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Comparison of strategies for enhancing RNA interference efficiency in *Ostrinia nubilalis*

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Abstract

BACKGROUND: Targeting insect-specific genes through post-transcriptional gene silencing with RNA interference (RNAi) is a new strategy for insect pest management. However, lepidopterans are recalcitrant to RNAi, which prevents the application of novel RNAi technology to many notorious pests, including *Ostrinia nubilalis* (ECB). Strategies for enhancing RNAi efficiency, including large doses of double-stranded RNA (dsRNA), nuclease inhibitors, transfection reagents, and nanoparticles, have proved useful in other insects exhibiting substantial dsRNA degradation, a major mechanism limiting RNAi efficacy. To determine if similar strategies can enhance RNAi efficiency in ECB, various reagents were tested for their ability to enhance dsRNA stability in ECB tissues, then compared for their effectiveness in whole ECB.

RESULTS: *Ex vivo* incubation experiments revealed that Metafectene Pro, EDTA, chitosan-based dsRNA nanoparticles, and Zn²⁺ enhanced dsRNA stability in ECB hemolymph and gut content extracts, compared to uncoated dsRNA. Despite these positive results, the reagents used in this study were ineffective at enhancing RNAi efficiency in ECB *in vivo*. To reduce assay time and required dsRNA, midguts were dissected and incubated in tissue culture medium containing dsRNA with and without reagents. These experiments showed that RNAi efficiency varied between target genes, and nuclease inhibitors improved RNAi efficiency for only a portion of the refractory target genes investigated *ex vivo*.

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CONFLICT OF INTEREST DECLARATION

The authors have no conflicts of interest to declare.

SUPPORTING INFORMATION

Cooper et al., RNAi efficiency in *Ostrinia nubilalis*

CONCLUSION: These results indicate that enhancing dsRNA stability is insufficient to improve RNAi efficiency in ECB and suggests the existence of additional, complex mechanisms contributing to low RNAi efficiency in ECB.

Keywords

RNAi; European corn borer; double-stranded RNA stability; feeding; injection; tissue culture

1. Introduction

Targeting insect-specific genes through post-transcriptional gene silencing with RNA interference (RNAi) is a new strategy for insect pest management.¹ RNAi-mediated crop protection strategies, including transgenic plants, baits, and sprays, are valuable approaches for sustainable agriculture and food security because they provide new approaches to address yield loss,² they offer a novel mechanism of action to combat pesticide resistance,³ and they provide greater species-specificity to reduce off-target environmental effects.⁴ Unfortunately, RNAi technology is currently hindered by inefficient and highly variable results when different insects are targeted, especially lepidopterans.^{5,6} Given that lepidopterans are among the most devastating pests of crops, forests, and stored products, basic research is needed to elucidate and overcome factors limiting RNAi efficiency in agriculturally relevant lepidopteran pests, such as the European corn borer (ECB), *Ostrinia nubilalis* (Hübner).^{7,8}

The ECB is a small, light brown, crambid moth native to Europe that was first introduced into the United States in the early 1900s and is now established in North America.^{9,10} ECB is one of the most damaging pests of corn in North America and Europe,¹⁰ and a variety of methods have been employed to limit crop loss, including genetic modification of corn and conventional insecticides. Corn plants have been genetically engineered to resist ECB feeding through recombinant expression of Bt Cry toxins from *Bacillus thuringiensis*.^{9,11} Unfortunately, resistance to these methods of control has already been documented.^{12,13} Therefore, new approaches that exploit novel modes of action are badly needed for ECB to combat insecticide resistance and prolong the use of Bt Cry toxins. However, before RNAi-based pest management strategies can be developed for ECB, the molecular mechanisms limiting RNAi efficiency in ECB and other lepidopteran pests must be understood and overcome.

