Title: KNOCKDOWN OF NUCLEASE ACTIVITY IN THE GUT ENHANCES RNAi EFFICIENCY IN THE COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA*, BUT NOT IN THE DESERT LOCUST, *SCHISTOCERCA GREGARIA*

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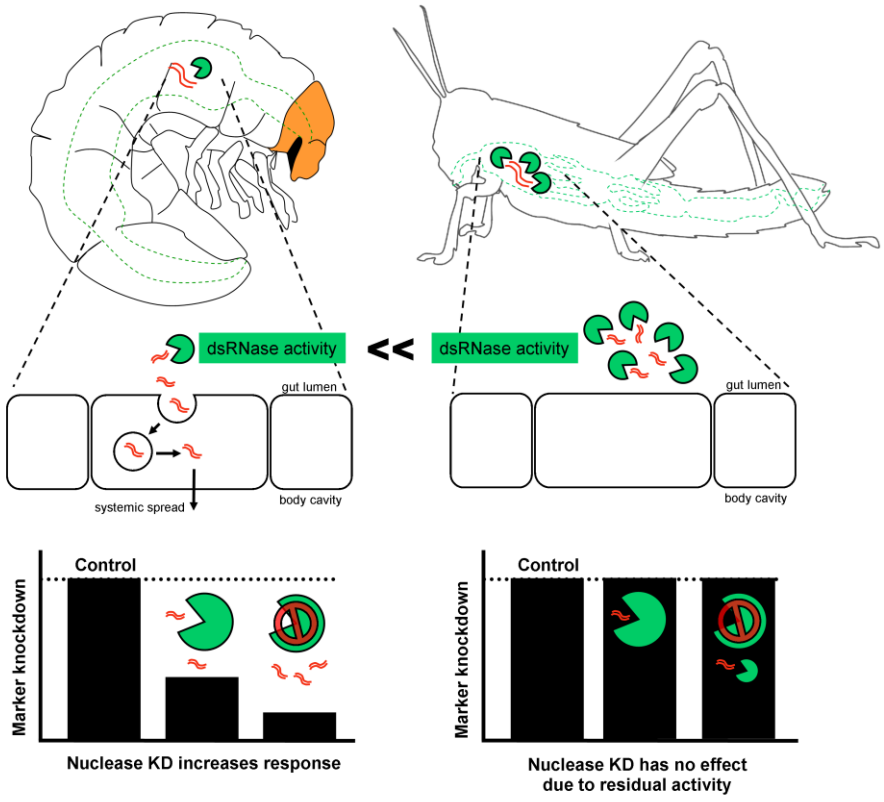
Abstract:

The responsiveness towards orally delivered dsRNA and the potency of a subsequent environmental RNA interference (RNAi) response strongly differs between different insect species. While some species are very sensitive to dsRNA delivery through the diet, others are not. The underlying reasons for this may vary, but degradation of dsRNA by nucleases in the gut lumen is believed to play a crucial role. The Colorado potato beetle, *Leptinotarsa decemlineata*, is a voracious defoliator of potato crops worldwide, and is currently under investigation for novel control methods based on dsRNA treatments. Here we describe the identification and characterization of two nuclease genes exclusively expressed in the gut of this pest species. Removal of nuclease activity in adults increased the sensitivity towards dsRNA and resulted in improved protection of potato plants. A similar strategy in the desert locust, *Schistocerca gregaria*, for which we show a far more potent nuclease activity in the gut juice, did however not lead to an improvement of the RNAi response. Possible reasons for this are discussed. Taken together, the present data confirm a negative effect of nucleases in the gut on the environmental RNAi response, and further suggest that interfering with this activity is a strategy worth pursuing for improving RNAi efficacy in insect pest control applications.

Key words: RNA interference; RNAi; Colorado potato beetle; desert locust; dsRNase; nuclease; dsRNA



1. INTRODUCTION

Over the past decade, RNA interference (RNAi) has been rapidly gaining attention as a loss-of-function research tool, but it also shows great potential for therapeutic applications in the treatment of many diseases, and as a novel biological control mechanism for the protection of crops against different pests (Bellés, 2010; Burand and Hunter, 2013; Huvenne and Smagghe, 2010; Kim and Rossi, 2008; Price and Gatehouse, 2008). Its intracellular mode of action is highly conserved and well described (Hammond, 2005; Vodovar and Saleh, 2012). The inherent sequence specific nature of the mechanism allows for selectively targeting organisms, such as insect pests, by optimizing dsRNA fragments corresponding to species-specific gene sequences. It is obvious that the RNAi technology, allowing for *in vivo* post-transcriptional silencing of essential genes, thereby causing mortality with little effect on non-target species, gained significant interest in pest management research over the past years (Yu et al., 2013; Zhang et al., 2013). Indeed, several studies have demonstrated that this technique shows great potential for the development of novel biological strategies for selectively controlling agricultural pests (Baum et al., 2007; Zhu et al., 2011). Nevertheless, an important aspect in exploiting the RNAi response remains the introduction of dsRNA in the cells, and while the intracellular mechanisms appear highly conserved, the degree of sensitivity towards RNAi varies strongly among insects, with many (economically important) species being refractory to environmentally delivered dsRNA (Scott et al., 2013; Swevers et al., 2013b; Wynant et al., 2014a). Low sensitivity to RNAi is often observed in dipteran and lepidopteran species, although gene silencing in the latter has been reported in, amongst others, *Plutella xylostella*, *Spodoptera exigua* and *Manduca sexta* (reviewed by Terenius et al., 2011). In contrast, many members of the Hemiptera, Orthoptera and Coleoptera seem to be more responsive, making RNAi more feasible for pest control in these orders (Baum et al., 2007; Gong et al., 2014; Santos et al., 2014). However, there does not appear to be a clear rule to determine the sensitivity of an insect species. In addition, the method of dsRNA delivery is of crucial importance. In order to use RNAi as an efficient method in the control of insect pests, a potent knockdown through feeding of dsRNA is typically required. Yet, in several insect species, injection of naked dsRNA into the body cavity can successfully trigger RNAi, while feeding of the same dsRNA is less efficient, or completely ineffective. This was for instance observed in locusts, which are highly sensitive to injected dsRNA, while orally delivered dsRNA does not induce an efficient RNAi response (Luo et al., 2013; Wynant et al., 2014b).

Low efficacy of orally delivered dsRNA is often attributed to extracellular degradation in the gut lumen. In the digestive juice of larvae of the silkworm, *Bombyx mori*, an alkaline nuclease that could digest dsRNA has been isolated (Arimatsu et al., 2007). Later, high nuclease activity also has been demonstrated in the gut juice of locusts (Luo et al., 2013; Wynant et al., 2014b), as well as in salivary secretions of the pea aphid, *Acyrthosiphon pisum* (Christiaens et al., 2014), and the tarnished plant bug, *Lygus lineolarus* (Allen and Walker, 2012), to name a few. It is therefore very plausible that dsRNA degradation in the alimentary tract of insects contributes to a lowered RNAi efficiency upon feeding on dsRNA. This prompted us to hypothesize that removal of dsRNase activity in the insect midgut would have a beneficial effect on the RNAi potency, and could be sufficient to generate a more efficient knockdown through feeding in insect species that are less sensitive to orally induced

RNAi, but nonetheless possess the necessary cellular dsRNA uptake mechanisms and the subsequent means to generate a full RNAi response.

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, is a major crop pest of potato plants in large parts of the world, including North-America, Europe, and Asia. It is characterized by the ability to quickly develop resistance to a wide range of chemical pesticides, urging the need to develop alternative management strategies (Zhu et al., 2011). Several studies have shown that CPB is able to take up dsRNA from the gut lumen and subsequently induce a potent systemic RNAi response, suggesting that RNAi could be a feasible novel control method for this insect pest (Cappelle et al., 2016; Zhu et al., 2011). Until now no high nuclease activity has been demonstrated in the gut juice of CPB. Nevertheless, a survey of the gut transcriptome revealed the presence of potential DNA/RNA-non specific nuclease encoding genes (Swevers et al., 2013a). Considering the above, CPB constitutes an excellent insect model to study the involvement of extracellular nuclease degradation of dsRNA on the efficiency of the environmental RNAi response. Therefore, we aimed at identifying and further characterizing nuclease genes in CPB. Furthermore, we tried to deliver proof for extracellular dsRNA degradation in the gut lumen as a major contributor determining RNAi efficiency by knocking down the responsible nuclease encoding transcripts, and by simulating increased degradation by orally administering predigested dsRNA. Finally, we tried a similar approach in the desert locust, *Schistocerca gregaria*, an infamous pest insect, capable of forming huge devastating swarms when in the gregarious phase (Verlinden et al., 2009). Four nuclease genes were previously characterized in this species (Wynant et al., 2014b). Here, they were knocked down simultaneously in an effort to improve the RNAi sensitivity upon feeding of dsRNA.

2. MATERIAL AND METHODS

2.1 In silico detection and phylogeny

A tBLAST-N search using different insect dsRNase protein sequences as a query was used to search a combined assembly of three CPB data sets containing all available transcript sequences from adults, larvae and larval midguts (Kumar et al., 2014) (http://www.bio.unipd.it/~grapputo/CPB-Webpage). Retrieved transcripts were further assembled into contigs using Geneious. Obtained full length contig sequences were translated into amino acid sequences using the Expasy translate tool. For a multiple sequence alignment, two dsRNase sequences from *Tribolium castaneum* and one from *Locusta migratoria* were retrieved through BLAST searches against public databases on the NCBI website. In addition, *Sg_dsRNase1* and *Bm_dsRNase1* were selected from *S. gregaria* and *B. mori* based on previously published research (Liu et al., 2012; Wynant et al., 2014b). Translated protein sequences were aligned with the MUSCLE alignment software and manually verified. Signal peptides were predicted using SignalP 4.1 (Petersen et al., 2011), and possible disulfide bonds were predicted using the DiANNA web server (Ferrè and Clote, 2005).

