

# Liposome encapsulation and EDTA formulation of dsRNA targeting essential genes increase oral RNAi-caused mortality in the Neotropical stink bug *Euschistus heros*

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## Abstract

**BACKGROUND:** The Neotropical stink bug *Euschistus heros* is a major pest in soybean fields. Development of highly species-specific pesticides based on RNA interference (RNAi) could provide a new sustainable and environmentally friendly control strategy.

**RESULTS:** Here, the potential of RNAi as a pest control tool against *E. heros* was assessed. First, target gene selection using a microinjection approach was performed. Seven of the 15 candidate genes tested exhibited > 95% mortality after hemolymph injection of 27.5 ng dsRNA. Subsequently, dsRNA was administered orally using different formulations: naked dsRNA, liposome-encapsulated-dsRNA and dsRNA formulated with EDTA. Liposome-encapsulated dsRNA targeting *vATPase A* and *muscle actin* led to significant mortality after 14 days (45% and 42%, respectively), whereas EDTA-formulated dsRNA did so for only one of the target genes. *Ex vivo* analysis of the dsRNA stability in collected saliva indicated a strong dsRNA-degrading capacity by *E. heros* saliva, which could explain the need for dsRNA formulations.

**CONCLUSION:** The results demonstrate that continuous ingestion of dsRNA with EDTA or liposome-encapsulated dsRNA can prevent dsRNA from being degraded enzymatically and suggest great potential for using these formulations in dsRNA delivery to use RNAi as a functional genomics tool or for pest management of stink bugs.

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**Keywords:** *Euschistus heros*; RNA interference; dsRNA ingestion; Lipofectamine<sup>®</sup> 2000; EDTA; Hemiptera, Pentatomidae

## 1 INTRODUCTION

Soybeans are extensively grown in Brazil and are one of the most important agricultural commodities.<sup>1</sup> In the season 2016/2017, 60.9 million hectares were used for the cultivation of grains in Brazil, and 55.7% of this area was cropped with soybean, producing a yield of ~ 114.1 million tons of soybean pulses.<sup>2,3</sup> These soybean yields would be even higher if yield losses caused by insect pests could be reduced. In Neotropical regions, especially Brazil, the stink bug complex (Hemiptera: Pentatomidae) is the most destructive pest in soybean fields. Among these herbivorous pentatomids, the Neotropical brown stink bug, *Euschistus heros* (Fabricius) is most representative of this insect pest complex.<sup>4</sup> *E. heros* damages soybeans by inserting its mouthparts into the pods while probing the seeds, causing seeds to become dry and wrinkled, resulting in abnormal maturity of the plants and a reduction in their oil content and germination rates, all of which affect the production and quality of the seeds.<sup>5,6</sup>

Around the world, control of seed-sucking stink bugs remains heavily dependent on insecticides. For instance, despite the

known benefits of using integrated and more sustainable practices, control of soybean insect pests still relies on insecticide applications at a rate of more five times per season.<sup>4,7,8</sup> In Brazil, neonicotinoids and pyrethroids have become the most common insecticides used to control *E. heros*.<sup>5,9,10</sup> The restricted number of chemicals registered for control of these insects and the indiscriminate use of this control practice will certainly lead to substantial biodiversity losses in agricultural landscapes, selection for insecticide-resistant populations, resurgence and/or outbreaks of insect pests, and impairment of non-target organisms, including humans.<sup>4,10–14</sup>

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Double-stranded RNA (dsRNA)-mediated gene silencing, commonly referred to as RNA interference (RNAi), is becoming a widely used tool to knockdown sequence-specific target genes in insects; it also has a great potential to combat insect pests.<sup>15–18</sup> Because there are no reports of insecticidal proteins such as *Bt* toxins with activity against pentatomid stink bugs,<sup>19,20</sup> RNAi offers a potential novel approach to control this insect pest complex.<sup>21,22</sup> To induce an RNAi response in the insect, dsRNA can be delivered into the body via ingestion, soaking or microinjection.<sup>23–25</sup> Previous experiments have shown that RNAi is effective against *E. heros* via injection of chromatin-remodeling ATPase dsRNAs,<sup>21</sup> and against the brown marmorated stink bug *Halyomorpha halys* (Stål) via injection of *Hha-catalase* dsRNA<sup>26</sup> and ingestion,<sup>27</sup> demonstrating the existence and functionality of the RNAi machinery in pentatomid bugs.

For a role in crop protection, oral uptake of dsRNA and subsequent efficient uptake in the gut have to be evaluated.<sup>25</sup> Supplying dsRNA in artificial diet resulted in the knockdown of targeted genes in different species of Hemiptera including the small brown planthopper, *Laodelphax striatellus* (Fallén),<sup>28</sup> the brown planthopper, *Nilaparvata lugens* (Stål),<sup>29–32</sup> the corn planthopper, *Peregrinus maidis* (Ashmead),<sup>33</sup> the white-backed planthopper, *Sogatella furcifera* (Horvath),<sup>28,34</sup> the kissing bug, *Rhodnius prolixus* (Stål),<sup>35</sup> the pea aphid, *Acyrtosiphon pisum* (Harris),<sup>36–38</sup> the wheat aphid, *Sitobion avenae* (Fabricius),<sup>39,40</sup> the potato/tomato psyllid, *Bactericera cockerelli* (Sulc),<sup>41</sup> the Asian citrus psyllid, *Diaphorina citri* (Kuwayama),<sup>42,43</sup> the brown marmorated stink bug, *Halyomorpha halys* (Stål)<sup>27,44</sup> and the tobacco whitefly, *Bemisia tabaci* (Gennadius).<sup>45</sup> However, oral delivery in some hemipteran pests, such as the tarnished plant bug *Lygus lineolaris* (Palisot de Beauvois) and the pea aphid *A. pisum*, is confounded by high ribonuclease activity in the saliva which degrades the dsRNA.<sup>46,47</sup> This degradation of the nucleic acids leads to an impaired RNAi response in an organism unless a high dose of dsRNA or siRNA is used.<sup>48</sup> To enhance the stability of dsRNA and enhance cellular uptake, transfection reagents have been used to encapsulate dsRNA in cationic liposome complexes that slow down the degradation and increase the effectiveness of RNAi silencing in fruit flies<sup>38,49</sup> and in German cockroach.<sup>50</sup> Another alternative method of dsRNA delivery is use of the chelating agent EDTA that can act as a protein inhibitor of the nucleases present in the saliva, as recent studies have been reported that EDTA may inhibit nuclease digestion of DNA in blood samples.<sup>51</sup>

The purpose of this study was to assess the potential of RNAi as a tool for pest control, using injection and oral delivery of dsRNA in *E. heros*. First, screening was undertaken of different target genes encoding proteins that are essential for growth and development by microinjection into the hemolymph of *E. heros* nymphs. Furthermore, feeding trials were conducted using different formulations of dsRNA: naked dsRNA, EDTA and the transfection reagent Lipofectamine® 2000. Additionally, an attempt was made to evaluate dsRNA stability in the saliva for possible degradation upon oral delivery.

## 2 MATERIALS AND METHODS

### 2.1 Insects

Adults and nymphs of *E. heros* were obtained from a mass-rearing colony kept under controlled laboratory conditions ( $25 \pm 2$  °C,  $60 \pm 10\%$  relative humidity, and an L/D photoperiod of 14:10 h). To prevent diapause, artificial lighting was maintained between 08.00 h and 22.00 h, and all developmental stages of *E. heros*

were mass-reared in plastic boxes following previously described methods.<sup>52–54</sup> Briefly, the insects were fed *ad libitum* with a mixture of fresh green bean pods, *Phaseolus vulgaris* (L.), raw shelled peanuts, *Arachis hypogaea* (L.), and sunflower seeds, *Helianthus annuus* (L.), in addition to water. The plastic boxes were cleaned, and supplies were replenished at 2-day intervals. Eggs were removed from pieces of gauze placed inside the mass rearing boxes and transferred to plastic Petri dishes containing a piece (~3 cm) of green bean pod, and second-instar nymphs were transferred to plastic boxes and reared until they reached adulthood.

