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Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: Evidence from *Manduca sexta* and *Blattella germanica*

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ABSTRACT

RNA interference (RNAi) is a specific gene silencing mechanism mediated by double-stranded RNA (dsRNA), which has been harnessed as a useful reverse genetics tool in insects. Unfortunately, however, this technology has been limited by the variable sensitivity of insect species to RNAi. We propose that rapid degradation of dsRNA in insect hemolymph could impede gene silencing by RNAi and experimentally investigate the dynamics of dsRNA persistence in two insects, the tobacco hornworm, *Manduca sexta*, a species in which experimental difficulty has been experienced with RNAi protocols and the German cockroach, *Blattella germanica*, which is known to be highly susceptible to experimental RNAi. An *ex vivo* assay revealed that dsRNA was rapidly degraded by an enzyme in *M. sexta* hemolymph plasma, whilst dsRNA persisted much longer in *B. germanica* plasma. A quantitative reverse transcription PCR-based assay revealed that dsRNA, accordingly, disappeared rapidly from *M. sexta* hemolymph *in vivo*. The *M. sexta* dsRNAse is inactivated by exposure to high temperature and is inhibited by EDTA. These findings lead us to propose that the rate of persistence of dsRNA in insect hemolymph (mediated by the action of one or more nucleases) could be an important factor in determining the susceptibility of insect species to RNAi.

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1. Introduction

RNA interference (RNAi) is a term used to describe a number of gene silencing phenomena characterized by the specific binding of short RNAs (20–30 nucleotides in length) to target sequences in a process mediated by Argonaute family proteins (Zaratiegui et al., 2007; Obbard et al., 2009). In exogenous (or “classical”) RNAi the presence of foreign double-stranded RNA (dsRNA) molecules in a cell leads to the destruction of the cognate endogenous messenger RNA (mRNA). The process has been investigated in some detail in the dipteran *Drosophila melanogaster*, where the following steps have been shown to occur. Exogenous dsRNAs are processed by Dicer-2, a type III RNase III enzyme, into RNA duplexes 21–23 nucleotides in length with 2 nucleotide long 3′ overhangs and 5′ phosphate and 3′ hydroxyl termini named siRNAs (Ding and Voinnet, 2007), which are loaded into the RNA-induced silencing complex (RISC) by Dicer-2 and R2D2 (Liu et al., 2003). One siRNA strand is released

in an ATP-dependant manner and mRNA is cleaved by the RNase-H-like Argonaute-2 following the binding of the remaining siRNA to its complementary sequence (Tolia and Joshua-Tor, 2007).

RNA interference (RNAi) can be a useful tool for the functional characterization of newly identified genes in insects, since one can interfere with the expression of a specific gene by administering the cognate dsRNA. Unfortunately, however, not all insect species are equally susceptible to RNAi and some insects appear to be relatively or entirely insensitive (Terenius et al., 2011; Bellés, 2010). Because dsRNA is typically introduced by injection into the hemolymph and since both dsRNA processing and the subsequent targeting and degradation of mRNA are cellular processes, it follows that for RNAi to be successful dsRNA must persist for sufficiently long in the hemolymph to allow its uptake into cells. Therefore, rapid degradation of dsRNA in the hemolymph could result in insufficient uptake and processing of the dsRNA, and consequently limit the success of RNAi experiments.

Here, we present data concerning the persistence of dsRNA in the hemolymph plasma of *Manduca sexta*, a lepidopteran in which RNAi has been previously achieved (Eleftherianos et al., 2009), but

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which we have subsequently found to be variably sensitive to RNAi, and *Blattella germanica*, a dictyopteran which is reproducibly highly sensitive to RNAi (e.g. Ciudad et al., 2006, 2007; Martín et al., 2006; Maestro and Bellés, 2006; Cruz et al., 2006, 2008; Mané-Padrós et al., 2008; Revuelta et al., 2009; Suazo et al., 2009; Gomez-Orte and Belles, 2009; Guo et al., 2010; Huang and Lee, 2011). We report that exogenous dsRNA is rapidly degraded in the plasma of *M. sexta*, but that little degradation of dsRNA occurs in *B. germanica* plasma.

2. Methods

2.1. Insects

Larvae of the tobacco hornworm, *M. sexta* (Lepidoptera: Sphingidae), were reared at the University of Bath according to Bell and Joachim (1976) and Reynolds et al. (1985). Caterpillars were kept at 25 °C, 50% humidity and a photoperiod of 17 h light:7 h dark. Artificial diet was prepared according to the recipe of Yamamoto (1969) as modified by Bell and Joachim (1976). Adult German cockroaches, *B. germanica* (Dictyoptera, Blattellidae), were obtained from a colony in Barcelona, fed on Panlab dog chow and water, and reared in complete darkness at 30 ± 1 °C and 60–70% relative humidity.