Phylogenetic analysis was performed by using the tBLAST-N algorithm to search all public NCBI databases, using multiple insect dsRNase protein sequences as a query. Retrieved sequences were scanned to confirm the presence of a DNA/RNA non-specific endonuclease domain by the NCBI conserved domain scan (Marchler-Bauer et al., 2015). The translated coding regions of 45 selected sequences were aligned with the MUSCLE alignment software, manually verified and trimmed to obtain aligned regions with the highest homology. A full list of the sequences that were used can be found as Supplementary data S1. Phylogenetic analysis was performed using MEGA (Tamura et al., 2011). A maximum likelihood tree was constructed using a WAG substitution model and was tested by the bootstrap method, using 100 replications. Gaps were partially deleted with a site coverage cutoff of 95%. The phylogenetic tree was constructed using a bacterial DNA/RNA non specific endonuclease from *Salmonella enterica* as root.

2.2 Rearing of animals and sample collection

Colorado potato beetles (*L. decemlineata*) were reared on potato plants at controlled temperature (25°C), relative humidity (40%), and a 14h photoperiod. Larvae were developmentally synchronized at the moment of hatching, and 4th instar larvae or adults were used for experiments. To collect samples for total RNA extraction, larvae or adults were snap frozen in liquid nitrogen, and stored at -80°C until further use. For tissue distribution analysis, animals were dissected to collect separate tissues (head, gut, remainder of the body) before snap freezing. Locusts (*S. gregaria*) were reared under crowded conditions with controlled temperature (32 °C), relative humidity (40-60%), and a 14h photoperiod. They were fed daily with fresh cabbage leafs. Locusts were further developmentally synchronized by transferring them to a different cage immediately after the day of the final molt. For extraction of total RNA, guts of the animals were dissected and transferred to liquid nitrogen. Samples were stored at -80°C. In order to obtain biologically active gut juice, both CPB and locusts were

stimulated to vomit, so that regurgitated gut juices could be collected. Gut juice samples were stored at -20°C until further use.

2.3 Synthesis of dsRNA

Double stranded RNA fragments targeting *Ld_vATPaseE* (432 bp), *Ld_arf4* (631 bp), *Ld_dsRNase1* (608 bp), *Ld_dsRNase2* (636 bp), *Ld_Lethtgt* (1505), *Sg_dsRNase1* (616 bp), *Sg_dsRNase2* (386 bp), *Sg_dsRNase3* (536 bp), *Sg_dsRNase4* (686 bp), and *Sg_tubu* (564 bp) were prepared using the MEGAscript RNAi kit (Ambion). In brief, DNA templates containing T7 RNA polymerase promoter sequences on both ends were generated in a PCR reaction. These were subsequently used in a transcription reaction with the T7 RNA polymerase to generate dsRNA fragments. Remaining DNA and any single stranded RNA was removed with nuclease digestion. Finally, the dsRNA is purified by a solid-phase adsorption purification protocol, according to the manufacturers' instructions (Ambion). All primer sequences are presented in Supplementary data, Table S2. The PCR reactions were performed using the REDTaq ready mix (Sigma). Concentration of dsRNA was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific). The purity and integrity of PCR products and purified dsRNA fragments were assessed by performing 1% agarose gel electrophoresis.

2.4 In vitro degradation of dsRNA

To estimate nuclease activity in collected midgut juice samples, different dilutions were prepared using ringer solution (8.77 g/l NaCl, 0.19 g/l CaCl2, 0.75 g/ l KCl, 0.41 g/l MgCl2, 0.34 g/l NaHCO3, 30.81 g/l sucrose, 1.89 g/l trehalose, pH 7.2) and incubated for ten minutes with 250 ng of dsRNA. The incubation was performed at 25°C or 30°C, and at pH 6 or 7.5, for *L. decemlineata* or *S. gregaria*, respectively, corresponding to their optimal breeding temperature and pH of their gut lumen. Next, 6X loading dye was added to the samples and the dsRNA integrity was analyzed by means of 1% agarose gel electrophoresis. As a positive control, 250 ng of dsRNA was incubated in buffer. The total protein content of the gut juice samples was measured using the bicinchoninic acid (BCA) asay (Walker, 1994).

2.5 Marker gene selection

Depending on the experimental setup different genes were knocked down throughout this work. To assess sensitivity of the RNAi response in larval CPB, the expression of vATPase subunit E was targeted. Knockdown of this gene has been shown before to induce a potent gene knockdown and to induce larval mortality through feeding (Zhu et al., 2011). For further studies, *Ld_arf4* was selected as a marker gene. The *arf4* gene codes for ADP-ribolysation factor 4, which is ubiquitous in cells of all tissues. Therefore it forms a good marker for assessing the systemic knockdown efficiency in whole body after environmental uptake of dsRNA from the lumen of the gut on a transcript level. In addition, a lethal target gene (*Ld_Lethtgt*) was chosen. A knockdown of this transcript was shown to halt food intake and induce mortality in larvae as well as adults (Raemaekers et

al., 2014), making this gene an excellent marker to further study the phenotypic effects of a nuclease knockdown in adult beetles.

For experiments with the desert locust, *Sg_tubu* was used as a marker gene to assess knockdown efficacy after feeding. It encodes the α-tubulin subunit of microtubules, which are part of the cytoskeleton of eukaryotic cells. The *Sg_tubu* dsRNA fragment was shown before to be able to induce a potent systemic knockdown after injection (Wynant et al., 2012), which makes it a good marker to assess a potential knockdown after ingestion of dsRNA in *S. gregaria*.

2.6 Feeding and injection of dsRNA

Larvae and adults of CPB were fed dsRNA that was administered via potato leaf disks of around 6 mm in size. A total volume of 10 µl was typically applied on the disks. The disks were air-dried before being fed to individual insects. Doses of dsRNA that were applied were 500, 50, 5, 1 ng to determine RNAi sensitivity in third instar larvae, 100 ng undigested or predigested dsRNA, to assess the effects of predigestion, and 500, 50, and 5 ng for adult beetles after knockdown of nuclease transcripts. Knockdown of the nucleases themselves was obtained by administering 700 ng of dsRNA directed against both transcripts, *Ld_dsRNase1* and *Ld_dsRNase2*, twice, once before pupation, and once right after the emergence of the adults. After the disks were finished the animals were always transferred to plastic cages with fresh potato leaf material for the remainder of the time. In case of adult feeding experiments after nuclease knockdown, mortality was scored and potato plants were investigated for signs of feeding and egg-laying. As a control for all dsRNA treatments, *dsgfp* was used.

Locusts were first injected and then fed dsRNA. In case of injections adult locusts were injected ventrally between two segments of the abdomen with 6 µl of dsRNA. The solution contained 250 ng against all known nuclease genes from *S. gregaria*, *Sg_dsRNase1*, *Sg_dsRNase2*, *Sg_dsRNase3*, and *Sg_dsRNase4* (Wynant et al., 2014b), so a total of 1 µg was injected. To assess the subsequent feeding response, locusts were orally administered 10 µl of dsRNA (20 µg) by means of a micropipette, after which the animals were allowed to feed on fresh cabbage leafs. Care was taken that the animals did not vomit after orally delivering the dsRNA. As a control for dsRNA treatments, locusts were given *dsgfp*.

2.7 Total RNA extraction and cDNA synthesis

Total RNA from whole body CPB larvae was extracted utilizing the SV96 Total RNA isolation system (Promega) according to the manufacturers' instructions. Because of size, adult CPB and *S. gregaria* samples were processed using the Lipid tissue total RNA extraction kit (Qiagen), also according to the manufacturers' instructions. In all cases DNaseI treatment was performed to remove traces of genomic DNA contamination. RNA concentration and quality was assessed using a Nanodrop spectrophotometer and 1% agarose gel electrophoresis. Equal quantities of RNA were used as template to produce cDNA. Copy DNA synthesis was

performed using the Primescript First Strand cDNA synthesis kit (Takara Bio), following the manufacturers' protocol.

2.8 Quantitative real time qRT-PCR

The Primer3plus web tool was used to design quantitative real time qRT-PCR primers. All primer sequences are displayed as Supplementary data in Table S2. Primers were validated with a standard curve based on a serial dilution of cDNA to determine primer annealing efficiency. Each qRT-PCR reaction was performed in duplicate and contained 5 µl SYBR green solution (Invitrogen), 0.5 µl of forward and reverse primer (10mM) (Sigma), 2 µl milliQ water and 4 µl of cDNA. The PCR reaction was performed in a 96 well plate and analyzed by the StepOne System (ABI Prism, Applied Biosystems). Relative expression levels were calculated using the delta delta Ct method (Livak and Schmittgen, 2001). To correct for sample variation, expression was normalized against the geometric mean of two stably expressed reference genes (Vandesompele et al., 2002). Expression in CPB was normalized against *Ld_arf1* and *Ld_rp4* (Shi et al., 2013), expression in *S. gregaria* was normalized against *Sg_rp49* and *Sg_gapdh* (Van Hiel et al., 2009).

2.9 Statistical analysis

Data were tested for normality using the Shapiro-Wilk normality test (Razali and Wah, 2011). Significant differences in gene expression were determined by t-tests, when comparing two groups, or when comparing multiple groups, by ANOVA with Tukey comparisons for post-hoc tests. All statistical analyses were performed in Graphpad Prism 6 software.