### 2.2 Candidate genes ortholog identification

Lethal candidate genes were selected according to the database of essential genes in *Drosophila melanogaster* (<http://tubic.tju.edu.cn/deg/>), large-scale screens for target genes in coleopterans,<sup>55,56</sup> published RNAi research in *E. heros*<sup>21</sup> and the target gene list used and published previously.<sup>57</sup> Candidate genes were identified with tBLASTn searches in *E. heros* pooled transcriptome using the sequences of *Tribolium castaneum* (Herbst) (Table 1).

### 2.3 cDNA preparation and dsRNA synthesis

*E. heros* total RNA was isolated from second-instar nymphs using a RNeasy kit (Qiagen, Hilden, Germany). The cDNA was reverse transcribed from 500 ng of total RNA template and oligo(dT) primer using SuperScript III First-Strand synthesis (Invitrogen, Merelbeke, Belgium). Primers were designed using the web application E-RNAi v. 3.2 and T7 promoter sequences were placed at the 5'-ends of both forward and reverse primer, to enable dsRNA transcription (Table 2). DNA templates were amplified using *Taq* DNA polymerase (Invitrogen) using 500 ng of cDNA as a template. For the negative control, a green fluorescent protein (GFP) fragment was amplified from a plasmid containing a GFP insert (Genbank ID: NC\_011521.1). DNA templates were purified using Wizard SV gel and a polymerase chain reaction (PCR) clean-up system (Promega, Madison, WI, USA). The dsRNAs were synthesized using 2 µg of PCR product as template with the MEGAscript RNAi kit (Ambion, Austin, TX, USA). Nuclease-free water was used for dsRNA elution. The dsRNA was quantified on a NanoDrop ND-1000 s (Nanodrop Technologies, Wilmington, DE, USA) at 260 nm and analyzed by gel electrophoresis to determine purity. The dsRNAs were diluted to 500 ng µL<sup>-1</sup> in nuclease-free water (Table 2).

### 2.4 dsRNA injection

Nano-injection of *E. heros* was performed using a nanoinjector (FemtoJet, Eppendorf, Hamburg, Germany), equipped with an injection needle prepared with glass capillary tubes. Bioassays were carried out with 0–1-day-old second-instar nymphs of *E. heros*. Cages having a large number of first-instar nymphs were selected and second-instar nymphs removed. The cages were kept in isolation for the emergence of new second-instar nymphs. Insects were anesthetized with diethyl ether for 2 min and immobilized in an agarose plate at 1.5%. Each nymph was injected with 54.75 nL of 500 ng µL<sup>-1</sup> dsRNA solution (i.e. 27.4 ng dsRNA in 1–1.5 mg insect). The injection site was the ventral metathoracic region near the hind coxa. dsRNA targeting GFP was used as a negative control. Twenty-five nymphs were injected per treatment. After injections, stink bugs were rested for 2 h before being moved to green bean slices (5 × 5 mm) in Petri dishes. Nymphs were evaluated phenotypically every day for 14 days. To measure

**Table 1.** Description of candidate genes for RNAi studies in *E. heros*

Gene symbol	Tribolium homolog	E value	Identity (%)	Locus description	Source
<i>cact</i>	TC002003	3E-53	41	NF-kappa-B inhibitor cactus-like	55
<i>Spr54k</i>	TC002574	5E-103	83	signal recognition particle 54 kDa protein	55
<i>pp1α96a</i>	TC015321	1E-94	49	serine/threonine-protein phosphatase 2A catalytic subunit beta isoform	55
<i>inr-a</i>	TC008263	4E-44	43	kinesin-like protein KIF17	55
<i>Rpn7</i>	TC006375	4E-65	40	26S proteasome non-ATPase regulatory subunit 6	55
<i>gw</i>	TC006679	1E-35	49	protein Gawky	55
<i>βcop</i>	TC013867	2E-32	52	coatomer subunit beta	56
<i>vATPase A</i>	TC008354	0	92	V-type proton ATPase catalytic subunit A	56
<i>ATPsynβ</i>	TC015322	2E-171	79	ATP synthase subunit beta, mitochondrial	57
<i>syb</i>	TC015183	2E-28	69	synaptobrevin-1-like	57
<i>γcop</i>	TC011806	3E-56	53	coatomer subunit gamma	57
<i>Taf-1</i>	TC002507	2E-34	58	transcription initiation factor TFIID subunit 1	57
<i>Prosa2</i>	TC004868	1E-141	81	proteasome subunit alpha type-2	57
<i>RpS13</i>	TC004990	1E-90	91	40S ribosomal protein S13	57
<i>act-2</i>				Muscle actin	21

**Table 2.** Primers used for dsRNA synthesis. Only gene-specific parts of the primer are listed. These are preceded by the T7 adaptor TAATACGACTCACTATAGGG for dsRNA synthesis

Gene symbol	Forward primer	Reverse primer	Amplicon size
<i>Cact</i>	CAACGTTTCAGATCAAGCGA	TAGGGTAGGTGGCTCAGGAA	397
<i>Spr54k</i>	TTCACCATGCGGGATATGTA	TCATTTTCGCCATTTGTTGA	418
<i>pp1α96a</i>	TGCCATTGACAGCTCTCGTA	CGTGTGGTTCTCCTCTCCTC	441
<i>inr-a</i>	GTTGGCATAGCGAAGGGTAG	TTTAATGAGCAAGGGATGGG	433
<i>Rpn7</i>	GCTGTTTTCGCCCTGTTTAG	TGCCAGAGCGACTAGGAAAT	417
<i>Gw</i>	TGCAACTCAGCCAGGTTCTA	ATCTCTGCCACAAAGAAG	391
<i>Bcop</i>	CCTCGAGTTGTTGGCCTATG	AGCTGTTTTCGGCTGCTTAG	366
<i>vATPase A</i>	TGCCTGCTGACAGTGGTTAC	TGAGTAGCTTGGCGATTCA	444
<i>ATPsynβ</i>	GGGTACCATGCAGGAAAGAA	TCAAGGGGTACAAGTTTGCC	438
<i>Syb</i>	AGCAACTTTGAAGGCAACCA	TTTTTCAACCACATGTTCCG	343
<i>Γcop</i>	GCAGCACCAAAGTTGCTTTT	TGTTCAGAAGTTCGGATCCC	436
<i>Taf-1</i>	AATCAGCCTGGTCGTGTTTT	GTGGGAGGAGTCGTAGGGTT	425
<i>Prosa2</i>	CAACATTTAGCCCTCTGGA	TTTCCAAGCAAAGTATGCC	446
<i>RpS13</i>	ATCATGGGTCGTATGCACG	CCGCCCTGGTTTTGTAGTA	404
<i>act-2</i>	GATGACCCAGATCATGTTGAGAC	CAAGATTCATACCCAAGAAGGAAG	462
<i>GFP</i>	TACGGCGTGCAGTGCT	TGATCGCGCTTCTCG	455

mortality due to expression silencing, survival among injected individuals was compared using 23–25 nymphs (25 total minus the dead insects at 24 h post injection). The injection bioassay was repeated twice, each with independent sets of insects. Cumulative mortality was compared using Kaplan–Meier survival curves and the log-rank test (PROCLIFETEST, 2008; SAS Institute, Cary, NC, USA). Insects that remained alive at the end of the bioassay were censored for the analyses. Overall similarity among the survival and median survival times (i.e. LT<sub>50</sub> values) was tested using the log-rank test, and pairwise comparisons among the curves were tested using Holm–Sidak’s test ( $P < 0.05$ ).

For the molecular analysis, each nymph was injected with 52.3 nL of 500 ng μL<sup>-1</sup> dsRNA solution for the target genes *vATPase A* and *act-2*. dsRNA targeting GFP was used as negative control. Sixteen nymphs were injected per treatment. *E. heros* nymphs were collected at 48 and 72 h post injection. At each time point, eight nymphs from each treatment (divided into two biological samples of four individuals each) were collected and total RNA was extracted from the whole insect body.