2.2. dsRNA synthesis

Double-stranded RNA (dsRNA) for *M. sexta moricin* and for eGFP was synthesised by PCR and *in vitro* transcription as described in Clemens et al. (2000). PCR using primers with terminal 5' T7 promoter sites generated a product used as a template for *in vitro* transcription using the T7 "Megascript" kit (Ambion). Primer sequences were (T7 sites in bold) *Moricin_F*: 5'-**TAA TAC GAC TCA CTA TAG GGA GA** ATA TTG CTG GCA CCA CAC A C-3' and *Moricin_R*: 5'-**TAA TAC GAC TCA CTA TAG GGA GA** AAG ATT CCG AAG GGA GAA CG-3'; *eGFP_T7_F*: 5'-**TAA TAC GAC TCA CTA TAG GGA GAC** CCT GAA GTT CAT CTG CAC CA-3' and *eGFP_T7_R*: 5'-**TAA TAC GAC TCA CTA TAG GGA GA** GAA CTC CAG CAG GAC CAT GT-3'. Transcription was performed according to the kit instructions except that the reaction was allowed to proceed overnight. Prepared dsRNA was treated with DNase, precipitated with LiCl and resuspended in DEPC-treated water.

Vitellogenin dsRNA was prepared for injection into *B. germanica* as described by Martín et al. (2006). A 732 bp fragment of vitellogenin from amino acid 746–990 (GenBank ID: CAA06379) was cloned into the pSTblue-1 vector (Novagen). The plasmid was digested overnight at 37 °C in separate reactions with SalI and BamHI enzymes (Promega) and the linearized plasmids used as templates for *in vitro* transcription (using T7 or SP6 RNA polymerase) to generate single-stranded RNA. To prepare dsRNA, equimolar amounts of sense and antisense RNAs were mixed, heated for 5 min at 90 °C and cooled down slowly to room temperature. A control dsRNA consisting of 92 bp of non-coding sequence from the pSTblue-1 vector, was also produced.

2.3. RNAi experiments

Newly emerged fifth instar *M. sexta* larvae were injected (in the hindmost segment of the larva) with either 50 µl DEPC water (control) or 50 µl DEPC water containing 100 ng *moricin* dsRNA or 100 ng dsRNA for eGFP (as a dsRNA control). Six hours post injection the insects were injected with either PBS or PBS containing approximately 5 × 10⁵ DH5α *Escherichia coli* cells. The bacterial culture was prepared by inoculating 5 ml LB liquid media with 5 µl of a 20% glycerol stock culture and incubating for 16 h in a

shaking incubator at 37 °C and ~220 rpm. The cells were washed with sterile PBS and their concentration adjusted. The number of injected bacterial cells was confirmed by making serial dilutions of the injection solution and plating the dilutions onto 1.5% agar plates, followed by incubation at 37 °C overnight. Sixteen hours after injection with PBS or *E. coli*, fat body tissue was collected from the insects and subjected to qPCR analysis to determine *moricin* transcript levels.

Newly emerged adult *B. germanica* females were injected with 1 µl dsRNA for *vitellogenin* adjusted to a final concentration of 1 µg µl⁻¹ in Ringer saline. Five days after injection fat body was dissected out, total RNA was extracted from the tissue, treated with DNase and subjected to quantitative reverse transcription PCR (q-RT-PCR) to quantify *vitellogenin* transcript levels. The phenotype of the treated insects was also analyzed. The ovaries were dissected out and ovariole length measured using an ocular micrometer adapted to a stereomicroscope.

2.4. q-RT-PCR

Total RNA was extracted from *B. germanica* and *M. sexta* fat body by phenol–chloroform extraction with TRI reagent or using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma). Extracted RNA was treated with RNase-free DNaseI (Ambion) and quantified using either a Qubit® 2.0 Fluorometer (Invitrogen) or a Nanodrop Spectrophotometer (ThermoScientific). Complementary DNA (cDNA) synthesis was performed using MMLV reverse transcriptase (Promega) or a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) with random hexadeoxynucleotide primers. Real-time PCR was carried out using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and iTaq SYBR Green Supermix (Bio-Rad) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye. PCR reactions were carried out in duplicate using 7.5 pmol specific primers and approximately 5 ng cDNA in a total volume of 15 µl. Actin-5c (Accession number AJ862721) was used as the internal control gene for *B. germanica* reactions and ribosomal protein S3 (rpS-3; GI: 527679) was used as the internal control for *M. sexta*. Primer pairs were validated by standard curve analysis and primer sequences are presented in Table 1 in the Supplementary information.

2.5. Ex vivo dsRNA degradation assay

Hemolymph was collected in individual pre-chilled 1.5 ml microcentrifuge tubes containing a few grains of phenylthiourea (PTU) to inhibit melanisation (Arakawa, 1995). Hemocytes were removed by centrifugation at 1000g for 8 min at 4 °C. One microlitre eGFP dsRNA solution (containing 200 ng dsRNA) was mixed with 3 µl hemolymph plasma in a 1.5 µl microcentrifuge tube and incubated at room temperature for a number of hours. In a control experiment, dsRNA was incubated with DEPC-treated water and PTU for the same time as the longest experimental incubation. After incubation in hemolymph plasma the dsRNA was recovered using an RNeasy Mini Kit (Qiagen): bound dsRNA was eluted into 30 µl DEPC-treated water and a 10 µl aliquot mixed with 2 µl loading dye and run on a 1% agarose EtBr gel. Visualization of the gel using a UV transilluminator allowed analysis of the integrity of dsRNA.

2.6. In vivo dsRNA detection assay

q-RT-PCR was used to detect injected eGFP dsRNA in insect hemolymph. Initial experiments confirmed that q-RT-PCR, which is typically used for the detection of single-stranded messenger RNA (mRNA), could also be used to detect double-stranded RNA.

