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A gene knock-down approach in *Tribolium castaneum* to study survival and priming towards *Bacillus thuringiensis* var. *tenebrionis*

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Abstract: Insects possess an array of defense molecules allowing them to fight infections. They can also show a form of immune memory, named priming. However, the involvement of insect immune defense mechanisms in priming is still relatively unclear, since invertebrates lack the molecular machinery present in vertebrates to build an immune memory. In the red flour beetle *Tribolium castaneum*, larvae can be primed via the oral route with *Bacillus thurigiensis* var. *tenebrionis (Btt)*. This results in changes in the expression of a large number of genes, among which some belong to families of ancient innate immune defense genes. In the present work, we tested whether three chosen candidate genes (a Thaumatin, a C-type Lectin and an Osiris-like gene) might be involved in the survival to a *Btt* exposure, as well as in the priming phenotype. We assessed changes in their expression over time and according to the priming treatment, knocked them down individually by RNA interference (RNAi), and observed how it affected survival upon challenge. The quantification of gene expression patterns in our larvae with RT-qPCR showed that up- and/or down-regulation of the genes, after the priming treatment, was quite volatile and time-dependent. Upon knock-down, we neither observed the expected decrease in survival to *Btt* nor a reduced priming phenotype. We conclude that knocking down genes individually is probably insufficient to affect survival and priming in our system. This gives us insight into the complexity of the molecular processes underpinning priming, and questions the usefulness of RNAi of single genes for studying resistance and priming in our host-pathogen system.

Keywords: Priming; RNAi; *Tribolium castaneum*; Host-parasite interaction; *Bacillus thuringiensis*

Introduction

Parasites and pathogens are ubiquitous and can have detrimental effects on the survival and reproduction of their hosts [1]. Defense mechanisms, which improve host survival in the case of an infection, have been under strong evolutionary pressure since the appearance of the first eukaryotic cells [2]. However, adaptation to parasites can also occur within an individual's lifetime through a plastic enhancement of the immune response and other life history traits. In vertebrates, this is achieved by adaptive immunity, which increases host fitness upon the secondary encounter of a pathogenic agent. The adaptive immune response relies on

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the production of specific cells and antibodies, which are more specifically directed against a pathogen that has been encountered before, allowing vertebrates to react more effectively against a pathogen in case of re-infection, increasing the host's survival probability [3].

Invertebrates, on the other hand, lack the immune effectors of the adaptive immune system [4], and rely on their innate immune systems to fight pathogens and parasites. The discrimination between self from, potentially harmful, non-self-components is mainly mediated by germline encoded pattern recognition receptors (PRRs) [5], which recognize conserved pathogen associated molecular patterns (PAMPs) on the surface of invading microorganisms. Nevertheless, several invertebrate species have shown an increased survival upon secondary pathogenic exposure, a phenomenon that has been called priming, and can even be specific [6,7]. The evidence for immune memory across invertebrates focuses mainly on insects and crustaceans, however, there is also some evidence of priming in a few taxa within the Lophotrochozoa, mostly molluscs [8].

Some studies are starting to shed light on the molecular underpinnings of priming. To this day, several authors have seen upregulation of genes with a putative immune function, such as components of the Toll pathway and changes of phagocyte populations in *Drosophila melanogaster [9]*. However, it is still not clear whether some genes, although upregulated following primary exposure to PAMPs, are directly involved in increasing host survival upon secondary exposure. Up to now, work on invertebrate immune priming has been mostly descriptive of the priming phenotype, while functional approaches using candidate gene knock-outs or knock-downs have rarely been used (but see [10]). Analysing the relation between gene expression and survival probability of an organism during infection in the presence or absence of priming will give us more information about which genes are potentially involved in the defence mechanism of this organism's immune system.

While most priming studies use septic infections, oral infection routes have also been used to study immune priming in several species [11,12]. In infections *via* the septic route, pathogens such as vegetative bacterial cells are directly introduced into the insect's body cavity by pricking the intersegmental membrane with a contaminated needle [8]. Infections *via* the oral route, on the other hand, are usually achieved by exposing hosts to pathogen-contaminated diets [13] or the ingestion of bacterial cues, using filtered medium supernatants from bacterial spore cultures [14]. Despite evidence suggesting that septic injury can occur in nature [15], most pathogens enter their hosts *via* the oral route [8]. In this work, we focus on a pathogenic exposure *via* the oral route. Priming has been observed in the red flour beetle *Tribolium castaneum* upon oral and septic exposure to bacterial culture supernatants [14,16,17,18]. Previous work has shown that ingestion of bacterial cues derived from spore culture supernatants of the pathogenic strain *B. thuringiensis* var. *tenebrionis (Btt)* induced regulation of a large set of genes, including many immune genes [19]. In the present study, we take advantage of this well-described host-pathogen system to investigate the role of a subset of three genes: one PRR, one putative effector, and one gene with unknown function, which has previously shown involvement in immune responses and insect development.

As a PRR, we chose a C-type Lectin (TC 003708). C-type Lectins (CTL) belong to a large superfamily and are widely distributed in almost all organisms. They function as PRRs by binding to polysaccharide chains on the surface of pathogens [20,21] and play a role in phagocytosis [22] and the agglutination of bacteria and yeast [23] via opsonization [21]. A recent study demonstrated the role of a CTL in *T. castaneum* for innate immunity [24]. Moreover, CTLs were

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shown to be involved in the survival and colonization of gut bacteria, in both the mosquito *Aedes aegypti* and *Culex pipiens pallens* [25]. CTLs are supposed to enable recognition specificity in invertebrate immunity [26], which is an intensively studied aspect in *T. castaneum* immune priming [16,27,28].