3. RESULTS

3.1 Identification and sequence analysis of nuclease genes

A combined assembly of three CPB data sets containing transcript sequences from whole adult, whole larvae and larval midguts (Kumar et al., 2014), was searched for transcripts encoding possible nucleases. Five transcript sequences were retrieved (Ld_rep_c46402, Ld_rep_c32902, Ld_rep_c29028, Ld_repc25652, and Ld_c8070). The first two and the other three together could be further assembled into two different sequences, which were named *Ld_dsRNase1* and *Ld_dsRNase2*, respectively. Both nucleotide sequences span the entire open reading frame of the protein, and code for a single DNA/RNA non specific endonuclease domain. In addition, both proteins contain a predicted signal peptide, suggesting secretion to the extracellular medium, in accordance with their presumed function as nucleases that are active in the gut lumen. The full nucleotide and corresponding protein sequences were uploaded to GenBank (accession numbers KX652406 and KX652407, *Ld_dsRNase1* and *Ld_dsRNase2*, respectively), and can be found as supplementary data (Supp. Fig. S3). In Figure 1 a multiple sequence alignment is presented comparing the predicted amino acid sequences with two predicted nuclease sequences that were deduced from public databases of *Tribolium castaneum* (*Tc_dsRNase1, Tc_dsRNase2*; accession numbers XP_015840884 and XP_970494), a nuclease sequence identified from *Locusta migratoria* EST sequences (accession number KX652408), Sg_dsRNase1 from *S. gregaria* (accession number AHN55088; Wynant et al., 2014b), and the sequence from the alkaline nuclease that was purified from *Bombyx mori* gut juice (accession number AB254196; Arimatsu et al., 2007). Ld_dsRNase1 and Ld_dsRNase2 are similar and share 56% amino acid sequence identity. Both of them are most related to Tc_dsRNase2. Both locust sequences are also highly related, sharing 65% identity. The presence of eight conserved cysteine residues in all sequences points to possible disulfide bonds that could confer increased protein stability and similar protein architecture. For Ld_dsRNase1 possible bonds were predicted to be formed between cysteine residues 26-68 (1-2), 88-382 (3-8), 108-357 (4-7), and 123-148 (5-6). However, for Ld_dsRNase2 they were predicted between residues 24-66 (1-2), 86-146 (3-6), 106-356 (4-7), and 121-381 (5-8).

Additionally, a phylogenetic tree was constructed using 41 putative insect dsRNase sequences, retrieved from sequence data from 26 different insect species that belong to five different major insect orders (Lepidoptera, Diptera, Coleoptera, Orthoptera, and Hemiptera) (Figure 2). The insect sequences were supplemented with three homologous sequences identified from decapods. The tree was rooted using a bacterial putative DNA/RNA non-specific endonuclease from *S. enterica*. All sequences were confirmed to contain at least one DNA/RNA non-specific endonuclease domain by a NCBI conserved domain scan. In general, most sequences cluster in monophyletic groups that are well supported by high bootstrap values, with the exception of a putative dsRNase from Anopheles gambiae, which appears to cluster within the hemipteran sequences. Additionally, three coleopteran sequences also appear more closely related to the hemipteran cluster compared to the other coleopteran sequences. From the phylogenetic analysis it is clear that Ld_dsRNase1 and Ld_dsRNase2 are more closely related to each other than to sequences from related Coleoptera, indicating the duplication event of this gene likely occurred after the divergence from the common ancestor.

3.2 Nuclease expression and activity in the gut of CPB

The relative transcript profile for both identified nucleases was determined for the gut, head, and remainder of the body of larval CPB. Expression occurs predominantly in the gut for both *Ld_dsRNase1* and *Ld_dsRNase2* (Figure 3A-B), further suggesting that these enzymes are involved in extracellular nucleic acid digestion in the gut lumen. In addition, transcript levels were compared between larvae and adults. The results clearly show higher expression levels in adults, indicating that larvae might be more susceptible towards orally induced (environmental) RNAi (Figure 3C-D).

To investigate if CPB effectively possesses active dsRNA digesting enzymes in its gut lumen that might influence the RNAi response, dsRNA was incubated with a dilution series of collected gut juice *in vitro* (Figure 4). To have a better idea on the potency of the degradation, it was compared with a dilution series of gut juice from *S. gregaria*, known for its very high nuclease activity and inability to generate an oral RNAi response (Wynant et al., 2014b). After incubation for ten minutes, the dsRNA had disappeared at much higher dilution factors in *S. gregaria* compared to *L. decemlineata*. Nevertheless, clear degradation of the dsRNA occurred in the gut juice of the CPB as well, indicating the presence of active nucleases in the gut lumen. In addition, in accordance with the expression data, adult CPB gut juice appeared more potent compared to larval gut juice. A 50-fold dilution of adult gut juice was still capable of degrading the dsRNA, while in a 50-fold dilution of larval gut juice the dsRNA was still clearly visible. For *S. gregaria*, no difference in potency between larval and adult gut juice could be observed. A 500-fold dilution showed a very faint band after incubation in gut juice from both developmental stages, and the dsRNA remained fully intact only after incubation in a 1000-fold dilution (Figure 4). By comparing dilution series of pure gut juice, which contains a complex mixture of proteins, the physiological conditions of the gut lumen between the two species are compared in the most natural way. However, additionally, the total protein content of the undiluted gut juice samples was measured and averaged at 135 mg/ml for both *S. gregaria* adults and L4 larvae, while *L. decemlineata* larval gut juice averaged around 96 mg/ml and that of adults at 92 mg/ml.

3.3 The RNAi response in CPB is time and concentration dependent

Prior to studying the effect of nuclease activity in more detail, a reference frame for the sensitivity and efficiency of the environmental RNAi response was established. Third instar CPB larvae were fed different concentrations of marker dsRNA (*Ld_vATPase*). Subsequently, whole larvae were collected at two different time points, 24 hours and 3 days after feeding, and transcript levels were determined. The results show that 24 hours after ingestion of the dsRNA, transcript levels were significantly reduced with 89%, 79%, and 64% for 500, 50, and 10 ng of dsRNA, respectively (Figure 5). Administration of 1 ng of dsRNA per individual did not significantly reduce the transcript levels compared to the controls, although a trend was visible. Extending the incubation time to 3 days further reduced the transcript levels to 95%, 96%, and 84% for 500, 50, and 10 ng, while 1 ng still did not have a significant effect on the transcript levels (Figure 5).

3.4 Predigesting of dsRNA in gut juice lowers the RNAi efficiency of CPB larvae

Next, larvae of the CPB were fed a leaf disk containing dsRNA that was predigested through incubation with a diluted solution containing collected midgut enzymes. Incubating the dsRNA for 1 hour clearly digested it (Figure 6). However, a smear was still visible, suggesting that only partial degradation occurred at this point. The approximate size of the degraded dsRNA fragments that were applied on the leaf disk can be estimated between 100 bp and 200 bp, which is still above the known threshold for cellular uptake, which has been indicated to be around 30 bp in *T. castaneum* (Miller et al., 2012) and in *Drosophila* S2 cells (Saleh et al., 2006). Incubating the dsRNA for a longer period completely removed the smear, showing complete digestion after 16 hours. Consequently, 1 hour of pre-digestion of the dsRNA significantly lowered the knockdown efficiency to 42%, 24 hours after ingestion of the equivalent of 100 ng of dsRNA, compared to feeding of 100 ng undigested dsRNA, which induced a whole body knockdown of 70%. Increasing the incubation time further reduced the RNAi efficiency, up to a point that a knockdown could no longer be detected (Figure 6). Control animals received 100 ng of dsgfp that was incubated for 16 hours to rule out any effects of the ingestion of dsRNA or the diluted solution of gut juice.

3.4 Knockdown of nuclease genes sensitizes the RNAi response in CPB adults

To further assess the effects of nuclease activity in the gut on the sensitivity of the RNAi response, an RNAi-on-RNAi approach was applied, where the effect of a knockdown of a target gene was studied by assessing the efficiency of a subsequent marker gene knockdown. The expression of both *Ld_dsRNase1* and *Ld_dsRNase2* was targeted simultaneously by feeding last instar larvae, which were close to pupation, with a leaf disk containing a dose of 700 ng of dsRNA for each of both nuclease genes. The same treatment was repeated immediately after emergence of the adults, to ensure maximal knockdown of the nucleases at the protein level in the adult stage. Adults were chosen because the expression *of Ld_dsRNase1* and *Ld_dsRNase2* was higher at this stage and nuclease activity in the gut juice was clearly stronger. Three different concentrations of marker gene dsRNA were subsequently administered through another leaf disk. Transcript levels were measured 24 hours after ingestion. Two distinct marker genes were used. In addition to *Ld_arf4*, which was solely used for qRT-PCR analysis, a second marker gene (*Ld_lethtgt*) was selected. Knockdown of this transcript leads to halting food intake, and is capable of inducing mortality in both CPB larvae and adults (Raemaekers et al., 2014).

Both nuclease genes were significantly knocked down. Transcript levels were reduced with 84% and 86% for *Ld_dsRNAse1* and *Ld_dsRNase2*, respectively (Figure 7A-B). Moreover, the removal of nuclease expression positively influenced the knockdown of the marker genes 24 hours after uptake of the dsRNA, especially at the lowest concentration of dsRNA. Ingestion of 500 ng of *Ld_arf4* dsRNA by control animals resulted in a 60% reduction in transcript level, compared to 65% after nuclease knockdown. The difference increased to 11% for 50 ng and 21% for 5 ng, corresponding to a knockdown increase from 49% to 51%, and 30% to 51%, respectively (Figure 7C). Ingestion of 5 ng of *Ld_lethtgt* dsRNA in control animals resulted in a knockdown of 38%, compared to 57% after nuclease knockdown, which accounts for a difference of 19%. After ingestion of 50

ng, the transcript levels were reduced with 47% and 63%, for control and nuclease knockdown animals, respectively; a 16% difference. For 500 ng, knockdown values averaged at 68% and 70% (Figure 7D).