The step in the q-RT-PCR protocol directly preceding the reverse transcription reaction involves heating RNA to 75 °C for 10 min to denature RNA secondary structure. This heating step separates double-stranded RNA into single-stranded molecules, allowing reverse transcription of the RNA (separation of RNA strands was confirmed using gel electrophoresis; data not shown).

We calibrated the qPCR detection assay by spiking hemolymph plasma with a range of known doses of dsRNA for eGFP. It was necessary to add 10 µg Poly-cytidylic-inosinic acid potassium salt (Poly(I:C); supplied by Sigma) to the hemolymph plasma to act as a carrier in the extraction of RNA. q-RT-PCR was carried out as previously described using primers eGFP_qPCR_F: 5'-CCG ACC ACT ACC AGC AGA AC-3' and eGFP_qPCR_R: 5'-TTG GGG TCT TTG CTC AGG-3'. The calibration experiment gave rise to a formula capable of quantitating dsGFP levels in plasma for a given qPCR signal (Fig. 3a).

The formula derived from the calibration experiment was used to quantify eGFP dsRNA in hemolymph plasma. Newly emerged fifth instar *M. sexta* larvae were injected with 4 µg dsRNA for eGFP and their hemolymph removed after incubation for a number of hours at 25 °C. Hemocytes were removed by centrifugation at 1000g for 8 min at 4 °C. Ten micrograms Poly(I:C) was once again added to the hemolymph plasma to act as a carrier during the extraction of RNA. Total RNA was extracted from the plasma, treated with DNase and an aliquot reverse transcribed, as previously described. q-RT-PCR was performed on a dilution of the resulting cDNA and Ct values obtained from the qPCR reactions were used to calculate the quantity of dsRNA in the plasma using the formula derived from the calibration experiment (and any dilution factors).

3. Results

3.1. *B. germanica* and *M. sexta* are differentially sensitive to RNAi

We conducted RNAi experiments in *B. germanica* and *M. sexta* in order to demonstrate that they are differentially susceptible to RNAi and found that, at best *M. sexta* is variably sensitive to RNAi, whilst *B. germanica* is highly sensitive to RNAi. The details of the RNAi experiments are presented in the [Supplementary information](#) and the data are shown in [Figs. S1 and S2](#). The poor RNAi response of *M. sexta* to the injected dsRNA was observed over a range of doses both greater and smaller than that administered to *B. germanica* (see the [Supplementary information](#) for details).

3.2. dsRNA is degraded in *M. sexta* hemolymph plasma but not in plasma from *B. germanica*

In order to investigate the persistence of dsRNA in insect hemolymph, we developed an *ex vivo* assay, where dsRNA for eGFP was incubated *ex vivo* with hemolymph plasma (cell-free hemolymph) and the integrity of the dsRNA subsequently analyzed on a 1% agarose gel. Incubation of dsRNA in hemolymph plasma isolated from newly emerged fifth instar *M. sexta* larvae resulted in weakening and smearing of the dsRNA band on the gel, which can be interpreted as degradation (Fig. 1a). Smearing and weakening of the dsRNA band began to occur after incubation in plasma for just 1 h; after 3 h the dsRNA band had almost entirely disappeared. The outcome of an experimental control, where eGFP dsRNA was incubated in water for 3 h (Fig. 1a: H₂O, $t = 3$), was that the dsRNA retained its integrity, allowing us to conclude that the dsRNA did not spontaneously degrade under the experimental conditions utilized, as well as eliminating the possibility that the agent responsible for the dsRNA degradation originated from a contaminant in the dsRNA preparation, rather than the insect plasma itself.

When the *ex vivo* assay was conducted using plasma from the RNAi-susceptible species, *B. germanica*, eGFP dsRNA degradation occurred at a much lower rate than in the RNAi-resistant insect *M. sexta* (Fig. 1b). In contrast to the marked smearing and weakening of the band of dsRNA observed following incubation in *M. sexta* plasma, a discrete band of dsRNA was still present following incubation in hemolymph plasma from *B. germanica* adult females at all time points sampled. Some loss of intensity of this band was observed, but this was not obviously progressive with time, and a clear band of dsRNA was still present even after incubation for 24 h.

3.3. A heat-labile protein with a requirement for cations degrades dsRNA in *M. sexta* hemolymph plasma

The ability of *M. sexta* hemolymph plasma to degrade dsRNA was strongly inhibited by pre-heating the plasma to 100 °C for 10 min (Fig. 2a), suggesting that a heat-labile enzyme is responsible for the degradation. Degradation of dsRNA was also inhibited by addition to the plasma of the divalent cation-chelating agent EDTA (Fig. 2b). The effect of EDTA was dose-dependent: 20 mM and 10 mM EDTA completely inhibited dsRNA degradation (Fig. 2b) whereas the addition of 5 mM EDTA only partially inhibited degradation (data not shown). These results provide evidence that a metal-dependent enzyme is responsible for degradation of dsRNA in the hemolymph plasma. Further experiments showed that when plasma was passed through 5 and 10 kDa filters dsRNA-degrading activity was retained in the fractions retained in the filters, whilst the flow-through had no degrading activity (data not shown). This result is consistent with enzymatic dsRNA degradation.