### 2.5 Feeding of dsRNA in an artificial diet

For the feeding assays, dsRNA was mixed with a diet, provided by Bayer AG, adapted from a liquid meridic diet for rearing of *Lygus hesperus* (Knight) (modified after Debolt<sup>58</sup>). The effectiveness of different formulations of dsRNA, naked dsRNA, the transfection reagent Lipofectamine 2000 (Invitrogen) and the protein inhibitor EDTA, were tested. Bioassays were carried out with 0–1-day-old second-instar nymphs of *E. heros*. To feed the stink bugs, Parafilm® (Bemis NA, Neenah, WI, USA) was folded with a soldering iron to form small sachet pockets. Sachet-making was carried out aseptically using UV-sterilized Parafilm to minimize the possibility of degradation in test samples and to prevent microbial contamination. The exposure to the amended diet was 6 days, the sachet was replaced on day 3. After the exposure period, the artificial diet was substituted with green bean slices (5 × 5 mm), plant matter was changed daily. Nymphs were evaluated phenotypically every day for 14 days, and weights were measured on days 4, 7 and 14 after the beginning of the feeding assay. Survival was analyzed using Kaplan–Meier survival curves and the log-rank test.

**Table 3.** Primers used for qRT-PCR assay and primer efficacy results

Target gene	Forward primer	Reverse primer	Amplicon size	Efficiency body (%)	Efficiency guts (%)
<i>vATPase A</i>	TGCCTGCTGACAGTGGTTAC	CCCTCCCTCTCTGGATTACC	103	97.6	88.9
<i>act-2</i>	ATCACCAACTGGGACGACAT	GAGCCTCAGTCAGGAGGATG	100	94.9	94.9
Reference gene	Forward primer	Reverse primer	Amplicon size	Efficiency body (%)	Efficiency guts (%)
<i>ARL2</i>	GGTTGGCATTCTTCAGTTGG	GCGCAATCGTAACTGGTACA	101	86.7	95.2
<i>ARP8</i>	TGCCATTCTTGCTTGCTCTTG	GGCCCTCTTCTCGTATCAA	96	103.7	102.5
<i>UB4A</i>	AGCTTCATCGAGCAGGAAAA	CTCGTGAGCGGAAACTAAC	100	93.4	92.5

For naked dsRNA, nymphs were fed *ad libitum* on a sachet containing 40  $\mu\text{L}$  of artificial diet amended with dsRNA. The final concentration of dsRNA in the diet was 320  $\text{ng } \mu\text{L}^{-1}$ , using *act-2* and *vATPase A* as target genes and GFP as control. In total, 20 nymphs were used per treatment in each replicate, and two replicates were performed.

For all assays where Lipofectamine 2000 was used, the mixture with dsRNA was prepared as follows: 10.7  $\mu\text{L}$  of dsRNA (3  $\mu\text{g } \mu\text{L}^{-1}$ ) was mixed with 4.3  $\mu\text{L}$  of nuclease-free water and 1  $\mu\text{L}$  of Lipofectamine 2000. The mixture was incubated at room temperature for 5 min and then mixed with the artificial diet. Sachets containing 30  $\mu\text{L}$  of artificial diet amended with freshly prepared lipoplex solution (dsRNA with liposome) were used in the feeding assays. The final concentration of dsRNA in the diet was 300  $\text{ng } \mu\text{L}^{-1}$ , using *act-2* and *vATPase A* as target genes and GFP as control. Twenty nymphs were used per treatment in each replicate, and two replicates per treatment were performed.

For the EDTA assays, a concentration of 3% w/v was used, based on a suggested concentration by the EPA for agricultural products. Nymphs were fed *ad libitum* on a sachet 30  $\mu\text{L}$  of artificial diet amended by the addition of dsRNA and EDTA. The final concentration of dsRNA in the diet was 300  $\text{ng } \mu\text{L}^{-1}$ , using *act-2* and *vATPase A* as target genes and GFP as control. Another control group without dsRNA treatment was fed with the same amount of EDTA solution. Twenty nymphs were used per treatment in each replicate, and two replicates per treatment were performed.

For expression analysis of feeding of dsRNA, bioassays were carried out with 0–1-day-old fourth-instar nymphs of *E. heros*. Naked dsRNA, and mixtures of dsRNA with Lipofectamine 2000 and EDTA 3% w/v were mixed with the artificial diet. Sachets containing 30  $\mu\text{L}$  of the diet amended with dsRNA were made aseptically using UV-sterilized Parafilm. The exposure times were 24, 72 and 120 h using *act-2* and *vATPase A* as target genes and GFP as the control. The final concentration of dsRNA in the diet was 300  $\text{ng } \mu\text{L}^{-1}$  for naked dsRNA, Lipofectamine 2000 and EDTA. Total RNA was extracted from the guts after continuous feeding with dsRNA, each treatment contained four biological samples of three pooled guts. For dissection, larvae were first chilled on ice, the posterior and anterior ends were removed, and entire guts were excised.

## 2.6 Ex vivo saliva degradation assays

To collect watery saliva from *E. heros*, adult insects were chilled on ice for  $\sim 5$  min, then placed ventral side up and observed with a dissecting microscope. As the bugs returned to room temperature, watery saliva was secreted from the tip of the beak.<sup>59</sup> This saliva was collected with a Microloader™ pipette tip (Eppendorf). After

collection, the saliva was expelled into a 1.5 mL tube and stored on ice until enough saliva was collected. For the digestion assay, 20  $\mu\text{L}$  of a 200  $\text{ng } \mu\text{L}^{-1}$  dsRNA solution was incubated in 2  $\mu\text{L}$  of RNase-free water, 2  $\mu\text{L}$  of saliva or 2  $\mu\text{L}$  of saliva diluted at 1:10. dsGFP and ds*act-2* were used as dsRNA for degradation assays. To test the ability of EDTA to inhibit the enzymatic activity of nucleases in the saliva of *E. heros*, 1  $\mu\text{L}$  of 10 mM EDTA was added to the mixture before incubation. All digestions were performed at 25 °C. Aliquots of 5  $\mu\text{L}$  were collected after 10, 30, 60 and 120 min, and added to the same volume of EDTA (10 mM) to stop the enzymatic reaction and run on a 1.5% agarose gel.

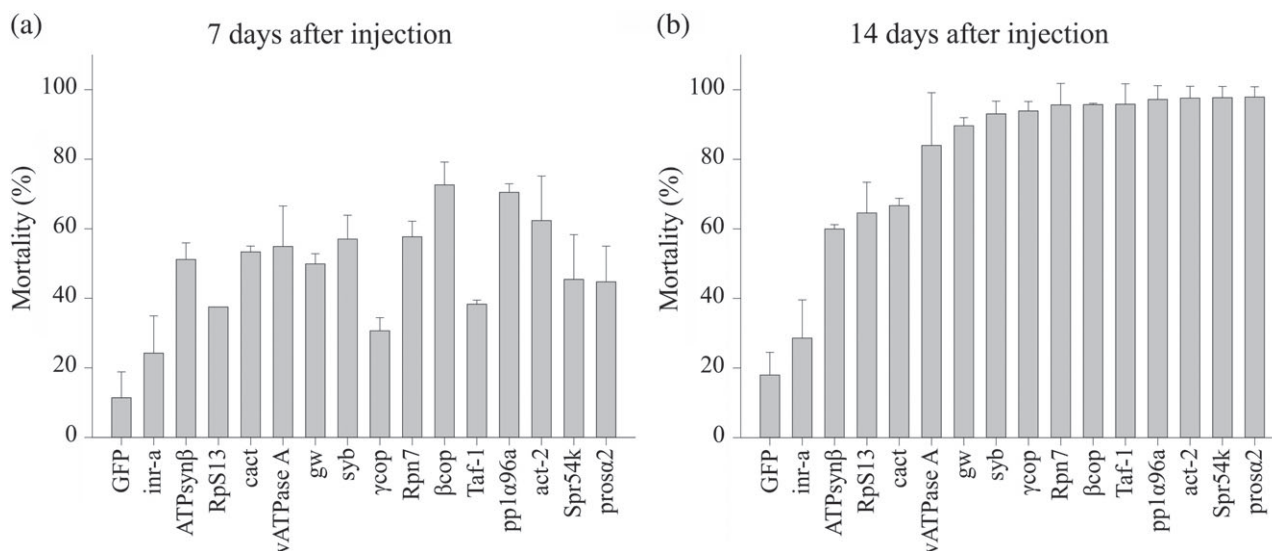
To evaluate the ability of Lipofectamine 2000 to protect dsRNA against degradation by nucleases present in the insect saliva, a similar experiment was undertaken. Twenty microliters of dsRNA of 200  $\text{ng } \mu\text{L}^{-1}$  (with Lipofectamine 2000, same ratio as before) was incubated in 2  $\mu\text{L}$  of RNase-free water, 2  $\mu\text{L}$  of saliva or 2  $\mu\text{L}$  of saliva diluted at 1:10. Aliquots of 5  $\mu\text{L}$  were collected after 10, 30, 60 and 120 min. To stop the enzymatic reaction and disassemble the dsRNA complexes, 7.5  $\mu\text{L}$  of SDS 1% w/v was added, and the mixture posteriorly run on 1.5% agarose gel.

