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# Evolution of defence cocktails: antimicrobial peptide combinations reduce mortality and persistent infection

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**Abstract**

The simultaneous expression of costly immune effectors such as multiple antimicrobial peptides is a hallmark of innate immunity of multicellular organisms, yet the adaptive advantage remains unresolved. Here we test current hypotheses on the evolution of such defence cocktails. We use RNAi gene knock-down to explore, the effects of three highly-expressed antimicrobial peptides, displaying different degrees of activity *in vitro*

against *Staphylococcus aureus,* during an infection in the beetle *Tenebrio molitor*. We find that a defensin confers no survival benefit, but reduces bacterial loads. A coleoptericin contributes to host survival without affecting bacterial loads. An attacin has no individual effect. Simultaneous knock-down of the defensin with the other AMPs results in increased mortality and elevated bacterial loads. Contrary to common expectations, the effects on host survival and bacterial load can be independent. The expression of multiple AMPs increases host survival, and contributes to the control of persisting infections and tolerance. This is an emerging property that explains the adaptive benefit of defence cocktails.

**Introduction**

The timing and magnitude of an immune response is under selection to minimize fitness costs of infections and costs of immune defence (Schmid-Hempel 2005; Schulenburg *et al.* 2009; Cressler *et al.* 2014). During an immune response, several immune effectors are simultaneously or sequentially expressed (Dangl & Jones 2001; Kounatidis & Ligoxygakis 2012; Mills *et al.* 2015). Hence pathogens simultaneously and sequentially encounter several immune effectors during the course of an infection.

A prominent feature of the insect immune defence is the synthesis and release of multiple antimicrobial peptides (AMPs) (Bulet & Stöcklin 2005; Rolff and Schmid-Hempel. 2016). Such defence cocktails often contain a high abundance of AMPs that are not known to be effective against the infectious agent, (Yokoi *et al.* 2012; Barribeau *et al.* 2014; Makarova *et al.* 2016), despite the capacity of the insect immune system to mount specific immune responses (Kounatidis and Ligoxygakis 2012) . Also, the expression of AMPs comes at a metabolic cost (Poulsen *et al.* 2002; Johnston *et al.* 2015).

To explain this *a priori* non-adaptive response to infections, it has been proposed that

the simultaneous action of several AMP classes makes bacterial killing more efficient (Lazzaro 2008). *In vitro* studies have highlighted that the susceptibility of microorganisms is greater to combinations than to individual AMPs (Rahnamaeian *et al.* 2015; Marxer *et al.* 2016; Yu *et al.* 2016) lending support to this hypothesis. This adaptive hypothesis has not been tested in the natural environment of pathogens, the host.

In the mealworm beetle *Tenebrio molitor,* experimental infection with *S. aureus causes* upregulation of AMP gene expression for at least seven days (Johnston *et al.* 2013) and AMPs are present at elevated levels for at least 21 days in the haemolymph (Makarova *et al.* 2016). These highly expressed AMPs include the defensin Tenecin 1, which is active against *S. aureus in vitro (Moon & Al 1994),* the coleoptericin Tenecin 2, not active against *S. aureus* (Roh *et al.* 2009) (O. Makarova, J. Rolff unpublished) and the attacin Tenecin 4 (Chae *et al.* 2011), which shows high activity against *E. coli* and weak activity against *S. aureus in vitro*.

Here, we remove components of a naturally selected defence cocktail and ask how this changes host survival and bacterial persistence within the host. We use a gene knock-down approach in the mealworm beetle *T. molitor* to investigate the temporal dynamics, and interactions of a potent defensin and the nature of it's interactions with two other AMPs. We used *Staphylococcus aureus* as our model bacterium, an opportunistic pathogen that causes persistent infections following experimental inoculation in *T. molitor* (Haine *et al.* 2008).

We (a) assessed the effect of AMP knock-downs on the survival of the beetles, and (b) on the survival of bacteria within the beetles over a time course of 14 days. We first knocked down the expression of the abundant defensin Tenecin 1 which is highly active against *S. aureus in vitro*. We then knocked down Tenecin 1 in combination with either

the coleoptericin Tenecin 2 or the attacin Tenecin 4. Finally, we performed single knock-downs of Tenecin 2 and Tenecin 4 to explore their contributions to the combined effects.