3.4. dsRNA disappears from *M. sexta* hemolymph *in vivo*

Following the observation that dsRNA is rapidly degraded in *M. sexta* hemolymph plasma, we wanted to study the survival of dsRNA *in vivo* and developed an assay based on the detection of injected dsRNA by q-RT-PCR. An absolute quantitation approach was employed; we calibrated the assay using hemolymph plasma spiked with known quantities of eGFP dsRNA (Fig. 3a). Using this technique dsRNA was found to rapidly disappear from the hemolymph plasma (Fig. 3b; note the logarithmic scale). By 3 h after initiating the experiment, the level of dsRNA had fallen to the detection limit of the assay. Linear regression analysis of the log transformed data (from the experiment denoted in the figure by the open diamonds) showed that there was a significant negative logarithmic relationship between dsRNA levels and the time that had elapsed after dsRNA injection ($t = -8.189$, $p < 0.001$, $y = -0.66916x - 0.39316$). We calculated the half-life of dsRNA in the hemolymph (using the mean values for 0 h and 3 h from the experiment depicted in Fig. 3b with open diamonds) as 27 min.

3.5. Identification of *M. sexta* DNA/RNA non-specific nucleases

We took a candidate approach to identify the gene responsible for dsRNA degradation in *M. sexta* hemolymph. A 43 kDa enzyme with dsRNase activity is known to be present in another lepidopteran insect, the silkworm *Bombyx mori* (Arimatsu et al., 2007). This protein belongs to the DNA/RNA non-specific nucleases family, is apparently expressed only in midgut epithelial cells, and is secreted into the gut lumen as a digestive enzyme.

We found two sequences with a high degree of similarity to the silkworm DNA/RNA non-specific nuclease in a *M. sexta* EST library and isolated 1547 bps of *M. sexta* nuclease 1 (*MsN1*) and 1543 bps of *M. sexta* nuclease 2 (*MsN2*). Both sequences are predicted to contain open reading frames (ORFs) encoding 447 amino acid residues

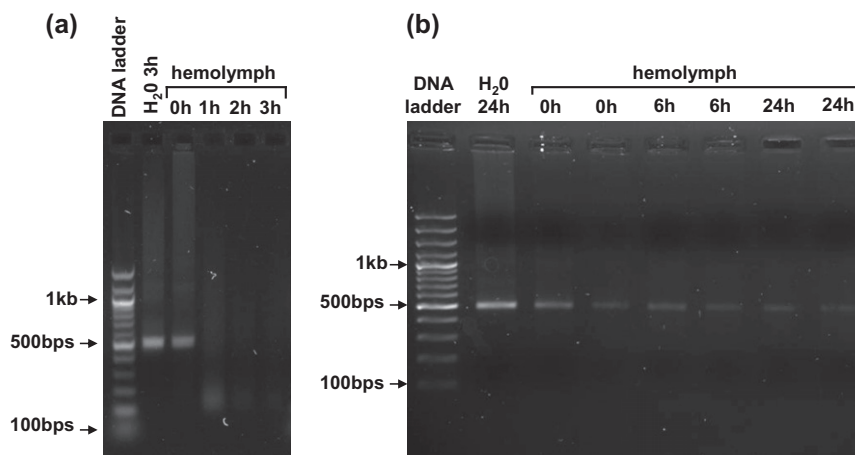


Fig. 1. *Ex vivo* degradation of dsRNA using hemolymph from (a) *Manduca sexta* and (b) *Blattella germanica*. An amount of 200 ng dsRNA for eGFP (total 1 μ l volume) was incubated with either 3 μ l DEPC-treated water (H_2O) or 3 μ l cell-free hemolymph for t hours after which it was recovered using an RNeasy kit (Qiagen) and run on a 1% agarose EtBr gel. Hemolymph was prepared from (a) *M. sexta* fifth instar larvae and (b) *B. germanica* adult females.