Investigations of molecular mechanisms influencing RNAi efficiency reveals five main factors limiting RNAi efficiency in insects: instability of dsRNA in body fluids, inadequate internalization of dsRNA, deficient core RNAi machinery, insufficient systemic spreading of the silencing signal, and refractory target genes.^{1,14} To combat these limitations in RNAi efficiency, there is a growing suite of strategies and technologies for dsRNA delivery to enhance RNAi efficiency in RNAi-refractory insects, including *Drosophila suzukii*,¹⁵ *Spodoptera exigua*,^{16,17} *Hyphantria cunea*,¹⁸ and *Anopheles gambiae*.¹⁹ These strategies include large doses of double-stranded RNA (dsRNA), repeated exposure to dsRNA, cationic liposomes, and dsRNA-nanoparticles, among others.^{1,14} Thus, it may be possible to enhance RNAi efficiency (i.e., suppression at a given dose of dsRNA) in ECB by optimizing

dsRNA delivery procedures and reagents. However, strategies for enhancing RNAi efficiency in ECB have not yet been evaluated.

In the present study, various strategies, procedures, and reagents were tested for their ability to enhance dsRNA stability and RNAi efficiency in ECB. *Ex vivo* incubation experiments were used to identify transfection reagents, nanoparticles, and nuclease inhibitors that enhanced dsRNA stability in ECB gut contents (GC) and hemolymph (HE). These reagents, as well as a peritrophic matrix (PM) disrupting reagent, were then tested on a variety of ECB developmental stages to determine if they could enhance oral or injected RNAi efficiency *in vivo*. An assay using midgut tissue culture was also used for the rapid comparison of RNAi efficiency among different target genes. Together, these findings support the hypothesis that RNAi efficiency in ECB is limited by multiple mechanisms and cannot be enhanced using individual strategies or reagents.

2. Materials and Methods

2.1 Insect rearing

The ECB used in this study originated from French Agricultural Research (Lumberton, MN, USA) and were continuously reared in the laboratory at Kansas State University, Manhattan, KS, as described in Cooper et al.²⁰

2.2 Preparation of dsRNA and reagents

To obtain dsRNA for stability enhancement experiments and RNAi assays, dsRNA targeting either an endogenous gene encoding lethal giant larvae protein (*OnLgl*; MT467568) from the ECB or an exogenous gene encoding enhanced green fluorescent protein (*GFP*, LC336974.1) were synthesized, purified, and assessed as described in Cooper et al.²⁰ The primer sequences for dsRNA synthesis are listed in Table S-1. *OnLgl* was selected as the target gene because it is a single copy gene with an essential role in cell proliferation,²¹ and it is expressed in all developmental stages and tissues of ECB. In addition, RNAi of *TcLgl* led to efficient and rapid suppression of transcript levels that lasted over 10 days and resulted in molting defects and mortality in all developmental stages of *Tribolium castaneum* tested.²¹ *GFP* was selected as a non-target control to account for non-specific changes in gene expression associated with dsRNA exposure.²² To form chitosan-based dsRNA nanoparticles (NP dsRNA), ethanol precipitated dsRNA was first purified with the MEGAclean Transcription Clean-Up Kit (Invitrogen, Carlsbad, CA) and eluted in nuclease-free water. Eight micrograms of dsRNA (in 8 μ l water) were then combined with 2 μ l (8 μ g/ μ l) proprietary chitosan-based polymer in 1% acetic acid solution, and 198 μ l of 1% acetic acid. This solution was incubated for three hours at room temperature to allow NP dsRNA to form. After incubation, newly formed NP dsRNA was pelleted out of solution by centrifuging at 16,000 \times g for 30 min. The supernatant was then transferred to another tube for quantification. Unincorporated dsRNA remaining in the supernatant was quantified with a NanoPhotometer (Implen, Westlake Village, CA, USA), and subtracted from the starting quantity, to obtain the amount of NP dsRNA in the pellet. The dsRNA incorporation efficiency was then calculated, and water added to the NP dsRNA pellet to reach the desired

final concentration. The NP dsRNA solution was stored at 4°C, and a hand-held tissue homogenizer was used to fully resuspend the solution prior to use.