## Material and methods

*Insect rearing*

*Tenebrio molitor* larvae were bought from a supplier and maintained in plastic boxes (18x18x8 cm) in the dark at 25°C and at a density of 500 beetles in 400g of wheat bran, rat chow, and fed every other day with pieces of apples. Every second day all pupae were collected and sexed. Only the females were kept for further experiments to reduce variation caused by sexual dimorphism in immune function (Rolff *et al.* 2005). After emergence, the females were kept individually in grid boxes filled with wheat bran and a piece of filter paper and were supplied every second day with a piece of apple. All experimental treatments were performed 9 to 11 days after emergence to ensure sexual maturity. Only female beetles within a weight range from 0. 120 g to 0. 165 g were used. The survival experiments were carried in parallel and included the same cohorts of beetles as the assessment of the bacterial load in the hemolymph. The layout of the experiments is illustrated in the Supporting Information S1.

*Gene knock-down by RNA interference*

Double-stranded RNA injection was used to knock-down gene expression by RNA interference, previously shown to be efficient in *Tribolium* and *T. molitor (Fabrick et al. 2009; Miller et al. 2012)*.

As template for dsRNA synthesis we used synthetic constructs (the full sequences and primers are presented in the Supporting Information S2). For procedural control we used RNAi based on *Galleria mellonella* lysozyme cDNA, which has no homology of sequence with any known gene of *T. molitor (Johnston & Rolff 2015)*. We amplified these templates 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 by running it 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 manufacturers 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 $CaCl_2$, 1.3 mM KCl, 2.3 mM $NaHCO_3$). Before being used, the RNA was annealed by heating it up at 90°C and allow it to slowly cool down, in order to obtain dsRNA. In the case of the knock-down of single genes, we injected 500 ng of dsRNA at a concentration of 100 ng/µl in 5µL of insect Ringer solution. In the case of double knock-downs, we injected the same absolute quantity of dsRNA (100µg/µL) for each gene in the same total volume of Ringer, resulting in a concentration of 200ng/µl of dsRNA in 5 µL of Ringer solution and an absolute quantity of 1000 ng of dsRNA. The procedural control received an injection of *Galleria mellonella* lysozyme dsRNA, at the same concentration as in the corresponding experimental groups, therefore the two groups differ whether they belong to the single or double knock-down experiment. In the single knock-down experiments they are called „single-dose control“ whereas they are called „double-dose control“ in the double knock-down experiment. We adjusted the concentration of the dsRNA by diluting it in insect Ringer solution after annealing, and stored this solution at -80°C until the time of

injection. The beetles were chilled on ice before injection of dsRNA into the pleural under the right elytra using a pulled glass capillary. Care was taken to insert the needle under the pleural membrane parallel to the antero-posterior axis of the beetles to avoid piercing internal organs. While the single and double knock-down experiments required separate controls to ensure the same dosage per gene across treatments (see above), they were set up in a parallel fashion to avoid any temporal or population stock fluctuations that could be possibly confounding.

We checked that the gene knock-down was efficient by qPCR. The details and a dicussion on variation in efficiency are given in the supplemental information (Supporting Information S2 figures S1-S12).

*Bacterial culture and injection*

We use the strain JLA 513 of *Staphylococcus aureus*, derived from SH 1000 strain (Needham *et al.* 2004) and containing a Tetracyclin-resistance cassette inserted into its chromosome, which allows us to use selective media in our experiments. In both solid and liquid media, we used Tetracyclin at 5 µg/mL (Sigma).

A frozen stock of *S. aureus* was plated onto LB agar and incubated at 30°C for three days. For each batch of infection, we transferred 3 randomly selected colonies from this plate into LB medium and incubated them overnight at 25°C with shaking, until the optical density of the culture reached $OD_{600}$ = 0.95, which corresponds to approximately $10^9$ CFU/mL. The bacterial suspension was then washed in insect Ringer solution three times, and 5 µl of the resulting suspension were injected into each beetle. Each beetle received an injection of approximately $4.10^6$ CFU which was confirmed by dilution

plating of the inoculum at the time of injection. We chose this inoculum size in order to maximize the probability of detecting CFUs at later time points, and we have shown previously that the magnitude of AMP upregulation in *T. molitor* does not differ between beetles injected with $10^4$ or $10^7$ CFU (Dorling *et al.* 2015). The injections were performed similarly to the dsRNA injections but under the left elytra, to avoid trapping the bacteria in a potential clot resulting from the wound-repair process (Karlsson *et al.* 2004). Any beetle from which haemolymph leaked out was discarded from the experiment. The beetles were then returned to the standard rearing conditions. Beetles received the bacterial injection three days after dsRNA injection.