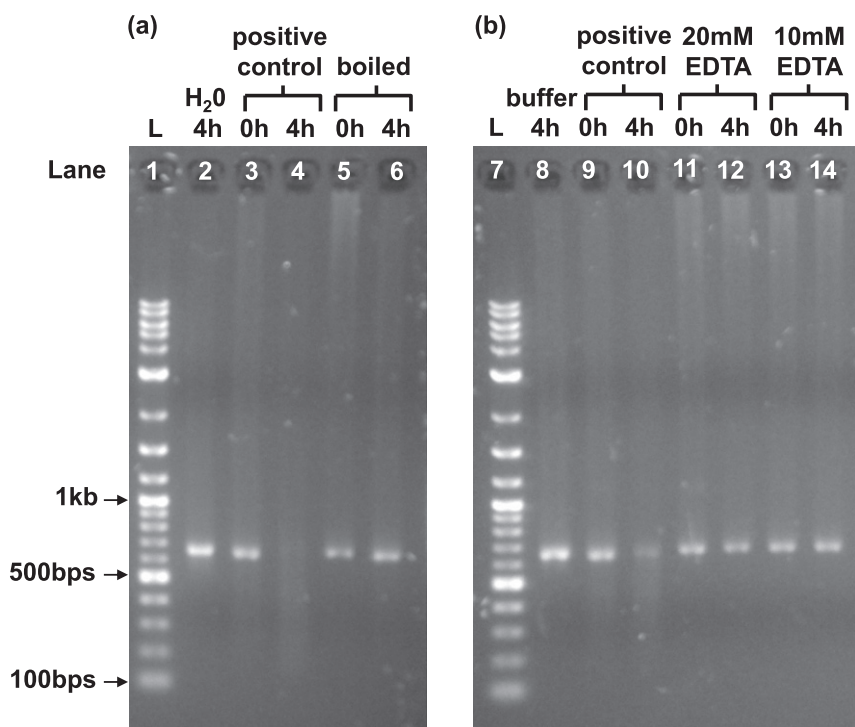


Fig. 2. *Ex vivo* degradation of dsRNA by *Manduca sexta* hemolymph. Two hundred nanograms eGFP dsRNA was incubated with 3 μ l *M. sexta* cell-free hemolymph for t hours after which it was recovered using an RNeasy kit (Qiagen) and run on a 1% agarose EtBr gel. (a) Degradation of dsRNA is abolished by heat treatment. Lane 1 contains a DNA ladder, lane 2 contains dsRNA incubated with DEPC-treated water for 4 h, lanes 3 and 4 contain dsRNA incubated with cell-free hemolymph for 0 and 4 h, respectively and lanes 5 and 6 contain dsRNA incubated with cell-free hemolymph that had previously been heated to 100 $^{\circ}$ C for 5 min for 0 and 4 h, respectively. (b) Degradation of dsRNA is abolished by divalent ion chelation using EDTA. Lane 7 contains a DNA ladder, lane 8 contains dsRNA incubated with MOPS buffer (40 mM MOPS, pH 7.5) for 4 h, lanes 9 and 10 contain dsRNA incubated with cell-free hemolymph and MOPS buffer for 0 and 4 h, respectively, lanes 11 and 12 contain dsRNA incubated with cell-free hemolymph and MOPS buffer with 20 mM EDTA for 0 and 4 h, respectively and lanes 13 and 14 contain dsRNA incubated with cell-free hemolymph and MOPS buffer with 10 mM EDTA for 0 and 4 h, respectively.

and containing (like the *B. mori*, *Spodoptera littoralis* and *Spodoptera frugiperda* DNA/RNA non-specific nuclease protein sequences) a single DNA/RNA non-specific endonuclease domain as well as a signal peptide (identified using SignalP: <http://www.cbs.dtu.dk/services/SignalP/>). The *M. sexta* deduced sequences show high amino acid identity with the other lepidopteran sequences; MsN1 has 76%, 72% and 71% identity with the *B. mori*, *S. littoralis* and *S. frugiperda* sequences and MsN2 66%, 68% and 68% identity.

A phylogenetic tree constructed by the neighbour-joining method using ClustalW software revealed that the *M. sexta* nuclease proteins cluster, as one would expect, with the other lepidopteran DNA/RNA non-specific nucleases (Fig. 4). The MsN1 predicted protein appears to be most closely related to the *B. mori* protein, whilst the MsN2 predicted protein does not cluster with the *B. mori* protein, but is instead placed on a branch of its own (Fig. 4), reflecting its lower amino acid identity with the *B. mori* DNA/RNA non-specific