As an immune effector, we picked a Thaumatin, named PRP5 (TC 000516). First described in plants, Thaumatins show *in vitro* anti-fungal activity, and have been shown to be upregulated following an infection [29]. It has been proposed that Thaumatins may exist as ancient antimicrobial peptides (AMPs) that either have been lost in most insect species or that have evolved independently in aphids and beetles. In *T. castaneum*, Thaumatins have been identified as effectors also against fungi by inhibiting the spore germination of filamentous fungi [30]. Their activity against *B. thuringiensis* is unknown, both *in vitro* and *in vivo*.

Lastly, we picked an Osiris-like gene (Osiris 16-like, TC012679). The Osiris gene family has evolved by gene duplication and divergence events, very early in the radiation of insects, and has been subject to strong selection in all insects ever since [31]. The exact function of Osiris genes is still unknown, though Osiris genes seem to be developmentally regulated. For example, in bumble bees and ants, the expression of most of the Osiris genes is very low in the larval stage and increases in pupae [32]. In *Drosophila melanogaster*, Osiris gene expression has been shown to be induced in the early stages of intestinal colonization by entomopathogenic bacteria, which could indicate an involvement in the maintenance of gut homeostasis during an oral infection [33].

The genes studied in this work were chosen from a pool of genes that had been shown to be upregulated, upon priming against *Btt*, in an RNAseq study carried out by Greenwood et al. [19]. We assessed how priming influenced the expression pattern of these genes over time, and checked whether the patterns found by Greenwood et al. [19] by RNAseq could be confirmed by qPCR. We used a gene knock-down approach by RNA interference of these three genes, in order to observe how their expression influences the priming phenotype. In parallel, we assessed how the knock-down of each one of the genes influenced the expression of all three of them. If they were involved in the defense against *Btt*, we expect survival of *T. castaneum* larvae to decrease as a result of their knock-down. If they were involved in increasing the protection of larvae upon secondary exposure, we expect the priming phenotype to be at least partially reduced by their experimental down-regulation.

Materials and Methods

Insect maintenance

The experiments were performed with the strain Cro1 of *T. castaneum*. This strain was collected from the wild in Croatia in 2010 [34]. The stock populations were kept in plastic boxes (29 x 20 x 9cm) filled with heat sterilized (75°C) organic wheat flour (type 550) and 5 % of brewer's yeast, in climate chambers at 30 °C, 70 % humidity, and a 12 h light/dark cycle. The experimental insects were obtained from approximately 200 adult beetles, transferred in a fresh plastic box containing the aforementioned substrate, in the same experimental conditions, and left to breed and lay eggs for 6 hours. Experimental larvae were retrieved 14 days after the beginning of the egglay by sieving the substrate. Injections were performed on the day of collection, which we called day 0. Priming was performed on day 1, when the larvae were 14 days old, whereas bacterial exposures was performed on day 5, with larvae aged 20 days (see below, Figure 1). The beetle larvae were exposed to *Bacillus thuringiensis* var. *tenebrionis* as in previous studies carried out in our laboratory [14].

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Generation of dsRNA and gene knock-down by dsRNA injection

The C-type Lectin (TC 003708) and the Osiris (TC012679) dsRNA were ordered from Eupheria Biotech (Dresden, Germany) at a concentration of 1000 ng/μL, and delivered suspended in injection buffer (1.4 mM NaCl, 0.07 mM Na_2HPO_4, 0.03 mM KH_2PO_4, 4 mM KCl, 1 % (v/v) phenol red, pH 7.2). Since the Thaumatin (TC 000516) gene dsRNA was not available from Eupheria, we synthesized it from a synthetic DNA construct ordered from Eurofins Operon (Eurofins Genomics Germany GmbH, Ebersberg, Germany). The control dsRNA, from the enhanced Green Fluorescent Protein (eGFP), was generated from a DNA sequence cloned into *E. coli* . The sequences of the injected constructs can be found in supplemental material S3. For these last two genes, the template for dsRNA synthesis was amplified from the constructs by PCR (GoTaq G2 Flexi DNA Polymerase kit, Promega, Madison, USA) 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, 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 checked once again product integrity and size before purifying the RNA with a phenol-chloroform extraction followed by isopropanol precipitation and DNase digestion (TURBO DNase from 5x MEGAscript T7 Kit, Thermo Fisher Scientific, Waltham, MA USA), and resuspended the pellet in injection buffer. We adjusted the concentration to 1000 ng/μL using a Nanodrop (IMPLEN, München, Germany). Before usage, the RNA was annealed, at 90°C, and allowed to slowly cool down, in order to obtain dsRNA.

DsRNA was injected into the body cavity of 14 days old larvae (age after the beginning of the egg lay). The injections were performed with the Nanoject II (Drummond) set to deliver 27.6 nL per injection *via* a pulled capillary, corresponding to an amount of 27.6 ng dsRNA transferred into each larva. Before the injections, batches of 30 larvae were kept on ice (covered with two layers of aluminium foil). After five minutes they were glued on glass slides with “TESA Posterstripes” (TESA, Norderstedt, Germany) and kept on ice for another two minutes, prior to injection. Four times 30 to 35 larvae, per gene knock-down treatment, were injected between the 3rd and 4th abdominal segments. Approximately 3000 larvae were injected in total. Care was taken that no fluid leakage or internal organ perforation took place. The larvae were then carefully removed from the slides and placed individually into 96-well plates filled with a few grams of flour. The plate was covered with two layers of tape (TESA, Norderstedt, Germany), into which holes were pricked. We used one plate per batch of 30 larvae that had been injected with the same needle, in order for one plate to contain the variance caused by the needle quality. All the larvae were kept in the same groups of around 30 larvae per 96 well plate, continuously belonging to the same experimental batch. This whole process was replicated 6 times, on 6 different days and with 6 different egg lays. A non-injected control was included in the last two replicates. On the day following the injection larvae survival was checked. The knocked-down larvae (together with naive larvae, i.e not injected) that had survived the manipulation, were transferred to 96 well plates according to their priming treatment.