To form Lipofectamine RNAiMax dsRNA lipoplexes (Lipo dsRNA), two mixes were created and then combined. Mix 1 contained 1 µl of Lipofectamine RNAiMax reagent (Invitrogen) and 7 µl of buffered sucrose. Mix 2 contained 32 µg of dsRNA and enough buffered sucrose to bring the final volume to 8 µl. Mix 1 and 2 were combined together, vortexed, spun down and incubated at room temperature for 30 min to allow Lipo dsRNA lipoplexes to form. Buffered sucrose solution contained 2% sucrose, 10 mM Tris, (pH 7.5), and 0.5 mM spermidine.¹⁵ Lipo dsRNA solution was stored at 4°C.

To form Metafectine Pro dsRNA lipoplexes (Meta dsRNA), 3.5 µl of Metafectene Pro (Biontex Laboratories GmbH, Munchen, Germany) was mixed with 3.5 µl of nuclease-free water containing 14 µg of dsRNA. The solution was then vortexed, spun down, and incubated at room temperature for 30 min to allow Meta dsRNA liposomes to form. Meta dsRNA solution was stored at 4°C.

Nuclease inhibitor solutions were prepared by dissolving powdered ethylenediaminetetraacetic acid (EDTA), Zn²⁺, Mn²⁺, or Co²⁺ in 1X phosphate-buffered saline (PBS) to create 25–100 mM (w/v) stock solutions for use in subsequent assays.

2.3 dsRNA-stability assays

To assess which of the candidate reagents could enhance dsRNA stability in ECB tissues, *ex vivo* incubation experiments were performed using hemolymph (HE) and gut contents (GC) harvested from fifth-instar larvae following the detailed protocols described in Cooper et al.²⁰ In brief, after harvesting tissues from the ECB larvae, PBS was used to normalize the total protein content between biological replicates (each containing tissue pooled from 15 individuals). One microliter containing 1 µg of coated or uncoated 300 bp ds*GFP* (i.e., naked ds*GFP*, Lipo ds*GFP*, Meta ds*GFP*, or NP ds*GFP*) was then incubated with 2.7 µl of the normalized tissue extracts (or PBS for control incubations), and enough PBS and/or nuclease inhibitor (i.e., EDTA, Zn²⁺, Mn²⁺, or Co²⁺) to reach a final volume of 14 µl. After incubating at room temp for 30 min, the reactions were quenched by either adding 2.8 µl of 50 mM EDTA or by heating to 65°C for 10 min. The remaining dsRNA in each sample was then converted to cDNA and quantified with RT-PCR (see Table S-1 for primers used). Cycle threshold (Ct) values were converted to the amount of ds*GFP* in nanograms based on standard curves (Fig S-1) as previously described.²⁰ Experiments followed a two-way factorial treatment design to allow for assessment of significant effects on dsRNA stability due to reagent (present or absent), incubation fluid (tissue extract or PBS) and/or interaction between both factors. Fold-increases in dsRNA stability due to each reagent were calculated by taking the mean amount of dsRNA in the “dsRNA plus reagent in tissue extract” treatment divided by the mean amount of dsRNA in the “dsRNA in tissue extract” treatment.

2.4 RNAi bioassays

To compare strategies for enhancing RNAi efficiency in ECB, dsRNA, in combination with candidate RNAi-efficiency enhancing reagents, were fed or injected into numerous ECB developmental stages. DsRNAs (500 bp) targeting either the endogenous *OnLgl* gene or the

exogenous *GFP* gene were used for all bioassays. Experiments were generally designed in a two-way factorial treatment structure so that significant effects on relative gene expression, weight, and survivorship due to dsRNA (e.g., ds*OnLgl*, ds*GFP*, water), reagent (e.g., 0, 10, 20 mM Zn²⁺), or interaction between both factors could be investigated. Optimization experiments (data not shown) were performed using the reagents alone to determine the highest dose the insects could tolerate without negative effects on body weight and survivorship.