## 2.7 Real-time quantitative PCR

The RNeasy Mini Kit (Qiagen) was used for RNA extraction following the manufacturer's instructions. The total RNA was purified with DNase treatment and removal reagent using a turbo DNA-free kit (Invitrogen) following the suppliers' recommendations. After DNase I treatment (Ambion), RNA was quantified using a NanoDrop ND-1000 (Nanodrop Technologies) and verified by 1.5% agarose gel electrophoresis. The cDNA was reverse transcribed from 500 ng of total RNA template and oligo (dT) primer using SuperScript III First-Strand synthesis (Invitrogen).

*E. heros* qRT-PCR specific primers were designed using Primer3 Plus free-software (Table 3). The reference genes *ARL2*, *ARP8* and *UB4A* exhibit the most stable expression following the dsRNA treatment in the stink bug *H. halys*, making these genes appropriate for qRT-PCR data normalization for gene silencing analysis.<sup>26</sup>

The qRT-PCR reactions were performed in the CFX 96TM real-time system (Bio-Rad, Hercules, CA, USA) with SYBR green dye as the fluorescence reporter for each elongation cycle. The primers used in the analysis were validated with a standard curve based on a serial dilution of cDNA to determine the primer annealing efficiency and a melting curve analysis with temperature range from 60 to 95 °C. The reaction included 10  $\mu\text{L}$  of GoTaq qPCR Master Mix for Dye-Based Detection (Promega), 0.5–1  $\mu\text{L}$  of 10  $\mu\text{M}$  forward primer (Invitrogen), 0.5–1  $\mu\text{L}$  of 10  $\mu\text{M}$  of reverse primer (Invitrogen), 0–1  $\mu\text{L}$  of nuclease-free water and 8  $\mu\text{L}$  of cDNA (dilution 1:100), in a total volume of 20  $\mu\text{L}$ . The amplification conditions



**Figure 1.** Mortality of second-instar nymphs of *Euschistus heros* after injection of double-stranded (ds)RNA. Injection with dsRNA targeting green fluorescent protein (GFP) was used as a control. Values represent the mean mortality percentages  $\pm$  SEM ( $N = 50$ ) and standard errors from two independent replicates. (a) Seven days post injection and (b) 14 days post injection.

were 3 min at 95 °C followed by 39 cycles of 10 s at 95 °C and 30 s at 60 °C. The reactions were set-up in 96-well format Microseal PCR plates (Bio-Rad) in duplicate. The endogenous controls, ribosomal protein *ARL2*, *ARP8* and *UBE4A*, were used for normalization of the data. Appropriate controls, no-template control and no-reverse transcriptase control, were also included in the assay. Relative expression values of genes in biological samples were calculated using the equation ratio  $2^{-\Delta\Delta Ct}$ .<sup>60</sup>

### 3 RESULTS

#### 3.1 dsRNA injection

The insecticidal activity of 15 dsRNAs targeting essential genes was studied following direct injection in second-instar larvae of *E. heros*. dsRNA injection of 27.5 ng mg<sup>-1</sup> body weight (~1.0 mg) led to significant differences in survival (log-rank test:  $\chi^2 = 349.358$ ,  $df = 16$ ,  $P < 0.001$ , Fig. 1). Of the 15 candidate genes tested, silencing of the genes *prosa2*, *Spr54k*, *act-2*, *pp1α96a*, *taf-1*, *γcop* and *rpn7* gave mortality rates of > 95%. After 7 days comparing both repetitions, the injection of dsRNA against genes *βcop*, *pp1α96a*, *act-2*, *rpn7*, *syb*, *vATPase A*, *cact* and *ATPsynβ* produced > 50% mortality (Fig. 1a). These values differed from those obtained for dsGFP-treated insects ( $F > 39.87$ ,  $P < 0.001$ ). At 14 days, mortality reached 90–100% for silencing of the genes *prosa2*, *Spr54k*, *act-2*, *pp1α96a*, *taf-1*, *γcop*, *Rpn7*, *βcop* and *syb* (Fig. 1b), which was significantly different from the dsGFP control ( $F > 21.3$ ,  $P < 0.001$ ).

The survival curves for silencing of the genes *prosa2*, *spr54k*, *act-2*, *pp1α96a*, *taf-1*, *βcop*, *rpn7*, *γcop*, *syb*, *gw* and *vATPase A* were not significantly different from each other, but differed from the values obtained for dsGFP-treated insects (Holm-Sidak's statistics  $< 23.9$ ,  $P > 0.0001$ , not shown). Only silencing of the genes *inr-a*, *ATPsynβ*, *RpS13* and *cact* showed a mortality rate < 70%. The survival rate for *inr-a* dsRNA-treated insects was not significantly different from values obtained for dsGFP-treated insects (Holm-Sidak's statistics  $< 0.18$ ,  $P = 1.00$ , not shown).

Figure 2 shows the survival curve over time for the three genes causing the highest mortality in the injection assays (*prosa2*, *Spr54k* and *act-2*) and *vATPase A*, a target gene commonly used

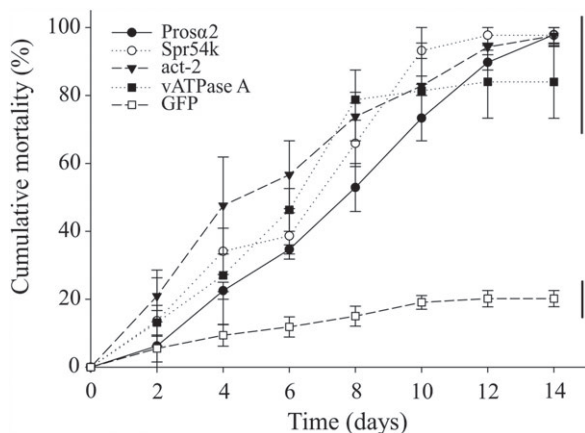
in RNAi feeding experiments and has been shown to be effective in causing mortality upon knockdown in many other insect species.<sup>41,45,56,61–64</sup> *dsact-2* caused early mortality, with a median survival time ( $LT_{50}$ ) of 3.0 days (95% confidential limits: 1.0–4.9) (Fig. 2). Silencing of the *prosa2*, *Spr54k* and *vATPase A* genes gave intermediate survival times, 6.0 days (95% confidential limits: 4.1–7.9) for *vATPase A*, and 7.0 days (95% confidential limits: 5.5–8.5) for *Spr54k* and *prosa2*. Normal development was observed in the control (dsGFP), whereas detrimental effects were demonstrated under target gene dsRNA treatments. These detrimental effects included lower mobility, lower weight, slower development and defects in molting. These results confirmed that *E. heros* is highly susceptible to RNAi even when low doses are injected. Based on these results, the choice was made to continue this investigation with *act-2* and *vATPase A*. The former was chosen purely based on the high and rapid mortality observed after injection of the specific dsRNA, whereas *vATPase A* was chosen partly based on the results of the injection assay, but also because it is a commonly used target gene in oral RNAi assays, often leading to a high mortality upon feeding the *dsvATPase*.