We assessed the survival of the beetles infected with *S. aureus*, and the survival of *S. aureus* in the beetle host. Hemolymph was flushed out of beetles at various time points after infection. Since it was a time consuming process, we chose to recover the hemolymph of infected beetles with *tenecin 1* gene expression knocked down, then of *tenecin 1* and *tenecin 2* or *tenecin 1* and *tenecin 4* gene expression knocked down over the full time course. This allowed us to identify the time points for which the knock-down treatments were different compared to control and the double knock-down treatments different from the single knock-down. We then focused on these time points to sample *tenecin 2* or *tenecin 4* knock-down beetles, in order to highlight interactions between Tenecin 1 and these latter AMPs.

*Survival experiments*

The survival of *S. aureus*-injected versus Ringer-injected experimental groups was recorded every second day for 35 days following injection. There were three sets of experiments in which one or two AMP genes were knocked down. In each of these sets

we included a group of full control beetles which received no dsRNA injection. This group is shared between the double knock-down groups and the single knock-down groups of tenecin 2 and tenecin 4, since they were performed simultaneously. The procedural control group however is different according to whether it is the procedural control of the single or double knock-down experiments, in order to account for the different quantity of dsRNA injected in the case of the single or double knock-downs (see above).

The first single knock-down experiment consisted of a full control, a single-dose control, and a *tenecin 1* knock-down (Ten1KD) treatment. The double knock-down experiment consisted of a full control, a double-dose control, a *tenecin 1* and *tenecin 2* knock-down (Ten1Ten2KD) treatment, and a *tenecin 1* and *tenecin 4* knock-down (Ten1Ten4KD) treatment. In parallel to this double knock-down experiment we ran two single knock-down experiments of tenecin 2 (Ten2KD) and tenecin 4 (Ten4KD), to check for the single effects of these two AMPs, alongside their corresponding single-dose control. Each of these groups was split into infected (*S. aureus* injected) or non-infected (Ringer injected) beetles. The final sample sizes are displayed in the Supporting Information S1 table S1.

*Bacterial load assessment*

The hemolymph of control and knock-down beetles was flushed out at different time points following *S. aureus* injection : 30 minutes, 6 hours, 1 day, 3, 5, 7 and 14 days. Beetles were sedated on ice in a glass tube containing a piece of cotton soaked in ethyl acetate for 10 minutes. They were then cleaned with 70 % ethanol to avoid any fungal or bacteria contamination from the cuticle. An incision at the end of the abdomen above the genitalia was performed with a scalpel, and a syringe loaded with 500 µl of

phosphate buffered saline (PBS) was inserted in the unsclerotised part between the head and prothorax. The content of the syringe was injected and the resulting solution harvested in a microcentrifuge tube. This solution was then diluted, depending on the concentrations of *S. aureus* expected to be recovered before being plated on selective LB agar. The plates were incubated for 48h at 30◦C, after which the number of colony forming units (CFU) were counted. Two dilutions per beetle were usually countable and were used as replicates. This experiment required more batches of beetles than the survival experiment, and the injection and recovery process was spread through time, in order to represent all batches in a given time point. The batches used in the survival experiments make up at least a third of the sample size of each treatment and at each time point in the bacterial load experiment.

Some beetles were already dead at the sampled time point or obviously moribund. The collection of their hemolymph confirmed that they contained a very high concentration of *S. aureus*. It is possible that such a high concentration could have been responsible for the death of the beetle. However, it is also possible that *S. aureus* could have been allowed to reach such high densities because of the death/weakening of the host. Since we could not disentangle these effects, we decided not to include such beetles in our results.