We chose 14 days old larvae for injection with dsRNA, because preliminary trials had shown that by this time they had reached a size at which injection did not increase mortality.

Microbial cultures and preparation of the priming and challenge diets

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The strain of *B. thuringiensis* var. *tenebrionis* used was ordered from the Bacillus Genetic Stock Center (catalogue: BGSC 4AA1). One aliquot of the general stock (kept at -80°C) was plated out on LB-Medium agar plates. Five colonies were taken to start an overnight culture which was on the next day transferred to a sporulation culture (ingredients of overnight and sporulation culture see S1).The sporulation culture was centrifuged at room temperature for 15 min (4500rpm). The supernatant was collected, filtered (pore size: 0.45μm (Clear-Line, Brumath, France); and pore size: 0.2μm (Whatman, Maidstone, UK)), insuring its sterility. The pellet containing the spores was mixed with flour and used for the challenge plates. To do so, the concentration of spores in the culture was assessed with a counting chamber, and adjusted to 1 x 10^{10} mL^{-1} with PBS. 10 mL of the *B. thuringiensis* spore and crystals suspension were mixed with 1.5 g of flour and then pipetted in each well of a 96-well plate (20 μL per well). The plates were covered with air permeable tape and dried at 30°C overnight.

Priming and challenge of T. castaneum larvae

Two batches of each injection treatment (including the control larvae) were transferred to 96-well plates containing the priming diet. The other half was transferred to the non-priming diet containing only Bt-Medium (see Fig.1). After 24h the larvae were transferred to new plates containing a mix of flour and PBS (10 mL of PBS and 1.5 g flour) for 4 days. At this time point, 5 larvae per treatment were snap frozen in liquid nitrogen for analysis of gene expression. The remaining larvae were transferred to challenge or non-challenge diet (see Fig.1). The survival of the larvae was tracked for the next seven days. The exact same priming experiment was performed with an additional batch of non-injected larvae, without challenge treatment, and therefore the survival was not tracked. The timepoint for freezing the larvae was the same as used in the previous batches, 4 days after priming. Additional samples were taken 24h after exposure to the priming diet to track the efficiency of the knock-down.

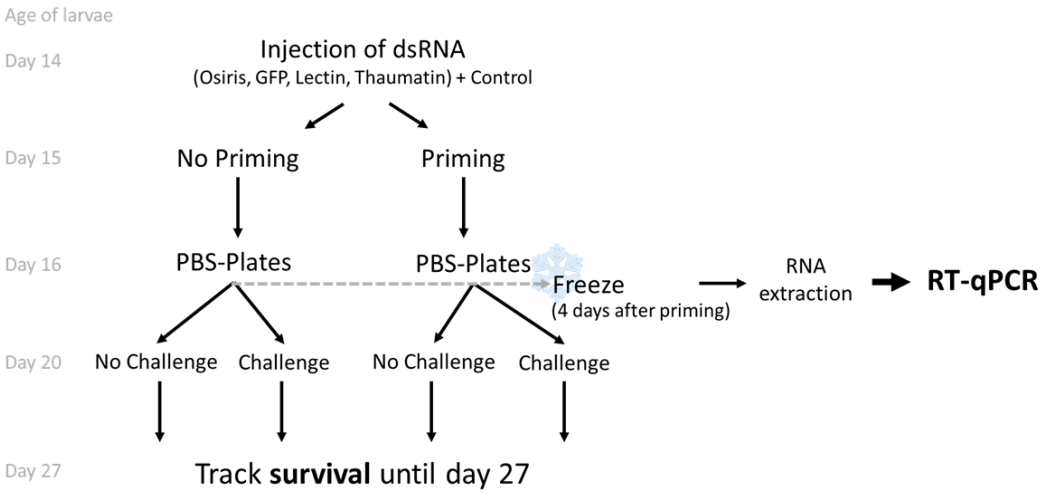

Figure 1. Overview of the experimental design. After the injection of dsRNA, the larvae were split into two groups. One group was placed on the priming diet and the other, the "priming control", was fed with *Btt* growth medium only, for 24h. Samples of each treatment were taken before the groups were split again, after spending four days on a PBS control diet. One half was exposed to the challenge diet (with bacterial spores) and the other half to the "challenge control" diet, *i.e* PBS flour discs. Afterwards, the survival of the larvae was tracked for the next 7 days.

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Assessment of gene expression by qPCR and verification of gene knock-down efficiency

We used RNA samples of individual larvae for reverse transcription prior to qPCR, in order to assess the effect of time on gene expression at both 24 hours and 4 days after priming, as well as to check whether the knock-down treatment affected the target gene expression 4 days after priming. After snap-freezing, samples were transferred to the -80ºC freezer. For processing, individual larvae were homogenized with a clean pestle while holding the bottom of the tube into liquid nitrogen. Trizol was subsequently added to the homogenate. The RNA of each frozen larva was extracted following a combined TRIZol / spin column protocol developed by Schulz, Buhr, & Kurtz (2018). The quality and the concentration of the RNA were controlled on a 1.5 % agarose gel, and its concentration assessed with a nanophotometer (IMPLEN, München, Germany). 100 ng of the extracted RNA were used as a template for reverse transcription. This step was performed using the RevertAid First Strand cDNA Synthesis Kit by Thermo Fisher Scientific (Waltham, MA USA) according to the manufacturer's instructions, using random hexamer primers. The qPCR was performed on a 3-fold dilution of the aforementioned cDNA, with each sample corresponding to one individual larva. Each well, of a 384 well plate, was loaded with 5 μL Kapa SYBR Fast (Kapa Biosystems, Sigma-Aldrich, Darmstadt, Germany), 0.3 μL of each forward and reverse primer (stock solution: 100μM, dilution 1:20), 3.4 μL of nuclease-free water and 1 μL of a 3-fold dilution of the aforementioned cDNA. Each sample was run in duplicates. The plate was covered with optical foil and analysed in the LightCycler 480 by Roche. (95°C 3min; 95°C 15min, 60°C 1min (40 cycles)). The Ct values were retrieved with the help of the LightCycler480 software (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). The sequence of the primers can be found in the Supplementary Table S2.