#### 3.2 Real-time quantitative PCR for the injection assays

To confirm knockdown of the genes *act-2* and *vATPase A* after dsRNA injection, a qRT-PCR analysis was performed using the cDNA of injected insects. For *act-2*, dsRNA injection reduced transcript levels by 69% ( $P = 0.002$ ) 48 h after injection and 59% ( $P = 0.032$ ) 72 h after injection (Fig. 3a). At 48 h after injection, *vATPase A* transcript levels were reduced by 54%, but were not significantly different from the control ( $P = 0.12$ ) (Fig. 3b). At 72 h after injection of dsRNA, *vATPase A* transcript levels were reduced by 90% ( $P = 0.019$ ) (Fig. 3b).

#### 3.3 Feeding of dsRNA in an artificial diet

Next, dsRNA was administered via feeding by mixing with the artificial diet using different formulations of dsRNA: naked dsRNA, dsRNA mixed with liposomal transfection reagent and dsRNA mixed with the chelating agent EDTA, which is able to inhibit nuclease activity. Because transcript silencing of the genes *act-2*

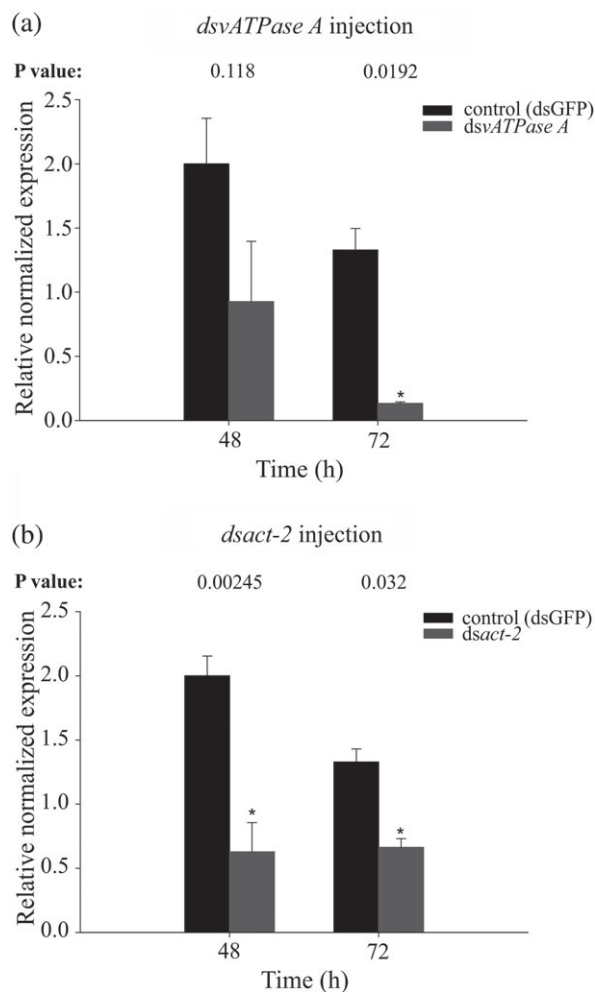


**Figure 2.** Cumulative mortality of *Euschistus heros* after injection of double-stranded (ds)RNA targeting a selection of target genes in second-instar nymphs. Injection with dsRNA targeting green fluorescent protein (GFP) was used as a control. The curves encompassed by the same vertical bar at the left side of the plot are not significantly different according to Holm-Sidak's test ( $P > 0.05$ ).

and *vATPase A* was confirmed with qRT-PCR after microinjection, these genes were selected for the artificial diet assay. For the naked dsRNA test, diet containing  $320 \text{ ng } \mu\text{L}^{-1}$  of dsRNA resulted in 33% mortality for *dsact-2* and 30% mortality for *dsvATPase A* (Fig. 4a). According to the survival analysis, only the observed mortality for *dsact-2* was significantly different from the control dsGFP (Holm-Sidak's statistics = 9.485,  $P = 0.023$ ). As a second formulation, the influence of the liposomic transfection agent on dsRNA delivery was tested. The diet containing  $300 \text{ ng } \mu\text{L}^{-1}$  of dsRNA combined with liposomes resulted in 45% mortality for *dsvATPase A* and 42% mortality for *dsact-2* after 14 days (Fig. 4b). According to the survival analysis, the observed mortality for *dsvATPase A* and *dsact-2* was significantly different from the liposome-coated dsGFP control (Holm-Sidak's statistics = 12.266,  $P = 0.007$ ). In the final treatment, EDTA was added to the artificial diet to test the influence on dsRNA delivery. EDTA is a chelating agent that could inhibit the enzymatic activity of nucleases present in insect saliva. After 14 days, the diet containing  $300 \text{ ng } \mu\text{L}^{-1}$  of dsRNA and EDTA at 3% (w/v) resulted in 51% mortality for *dsvATPase A* and 22% mortality for *dsact-2* (Fig. 4c). The observed mortality for *dsvATPase A* was significantly different from the EDTA-dsGFP and EDTA controls (Holm-Sidak's statistics = 52.739,  $P < 0.001$ ). Significant differences in weight between the liposomic and EDTA treatments for both target genes compared with controls were already recorded after 4 days of feeding (Fig. S1).

### 3.4 Ex vivo saliva degradation assays

An *ex vivo* assay with watery saliva of *E. heros* adults was conducted to evaluate whether dsRNA is degraded during extra-oral digestion. The saliva was found to rapidly digest dsRNA because after 10 min the dsGFP and *dsact-2* were completely degraded (Fig. 5a,b). To establish an effective concentration of saliva necessary for dsRNA digestion, raw saliva was diluted at concentrations of 1:10, 1:50, 1:200 and 1:1000. After 10 min at  $25^\circ\text{C}$ , the most concentrated sample resulted in complete digestion of the dsRNA, a 1:50 dilution resulted in a minimal degradation of dsRNA, and the dsRNA in the less concentrated saliva samples appeared intact (data not shown). Based on these results, a 1:10 dilution was used in the further experiments. With the 1:10 dilution, an incubation of 30 min resulted in a clear smear below the band representing

