tenecin 3, an insect antifungal protein isolated from *Tenebrio*
546 *molitor*, and its efficient purification. Mol. Cells 8:786-789.

547 Kim, D.H., Lee, D.G., Kim, K.L., Lee, Y., 2001. Internalization of Tenecin 3 by a fungal
548 cellular process is essential for its fungicidal effect on *Candida albicans*. Eur. J.
549 Biochem. 268:4449-4458.

550 Kirkland, B.H., Westwood, G.S., Keyhani, N.O., 2004. Pathogenicity of
551 Entomopathogenic Fungi *Beauveria bassiana* and *Metarhizium anisopliae* to Ixodidae
552 Tick Species *Dermacentor variabilis*, *Rhipicephalus sanguineus*, and *Ixodes scapularis*.
553 J. Med. Entomol. 41:705-711.

554 Lacey, L.A., Grzywacz, D., Sapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel,
555 M.S., 2015. Insect pathogens as biological control agents: back to the future. J.
556 Invertebr. Pathol. 132:1-41.

557 Lamberty, M., Caille, A., Landon, C., Tassin-Moindrot, S., Hetru, C., Bulet, P.,
558 Vovelle, F., 2001a. Solution structures of the antifungal heliomicin and a selected
559 variant with both antibacterial and antifungal activities. Biochemistry 40:11995-2003.

560 Lamberty, M., Zachary, D., Lanot, R., Bordereau, C., Robert, A., Hoffmann, J.A.,
561 Bulet, P., 2001b. Insect immunity. Constitutive expression of a cysteine-rich
562 antifungal and a linear antibacterial peptide in a termite insect. J. Biol. Chem.
563 276:4085-4092.

564 Lee, Y.J., Chung, T.J., Park, C.W., Hahn, Y., Chung, J.H., Lee, B.L., Han, D.M., Jung,
565 Y.H., Kim, S., Lee, Y., 1996. Structure and expression of the tenecin 3 gene in
566 *Tenebrio molitor*. Biochem. Biophys. Res. Commun. 218:6-11.

567 Lemaître, B., Reichhart, J.M., Hoffmann, J.A., 1997. *Drosophila* host defense:
568 differential induction of antimicrobial peptide genes after infection by various classes
569 of microorganisms. Proc. Natl. Acad. Sci. USA. 94:14614-14619.

570 Levashina, E.A., Ohresser, S., Bulet, P., Reichart, J.M., Hetru, C., Hoffmann, J.A.,
571 1995. Metchnikowin, a novel immune-inducible proline-rich peptide from *Drosophila*
572 with antibacterial and antifungal properties. Eur. J. Biochem. 233:694-700.

573 Li, Y., Zhao, P., Liu, S., Dong, Z., Chen, J., Xiang, Z., Xia, Q., 2012. A novel protease
574 inhibitor in *Bombyx mori* is involved in defense against *Beauveria bassiana*. Insect
575 Biochem. Mol.Biol. 42:766-775.

576 Liu, Z., Yuan, K., Zhang, R., Ren, X., Liu, X., Zhao, S., Wang, D., 2016. Cloning and
577 purification of the first termicin-like peptide from the cockroach *Eupolyphaga sinensis*.
578 J. Venom Anim. Toxins Incl. Trop. Dis. 22:5

579 Lopes, R.B., Laumann, R.A., Blassioli-Moraes, M.C., Borges, M., Faria, M., 2015. The
580 fungistatic and fungicidal effects of volatiles from metathoracic glands of soybean-
581 attacking stink bugs (Heteroptera: Pentatomidae) on the entomopathogen *Beauveria*
582 *bassiana*. J. Invertebr. Pathol. 132:77-85.

- 583 Lord, J.C., Anderson, S., Stanley, D.W., 2002. Eicosanoids mediate *Manduca sexta*
584 cellular response to the fungal pathogen *Beauveria bassiana*: a role for the
585 lipoxygenase pathway. Arch. Insect Biochem. Physiol. 51:46-54.
- 586 Lu, D., Geng, T., Hou, C., Huang, Y., Qin, G., Guo, X., 2016. *Bombyx mori* cecropin A
587 has a high antifungal activity to entomopathogenic fungus *Beauveria bassiana*. Gene.
588 583:29-35.
- 589 Makarova, O., Rodríguez-Rojas, A., Ervaci, M., Weise, C., Dobson, A., Johnston, P.R.,
590 Rolff, J., 2016. Antimicrobial defence and persistent infection in insects revisited.
591 Philos. Trans. R. Soc. Lond. B Biol. Sci. 371 (1965).
- 592 Marti, G.A., Scorsetti, A.C., Siri, A., Lastra, C.C., 2005. Isolation of *Beauveria*
593 *bassiana* (Bals.) Vuill. (Deuteromycotina: Hyphomycetes) from the Chagas disease
594 vector, *Triatoma infestans* (Hemiptera: Reduviidae) in Argentina. Mycopathologia
595 159:389-391.
- 596 Meyling, N.V., Thorup-Kristensen, K., Eilenberg, J., 2011. Below- and aboveground
597 abundance and distribution of fungal entomopathogens in experimental conventional
598 and organic cropping systems. Biol. Control 59: 180-186.
- 599 Miller, S.C., Miyata, K., Brown, S.J., Tomoyasu, Y., 2012. Dissecting systeminc RNA
600 interference in the red flour beetle *Tribolium castaneum*: parameters affecting the
601 efficiency of RNAi. PLoS One 7:e47431. doi: 10.1371/journal.pone.0047431.