Additionally, we flushed the hemolymph out of 1 Ringer injected single or double dose control and 1 Ringer injected KD beetle per flushing session: none of these samples yielded any colonies after plating. The final sample sizes are displayed in Supporting Information S1 table S2.

*Statistical analysis*

All data were analysed using R (Team 2008). Each model fit was checked using the « visreg » package (Breheny & Burchett 2013), in addition to checking for the distribution of the residuals.

In the survival experiments, we tested whether the infection and the knock-down treatment, as well as the interaction between them, explained the mortality of the beetles over 35 days. The hazards of the survival data were not proportional (checked with the « coxzph » function) and were thus analyzed with the « survreg » function of the « survival » package (Therneau 1999) using a t-distribution. Details of the statitstical testing for the differences between groups by comparing the estimates and p-values of each treatment to the intercept is summarized in the Supporting Information S3 tables S1, S3 and S5.

The bacterial loads from the beetles of the bacterial load assessment experiments were analysed using the « nlme » package (Pinheiro *et al.* 2013). We tested whether the number of colony forming units of *S. aureus* recovered from the beetles were explained by time and knock-down treatment, or their interaction. Because of the high dispersion of the data, the best fit was obtained after log-transformation of the number of CFU and by accounting for the heteroscedasticity of variances between each treatment with a Linear Model (LM) using the Generalized Least Squares method (« gls » function). In each case, the AICs (Akaike's Information Criterion) of all models possible including the null model were compared, and the model with the lowest AIC was used (Akaike 1976). Post-hoc comparisons can be performed by comparing the 95 % confidence intervals around the estimates of the models fitted to the bacterial load data: significant differences are detected when the 95 CI do not overlap on more than half of their length,

as advised by (Cumming 2013). The post hoc plots are presented in the Supporting Information S3 figures S1 and S2.

Since the single and double knock-down experiments each required a different procedural control, they could not be analysed together. However, effect size calculations are a reliable way to compare data across experiments (Cumming 2013). We used effect size comparisons in order to detect additive or non additive effects in the knock-down of several AMPs compared to single ones. To this end, we compared the effects of different experimental treatments compared to their respective controls across experiments. As effect size measurements we used odds ratios in the case of survival data, and Cohen's d for bacterial count data (Nakagawa & Cuthill 2007). Cohen's d can be split into three categories representing small (d = 0.2), medium (d = 0.5) and large (d = 0.8) effects. Similarly, odds ratios can reflect either small (OR = 1.68), medium (OR = 3.47) or large (OR = 6.71) effects (Nakagawa & Cuthill 2007).

**Results**

*Knock-down of a defensin does not influence host survival but influences the bacterial load*

We first followed the survival of Tenecin-1-knock-down versus control beetles for 35 days post injection. The knock-down (KD) of Tenecin 1 (Ten1KD) did not reduce survival compared to both single dose control and control (KD treatment*infection: Deviance = 2.25 ; p = 0.33 ; df = 2 , 130: KD treatment : Deviance = 1.40 ; p = 0.5 ; df = 2 , 133, Fig. 1a). *S. aureus* infection significantly decreased survival (Deviance = 15.53 ; p = $8.11e^{-5}$ ; df = 2 , 132, see Supporting Information S3 table S1 for post-hoc comparisons).

In single dose control beetles, bacterial load remained stable between 30 minutes and 1 day post infection (Fig. 1b) and then decreased. Bacterial loads in Ten1KD beetles followed the same pattern, but were significantly higher at day 7 compared to single dose control, and converged to the same level at day 14 (time*treatment : $F_{13,248}$ = 24.76 ; $p < 0.0001$. See Supporting Information S3 Figure S1 for post-hoc comparisons).

In Ten1KD beetles the dispersion of bacterial loads is significantly higher at day 7 (Fligner-Killeen test of homogeneity of variances : med $X^2$ = 9.88 ; p = 0.0017, Supporting Information S3 Table S2). This dispersion may reflect the variance in knock-down efficiency, but this is unlikely since the variance in the relative expression of tenecin 1 is higher in single dose controls than in Ten1KD beetles (Supporting Information S2 figure S1). This differential dispersion likely reflects that Ten1KD removes a constraint on the progression of the infection.