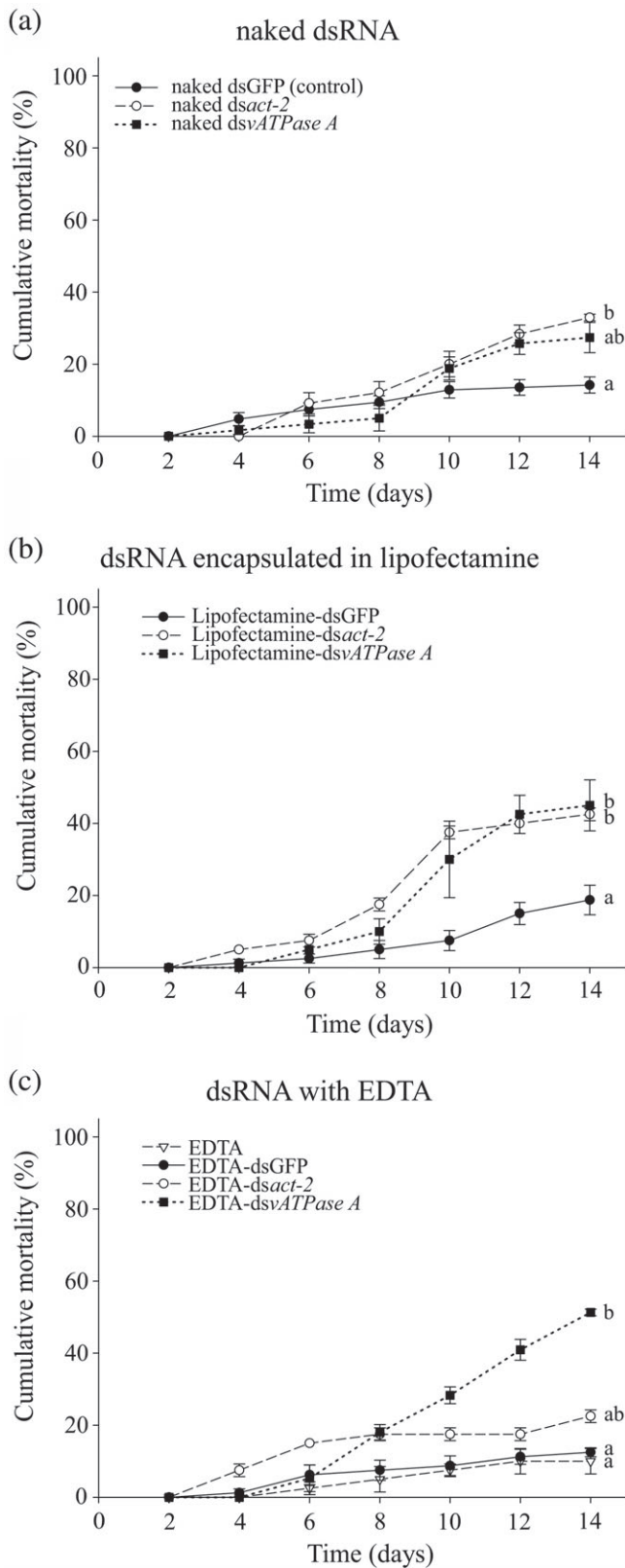


**Figure 3.** Knockdown of *act-2* and *vATPase A* in second-instar nymphs of *Euschistus heros* injected with target gene-specific dsRNA. Injection with double-stranded (ds)RNA targeting green fluorescent protein (GFP) was used as a negative control. (a) *vATPase A* and (b) *act-2*. As internal controls, *ARL2*, *ARP8* and *UBE4A* were used. Values are based on two biological samples and expressed as mean  $\pm$  SEM. Each sample contains four pooled insects.  $P$ -values were calculated by unpaired *t*-test.

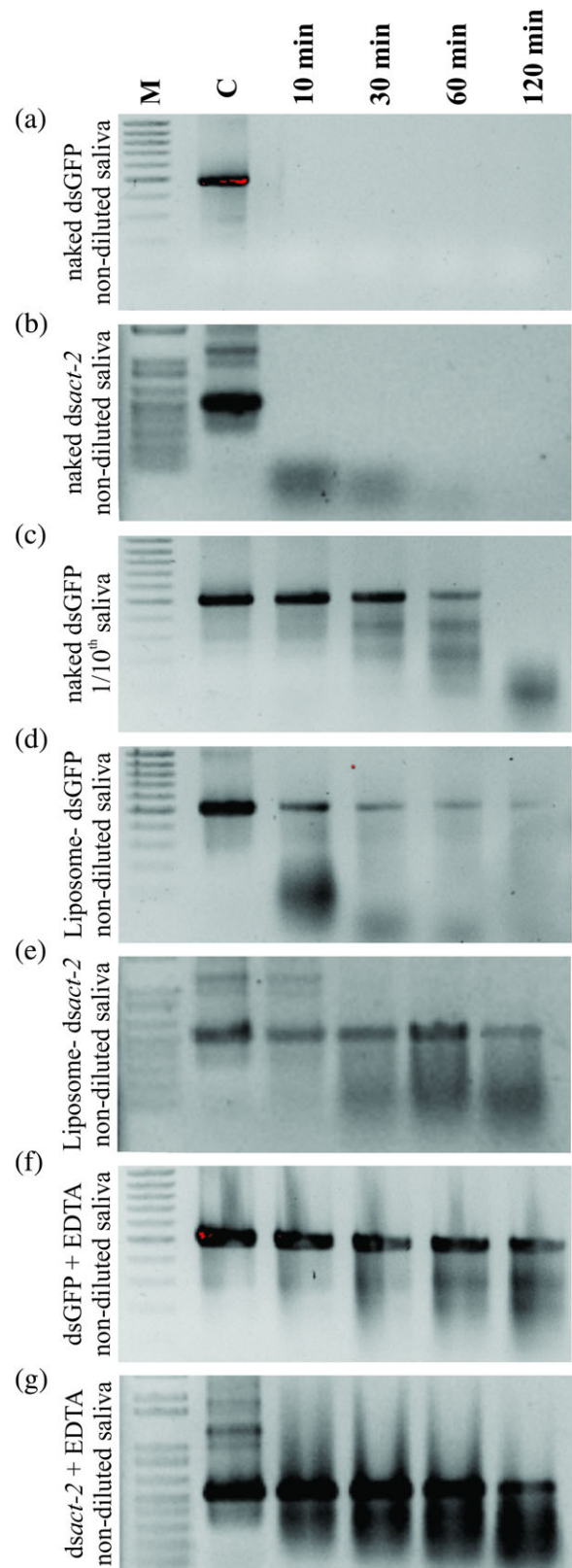
the intact dsGFP fragment, indicating digestion of the dsRNA. After 60 min of incubation, the dsGFP was partially degraded, and after 120 min the dsRNA was completely degraded (Fig. 5c). To examine whether liposomes and EDTA could protect dsRNA from degradation, similar experiments were set up to test the integrity of dsRNA, using gel electrophoresis after incubation in saliva. The liposomes were less effective in protecting the dsRNA against saliva degradation. After 30 min of incubation with non-diluted saliva, dsGFP and *dsact-2* were partially degraded (Fig. 5d,e); and after 120 min dsGFP was almost completely degraded (Fig. 5d), whereas for *dsact-2* a small band was still present (Fig. 5e). The EDTA protected the dsGFP and *dsact-2* longer against enzymatic degradation by the saliva with non-diluted saliva (Fig. 5f,g). dsGFP and *dsact-2* bands were still present after 2 h of incubation, despite signs of ongoing degradation illustrated by the smearing on the gel (Fig. 5g).

### 3.5 Real-time quantitative PCR for the feeding assays

To confirm the knockdown of *vATPase A* after dsRNA feeding with different formulations of dsRNA, qRT-PCR was performed using



**Figure 4.** Cumulative mortality of second-instar nymphs of *Euschistus heros* after feeding dsRNA with different formulations of double-stranded (ds)RNA: (a) naked dsRNA, (b) liposome-encapsulated dsRNA, and (c) EDTA formulation. Feeding with dsRNA targeting green fluorescent protein (GFP) was used as a negative control. The curves encompassed by the letter at the left side of the plot are not significantly different according to Holm-Sidak's test ( $P > 0.05$ ).