Statistical analysis

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Statistical analyses were performed with the R software [36]. In order to represent the full extent of the variation in gene expression data without any bias, we analyzed raw delta Ct values with Generalized Linear Models fitted with a negative binomial distribution. This takes into account overdispersion as well as heterogeneity in the variances between the treatments. We also chose to show relative gene expression data according to the two reference genes separately in order to verify the consistency of the results. The gene expression data comparing the 24 hours and 4 days after priming time points were analysed with a negative binomial regression using the MASS package [37]. The mean Ct value of 2 technical replicates per sample of 1 focal gene was the response variable to which we added 10, multiplied by 1000, and rounded, in order to fulfill the criteria of a negative binomial distribution without losing information. The time point (24 hours or 4 days) and the treatment (primed or non-primed) were the explanatory variables. The gene expression data comparing expression of one focal gene across different knock-down treatments in the presence or absence of priming was analysed similarly, with the mean Ct value of 2 technical replicates per sample of 1 focal gene being the response variable, and the knock-down treatment (naive, GFP, Lectin, Osiris & Thaumatin KD) and priming treatment (primed or non-primed) being the explanatory variables. The main body of the manuscript depicts gene expression data relative to the expression of ribosomal protein L13a (Rpl13a), whereas gene expression relative to Rp49 is presented in the supplementary material (S11 and S12). Knock-down efficiencies are presented in the supplementary material S4. The survival of the larvae was analysed with the "survreg" function of the survival package [38,39], fitted for a lognormal distribution (decided upon by using the "flexsurv" package [40], and according to the knock-down treatment, priming and challenge treatment as explanatory variables, together with maintenance plate as a frailty term. In each case, we built the most complex model, with all interactions included, as well as all the possible derived models and the null model, and compared their Akaike's Information Criterion (AIC). We kept as optimal models the ones with the lowest AICs [41].

Results

Effect of priming on gene expression

We assessed the effect of the exposure of *T. castaneum* larvae to the priming diet on the expression of the three genes of interest 24 hours and 4 days after exposure (Figure 2). Priming did not affect C-type Lectin gene expression neither over time (time*treatment: L ratio = 0.012; p = 0.91, df = 1,38) nor as a simple effect (treatment: L ratio = 0.0021; p = 0.96; df=1,40). However, C-type Lectin expression decreased over time (time: L ratio = 9.55; p = 0.002; df =1,40 see *post hoc* test in supplementary material S5). Regarding the Osiris gene, its expression

was higher at 24 hours in control larvae compared to primed ones, with large variation between the replicate samples. However, 4 days later, the Osiris gene expression level was similar between the two treatments (time*treatment: L Ratio = 7.01; p = 0.008, df = 1,38, see post hoc test in supplementary material S6). Finally, Thaumatin gene expression decreased over 4 days in control larvae, while it remained stable between 24 hours and 4 days in primed larvae, resulting in primed larvae having a higher Thaumatin gene expression 4 days post priming than control larvae (time*treatment, L Ratio = 3.94; p = 0.047; df = 1,38, see effect plot in supplemental material S7).