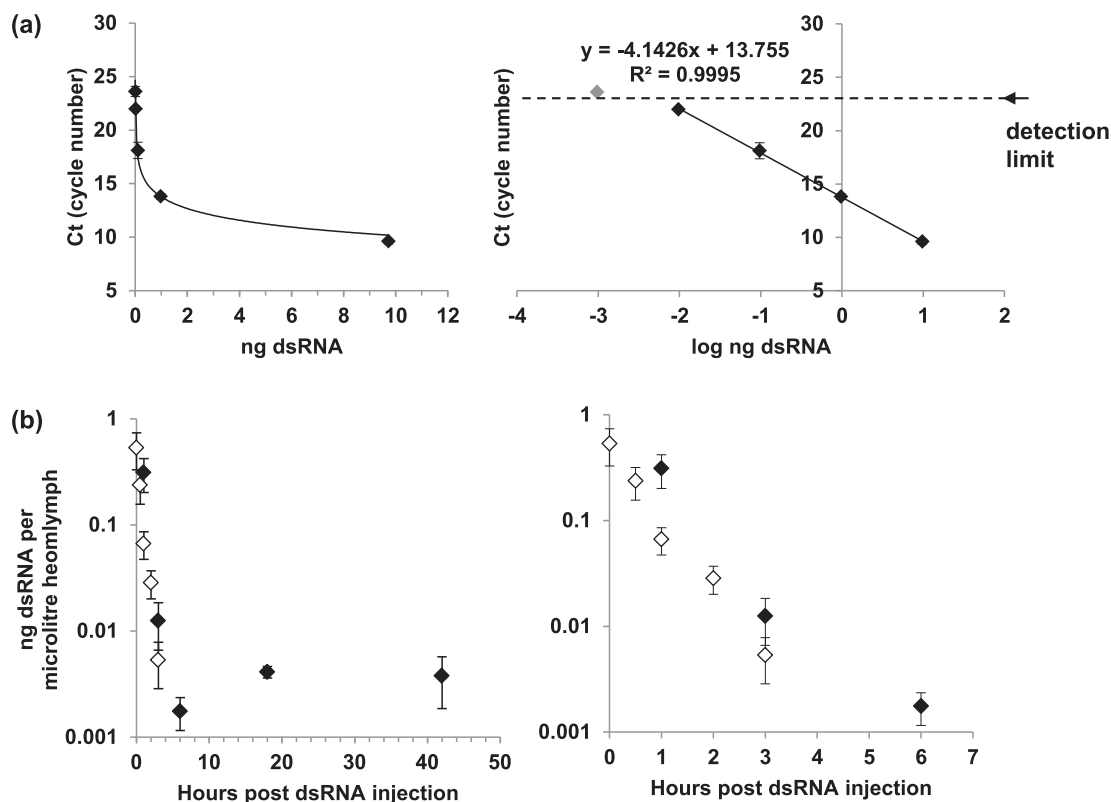


Fig. 3. Persistence of dsRNA in *Manduca sexta* hemolymph. (a) The assay was calibrated by spiking dissected plasma with a range of doses of eGFP dsRNA and immediately extracting and analyzing the plasma samples by q-RT-PCR. The qPCR output (Ct, or cycle number) was plotted against the quantity of dsRNA (left) and the log transformed quantity of dsRNA (right). One data point (in grey) was excluded from the fitted trendline because it fell below the detection limit of the assay. (b) Two persistence assays *in vivo* were conducted, where a dose of 4 μ g of eGFP dsRNA was injected into newly emerged fifth instar larvae and the hemolymph dissected after incubation for a number of hours ($n = 4$). Hemocytes were removed from the hemolymph by centrifugation and absolute dsGFP levels in the plasma quantified using the calibrated q-RT-PCR protocol. The first experiment (depicted with black diamonds) monitored dsRNA levels over 48 h, whilst the second experiment (open diamonds) concentrated on shorter time points. The two scatter plots depict the same data but the plot on the right displays data for the first 6 h only.

endonuclease (66% identity compared to 76% for MsN1). Interestingly, the MsN2 predicted protein still shares 75% amino acid identity with the *B. mori* protein when only the DNA/RNA non-specific endonuclease domain is analyzed, with much of the sequence variation occurring in the region outside this functional domain (MsN2 has only 54% identity with the *B. mori* protein in the regions not encoding the DNA/RNA non-specific endonuclease domain). In addition to having lower amino acid identity with the *B. mori* alkaline nuclease protein (compared with MsN1), the MsN2 predicted protein further differs from the *B. mori* protein in its isoelectric point (as predicted by the ProtParam tool found at <http://www.expasy.org/tools/protparam.html>). MsN2 has an isoelectric point (pI) of 7.97 compared to 9.48 for the *B. mori* protein and 9.32 for the MsN1 predicted protein (Fig. 4).

3.6. Expression of *M. sexta* DNA/RNA non-specific nucleases

q-RT-PCR experiments revealed that expression levels of the MsN1 and MsN2 transcripts in larval tissue (fat body, hemocytes and midgut) are extremely low (Fig. 5). Transcripts of MsN1 are present at resting levels approximately 1,000,000 times lower than the internal control gene, RPS-3, in fat body, 100,000 times lower than the internal control gene in hemocytes and 10,000 times lower than the internal control gene in midgut tissue. Transcripts of MsN2 are present in similar levels to those of MsN1 in the midgut, but were undetectable using our q-RT-PCR methodology in the fat body and hemocytes (and must therefore be expressed at levels more than 1,000,000 times lower than the internal control gene).

4. Discussion

Some insect species have been observed to be relatively or even totally insensitive to RNAi (Bellés, 2010; Terenius et al., 2011). We hypothesized that rapid degradation of dsRNA could be responsible for this insensitivity (as suggested by Bellés, 2010), and tested this hypothesis by evaluating the persistence of dsRNA in the hemolymph plasma of *B. germanica*, which is highly sensitive to RNAi, and in plasma from *M. sexta*, which we have found to be at best variably sensitive to RNAi. We found that synthetic dsRNA molecules are rapidly degraded *ex vivo* by an extracellular enzyme in *M. sexta* hemolymph plasma, whilst comparable degradation occurs either not at all or much less quickly in *B. germanica* plasma (Fig. 1). The *ex vivo* degradation of dsRNA by *M. sexta* plasma appears to be physiologically relevant, since we observed that injected dsRNA disappeared from *M. sexta* hemolymph plasma *in vivo* over a similar time scale to that seen *ex vivo* (Fig. 3).

We propose that deficient RNAi in *M. sexta* is caused at least in part by the rapid degradation of dsRNA in hemolymph plasma by an extracellular nuclease. This is not the first report linking nuclease activity with resistance to RNAi. Kennedy et al. (2004) identified a nuclease in the nematode worm, *Caenorhabditis elegans*, encoded by the eri-1 (enhanced RNAi-1) gene, which is expressed at high levels in the gonad and neurons of the worm, tissues which are unusually refractory to RNAi. They found that eri-1 mutant worms show an enhanced RNAi effect and accumulate more siRNAs than wild type animals, providing evidence for inhibition of RNAi by the eri-1 gene product in wild type worms. Although there are no examples known to us of insect nucleases that have been