Injection bioassays consisted of a single, high-dose injection of dsRNA, so comparisons of the enhancement of RNAi efficiency could be made using each of the dsRNA stabilizing reagents, as well as among developmental stages. First instar neonate larvae proved challenging to inject, so they were only used for oral RNAi. However, both delivery methods were tested on second instar larvae. To reduce the amount of dsRNA required for the assays, RNAi efficiency was compared among developmental stages using injection, rather than ingestion. Injection of dsRNA was performed on second-, third-, fifth-instar larvae, pupae, and adults using a Nanoinject II system (Drummond Scientific, PA, USA) coupled with a SYS-Micro4 controller (World Precision Instruments, Sarasota, FL, USA). Each 125-nl injection contained 500 ng of dsRNA with and without reagent delivered at the intersegmental membrane of abdominal segments A5–A6. An equal volume of nuclease-free water or PBS (pH 7.0) was used for buffer-only control injections. Twenty to twenty-five individuals were injected per replicate and either placed on artificial diet inside of 37-ml clear plastic cups sealed with oversnap caps (Frontier Agricultural Sciences, Newark, DE, USA) or placed inside of a covered 1-L glass beaker containing a 90 × 15 mm plastic petri dish with a cotton ball soaked in 20% sucrose/water solution. Three biological replicates, each containing 20–25 individuals, were injected 24–48 h apart. Insects were maintained under rearing conditions until sampling.

Oral RNAi bioassays for ECB were designed based on those used by Khajuria et al.,^{7,23} for young larvae continuously fed on a high dose of dsRNA. Ten micrograms of dsRNA with and without each reagent were applied to artificial diet. An equal volume of nuclease-free water or PBS (pH 8.0) was used for buffer-only controls. Optimization experiments were performed to determine how much diet 25 neonate larvae consume on average in the first 3 d of life, and then the next 3 d of life, to ensure that the full dose of dsRNA would be consumed. Nitrogen gas was used to dry dsRNA/reagent solutions (10 µg/larvae) onto 20 mg squares of artificial diet inside individual Bio-Assay Tray cells (Frontier Agricultural Sciences). Twenty-five larvae were then transferred into each prepared cell using a fine-point paintbrush and sealed inside with Bio-Assay Tray Lids (Frontier Agricultural Sciences). Three biological replicates of every treatment, each containing 25 individuals, were started 12–24 h apart. Larvae were allowed to feed under rearing conditions for three days. On the third day (once all the diet had been consumed), the larvae were transferred to a new cell containing a dsRNA/reagent-treated 40 mg diet square (10 µg/larvae), and then allowed to feed for another three days (until all diet was again consumed). Thus, about 20 µg of dsRNA was fed to each larva over a period of six days. To avoid negative effects on larvae due to starvation, additional untreated diet was added as needed if the larvae finished the treated diet before the 3 or 6 d period was over. Larvae were maintained under rearing conditions until sampling.

Sampling was performed 3 and 6 d after the start of dsRNA exposure as well as at other time points as needed to assess phenotypic effects. The average body weight was determined by weighing all the individuals from each replicate on a laboratory balance and then dividing by the total number of larvae weighed. Percent survivorship was calculated as the number of live individuals divided by the starting number of larvae times one hundred. For expression analysis, three individuals (the largest, the smallest, and an intermediate sized individual) from each replicate of each treatment were pooled at each time point and homogenized in TRIzol reagent (Invitrogen) and frozen at -80°C until further processing. The effects on transcript levels were calculated as described below.

2.5 RNAi tissue culture assays

To determine how RNAi efficiency for *OnLgl* compares to the RNAi efficiency for other target genes, a protocol for incubating excised ECB midguts in tissue culture media containing dsRNA was developed, and used to compare the RNAi efficiency of five additional target genes, encoding clathrin heavy chain (*Chc*), clathrin coat adaptor protein (*AP50*), ADP ribosylation factor 72A (*ARF72A*), V-type proton ATPase 16 kD proteolipid subunit (*Vha16*), and V-type proton ATPase subunit H (*VhaSFD*). To determine if enhanced stability of dsRNA in the ECB midgut is sufficient to enhance RNAi efficiency, midgut tissue culture assays were performed with and without the addition of Zn^{2+} , Mn^{2+} , and Co^{2+} in the medium. Only the divalent cations were tested on ECB midgut cultures because they were the most promising candidates, as EDTA and transfection reagents were toxic to larvae.