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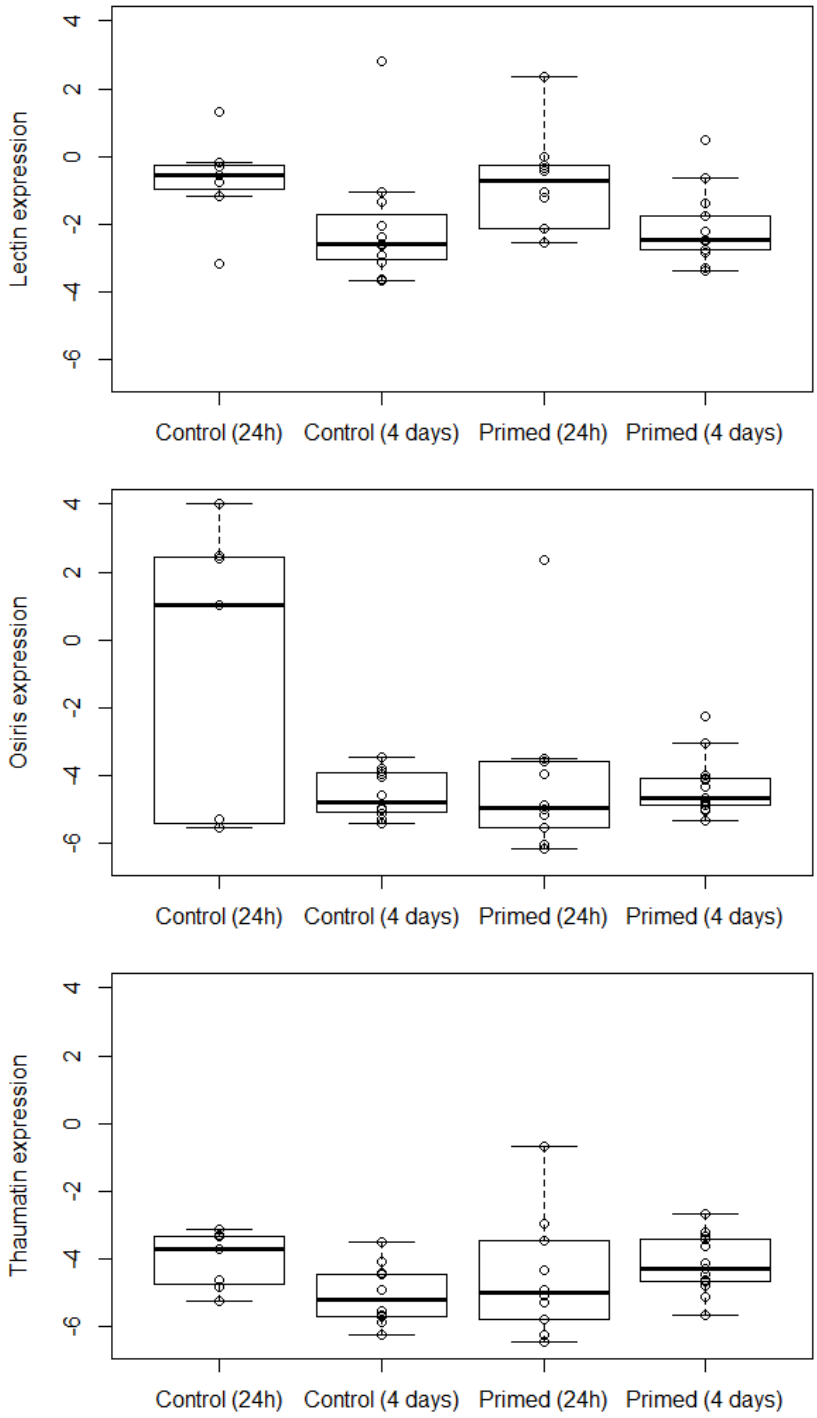

Figure 2. Effect of priming on gene expression levels of C-type Lectin (TC 003708), Osiris 16 like (TC012679) and Thaumatin (TC 000516). The boxplots depict the gene expression of individually extracted larvae, for the three focal genes C-type Lectin (TC 003708), Osiris 16 like (TC012679) and Thaumatin (TC 000516) relative to the expression of the housekeeping gene RpL13a. Each dot represents an individual larva (mean of 2 technical replicates). The boxplots represent the first to the third quartiles around the median (horizontal black line), while the vertical bars represent the 1.5 interquartile of the lower and upper quartiles. The results show the gene expression at two different timepoints. The first one, 24h after the priming (primed: n=10; control: n=7), the second one, 4 days after priming (primed: n=13; control: n=12), which means immediately before challenge.

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Effect of gene knock-down and priming on gene expression

We investigated how the knock-down by RNA interference affected the gene expression of our three focal genes (see Fig. 3). The results depict the gene expression of the three candidate genes in primed and non-primed larvae, 4 days after the priming treatment, i.e., the time point immediately before challenge.

Lectin gene expression was influenced by the injection treatment, as expected (Injection: Deviance = 62.35; $p < 0.001$; df = 4,132). Injection of dsRNA itself (eGFP, Osiris or Thaumatin) did not change Lectin gene expression, however, it was significantly downregulated upon injection of Lectin dsRNA (see supplementary material Figure S8 for post-hoc comparisons). It yielded a 4.3-fold down-regulation compared to the eGFP control (supplementary material Table S4).

The expression of the Osiris gene was influenced by the injection in interaction with the priming treatment (Priming*Injection: Deviance = 12.26; $p = 0.015$; df = 4,127). The injection of dsRNA in itself upregulated Osiris gene expression in primed larvae, since Osiris gene expression went up upon priming in the eGFP RNAi control treatment, and to a lesser extent in the Lectin knock-down treatments, but not in the Osiris knock-down treatment. This resulted in a significant downregulation of Osiris gene expression in the Osiris knock-down treatment compared to control, as expected. RNAi yielded a 1.36 and 2.7-fold reduction in control and primed larvae, respectively. Interestingly, Osiris gene expression was also down-regulated in the Thaumatin knock-down treatment in primed larvae (see supplementary material Figure S9 for post hoc comparisons).

We did not detect any significant down-regulation of the Thaumatin gene in the knock-down treatment compared to the eGFP treatment (see supplementary material Figure S10 for *post hoc* comparisons). However, Thaumatin gene expression was influenced by the injection treatment (Injection: Deviance = 11.26; $p = 0.025$; df = 4,132). More precisely, it was downregulated by the injection of dsRNA in general, since it was lower in the eGFP and Lectin treatments compared to the naive control. This was not the case for the Osiris gene knock-down.