Furthermore, from Figure 8A it is clear that higher concentrations of *Ld_lethtgt* dsRNA induced mortality, and that removal of nuclease activity increased the effect. After eight days 75% and 50% of the beetles that had ingested 500 and 50 ng of dsRNA, respectively, had died. However, from the beetles that had their nuclease activity removed, 82% were dead or moribund for both 500 and 50 ng. In addition, no more death events occurred after day 8 in control animals that received 50 and 500 ng. In the nuclease knockout condition, mortality further increased to 90% and 100%, respectively, after 14 days. Moreover, all control animals that only received 5 ng of dsRNA were still alive at day 8, and 90% survived the experiment, while from the nuclease knockout animals 45% had died after 14 days (Figure 8A).

After ingestion of the final leaf disk containing the target dsRNA, beetles were transferred to potato plants for the remainder of the experiment. At day four after ingestion of the *Ld_lethtgt* dsRNA, potato plants were replaced. Three days later the fresh plants were assessed for signs of feeding and egg laying. Beetles from both control conditions (regular control and nuclease knockdown control) behaved normally. Multiple egg layings occurred, and the plants were almost completely devoured (Supplementary Fig. S4). Knockdown of the nucleases had no apparent phenotypic effect on the animals. In contrast, all beetles that received 500 ng of *Ld_lethtgt* dsRNA had no longer consumed anything at this point, and had not laid any eggs (Supplementary Fig. S4). Control beetles that received 50 ng of dsRNA showed only very small signs of feeding, while nuclease knockdown animals did not consume anything at all, with very little to no egg laying in either condition (Supplementary Fig. S4). However, at the lowest concentration of 5 ng a clear difference could be observed. Control beetles clearly still had fed on the plant and deposited several egg clutches, while no egg laying or feeding could be observed in nuclease knockout beetles that had received only 5 ng of *Ld_lethtgt* dsRNA (Figure 8B).

Finally, we investigated the effect of a single dose of 500 ng marker gene dsRNA on the transcript levels of *Ld_dsRNase1* and *Ld_dsRNase2* in adult beetles after 24 hours, to evaluate if dsRNA exposure could quickly desensitize the animals by stimulating nuclease expression. No significant difference on the transcript levels could be observed (Supplementary Fig. S5).

3.5 Knockdown of nuclease genes in Schistocerca gregaria does not improve the environmental RNAi response

Four nuclease genes were previously characterized in the desert locust: *Sg_dsRNase1, Sg_dsRNase2, Sg_dsRNase3,* and *Sg_dsRNase4*. In an extensive tissue distribution they were shown to be expressed exclusively in the gut, and a knockdown of these dsRNases lowered the nuclease activity in the gut significantly, especially for *Sg_dsRNase2* (Wynant et al., 2014b). Here we simultaneously knocked down all four dsRNases, by injection of 250 ng of dsRNA for each nuclease. The procedure was repeated after four days to ensure maximal knockdown. Two days later, 20 µg of marker gene dsRNA (*Sg_tubu*) was orally administered. Quantitative RT-PCR results show that expression of all four nuclease genes was clearly suppressed (91%, 94%, 97%, and 96%) (Figure 9A). However, no effect on the transcript level of the orally administered marker could

be observed (Figure 9B). Injection of the same dsRNA did lead to effective down regulation of the corresponding transcript (Supplementary Fig. S6). Gut juice collected from dsRNase knockdown animals clearly showed less potent degradation of dsRNA compared to controls. Nevertheless, a 50-fold dilution of gut juice was still capable of degrading 250 ng of dsRNA in ten minutes (Figure 10), indicating the presence of residual nuclease activity in the gut.

4. DISCUSSION

We confirmed that the Colorado potato beetle, *L. decemlineata*, has a very sensitive environmental RNAi response through feeding. Ingestion of as little as 10 ng of dsRNA by L3 stage larvae was sufficient to generate a significant knockdown after 24 hours. This seems to be close to the required minimal dose. One ng no longer led to significant differences on the transcript level, although did show a trend for suppression. The knockdown increased after three days for all concentrations, thereby showing a clear dose and time dependency of the RNAi response in CPB.

It is difficult to directly compare the sensitivity of the observed response to previously published work on CPB, or to other insects that are known to be sensitive towards orally induced RNAi. In many cases quantities are used that appear (far) above the threshold. This ensures maximal knockdown of the target gene, which usually is the primary objective. Data on the exact amount of dsRNA that is necessary for a potent knockdown are often limited, and might be sequence dependent to some extent. To the best of our knowledge this is the first study describing the exact concentration dependency of naked dsRNA on the environmental RNAi response in CPB. In reports where the concentration dependency of RNAi was studied in insects, often different concentrations of dsRNA are administered without monitoring the exact intake of individuals. One recent study in CPB clearly describes a gradual decrease in transcript level depending on the administered dsRNA concentration, and also, interestingly, depending on the larval stage of the insect (Guo et al., 2015). However, dsRNA was applied via a leaf dip bioassay, using bacterial suspensions. In another example, in the Western corn rootworm, *Diabrotica virgifera*, a species that is regarded as highly responsive towards oral RNAi, a concentration of 100 ng of dsRNA topically applied on 200 µl of artificial diet was sufficient to generate a physiological response, while 10 ng appeared to be insufficient (Miyata et al., 2014). However, from both these examples, it is impossible to know exactly how much dsRNA was actually ingested by the animals. In another coleopteran species, *T. castaneum*, it was shown that dsRNA against a lethal target (*vATPase*) had an LC_{50} value of 2.5 µg/g diet (Whyard et al., 2009). In this regard, it is interesting to mention that we also tested the feeding response in *T. castaneum* (data not shown). Interestingly, a clear response could not be observed, suggesting that no potent environmental RNAi response exists in our lab strain of *T. castaneum*. It is noteworthy that this discrepancy was also mentioned by another recently published research paper (Miyata et al., 2014), while another research group more recently succeeded in delivering dsRNA through the diet of *T. castaneum* (Abd El Halim et al., 2016). This indicates that the ability to generate a potent environmental RNAi response in *T. castaneum*, and probably in other insects, might be strain dependent. Possible explanations for strain dependency of RNAi could be differences in nuclease activity, differences in the efficiency of dsRNA uptake from the gut, or even differences in viral load that could affect the RNAi response (Swevers et al., 2013b), although these possibilities should be investigated further.

It is obvious that the CPB possesses a sensitive feeding RNAi response. However, despite this sensitivity, we were able to detect nuclease activity in the gut, which was higher in adults compared to larvae. We identified two nuclease sequences that are specifically expressed in the gut and that are responsible for the digestive

dsRNA degrading activity. The two sequences are very similar at the amino acid level. A multiple sequence alignment including sequences from *T. castaneum*, *L. migratoria*, *S. gregaria*, and *B. mori* further shows that dsRNases possess eight conserved cysteine residues, suggesting the presence of multiple disulfide bonds. These bonds could increase the stability of the protein in the hostile environment of the gut lumen. A feature they appear to have in common with many other digestive enzymes in the gut, such as digestive proteases (Spit et al., 2014). Disulfide bond prediction was indecisive on the exact positioning of the bonds. A link between cysteine residues 1 and 2, and 4 and 7 was suggested for both proteins. However, the other two bonds were predicted between cysteine residues 3-8 and 5-6, or between residues 3-6 and 5-8, for Ld_dsRNase1 and Ld_dsRNase2, respectively. Determining the exact disulfide bond linkages by for example reduction studies may be necessary (Tang and Speicher, 2004), and will provide more insight into the structure-activity relationship of the dsRNases.

Detecting active nuclease activity in the gut of an insect species that is very sensitive towards orally delivered dsRNA raises some questions, since nuclease activity in the gut has often been suggested to be associated with resistance towards environmental RNAi through feeding (Luo et al., 2013; Wang et al., 2016; Yu et al., 2013; Zhang et al., 2013). Comparison of the dsRNA degrading activity in the gut juice of CPB and *S. gregaria*, which is insensitive towards orally delivered dsRNA, nonetheless showed that, while the beetles are able to degrade the dsRNA, the gut juice of locusts was far more potent in this regard. This is in full accordance with the hypothesis that the potency of nuclease activity in the digestive system and the sensitivity towards RNAi are correlated in insects. A dual approach was taken to further investigate the effect of the nuclease activity on the efficiency of the RNAi response. First, larvae of the CPB were fed with dsRNA that was predigested in diluted gut juice. This simulates an extracellular environment with an increased nuclease activity. Second, the nuclease activity in adults was lowered by RNAi of the two identified dsRNase genes. Both treatments had opposite effects, in line with the hypothesis. Increasing nuclease activity lowered the RNAi sensitivity, while decreasing it made the animals more sensitive towards orally delivered dsRNA. The latter was evidenced by both qRT-PCR analysis, and by a reduced food uptake and increased mortality in adult beetles that ingested dsRNA against a lethal target in addition to having both their dsRNase genes knocked down.

