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

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1. Introduction

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

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

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

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

However, insects also possess a battery of antimicrobial peptides (AMPs), small cationic peptides showing a relatively broad spectrum of antimicrobial activity ranging from Gram positive and/or negative bacteria, protozoans, viruses, and fungi (Bulet et 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 ; Fehlbaum 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 ; Fehlbaum 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 coleoptericin 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.

2. Materials and Methods

2.1. Insect rearing

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

2.2. Fungal cultures and conidia suspensions

A *Beauveria bassiana* strain (KVL 03-144) isolated from a natural infection of *Leptopterna dolobrata* (Homoptera: Miridae) from an agreoecosystem in Denmark (Meyling et al., 2009) was kept at -80˚C in a culture collection at the University of Copenhagen before cultivation. Isolates were cultivated on quarter-strength Sabouraud Dextrose Agar + Yeast 10 % (SDAY) and incubated for 10 days at 23°C to allow for sporulation. Conidia were collected by scrapping the surface of the culture with a sterile loop and transferred in 1 ml PBS with 0.05% of Triton-X. The resulting 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 µL 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%.

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 *tenencin 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

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

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: $1x10^5$, $5x10^5$ and $1x10^6$ /ml, corresponding to 50, 250, 500 conidia applied per
184 beetle, since previous laboratory experiments showed that these concentrations span
185 the LC50 ($3x10^5$ 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 $5x10^5$ 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.

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 Dextose 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

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

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

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

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

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 “coxzph” 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

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

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

3. Results

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

There was no effect of the concentration of conidia applied on the beetles for infection 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 $5x10^5$ 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