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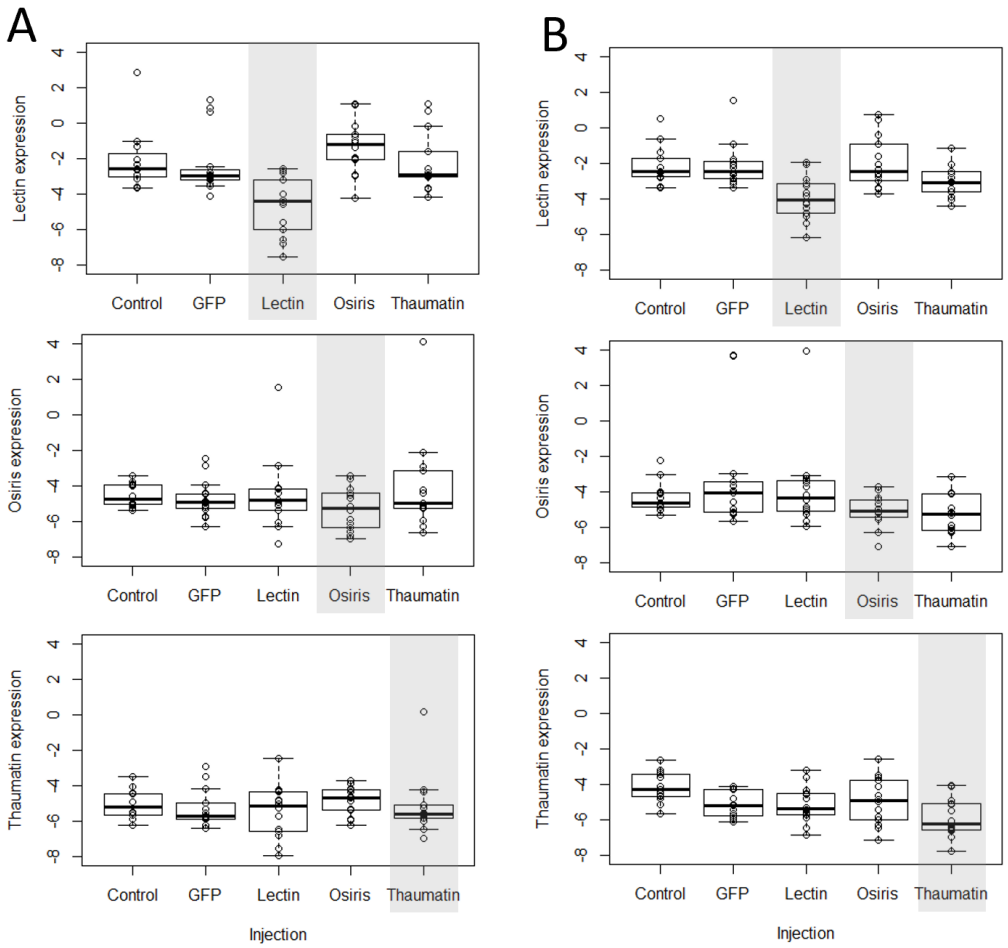

Figure 3. Effect of gene knock-down and priming on gene expression. The figure depicts the gene expression of the three candidate genes in knock-down and control larvae in the absence of priming (A) (eGFP n=15; Lectin/Osiris/Thaumatin n=14) or upon priming (B). (eGFP n=13; Lectin/Osiris/Thaumatin n=14) Each dot represents one individual larva (mean of 2 technical replicates). The boxplots represent the first to the third quartiles around the median (horizontal black line), while the vertical bars represent the 1.5 interquartile of the lower and upper quartiles. Highlighted in grey are the knock-down treatments corresponding to the gene whose expression is being measured. The gene expression shown is relative to the expression of the housekeeping gene RpL13a. The samples were taken four days after the priming diet was fed to the larvae, immediately before challenge. The data of the control larvae (primed (n=13) and control (n=12)) is the same as shown in figure 2. See supplementary material S4 for knock-down efficiencies.

Survival experiment

The knock-down treatments did not affect the survival of the challenged larvae differently upon priming compared to control (priming treatment*challenge treatment*knock-down treatment: Deviance = 5.87; p = 0.42; df = 10,459), indicating that in our setting, none of the gene knock-downs influenced the priming phenotype (see Fig. 4). Despite not being supported by model selection, there was an interaction between the priming and challenge treatments (priming treatment * challenge treatment: Deviance = 33.1; p= 0.013; df = 7,462), This indicates a weak priming effect, resulting in a slightly higher survival of primed larvae upon challenge. There was also a simple effect of both priming and challenge on survival. As expected, the challenge with *B. thuringiensis* significantly decreased the survival of the larvae (challenge treatment: Deviance = 342.08; p < 0.001; df = 4,465). Priming with *B. thuringiensis* supernatant had a positive effect on the survival of the larvae, regardless of their challenge regime (priming treatment: Deviance = 9.28; p = 0.0023; df = 4,465). The knock-down treatment

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did not affect the survival of the larvae (knock-down treatment: Deviance = 4.82; p = 0.31; df = 4,465).