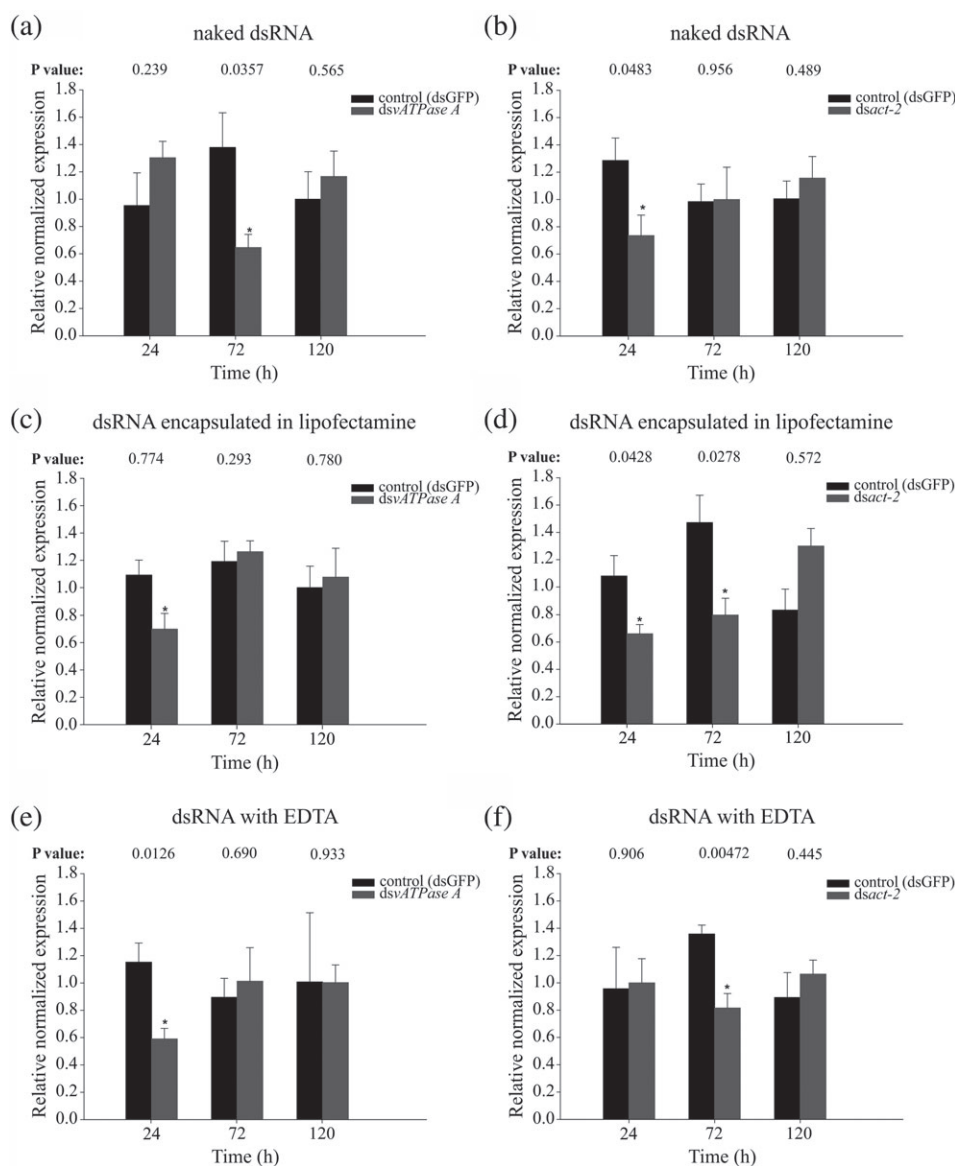
**Figure 5.** *Ex vivo* dsRNA degradation assay of different formulations of double-stranded (ds)RNA: (a) naked dsGFP non-diluted saliva, (b) naked *dsact-2* non-diluted saliva, (c) naked dsGFP in 1:10 diluted saliva, (D) dsGFP with liposome, (e) *dsact-2* with liposome, (f) dsGFP with EDTA and (g) *dsact-2* and EDTA. Watery saliva of *Euschistus heros* was extracted and incubated with 0.9  $\mu$ g of dsGFP per well for different time periods and run in 1.5% agarose gel. M = DNA ladder, C = 0.9  $\mu$ g of dsGFP at 2 h of incubation.

cDNA of dissected guts after 24, 72 and 120 h of feeding. A significant decrease in *vATPase A* and *act-2* mRNA levels was confirmed by qRT-PCR for naked dsRNA, EDTA-dsRNA and liposome-coated dsRNA (Fig. 6). For naked dsRNA, the *vATPase A* transcript levels were reduced by 53% after 72 h ( $P = 0.0357$ ; Fig. 6a) and *act-2* mRNA levels were reduced by 43% after 24 h ( $P = 0.0483$ ; Fig. 6b). The feeding of *dsvATPase A* lipoplexes resulted in a small significant reduction of 36% in the transcription ( $P = 0.0483$ ; Fig. 6d) compared with dsGFP lipoplex control. After feeding of lipoplexes with *dsact-2*, a significant reduction in *act-2* expression was observed at 24 h (39%;  $P = 0.0428$ ; Fig. 6e) and 72 h (46%;  $P = 0.0428$ ; Fig. 6e). A transcript depletion effect was observed after continuous feeding on dsRNA with EDTA; for *vATPase A*, transcript levels were lowered by 49% after 24 h ( $P = 0.0126$ ; Fig. 6g) and *act-2* transcript levels by 40% after 72 h ( $P = 0.00472$ ; Fig. 6h).

#### 4 DISCUSSION AND CONCLUSIONS

This study demonstrates that RNAi effects can be induced in *E. heros* by dsRNA microinjection and oral delivery. Recently,<sup>21</sup> it was reported that *E. heros* is highly sensitive to dsRNA by injection. The functionality of RNAi in *E. heros* was confirmed by the screening via microinjection of 15 potential genes. The class of target genes selected here represented varied functions such as signaling pathways, intracellular transport, degradation of proteins, cell energization, pH homeostasis, transcriptional regulation, protein synthesis and muscle movement. These genes were chosen based on the expectation that their knockdown results in a lethal phenotype.<sup>55,57</sup>

In this investigation, microinjection experiments were chosen for the first selection round, as only small amounts of dsRNA are needed for these experiments, and because even a dose of



**Figure 6.** Expression of *vATPase A* and *act-2* in fourth-instar nymphs of *Euschistus heros* after feeding on double-stranded (ds)RNA using different formulations: naked dsRNA, liposome-coated dsRNA and an EDTA formulation for 24, 72 and 120 h. Feeding with dsRNA targeting GFP was used as a control: (a) naked *dsvATPase A*, (b) naked *dsact-2*, (c) liposome-*dsvATPase A*, (d) liposome-*dsact-2*, (e) EDTA-*dsvATPase A* and (f) EDTA-*dsact-2*. *ARL2*, *ARP8* and *UBE4A* were used as internal controls. Values are based on four biological samples and expressed as mean  $\pm$  SEM. Each sample contains three pooled insect guts. *P*-values were calculated by unpaired *t*-test.

20 ng g<sup>-1</sup> of dsRNA of an essential gene (*actin*) led to higher mortality.<sup>21</sup> High insecticidal activity was observed, with almost total mortality for seven of the dsRNAs, targeting the genes *prosa2*, *Spr54k*, *act-2*, *pp1α96a*, *taf-1*, *γcop* and *rpn7*, at 14 days post injection compared with the low mortality (18%) in the control injected with dsGFP, although the dsRNAs against four other genes, namely *inr-a*, *ATPsynB*, *RpS13* and *cact*, failed to cause mortality over 70%. Interestingly, many of the selected genes that showed efficient silencing led to abnormal phenotypes such as a significant reduction in nymphal weight, reduction in mobility and molting abnormalities. Two target genes were then selected to continue the investigation in *E. heros* RNAi, namely *act-2* and *vATPase A*. The former encodes a muscle actin that is a crucial component for muscle contraction<sup>65</sup> and *vATPase A* is a catalytic subunit of the vacuolar-type proton ATPase. *vATPase* is an enzyme complex that functions to acidify intracellular organelles by pumping protons across the plasma membrane. This enzyme is also found in the plasma membrane of many animal cell types and is involved in pH homeostasis and ion transport.<sup>64,66</sup> *vATPase A* has revealed a high RNAi efficacy in impairing gene expression by ingestion in different insects including *Helicoverpa armigera* (Hübner),<sup>61</sup> *Dia-brotica* spp.,<sup>56,62</sup> *Cylas brunneus* (Fabricius),<sup>63</sup> *Leptinotarsa decemlineata* (Say),<sup>56</sup> *B. tabaci*<sup>45,64</sup> and *B. cockerelli*.<sup>41</sup> By contrast, *act-2* has been silenced only in *E. heros* by injection<sup>21</sup> and in *Homalodisca vitripennis* (Germar) cells by lipid-based transfection.<sup>65</sup>

First, RT-qPCR was used to confirm that significant transcript reduction in the expression of *act-2* and *vATPase A* (69% and 90%, respectively) was obtained after dsRNA injection. These results indicated that microinjection is a very promising technique for the study of functional genomics in *E. heros*.