602 Mylonaki, E., Podsiadlowski, L., Muhammed, M., Vilcinskas, A., 2016. Diversity,
603 evolution and medical applications of insect antimicrobial peptides. *Philos. Trans. R.*
604 *Soc. Lond. B Biol. Sci.* 371:20150290.

605 Ortiz-Urquiza, A., Keyhani, N.O., 2013. Action on the Surface: Entomopathogenic
606 Fungi versus the Insect Cuticle. *Insects* 4:357-374.

607 Pedrini, N., Ortiz-Urquiza, A., Huarte-Bonnet, C., Zhang, S., Keyhani, N.O., 2013.
608 Targeting of insect epicuticular lipids by the entomopathogenic fungus *Beauveria*
609 *bassiana*: hydrocarbon oxidation within the context of a host-pathogen interaction.
610 *Front. Microbiol.* 15:4-24.

611 Peschel, A., Sahl, H.G., 2006. The co-e-evolution of host cationic antimicrobial peptides
612 and microbial resistance. *Nat. Rev. Microbiol.* 4:529-536.

613 Poulsen, M., Bot, A.N., Nielsen, M.G., Boomsma, J.J., 2002. Experimental evidence for
614 the costs and hygienic significance of the antibiotic metapleural gland secretion in
615 leaf-cutting ants. *Behav. Ecol. Sociobiol.* 52:151-157.

616 R Development Core Team (2008). R: A language and environment for statistical
617 computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-
618 07-0, URL <http://www.R-project.org>.

619 Samuels, R.I., Charnley, A.K., Reynolds, S.E., 1988. The role of destruxins in the
620 pathogenicity of 3 strains of *Metarhizium anisopliae* for the tobacco hornworm
621 *Manduca sexta*. *Mycopathologia* 104:51-58.

622 Schmid-Hempel, P., 2005. Evolutionary ecology of insect immune defenses. *Annu. Rev.*
623 *Entomol.* 50:529-551.

624 Schuhmann, B., Seitz, V., Vilcinskas, A., Podsiadlowski, L., 2003. Cloning and
625 expression of gallerimycin, an antifungal peptide expressed in immune response of
626 greater wax moth larvae, *Galleria mellonella*. *Arch. Insect Biochem. Physiol.* 53:125-
627 133.

628 Souhail, A., Hiromasa, Y., Rahnamaeian, M., Giraldo, M.C., Takahashi, D., Valent, B.,
629 Vilcinskas, A., Kanost, M.R., 2016. Characterization and regulation of expression of an
630 antifungal peptide from hemolymph of an insect, *Manduca sexta*. *Dev. Comp.*
631 *Immunol.* 61:258-268.

632 Therneau, T., 2015. A Package for Survival Analysis in S. version 2.38,
633 <https://survival>.

634 Tian, C., Gao., B., Rodriguez Mdel, C., Lanz-Mendoza, H., Ma, B., Zhu, S., 2008. Gene
635 expression, antiparasitic activity, and functional evolution of the drosomycin family.
636 *Mol Immunol* 45:3909–3916.

637 Tzou, P., Reichhart, J.M., Lemaître, B., 2002. Constitutive expression of a single
638 antimicrobial peptide can restore wild-type resistance to infection in immunodeficient
639 *Drosophila* mutants. *Proc. Natl. Acad. Sci. USA.* 99:2152:2157.

640 Valero-Jiménez, C.A., Wieggers, H., Zwaan, B.J., Koenraadt, C.J., van Kan, J.A., 2016.
641 Genes involved in virulence of the entomopathogenic fungus *Beauveria bassiana*. J.
642 Invertebr. Pathol. 133:41-49.

643 Venables, W. N. & Ripley, B. D., 2002. Modern Applied Statistics with S. Fourth
644 Edition. Springer, New York. ISBN 0-387-95457-0.

645 Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag
646 New York.

647 Yang, W.Y., Wen, S.Y., Huang, Y.D., Ye, M.Q., Deng ,X.J., Han, D., Xia, Q.Y., Cao, Y.,
648 2006. Functional divergence of six isoforms of antifungal peptide Drosomycin in
649 *Drosophila melanogaster*. Gene 379:26-32.

650 Yang, Y.T., Lee, M.R., Lee, S.J., Kim, S., Nai, Y.S., Kim, J.S., 2017. *Tenebrio molitor*
651 Gram-negative-binding protein 3 (TmGNBP3) is essential for inducing downstream
652 antifungal Tenecin 1 gene expression against infection with *Beauveria bassiana* JEF-
653 007. Insect Sci. doi: 10.1111/1744-7917.12482.

654 Yokoi, K., Koyama, H., Minakuchi, C., Tanaka, T., Miura, K., 2012. Antimicrobial
655 peptide gene induction, involvement of Toll and IMD pathways and defense against
656 bacteria in the red flour beetle, *Tribolium castaneum*. Results Immunol. 2:72-82.

657 Yuan, Y., Gao, B., Zhu, S., 2007. Functional expression of a *Drosophila* antifungal
658 peptide in *Escherischia coli*. Protein Expr. Purif. 52:457-462.

659 Zanchi, C., Johnston, P.R., Rolff, J., 2017. Evolution of defence cocktails: Antimicrobial
660 peptide combinations reduce mortality and persistent infection. *Mol. Ecol.*
661 26:5334:5343.

662 Zasloff, M., 2002. Antimicrobial peptides of multicellular organisms. *Nature* 415:389-
663 395.

664 Zhang., Zhu, S.Y., 2009. Drosomycin, an essential component of antifungal defence in
665 *Drosophila*. *Insect Mol. Biol.* 18:549-556.