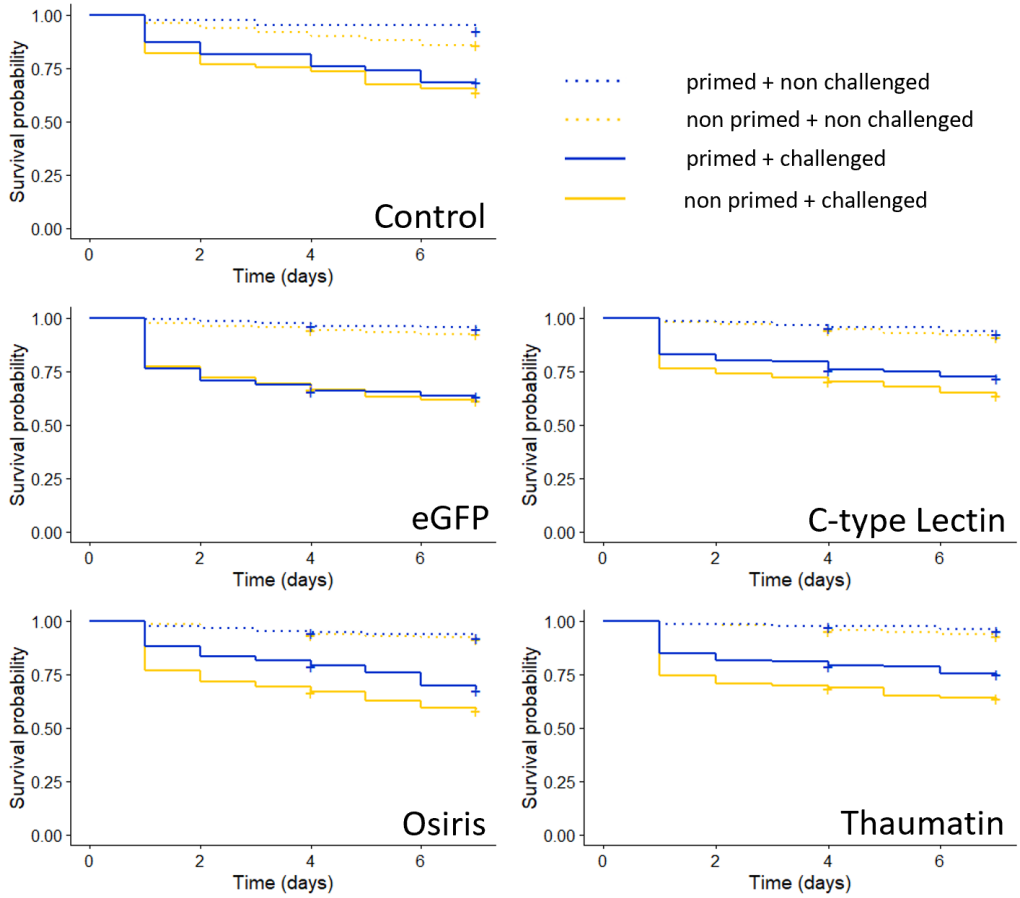

Figure 4. Survival of *T. castaneum* larvae upon exposure to *B. thuringiensis* var. *tenebrionis*, in the presence or absence of priming. This panel depicts the results of the survival experiment. Each graph shows one knock-down treatment (Control (n = 207), C-type Lectin (n = 518), Osiris (n = 533), Thaumatin (n = 497), eGFP (n = 524)). Data from primed larvae are shown in blue, non-primed ones in yellow. Additionally, the straight lines depict the challenged larvae and the dotted ones the non-challenged larvae. The survival was followed over 7 days.

Discussion

Here we investigated the role of three candidate genes for survival and priming of *T. castaneum* larvae upon oral exposure to the entomopathogen *B. thuringiensis tenebrionis*. We based our selection of candidate genes on a previous RNAseq study that had shown the upregulation indicated. Priming did not influence the expression of the C-type Lectin gene, while it did so for Osiris and Thaumatin genes, but in a time-dependent manner. The knock-down of these three genes yielded variable efficiencies and did not influence the priming phenotype.

C-type Lectin gene expression decreased over time and expression of the Osiris gene, on the other hand, was downregulated 24 hours after priming compared to control, turning back to a similar level between treatments after 4 days. Thaumatin gene expression level was similar between control and primed larvae 24 hours after priming. It decreased over 4 days in control larvae but remained stable in primed larvae, resulting in a relative upregulation compared to control 4

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days after priming. This is therefore the only gene for which we detected a significant up-regulation after priming.
Another Thaumatin gene was found strongly up-regulated upon septic priming in beetles derived from an experimental evolution for immune specificity [28]. This further supports a role of Thaumatins for immune priming, but also indicates that the different Thaumatins probably have specific functions. Greenwood et al. (2017) used a whole-transcriptome analysis to investigate the gene expression in pools of 32 larvae in each of their three replicates per treatment, whereas we extracted RNA from 10 to15 individual larvae per treatment. In general, measuring data from individual larvae is more informative then pooling samples together [42], as high variance within treatments cannot be detected in pooled samples [43].

The expression pattern of some immune genes in insects is known to be highly dynamic over time, and may depend on factors such as the number of copies or isoforms of the gene of interest [44]. In our study, we were able to additionally show that for Osiris and Thaumatin genes, these patterns changed when priming was applied. Using RNAi to knock down the three candidate genes, we found the most significant reduction for the expression of the C type Lectin gene. C-type Lectin (CTL) gene had a basal higher expression level compared to that of the Osiris and Thaumatin genes, independent of treatment and time point, which may have been the reason why we were able to decrease its expression more strongly. More generally, these results point to the value of validation of RNAseq results by qPCR, especially when RNAseq data is based on a small number of biological replicates that vary in gene expression.

We found the expression of the Thaumatin gene to be down-regulated upon the injection of dsRNA in general, but did not detect any further down-regulation upon injection of the corresponding gene target dsRNA. This could indicate that our construct for Thamatine gene was not efficient. Alternatively, it is possible that since the expression of the Thaumatin gene was already lowered by the simple injection of dsRNA, and a further down-regulation was undetectable.
The injection of the control dsRNA (eGFP) in primed larvae, resulted in an interaction between knock-down and priming treatments for this gene, leading to a higher detected knock-down efficiency for this gene. The fact that this effect was not present in the Osiris knock-down treatment shows that using RNAi for this Osiris gene is efficient. It is also likely that the low expression level of this gene in the non-primed control impairs our ability to detect a further down-regulation of this gene upon knock-down. We also found the expression of the Osiris gene to be upregulated upon dsRNA injection in primed larvae and the expression of the Thaumatin gene to be up-regulated in the Osiris knock-down treatment. This indicates a possible co-regulation of these two genes, in which Thaumatin could be a positive regulator of the Osiris gene, while Osiris could be a negative regulator of the Thaumatin gene. Alternatively, pleiotropic effects could connect the expression levels of different genes through potential, but yet unknown, physiological effects induced by the knock-down.