In contrast, a similar strategy of knocking down the four identified nuclease genes in *S. gregaria* had no effect on the sensitivity of a second RNAi response for a marker gene. Several possible explanations exist. Even though we obtained knockdown values of over 90% for all four *Sg_dsRNase* genes, some residual nuclease activity could still be detected in the gut juice. The knockdown only describes relative differences in transcript level, which does not reflect the absolute amount of transcripts that are left. In addition, the proteins may have a long half-life time. Alternatively, yet unidentified nuclease genes may be expressed in the locust gut, which were not targeted for knockdown by our treatment. Initial digestion in locusts occurs mainly in the foregut under the influence of regurgitated enzymes from the midgut and gastric caeca (Terra et al., 1994). Since the foregut is lined by a cuticle, which is considered impermeable for dsRNA, the residual nuclease activity, even though noticeably lower compared to controls, could still be strong enough to degrade the ingested dsRNA before the food bolus is passed on to the midgut, where the dsRNA would be taken up by the cells. This shows

the potential relevance of the feeding method and the digestion process in determining success of an environmental RNAi response. In the future, a more complete removal of the dsRNase activity in the gut by applying potent nuclease inhibitors or gene knock-out technologies like for example CRISPR/Cas9 (Doudna and Charpentier, 2014) might further improve our understanding of the exact role(s) of these enzymes. Apart from degradation in the digestive system, other research has shown the importance of functional uptake mechanisms for dsRNA. In insects, the primary mode of cellular uptake of dsRNA appears to be scavenger receptor-mediated endocytosis (Cappelle et al., 2016; Ivashuta et al., 2015; Li et al., 2015; Saleh et al., 2006; Ulvila et al., 2006; Wynant et al., 2014; Xiao et al., 2015; Yoon et al., 2016). Interestingly, also in adults of *S. gregaria*, inhibiting scavenger receptor function led to decreased sensitivity towards injected dsRNA (Wynant et al., 2014). However, in the current study, the dsRNA was delivered through the diet. Therefore, based on our results, it cannot be excluded that locusts simply do not possess the necessary mechanisms for uptake of dsRNA from the luminal side of the gut. Finally, it has been shown recently that even if the dsRNA is taken up efficiently by cells, incorrect further intracellular processing of the dsRNA molecules could also lead to reduced RNAi efficiency. This has been observed for lepidopteran cell lines derived from *Spodoptera frugiperda* and *Heliothis virescens*, where less efficient endosomal escape was suggested to lead to the accumulation of dsRNA in endosomal compartments, in contrast to cell lines from *T. castaneum* and *L. decemlineata*, where dsRNA was processed correctly (Shukla et al., 2016). However, since *S. gregaria* has been shown to possess a very potent systemic RNAi response upon injection (Wynant et al., 2012), it seems less likely that problems with intracellular processing would be responsible for the refractoriness towards orally delivered dsRNA in this species.Several reports mention that competition of dsRNA fragments might occur when they are administered together (Miller et al., 2012; Miyata et al., 2014; Tomoyasu et al., 2008). Nevertheless, here, simultaneous delivery of multiple dsRNA fragments resulted in potent knockdowns of all targeted genes at once, indicating that the RNAi machinery was not saturated, even with up to four dsRNA fragments in *S. gregaria*. It must however be noted that in order to minimize potential effects of dose and size dependent competition, we deliberately subjected the animals to the dsRNA treatment twice, administered the same concentration for all targets, and used dsRNA fragments of roughly a similar size. However, while the sequences of *Sg_dsRNase3* and *Sg_dsRNase4* show less similarity, the transcript sequences of *Sg_dsRNase1* and *Sg_dsRNase2* are very similar, and it has to be mentioned that previous research has shown that the dsRNA construct directed against *Sg_dsRNase1* might also influence the transcript levels of *Sg_dsRNase2* to some extend and vice versa (Wynant et al., 2014b). It is unclear if under other circumstances competition might occur. It would also be interesting to find out if more, and how many, genes can be targeted at once in a similar setup as applied here, before the RNAi machinery gets saturated. Yet, this number might still depend on the specific experimental circumstances.

In conclusion, the combined results deliver irrefutable proof that the environmental RNAi sensitivity in an insect is dependent on the potency of the dsRNA degradation in the gut lumen. The results also implicate that interfering with the nuclease activity, or protecting the dsRNA from degradation might be a good way to improve the RNAi response in some species. This could broaden the range of target pests, and reduce the economical and environmental costs of a dsRNA-based treatment, by lowering the necessary amount of active ingredient. However, the presented work also indicates that, next to many other possible mechanisms,

increasing the nuclease expression in the gut could be a very effective way of acquiring resistance of insect populations towards dsRNA. Nevertheless, while prolonged and continuous exposure might lead to this outcome, a single dsRNA exposure did not significantly increase the expression of nucleases in the gut of CPB. Finally, because degradation is independent of the dsRNA sequence it is important to assume the same initial mode of action for all naked dsRNA molecules, that will have similar cellular uptake mechanisms and survival properties in the gut lumen environment of the insect, even if the dsRNA molecules target completely different intracellular pathways. This is a valid concern, and will have to be carefully considered when developing novel pest control strategies based on the RNAi technology and when estimating the associated resistance risks.

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FIGURES

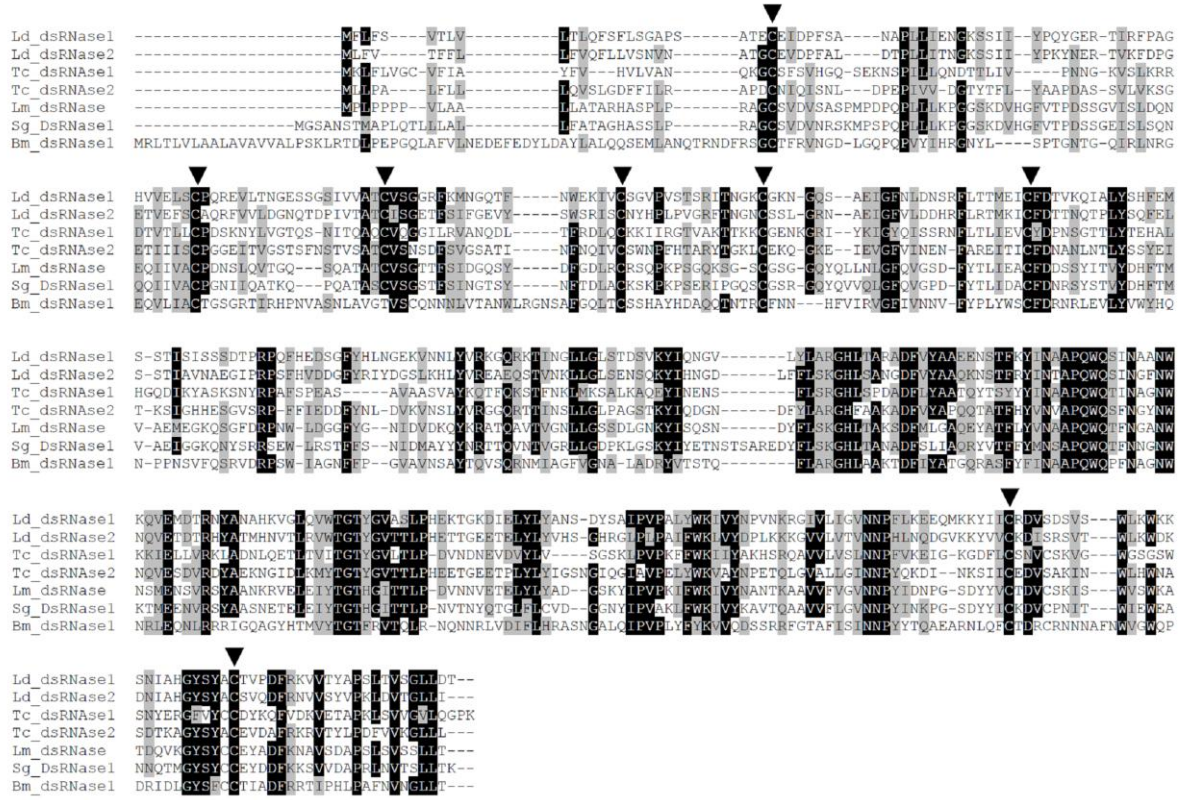

Figure 1**.** Multiple sequence alignment of identified Ld_dsRNase1 (KX652406) and Ld_dsRNase2 (KX652407) *from L. decemlineata* with Tc_dsRNase1 (XP_015840884) and Tc_dsRNase2 from *T. castaneum* (XP_970494), Lm_dsRNase from *L. migratoria* (KX652408), Sg_dsRNase1 (AHN55088) from *S. gregaria*, and Bm_dsRNase1 from *B. mori* (AB254196). Alignment was carried out using MUSCLE. Threshold for shading is set at 70% identity. Identical residues are shaded black, similar amino acids are shaded in gray. Conserved cysteine residues that may be used for disulfide bridge formation are indicated with an arrowhead.