*Simultaneous knock-down of two AMPs decreases survival and increases bacterial load*

Infected beetles with simultaneous knock-downs of Tenecin 1 and the coleoptericin Tenecin 2 (Ten1Ten2KD) or Tenecin 1 and the attacin Tenecin 4 (Ten1Ten4KD) show a very similar decrease in survival compared to double dose controls, with high mortalities between days 3-5 (KD treatment*infection : Deviance = 51.57 ; p = $7.08e^{-9}$ ; df = 7 , 192, Fig. 2a, Supporting Information S3 Table S3).

The bacterial loads displayed different temporal patterns in double dose control compared to Ten1Ten2KD and Ten1Ten4KD beetles (time*treatment : $F_{20,342}$ = 16.27 ; $p < 0.0001$, Fig. 2b), and differed at both days 7 and 14 (Supporting Information S3 Figure S2). The higher mortality observed on days 3-5 post infection in both knock-down treatments is not linked to an increase in mean bacterial load at day 3. The dispersion of bacterial loads is significantly higher in the knock-down treatments (Ten1Ten2KD vs

control : $X^2 = 14.28$ ; $p = 0.0002$ ; Ten1Ten4KD vs control $X^2 = 27.55$ ; $p < 0.0001$). In the Ten1Ten2KD this difference is only significant 14 days post infection (Supporting Information S3 Table S4). In the Ten1Ten4 KD, the difference is significant at 3 but not 5 days after infection. This indirectly suggests that the absence of increase in the mean bacterial load between Ten1Ten4KD and control could be due to a culling of the highest bacterial loads between these time points.

*The contributions of the coleoptericin and the attacin*

Given the result that the Ten1KD, i.e. the knock-down of a highly potent defensin, did not result in a survival cost, but the combination knock-downs did, we explored the effects of single Ten2KD and Ten4KD on host survival. We focussed on 3, 7 and 14 days post infection (KD treatment*infection : Deviance = 35 ; $p = 1.11e^{-5}$ ; df = 7,214). Only the Ten2KD treatment caused a significant reduction in survival of infected beetles (Fig. 3a, Supporting Information S3 table S5). Mortality occurred between days 3-5.

There was no effect of the knock-down treatment on bacterial loads either at 3, 7 or 14 days following infection (Fig. 3b), either in interaction with time (time*treatment : $F_{8,134} = 1.45$ ; $p = 0.24$) or alone (treatment : $F_{2,132} = 0.43$ ; $p = 0.65$). Only time post infection affected the bacterial load (time : $F_{2,132} = -6.98$ ; $p < 0.0001$).

*Quantifying the effects of AMP knock-downs across experiments*

We used effect sizes (Nakagawa & Cuthill 2007) to compare and quantify the strength of the biological effects across experiments that each have a different control (see methods), We used odds ratios to compare survival data and Cohen's d for bacterial loads. Ten1KD and Ten4KD result in much smaller effects on host survival post infection (Figure 1A, 3A, odds ratio Ten1KD = 2.46, Ten4KD = 1) than Ten1Ten4KD

(odds ratio = 9.75, Figure 2A). Ten2KD has a much smaller effect on host survival (odds ratio = 1.59, Figure 3A), compared to Ten1Ten2KD (odds ratio = 8.94, Figure 2A). These results suggest a non-additive effect of Ten1Ten4KD and Ten1Ten2KD on host survival post infection.

The effects on bacterial loads of Ten1KD (Figure 1B) and of Ten1Ten4KD (Figure 2B) at 7 days post infection are similar (Cohen's d Ten1KD = 0,85, Cohen's d Ten1Ten4KD = 0.88). Ten4KD has a negligible effect (Cohen's d = -0.44). At day 14 post infection, Ten1KD has a negligible effect (Cohen's d = 0.11) and Ten4KD has a medium effect (Cohen's d= 0.5 ) but Ten1Ten4KD has a much stronger effect (Cohen's d = 1.38) on bacterial load. This also suggests a non-additive effect of the knock-down of these two AMPs at day 14 post infection.

Ten2KD has a negligible effect on bacterial survival 7 days post infection (Figure 3B, Cohen's d = -0.33), and Ten1Ten2KD (Figure 2B, Cohen's d = 0.68) has a smaller effect than Ten1KD (Figure 1B). At 14 days post infection, Ten1KD and Ten2KD (Cohen's d = 0.39) have little to no effect on bacterial loads, whereas Ten1Ten2KD has a much stronger effect (Cohen's d = 0.8), suggesting non-additivity.