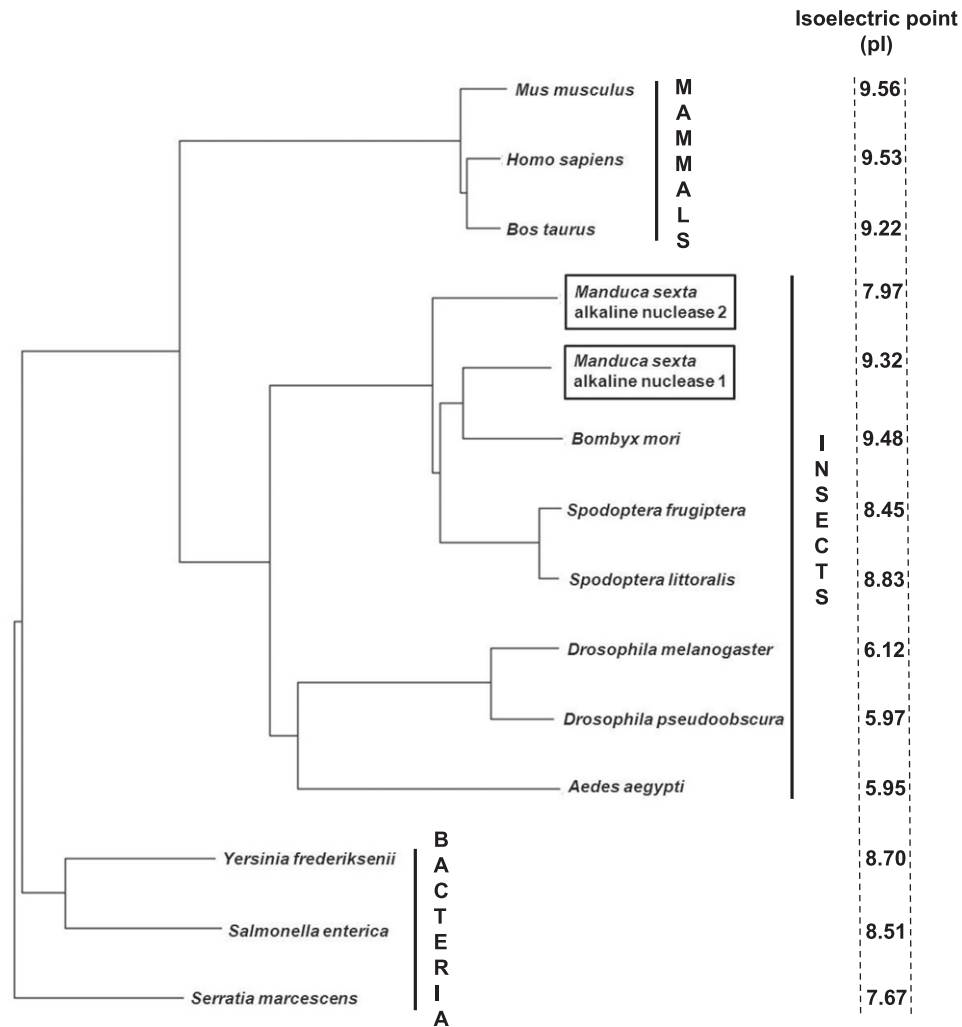


Fig. 4. Neighbor-joining tree showing the phylogenetic relationship of the newly identified *Manduca sexta* nuclease deduced amino acid sequences (*MsN1* and *MsN2*) with DNA/RNA non-specific nucleases. The amino acid sequences used for the analysis were from GenBank under following accession numbers: *Bombyx mori* (BAF33251.1); *Spodoptera littoralis* (CAR92522.1); *Spodoptera frugiperda* (CAR92521.1); *Serratia marcescens* (CAA00836); *Salmonella enterica*, putative DNA/RNA non-specific endonuclease (AAL91099); *Yersinia frederiksenii*, DNA/RNA endonuclease G (ZP_00829494); *Aedes aegypti*, putative deoxyribonuclease I (EAT42072); *Drosophila melanogaster*, CG3819-PA (AAF49206); *Drosophila pseudoobscura*, GA17708-PA (EAL30415); *Mus musculus*, endonuclease G (NP_031957); *Homo sapiens*, endonuclease G (AAH16351); *Bos taurus*, endonuclease G (P38447). The tree was rooted using the *S. marcescens* sequence as outgroup. The predicted isoelectric point (pI) of the protein sequences (calculated using the tool found at <http://www.expasy.org/tools/protparam.html>) is included in a column on the right.

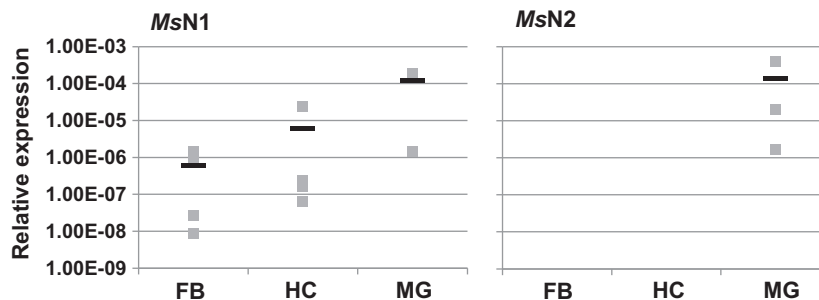


Fig. 5. Relative expression of the newly identified *MsN1* and *MsN2* mRNA in *Manduca sexta*. Relative mRNA levels in fat body (FB), hemocytes (HC) and midgut (MG) tissue from newly emerged fifth instar *M. sexta* larvae. The values for the four replicates are shown as grey squares and the mean is depicted with a black dash.

shown to interfere with RNAi, a 43 kDa enzyme with dsRNAse activity is known to be present in another lepidopteran insect, the silkworm *B. mori* (Arimatsu et al., 2007). A non-specific nuclease could be a candidate for the *M. sexta* hemolymph enzyme responsible for degradation of exogenous dsRNA, since in addition

to dsRNA, hemolymph plasma preparations also degrade ssRNA *in vitro* (data not shown).