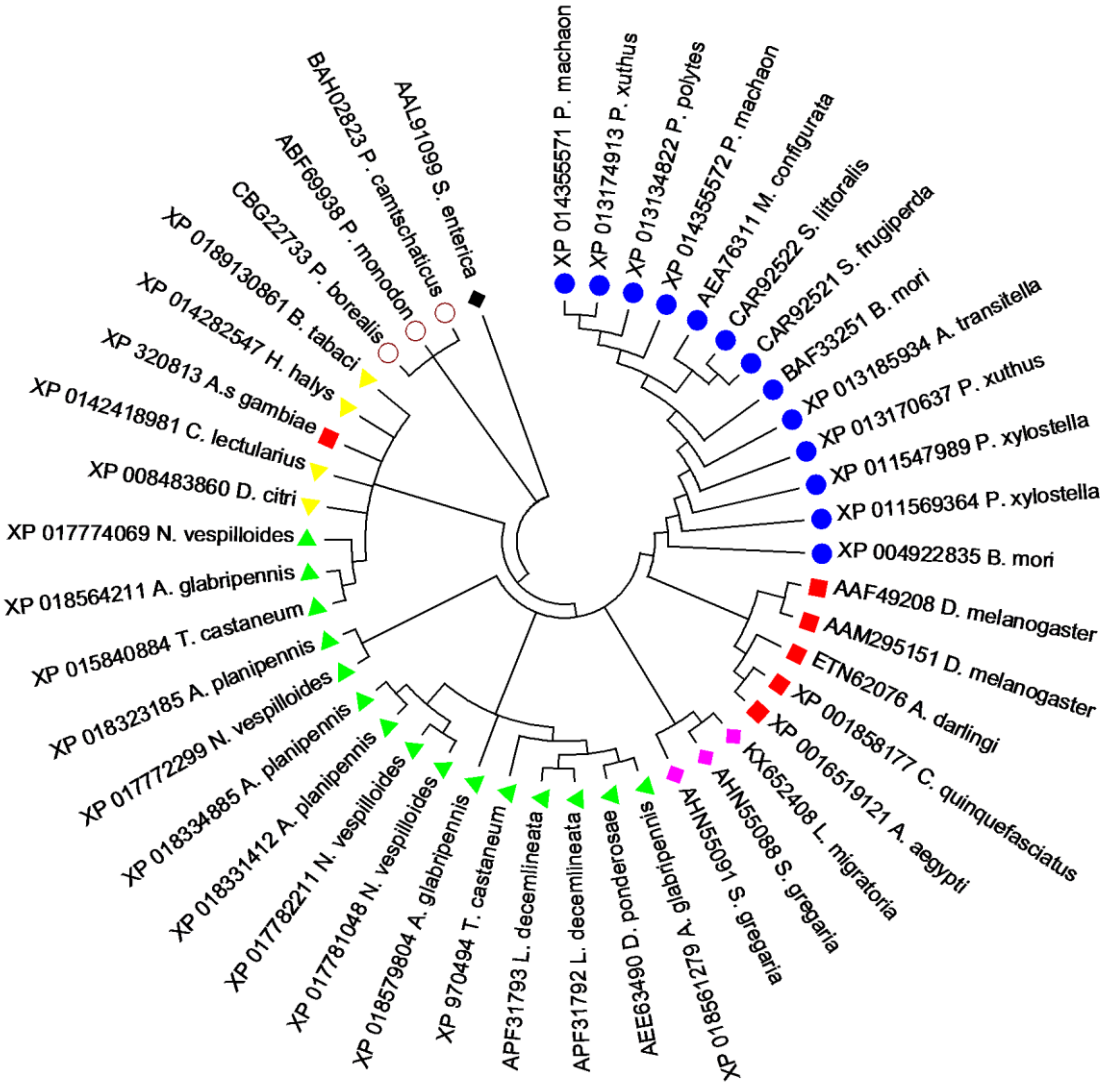

Figure 2**.** Circular maximum likelihood phylogenetic tree of putative dsRNase sequences from different insect orders, containing Ld_dsRNase1 and Ld_dsRNase2. The tree was rooted using a bacterial DNA/RNA non-specific endonuclease sequence and inferred from 100 bootstrap replicate. A condensed tree is presented, based on a bootstrap-value cutoff of 50. Accession numbers and species names are depicted. Sequences from Lepidoptera are indicated as blue circles, Diptera as red squares, Orthopera as purple diamonds, Coleoptera as green triangles, Hemiptera as yellow triangles, Decapoda as brown open circles, and the root of the tree as a black diamond.

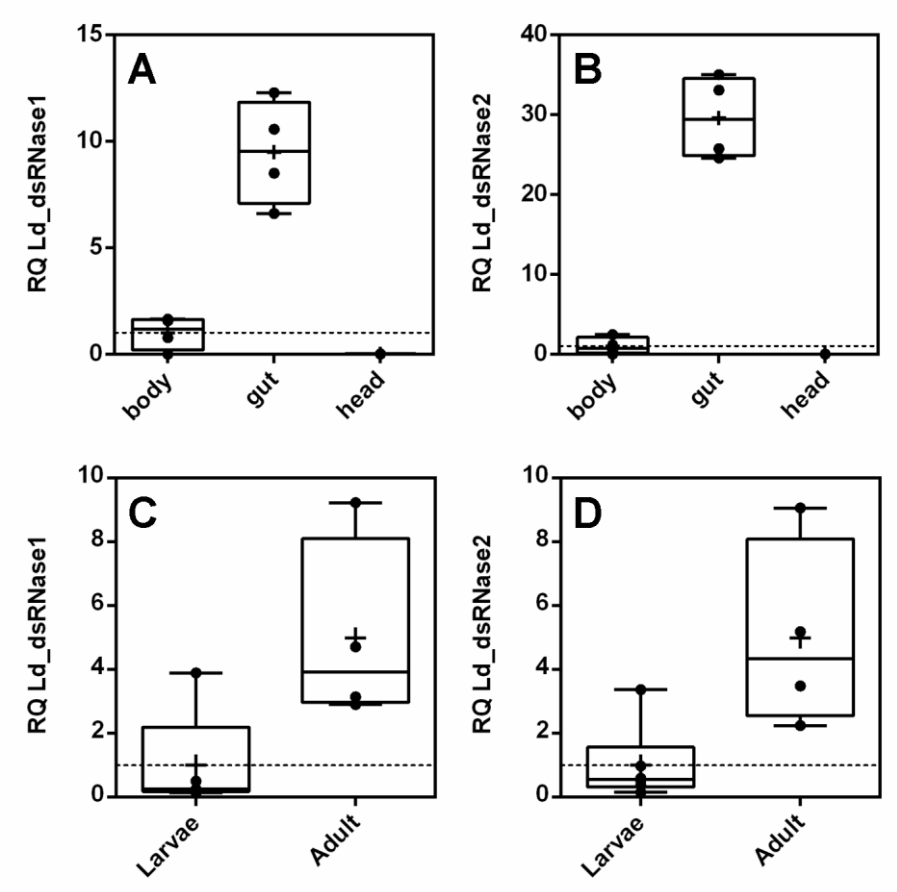

Figure 3**.** Expression of *Ld_dsRNase1* and *Ld_dsRNase2* in different body parts (**A,B**) and life stages of *L. decemlineata* (**C,D**). Relative transcript levels were normalized against two reference genes, *Ld_arf1* and *Ld_rp4*. Boxplots of the resulting data are presented (n ≥ 4). Means are indicated with a plus sign. For tissue distribution data, means were calculated against transcript levels in the body. Horizontal dotted line indicates RQ = 1. In case of larvae and adult expression comparison, the average relative quantity in the larvae has been set to 1.

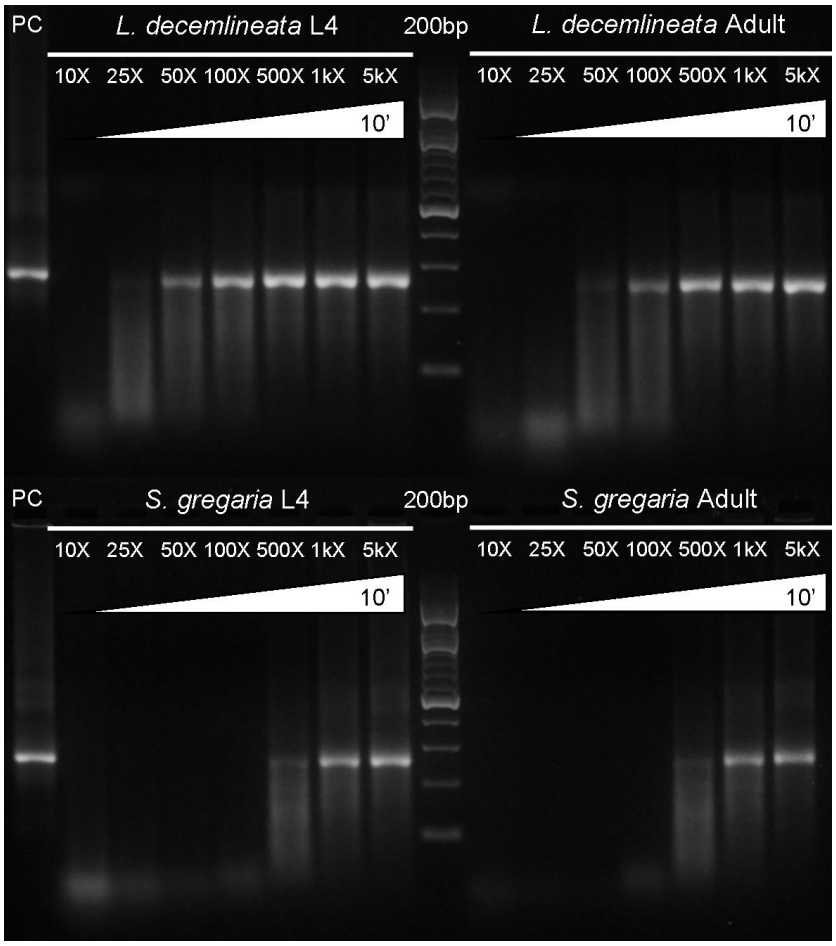

Figure 4**.** Incubation of 250 ng of dsRNA in a serial dilution of regurgitated gut juices for 10 min. Samples were analyzed by means of 1% agarose gel electrophoresis in the presence of a 200 bp DNA ladder. Nuclease activity in the gut juice of fourth larval instars and adult *L. decemlineata* (top) was compared to that of fourth larval instar and adults from *S. gregaria* (bottom). As a positive control (PC) naked dsRNA was incubated in buffer solution in the absence of nuclease enzymes.