**Discussion**

A prevailing view on insect immunity is that the combined effect of antimicrobial peptides determines host resistance to microbial infections (Lazzaro 2008; Rolff *et al.* 2016). The expression of several AMPs of the same class has also been proposed to provide redundancy to mitigate the impact of AMP resistance evolution (Lazzaro 2008). AMPs of the same class can have different spectra of activity, and AMPs of different classes often have overlapping spectra (Yi *et al.* 2014). This makes functional redundancy hard to predict. In our case, the knock-down of the only defensin known in

*T. molitor*, Tenecin 1 *(Johnston et al. 2013)*, surprisingly did not result in increased host mortality, contrasting with *in vitro* results on bacterial killing by Tenecin 1 (Moon *et al.* 1994). Tenecin 4 by contrast is not the only attacin present in *T. molitor* (Makarova *et al.* 2016) which could explain the absence of an effect on host mortality. We could have expected the same pattern for Tenecin 2 since two other coleoptericins have been identified in *T. molitor* (Johnston *et al.* 2013*),* instead of the increased mortality that we observed. Thus, similarly to Unckless and Lazzaro (2016) our data are consistent with the notion that the relationship between the defence phenotype of the host and apparent functional redundancy is more complex than previously thought.

Our data also highlight disparate results between *in vivo* and *in vitro* activites of AMPs. This could be caused by differences in the physico-chemical properties of the buffers used *in vitro* compared to host hemolymph. In the light of our results, however, it seems to be caused by interactions between AMPs with one another or other immune effectors that could mask their single effects. While Tenecin 1 and Tenecin 4 do not have an effect on host survival when knocked down separately, they produce a detectable effect when knocked down together. Moreover, effect sizes indicate non-additive effects of the knock down of multiple AMPs and possible synergism between AMPs. Since different controls were required in the single and double knock-down experiments (see above), we used effect sizes for comparison. Effect sizes are commonly used to compare data generated in different experimental settings in the context of meta-analyses (Nakagawa and Cuthill 2003), therefore we think they are a valid approach to study such interactions.

Though synergism is difficult to define (Baeder *et al.* 2016) we use the term loosely here to indicate that the effects of joint knock-downs of AMPs were stronger than the sum of their individual effects, which is common *in vitro (Yu et al. 2016).* Another possible effect is potentiation: an AMP shows no actitivity in isolation but only in presence of a second AMP. Such potentiating interactions have been previously described for insect AMPs *in vitro (P. Engstrom et al. 1984; Rahnamaeian et al. 2015)*. Potentiation could partially explain the results of our Ten1Ten4KD.

Our combined results are consistent with a high level of redundancy between AMPs of all classes of this defence system, where at least two components need to be removed to affect host survival, and to result in increased bacterial survival within the host (Lazzaro 2008). This threshold effect in the number of effectors required for host survival has been shown before for antibacterial compounds of the insect immune system (Clemmons *et al.* 2015). The actual nature of the interactions we studied here remains to be investigated *in vivo* .

The temporal dynamics of bacterial loads reported here, a sharp decline within the first 30 min with a much shallower decline later, are remarkably similar to the temporal dynamics of persisters (Brauner *et al.* 2016). At the current stage it is not certain that the surviving *S. aureus* are persister cells *sensu stricto (Lewis 2010)*. At later time points of the double knock-down experiment, however, the bacterial population constantly declines in control beetles but it is higher 7 days post infection than at day 5 in Ten1Ten4KD beetles (Figure 3 B). This pattern might be explained by either an escape of bacterial cells from host hemocytes (McGonigle *et al.* 2016) and/or cells exiting dormancy, or even the formation of persister cells (Sturm & Dworkin 2015). One other

possible explanation that currently cannot be ruled out is biofilm formation. Ten1KD beetles show a shallower decline in their bacterial populations. Such an infection trajectory highlights the potential importance of evolving long lasting immune responses as a way to avoid relapses of pathogenesis by dormant bacterial populations (Sturm & Dworkin 2015).