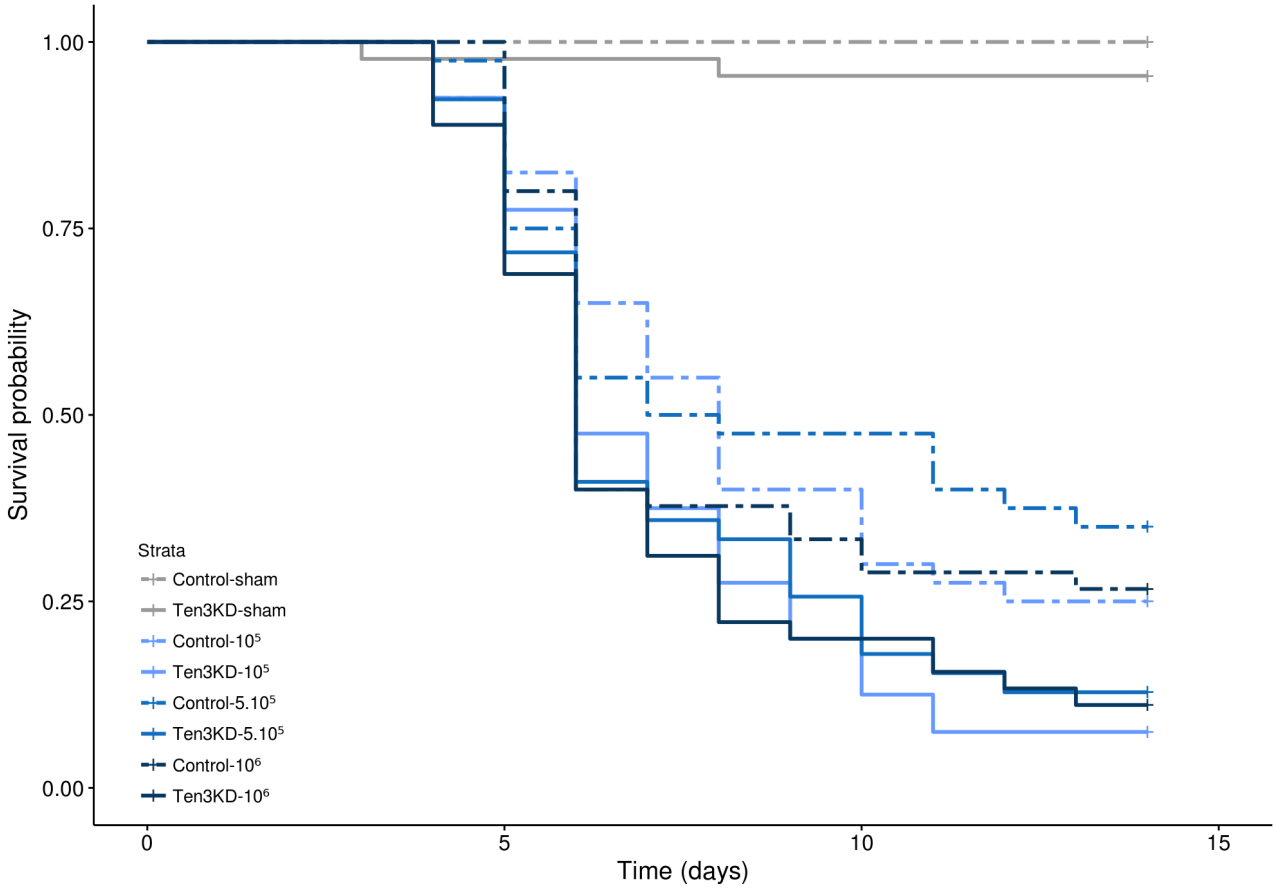

Figure 1 : Tenecin 3 improves the survival of *Tenebrio molitor* to *Beauveria bassiana*. Kaplan-Meier curve showing the survival over 14 days of the control (dashed lines) and Tenecin 3 knock-down (solid lines) beetles either sham (in grey) or exposed to *Beauveria bassiana* (in blue, lighter to darker shades indicating lower to higher concentrations : light blue = 10^5 conidia/mL ; medium blue = 5.10^5 conidia/mL ; dark blue = 10^6 conidia/mL). Only the knock-down treatment had a significant effect on survival of the beetles (dashed vs. solid lines, $X^2_{2,247} = 8.13$; $p = 0.017$).