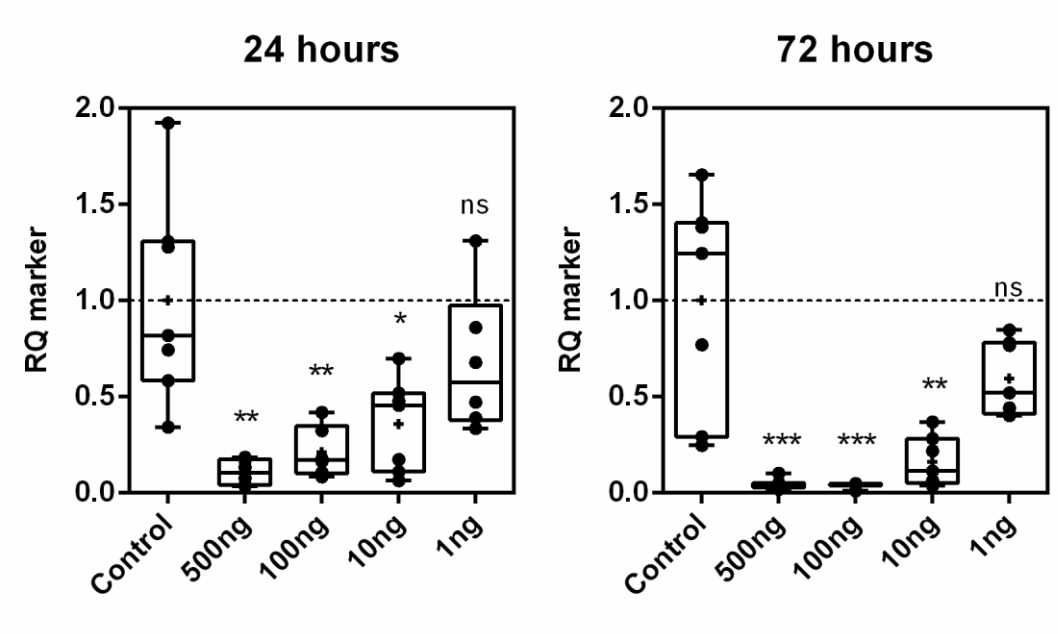

Figure 5**.** Concentration- and time dependency of the knockdown in larvae of *L. decemlineata*. Relative transcript levels of a marker gene (*Ld_vATPase*) were determined 24 and 72 hours after ingestion of the indicated amounts of dsRNA (1 ng to 500 ng). Control animals ingested 500 ng of dsgfp. Data were normalized against two reference genes, *Ld_arf1* and *Ld_rp4*, and mean control values were set to RQ = 1. Boxplots of the resulting data are presented (n ≥ 6) and means are indicated with a plus sign. Statistical differences were determined using ANOVA with post hoc tests (levels of significance are indicated by * for p ≤ 0,05, ** for p ≤ 0,005, *** for p ≤ 0,001, and ns for not significant).

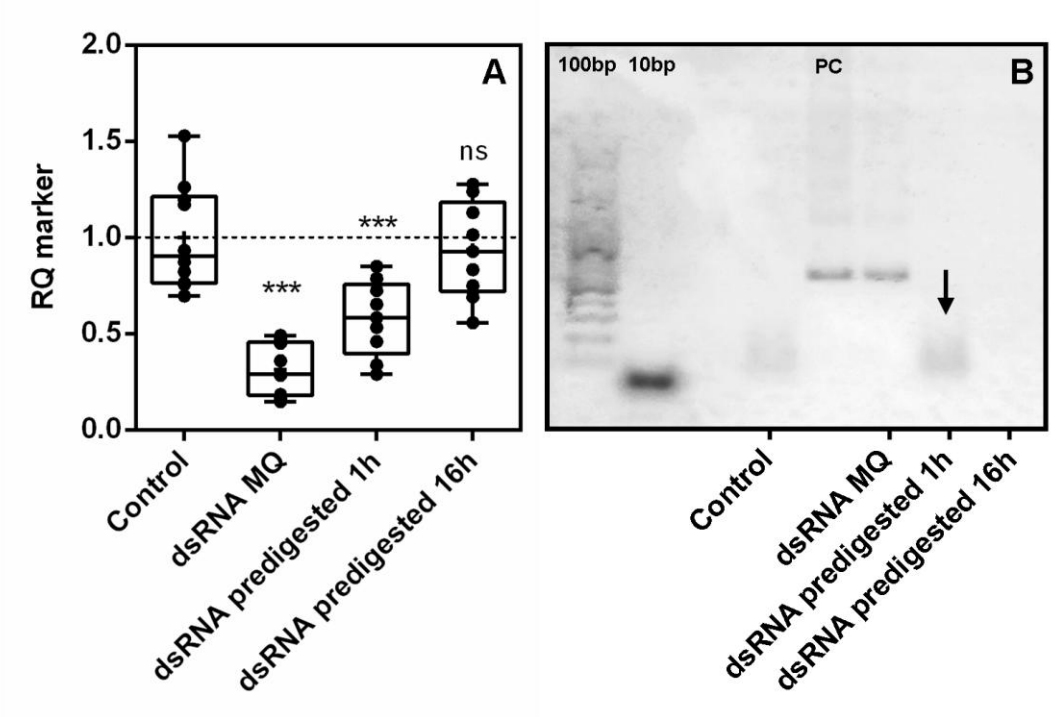

Figure 6**.** Predigestion of dsRNA lowers RNAi efficiency in *L. decemlineata* larvae. **(A)** An equivalent of 100 ng of marker gene (*Ld_arf4*) dsRNA was incubated in a diluted solution containing gut enzymes for 1 hour or 16 hours and fed to larvae. As a negative control, dsgfp was incubated for 1 hour in the same solution. Positive control knockdown values were determined by incubating dsRNA in MQ. Knockdown of gene expression was determined 24 hours after ingestion. Relative transcript levels were normalized against two reference genes, *Ld_arf1* and *Ld_rp4*. Data is presented as boxplots (n ≥ 10), means are indicated with a plus sign. Mean relative quantity (RQ) was set to 1 for the control animals. Statistical differences in gene expression were determined using ANOVA with post hoc tests (levels of significance are indicated by ** for p ≤ 0,005, *** for p ≤ 0,001, and ns for not significant). **(B)** Amount of dsRNA degradation in the samples after incubation was analyzed by means of 1% agarose gel electrophoresis. A 100 bp and 10 bp ladder were also added on the gel, in addition to 100 ng of untreated dsRNA as a positive control (PC). Arrow indicates smear of partially degraded marker dsRNA after 1 hour of incubation. Size of the partially degraded dsRNA can be estimated between 100 and 200 bp.

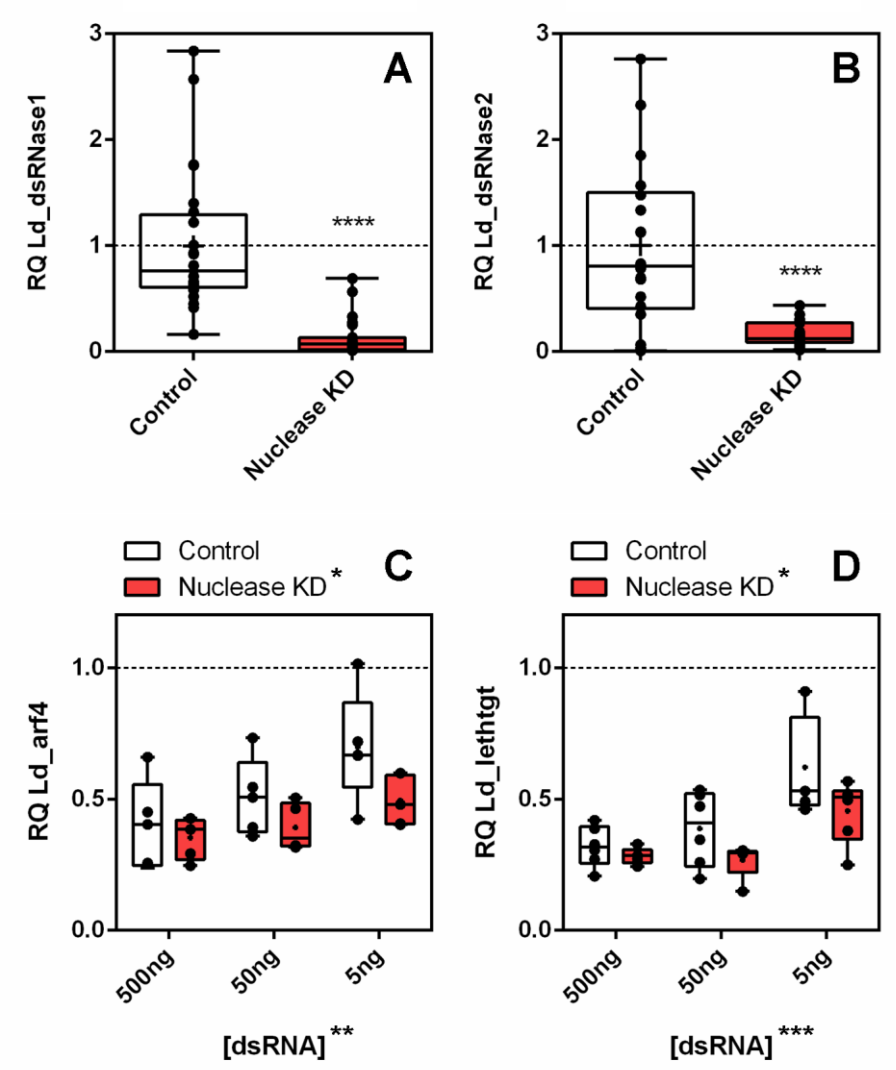

Figure 7**.** Effect of nuclease knockdown (KD) in *L. decemlineata* adults. Both *Ld_dsRNase1* **(A)** and *Ld_dsRNase2* **(B)** were significantly knocked down. Expression of both *Ld_arf4* and *Ld_lethtgt* was subsequently targeted in two independent experiments. Both *Ld_arf4* **(C)** and *Ld_lethtgt* **(D)** show improved RNAi efficiency after nuclease knockdown, 24 hours after ingestion of 50 ng and 5 ng of the marker dsRNA. Relative transcript levels were normalized against two reference genes, *Ld_arf1* and *Ld_rp4*. Boxplots of the data are presented (n ≥ 6) as compared to the control situation (RQ = 1), means are depicted as a plus sign. Statistical differences in gene expression were determined using t-tests to compare *Ld_dsRNase1* and *Ld_ dsRNase2* expression between the two groups, or two-way ANOVA for the comparison of marker gene expression between different administered concentrations and both groups. For both *Ld_arf4* and *Ld_lethtgt* both concentration of marker gene dsRNA and nuclease KD condition contribute significantly to the variation in marker gene transcript levels (levels of significance are indicated by * for p ≤ 0,05, ** for p ≤ 0,005, and *** for p ≤ 0,001).