The knock-down of the three focal genes did not significantly affect the survival of the larvae upon priming. We also did not find evidence for a negative effect of the knock-down of the focal genes on the survival of the larvae challenged with *Btt*, compared with control and eGFP-injected larvae.
This result might not be surprising in the cases of the C-type Lectin and Osiris, since they are not upregulated by the priming treatment, which could lead to the conclusion that they are not involved in the expression of this phenotype. How-

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ever, such a reasoning could lead to overlooking constitutively expressed genes which might play an important role in the survival to a pathogen [52].

C-type Lectins combine both receptor and effector functions, in the immune system. We thus may expect the knock down of this gene to have a negative effect on survival of the larvae, upon Btt exposure. CTLs are a superfamily of proteins found in most animals, with a large range of functions in immunity, development and homeostasis [45]. As PRRs, they play a central role in discerning pathogens [26], opsonization [21], nodule formation, agglutination, encapsulation, melanization, and prophenoloxidase activation, as well as in maintaining gut microbiome homeostasis [5]. CTLs have also been reported to play a role in regulating bacterial survival and colonization [46]. Several CTLs from lepidopteran insects, such as *Bombyx mori*, are the best described and characterized, with 23 CTL genes identified in *B. mori* using comparative genomic analysis [47]. Nevertheless, the knock-down of one single Lectin CTL5 (BmCTL5) using RNAi, revealed to be important for larvae of *B. mori* upon infection with the fungi *Beauveria bassiana* [46].

Several Thaumatins have previously shown antimicrobial functions [30]. For example, in the leaf insect, *Peruphasma schultei*, a Thaumatin gene was found to be one of the most upregulated immune effectors upon infection with several microbial elicitors, together with some other AMPs [30,48]. Our results do not point to an antibacterial function against *Btt* of the Thaumatin gene studied here, but further studies investigating their potential *in vivo* are needed.

The function of the genes of the Osiris family is still quite unclear. Cornman et al (2013) showed that the infection of honeybee larvae with the bacterial pathogen *Paenibacillus larvae* upregulated 11 genes with the Osiris domain, indicating important functions on the detoxification as reported already in *Drosophila sechellia* [49]. In *T. castaneum* Osiris genes are involved in chitin binding and deposition [32]. Chitin is the main constituent of the peritrophic matrix, which plays an important protective role against infections via the oral route by isolating food and toxins from the gut epithelium. Since the peritrophic matrix has been shown to be involved in the interaction between *B. thuringiensis* and its insect host [50], we expected a negative effect of the knock down of the expression of the Osiris gene on survival to the challenge.

It is important to point out that the efficiency of the knock down of the three genes of interest was relatively low. The efficiency assessed by qPCR might differ from the levels of the gene product present in the insect, and thus is difficult to assess. Regardless of this, we cannot exclude the possibility that the efficiency of the knock-down was not sufficient to yield an effect on survival, both in the presence or absence of priming. Since our results show that the individual knock-down of our focal genes affects the expression of other genes, it is also possible that a phenotypic effect on survival and/or priming would be masked. This is likely to be the case for any technique of gene silencing, whether this is due to non-specificity or pleiotropy.

The efficiency of the RNAi process could have probably been increased during the time interval, of 4 days, between the dsRNA injection and the exposure to the priming diet. We performed dsRNA injections at the earliest possible time point. Larvae have to enter the priming treatment at a maximum age of 15 days, in order to avoid pupation, which would render them resistant to Btt-caused mortality. Such constrains limit the applicability of RNAi in our system. On the other hand, RNAi has the advantage that it can be performed in a population with a genetically diverse background, which is important for ecological studies. Additionally, genes can be knocked down at a given time point, which avoids interfer-

ing with insect development in the eventuality of a pleiotropy of genes of interest.

Overall, our results are consistent with what was found by Tate et al. (2017) in the case of a trans-generational priming induced by a septic route in the *T. castaneum* - *Btt* system, in which the authors found that priming affects the temporal dynamics of gene expression. However, when the authors attempted to confirm the role of candidate genes, the knock-downs led to offspring death, potentially due to pleiotropic effects. Indeed, functional genetic approaches targeting immune priming remain challenging since it is a complex phenotype that likely depends on many genes, such that knocking down each one at a time might be insufficient. For example, in *Drosophila* the knock-down of single TEP (Thioester-containing protein) genes with redundant functions was insufficient, and a change in microbial infection was only observed upon knock-down of all 4 TEP genes simultaneously [51]. Tetreau et al. (2020) injected *Tenebrio molitor* individuals with a dsRNA cocktail of several candidate AMP genes to maximize the phenotypic effects of their knock-down. The problem of redundancy is particularly relevant as genes identified in transcriptomic studies as potentially involved in immune priming are often members of larger gene families, such as C-type Lectins or Osiris-like genes in our case. Nonetheless, seemingly weak effects observed in such studies can be evolutionarily highly relevant, as even small survival benefits might result in strong selection.

Conclusion: We explored the potential role of three candidate genes in priming and infection with *Btt*, in the insect host *T. castaneum*. Our results do not fully confirm the upregulation of the focal genes upon priming seen by RNAseq [19], which was our criterion for the choice of candidate genes: C-type Lectin (TC 003708), Thaumatin (PRR5, TC 000516) and Osiris (TC012679). Individual knock-down by RNA interference had a weak efficiency and did neither affect the survival nor priming of *T. castaneum* towards *Btt*. Further studies might therefore explore the role of genes, whether they are strongly up-regulated upon priming or not, and focus on knocking down those genes in combination. Large-scale RNAi screens with targeted candidate genes and CRISPR-Cas9 technology may provide powerful tools for future approaches.

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