We found two sequences with a high degree of similarity to the silkworm DNA/RNA non-specific nuclease in a *M. sexta* EST library. One characteristic of the *B. mori* nuclease identified by Arimatsu

et al. (2007) is its remarkably high isoelectric point (pI: the pH at which the protein carries no net charge) of 9.48, making it ideally adapted to function in the highly alkaline guts of lepidopteran larvae (with a luminal pH of between 9 and 12; Waterhouse, 1949; Dow, 1984).

Interestingly, whilst one of the *M. sexta* nucleases (*M. sexta* nuclease 1; *MsN1*) has a similar predicted isoelectric point to the silkworm protein (9.32), the other nuclease (*M. sexta* nuclease 2; *MsN2*) is predicted to have a pI of 7.97, leading to the hypothesis that this protein is active in hemolymph, which generally has a pH of around 6.7 (Dow, 1984). *M. sexta* nuclease 2 is, therefore, a potential source of the dsRNase activity we observed in *M. sexta* hemolymph plasma. Although our experiments revealed that this nuclease (*MsN2*) is expressed at minimal levels in *M. sexta* larval tissue (Fig. 5), we could not check whether the protein was nevertheless present in hemolymph (which might be the case if mRNA encoding it is expressed only at times other than those sampled, and the protein is long-lived). Moreover, information about the specific activity of the enzyme is lacking; it is possible that this may be high enough to give rise to the degrading phenotype at even very low levels. In any case, members of the DNA/RNA non-specific nuclease family should at least be considered as potential inhibitors of RNAi in insects. Interestingly, we could only find examples of the DNA/RNA non-specific nucleases in lepidopteran and dipteran species in the public databases, and not in other insect orders. These groups also happen to be those for which there are reports of insensitivity to RNAi (Terenius et al., 2011; Miller et al., 2008). Therefore, a phylogenetic restriction of the DNA/RNA non-specific nuclease family may be the explanation for observed differences in sensitivity to RNAi in different insect taxa.

What could be the evolutionary driving force for the high level of dsRNA-specific nuclease activity in the hemolymph of *M. sexta* compared to *B. germanica*? One possible explanation is that rapid degradation of dsRNA in hemolymph could represent an evolutionary response to a heavy viral load, and that German cockroaches and tobacco hornworms differ in the extent to which they are exposed to viruses. This is plausible, since dsRNA can be recognized as a viral molecular pattern (DeWitte-Orr and Mossman, 2010) and lepidopteran species like *M. sexta* may be particularly at risk from viral infections (Terenius, 2008). Although there are no recent studies systematically documenting viruses in different insect groups, one study in the 1980s reported that, whilst lepidopteran insects only make up 20% of all described holometabolous species, 70% of holometabolous species with recorded viral disease in their study belonged to the Lepidoptera (Martignoni and Iwai, 1986). Furthermore, there are many examples of Lepidoptera-specific viruses, including viruses from the genera Granulovirus (Baculoviridae) (Bilimoria, 1991; Miller, 1997). In contrast, few viruses are known to be associated with cockroaches (insects of the Order Blattodea). Evans and Shapiro (1997) reported that the densovirus (DNV; Parvoviridae, Densovirus) is the only virus infecting cockroaches. Although an entomopoxvirus has subsequently been isolated from *B. germanica* (Radek and Fabel, 2000), there is very little evidence of infection by viruses in cockroaches. Next generation sequencing technologies will facilitate the discovery and systematic documentation of insect viruses (Liu et al., 2011) and allow differences in viral load between different insect groups to be revealed.

Of course, it is possible that there are other explanations for the observed differences in dsRNase activity in the hemolymph of *M. sexta* and *B. germanica*, including that cockroaches, but not caterpillars, use dsRNAs as signaling molecules and therefore avoid the existence of dsRNase activity in the hemolymph.

Chronic infection with microorganisms may also lead to the constitutive expression of dsRNase activity. An example is known from a human cell line where expression of dsRNase activity was

traced to a mollicute bacterium (*Mycoplasma*) contaminant (Marcus and Yoshida, 1990). Mollicute infections can be apparently symptomless, uncultivable, and hard to detect. Many insects are known to harbour mollicute Spiroplasmas (Regassa and Gasparich, 2006). We used PCR with degenerate primers described by Sung et al. (2006) to detect potential mollicute infection in our culture of *M. sexta* with negative results (not shown) but we cannot say with confidence that mollicutes were absent.

In conclusion, we report dsRNA-specific nuclease activity in the hemolymph plasma of an RNAi-insensitive insect species, *M. sexta*, and speculate that rapid degradation of injected dsRNA may impair experimental RNAi in this insect. It is unlikely that the nuclease activity we report is restricted to *M. sexta* hemolymph, and rapid degradation of dsRNA in hemolymph may, therefore, be a widespread cause of insensitivity to RNAi in insects. Consequently, it may be useful to consider the stability of dsRNA in hemolymph plasma when performing RNAi experiments in insects and mitigate rapid degradation by either chemically modifying the dsRNA or identifying the nuclease responsible and interfering with its activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2012.05.013>.

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