3.2. Progression of the infection in the beetle host.

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

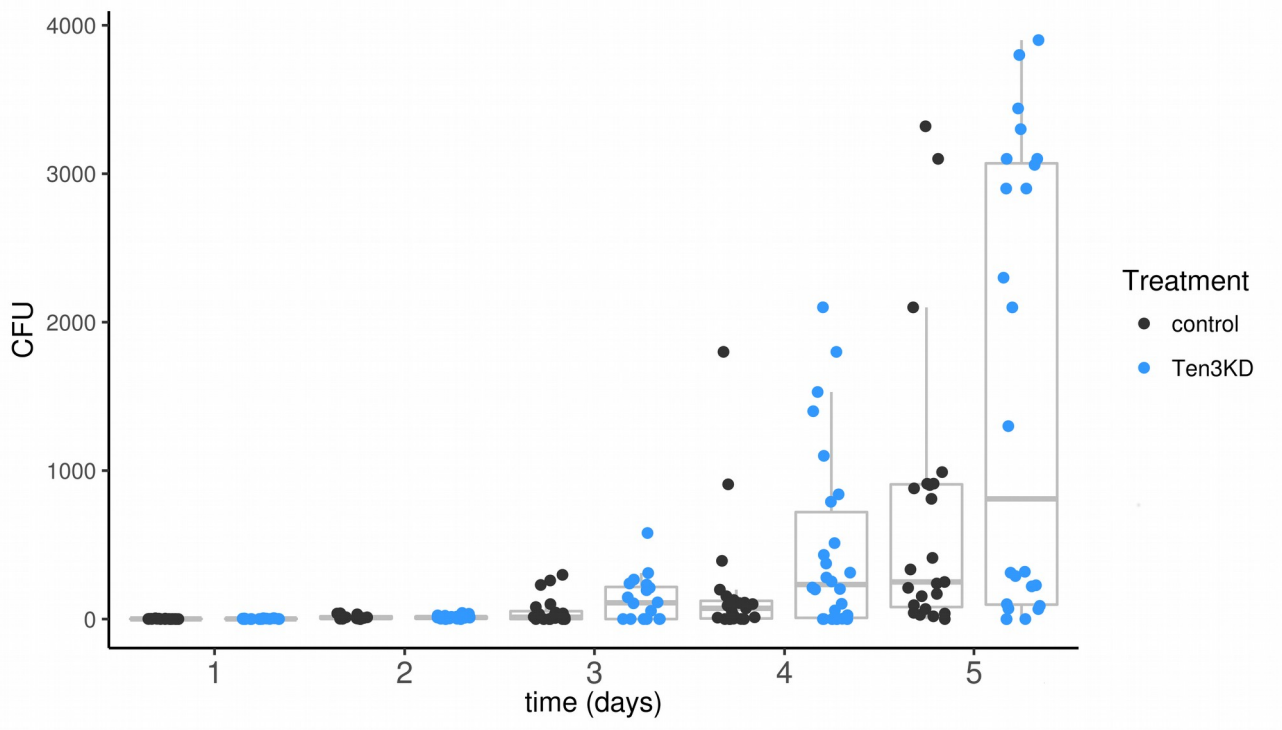

Figure 2 : Tenecin 3 decreases the fungal load of *Beauveria bassiana* in the hemolymph of *Tenebrio molitor*. Box-dotplot representing the number of CFU of *B. bassiana* recovered from the hemolymph of infected beetles according to the time after infection. Each dot represents a data point of control (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.

Moreover, an interesting pattern emerges from the distribution of our data. The quantity of hyphal bodies present in the beetles seems to evolve over time towards a

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.

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

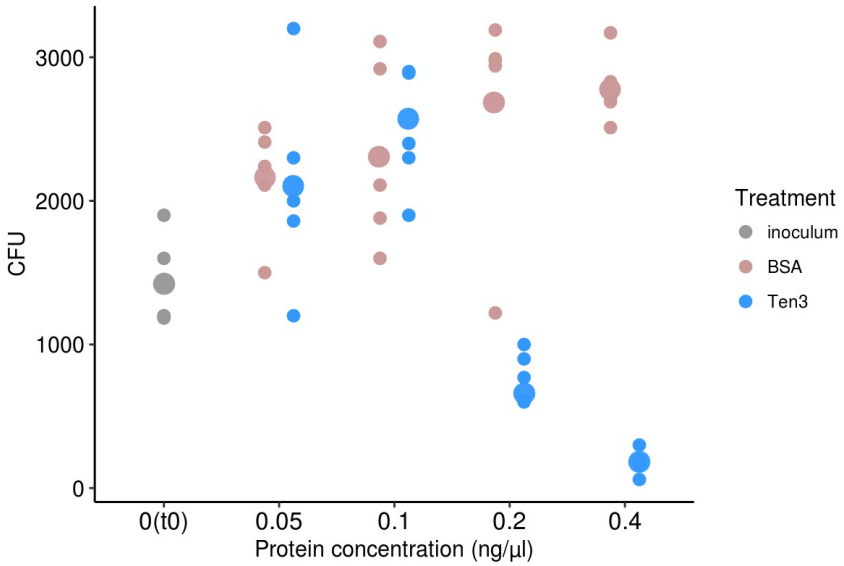

Figure 3 : Recombinant Tenecin 3 has an antifungal activity against the blastospores of *Beauveria bassiana in vitro*. Dotplot representing the number of CFU retrieved after *in vitro* incubation of blastospores for 6 hours with either Bovine Serum Albumin (BSA, in pink) or recombinant Tenecin 3 (in blue). Five replicates were performed, each dot represents a data point and the thicker dot represents the 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

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445

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