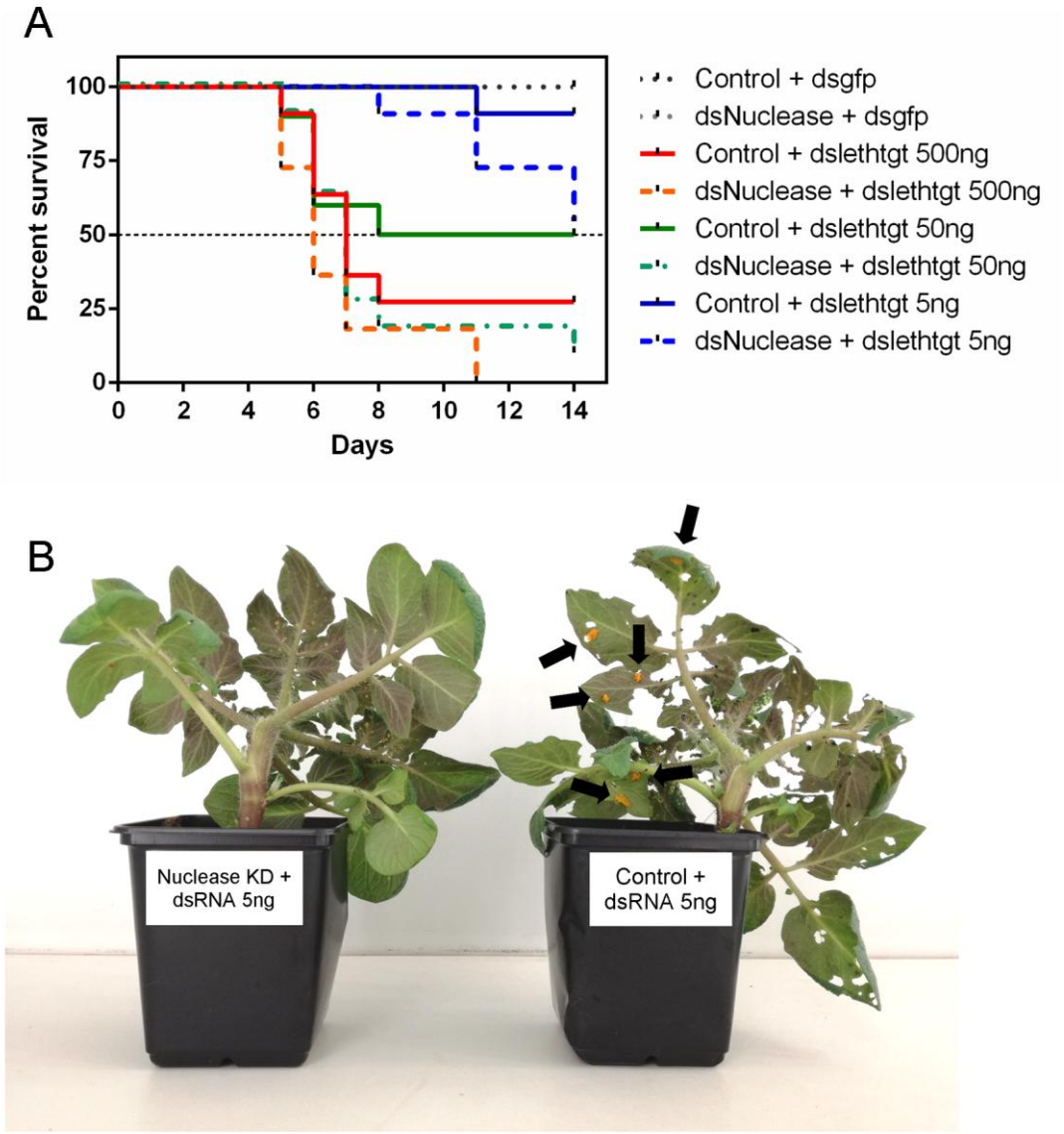

Figure 8**. (A)** Mortality curve of *L. decemlineata* adults after ingestion of 5 ng, 50 ng, or 500 ng of *Ld_lethtgt* dsRNA (dslethtgt), or 500 ng of gfp dsRNA (dsgfp), in both control animals (Control) or animals that received dsRNA against both *Ld_dsRNase1* and *Ld_dsRNase2* (dsNuclease). **(B)** Comparison of defoliation and egg laying between controls and nuclease knockdown animals that ingested 5 ng of *Ld_lethtgt* dsRNA. Deposited egg clutches are indicated by black arrows.

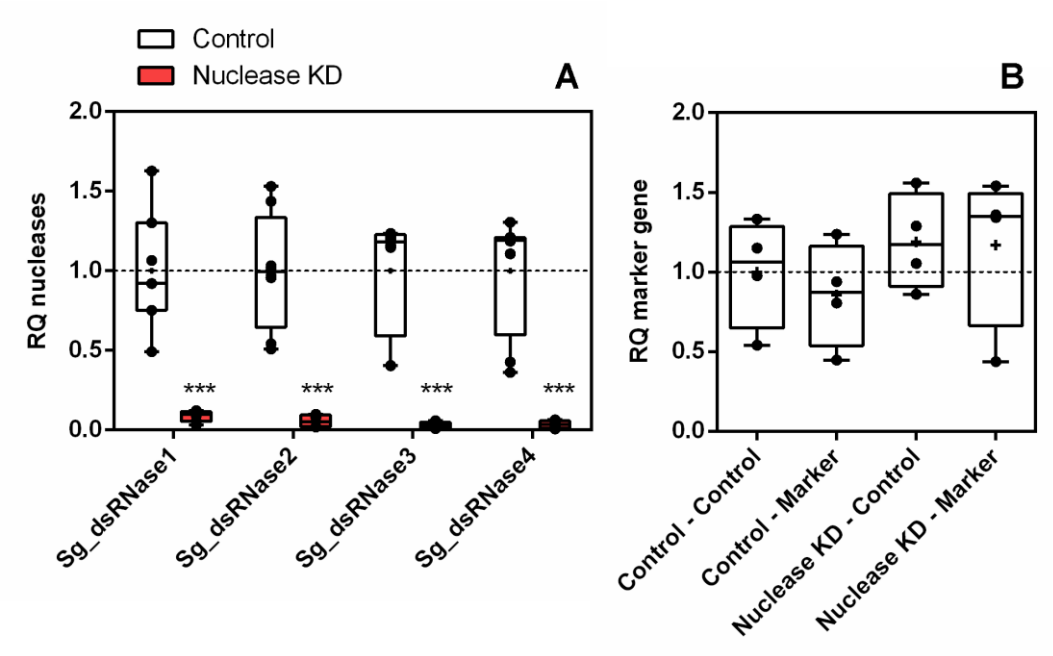

Figure 9**.** Effect of nuclease knockdown (KD) in *S. gregaria* adults. **(A)** All four identified *Sg_dsRNase* sequences were significantly knocked down by injection of a dsRNA mixture. **(B)** Subsequent feeding of 20 µg of marker gene (*Sg_tubu*) dsRNA did not lead to any significant knockdown of the marker gene. Relative transcript levels were normalized against two reference genes, *Sg_rp49* and *Sg_gapdh*. Boxplots of the data are presented (n ≥ 6). Means are depicted as a plus sign. For all independently targeted genes, mean relative quantity of the controls has been calculated to RQ = 1. Statistical differences in gene expression were determined using t-tests (levels of significance are indicated by *** for p ≤ 0,001).

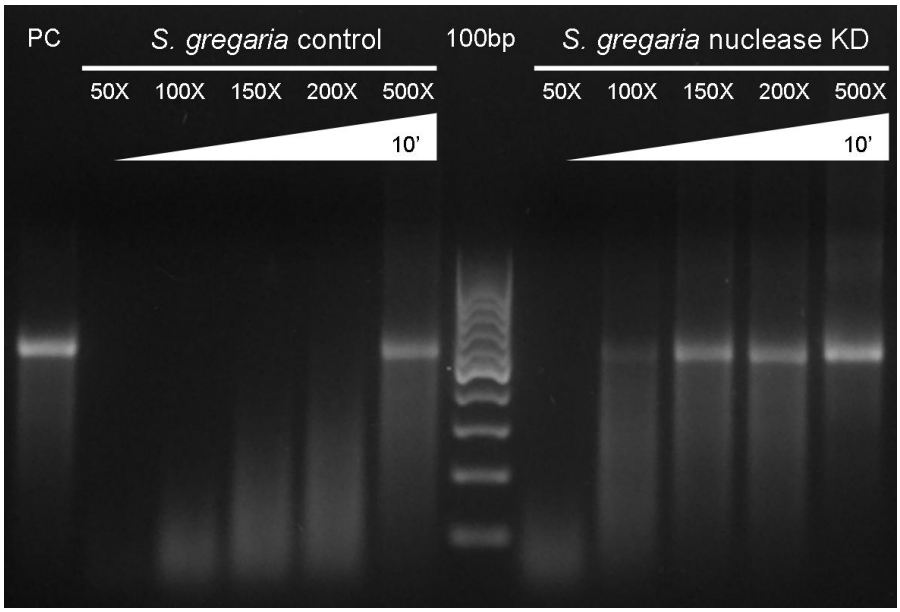

Figure 10**.** Incubation of 250 ng of dsRNA in a serial dilution (50x to 500x) of regurgitated gut juices from *S. gregaria* for 10 min. Samples were analyzed by means of 1% gel electrophoresis in the presence of a 100 bp DNA ladder. Nuclease activity in the gut juice of dsgfp treated locusts (control) was compared to that of locusts that had a simultaneous knockdown of *Sg_dsRNase1, Sg_dsRNase2, Sg_dsRNase3, and Sg_dsRNase4* (nuclease KD). As a positive control (PC) naked dsRNA was incubated in buffer solution in the absence of nuclease enzymes.