Because injection of dsRNA is not possible under field conditions, oral delivery and uptake of the dsRNA in the gut are very critical.<sup>45</sup> Therefore, to examine the potential of RNAi for crop protection against *E. heros*, the toxicity through oral delivery of the two selected target genes was evaluated.

Although *E. heros* displayed an RNAi response that is highly sensitive to dsRNA injection, oral delivery of dsRNA appeared much less effective. In these feeding experiments, even with high dsRNA quantities applied, significant suppression of the *vATPase A* was detected in isolated guts within 72 h (53%) and no significant differences were observed in survival compared with controls (Fig. 4a). This lower response upon oral delivery of the dsRNA has been reported in other insects, including *L. lineolaris*,<sup>46</sup> *Locusta migratoria* (L.),<sup>67</sup> *Bombyx mori* (L.),<sup>68</sup> *Schistocerca gregaria* (Forsskål),<sup>69</sup> *Drosophila suzukii* (Matsumura),<sup>49</sup> *C. brunneus*,<sup>63</sup> *C. puncticollis*,<sup>70</sup> and in *Blattella germanica* (L.).<sup>50</sup> Possible explanations for the large discrepancy between RNAi efficiency through microinjection and oral delivery of dsRNA may be the amount of dsRNA ingested by the insect, the frequency of feeding, degradation of dsRNA in the digestive tract, cellular uptake in the gut and systemic transport to the target tissues.<sup>15,23,24</sup>

This premise was further studied to observe specifically whether the saliva of *E. heros* has ribonuclease activity. An *ex vivo* incubation test showed rapid degradation of dsRNA because within 10 min the dsRNA had essentially disappeared from the gels (Fig. 5a), indicating complete digestion to monomers. This finding is in agreement with the strong degradation due to nucleases in the saliva of in *L. lineolaris*<sup>46</sup> and *A. pisum*.<sup>47</sup> It is increasingly evident that non-specific nucleases in the gut lumen could contribute to the degradation of dsRNA in *B. mori*,<sup>68,71</sup> and *S. gregaria*,<sup>69</sup> and even in the highly oral RNAi sensitive *L. decemlineata*.<sup>72</sup> Unlike other insects that primarily digest the food internally, hemipterans use

digestive enzymes for extra-oral digestion to liquefy vegetal tissue before ingestion.<sup>59,73,74</sup> These nucleases have to be expressed in the salivary glands. Liu *et al.*<sup>68</sup> demonstrated that a DNA/RNA non-specific alkaline nuclease is in fact present in several different tissues of *B. mori* larvae including epidermis, fat body, thoracic muscles, Malpighian tubules, brain and silk glands. A recent study in *B. tabaci* identified three homologs of this protein and demonstrated that suppression of dsRNase genes resulted in an enhanced efficacy of RNAi against two other insect genes, *AQP1* and *SUC1*.<sup>75</sup> However, extra-oral degradation of dsRNA is not substantial in the whitefly, but dsRNA ingested by the whiteflies is subjected to non-specific degradation within the insect body,<sup>75</sup> indicating that different nucleases are responsible for the degradation of dsRNA by the saliva, making in turn that future research should identify these enzymes on a biochemical and genetic level.

An alternative approach is to protect the dsRNA for its delivery, and various delivery vectors including liposomes, polymers and nanoparticles have been developed to avoid these problems.<sup>76–78</sup> In *D. melanogaster* (Meigen) and other drosophilid species, a transfection reagent has been used to enhance the uptake of encapsulated dsRNA into the target cell, due to its efficient interaction with cell membranes and nucleic acids.<sup>38,49,76</sup> Recently, Lin *et al.*<sup>50</sup> demonstrated that liposomes can be a protective vehicle of dsRNA against the degradation that takes place in the midgut juice of *B. germanica*.<sup>50</sup> In this study, ingestion of liposome-encapsulated *dsvATPase A* increased the mortality 1.6-fold compared with naked *dsvATPase A* (45.0% and 27.4% mortality, respectively), and significant but incomplete gene suppression in the isolated gut tissues was observed 24 h after feeding (35%). For *act-2*, the liposome was able to increase mortality 1.3-fold compared with naked dsRNA and resulted in gene suppression of 39% at 24 h and 46% at 72 h after feeding. These observations could be linked to an improved or more rapid cellular uptake and a partial protective activity against degradation by the saliva (Fig. 5c). qPCR analysis showed a prolonged silencing at the *act-2* transcript level for liposome-dsRNA compared with the naked dsRNA. However, a similarly improved transcript reduction for the *vATPase* liposome-dsRNA was not observed. In general, the observations at the transcript level do not clearly support the increased effect of this formulation at the phenotypical level. One possible explanation is that the limited number of time points analyzed resulted in a hidden difference in silencing duration, which were not detectable. Although these results make it difficult to explain the phenotypical differences seen between naked dsRNA and the liposome-formulated molecule, it does indicate that gene silencing occurs, which can explain the observed mortality and link it to an RNAi effect. Additionally, previous studies showed the restrictive depleting effect in the midgut by dsRNA ingestion might not pass through the gut cells into the hemocoel to cause a strong silencing effect in other tissues of the German cockroach.<sup>50</sup>

This study also shows that the degradation of dsRNA in the saliva was inhibited in the presence of EDTA, a chelating agent for divalent cations (Ca<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup>). These results provide evidence that a metal-dependent enzyme is responsible for the degradation of dsRNA in saliva, as previously reported in the degradation in the hemolymph plasma of *Manduca sexta* (L.).<sup>79</sup> and in the midgut of *C. puncticollis*,<sup>70</sup> suggesting that EDTA could be used as a dsRNA protective agent for feeding assays. The ingestion of *dsvATPase A* amended with EDTA caused significant mortality (51%), but the formulation did not appear to affect the mortality upon *act-2* feeding. However, a significant decrease in weight was observed for both target genes using EDTA as a formulation

(Fig. S1). The expression level of *vATPase A* decreased 49% within 24 h when feeding EDTA-dsRNA, whereas gene silencing with naked dsRNA was only significantly reduced after the third day. By contrast, *act-2* gene silencing occurs later when EDTA was added to the dsRNA compared with naked dsRNA. Similar to the results for liposome formulation, the effect at the transcript level appeared to be difficult to reconcile with the significantly increased mortality for these formulations. In this study, the goal of transcript analysis was to confirm that gene silencing occurred but further investigations into these dynamics, for example by evaluating samples taken at many more time points, or by looking at multiple tissues rather than the gut only, may help us explain these observations. Because the insects lived for several days while consuming the dsRNAs, this observation suggested that mortality was a consequence of latent effects of the dsRNA, reducing gene function sufficiently to disrupt normal gut cell function, thereby leading to death of the growing insect.<sup>38</sup> In future studies, it will be of interest to examine whether the ingestion of dsRNA with these formulations leads to an RNAi response in the whole body.

To summarize, the results presented here reveal an RNAi effect in *E. heros* through injection and oral delivery, although less sensitive for the latter. Several target genes were identified in which low dsRNA dose injection lead to effective silencing and high mortality. The rapid dsRNA degradation that occurred during extra-oral digestion impaired the RNAi response in the feeding assays. Feeding of lipofectamine-encapsulated dsRNA increased mortality and protected the dsRNA against saliva degradation. Furthermore, this study presents a promising approach for dsRNA oral delivery, namely the use of EDTA to prolong the stability of dsRNA and induce stronger RNAi effects. However, EDTA could only effectively increase RNAi-caused mortality for one of our target genes, despite showing clear capabilities to protect the dsRNA targeting the other target gene in the *E. heros* midgut environment. Further research will have to elucidate the exact reasons for this observation.

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## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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