While our systematic study of persisting bacterial infections is limited to one pathogen in one host, the results of other studies are consistent with the patterns that we observed. Experimental infections with *E. coli* in honey bees (over 80hrs) and *Erwinia carotorova* in *Drosophila* (over 24 hrs) found similar patterns of temporal declines in bacterial loads (Gaetschenberger et al. 2013, Shia et al. 2009). Work in weevils has demonstrated that AMPs are involved in the maintenance and control of persisting infections by symbionts (Login et al. 2011).

An interesting observation from our experiments is that the increase in bacterial load caused by the knock-down of AMP gene expression is not responsible for the increased mortality in the host, as the decrease in host survival pre-dates the increase in bacterial load. This indicates that AMPs might also influence host fitness beyond their bactericidal activity. Tolerance has been defined as the ability of an organism to limit the infection below a damage threshold, which translates into improved host survival (Råberg *et al.* 2007, see alsoLouie *et al.* 2016). It is thus possible that AMPs also affect tolerance to an infection in a time-dependent manner, i.e. tolerance to infection would be higher at 7 than 3 days post infection.

Finally, our results show overall that reducing the expression of a few elements of a complex cocktail of immune effectors is sufficient to produce an effect on both bacterial

presence in the host and host survival, probably by two independent mechanisms. This highlights the adaptive significance of immune effector cocktails, which can be found across a wide range of taxa, from plants (Sels *et al.* 2008) to metazoan animals (Zasloff 2002) and even single cell organisms (Kroiss *et al.* 2010).

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Conceived the study and designed the experiments: CZ, PRJ, JR. Conducted the experiments: CZ, with contributions from PRJ. Analyzed the data: CZ. Wrote the paper: CZ, JR.

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**Data availability:** Data are available on dryad: doi:10.5061/dryad.33k09..

**Supporting Information S1, S2, S3**

## Figures

Fig. 1 Knock-down of the defensin Tenecin 1 does not increase host mortality post *S. aureus* infection but changes the temporal dynamics of *S. aureus* within beetles. **(A)** Kaplan-Meier curves show the survival of full control (grey), control (black) and *tenecin* 1 knock-down beetles (red) sham (dashed lines) or *S. aureus* infected (full lines) over 35 days post infection. **(B)** CFU recovered from 100µL of hemolymph extract of control (black) or *tenecin 1* KD-beetles (red). The boxes show the first to the third quartiles and the median. The bars indicate the 1.5 interquartile of the lower and upper quartiles. Each dot represents a data point (Figure S1 for post hoc comparisons).

**Fig. 2** Double-knockdown of the defensin Tenecin 1 and either the coloeptericin Tenecin 2 or the attacin Tenecin 4 results in significant mortality and more *S. aureus* in beetles.

**(A)** Kaplan-Meier curve showing the survival of full control (grey), control (black), Tenecin 1 and Tenecin 2 (orange) and Tenecin 1 and Tenecin 4 (purple) beetles either sham (dashed lines) or *S. aureus* infected (full lines) over 35 days post infection (Table S5 for post hoc comparisons.)

**(B)** Bacterial loads recovered from 100µL of hemolymph extract of control (black), *tenecin 1* and Tenecin 2 (orange) or Tenecin 1 and Tenecin 4(purple) knock-down beetles. The boxes show the first to the third quartiles and the median. The bars indicate the 1.5 interquartile of the lower and upper quartiles. Each dot represents a data point (Figure S2 for post hoc comparisons).

**Fig**. **3** Knock down of Tenecin 2 (coleoptericin) but not Tenecin 4 (attacin) reduces host survival. Both knock-downs do not influence bacterial load.

**(A)** Kaplan-Meier curve showing the survival of full control (grey), control (black), Tenecin 2 (yellow) and Tenecin 4 (blue) beetles either sham (dashed lines) or *S. aureus* infected (full lines) over 35 days post infection (Table S5 for post hoc comparisons).

**(B)** Bacterial loads recovered from 100µL of hemolymph extract of control (black), Tenecin 2 (yellow) or Tenecin 4 (blue) knocked down beetles. The boxes show the first to the third quartiles and the median. The bars indicate the 1.5 interquartile of the lower and upper quartiles. Each dot represents a data point.