Whole guts were dissected from fifth instar ECB larvae in PBS buffer (pH 8.0) and trimmed to remove the foregut and hindgut. Fifth-instar larvae were selected for this assay due to their large size because they were easiest to dissect and three pooled midguts provided ample tissue for RNA extractions. Excised midguts were gently massaged with forceps to remove the food bolus, and directly transferred to a 96 well tissue culture plate containing 50 μl aliquots of prepared insect medium. Prepared insect medium was comprised of 1 X Gibco Grace's Insect Medium (Fisher Scientific, Huston, TX, USA) containing 1% penicillin-streptomycin, nuclease inhibitor (i.e., Zn^{2+} , Mn^{2+} , or Co^{2+}), and various concentrations of dsRNA (50, 100, or 150 ng/ μl) for either the target gene or the *GFP*-control, all diluted with 1X PBS (pH 8.0). After dissections were completed, the 96-well plate was sealed with Parafilm and incubated at 25°C . After 24 h, three midguts were pooled four times from each treatment, homogenized in TRIzol (Invitrogen) and frozen at -80°C until further processing for reverse transcription quantitative PCR (RT-qPCR).

2.6 Analysis of transcript levels

To determine if RNAi-mediated suppression of the target gene occurred, relative expression analysis was performed with RT-qPCR. Total RNA was extracted from three pooled individuals (whole bodies) following the manufacturer's instructions and resuspended in DEPC-treated water. The quality and quantity of total RNA were assessed using a NanoPhotometer (Implen). One microgram of total RNA from each sample was treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) to remove genomic DNA, and then converted to cDNA using the EasyScript cDNA synthesis kit (Applied Biological Materials,

Richmond, Canada) following the manufactures instructions. The resulting cDNA was diluted 5-fold with nuclease-free water for use as template for RT-qPCR.

RT-qPCR was performed in accordance with MIQE guidelines²⁴ on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) using BrightGreen 2x qPCR master mix (Applied Biological Materials) in a 20- μ l reaction volume with the following program: 95°C for 3 min, 39 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by melt curve analysis to confirm amplicon specificity using a temperature range from 65 to 95°C. Primer sequences for RT-qPCR are included in Table S-1. The primer efficiency [$E=10^{(-1/\text{slope})}$] and percent primer efficiency [% $E=(E-1)*100$] were calculated using the standard curve method for each primer pair. The cycle threshold (Ct) was plotted against concentration for each primer set, and a regression line was generated to determine the slope and correlation confidence (i.e., R^2 value). All RT-qPCR reactions were performed on at least three biological replicates, each with two technical replicates. Technical replicates that had Ct values more than 0.5 apart were repeated. In each run, non-template (water only) control reactions were used as negative controls for each primer set.

Ct values were analyzed following the $\Delta\Delta C_T$ method.²⁵ First, the mean Ct values of all technical replicates were normalized to the geometric mean of ribosomal protein S3 gene (*Rps3*, DQ988989) and elongation factor-1 alpha gene (*Ef1a*, AF173392) to calculate ΔC_T . Then ΔC_T values were normalized to the mean of the calibrator treatment (i.e., either the ds*GFP* or buffer-only control). Finally, fold change for each biological replicate was calculated, subjected to statistical analysis, and the mean and standard error of each treatment graphed. Ct values over 32 were considered non-detectible, and a fold change of zero was used for analysis. NormFinder,²⁶ geNorm,²⁷ BestKeeper,²⁸ and RefFinder (<http://150.216.56.64/referencegene.php>) were used to verify the stability of reference genes across ECB tissues and developmental stages.²⁰ Percent suppression of the target gene was calculated as [(control-target)/control]*100].

In two of the bioassays, additional samples were collected at the 3, 6, 12, and 24 h time points to investigate short-term transcriptional responses of the target gene to dsRNA exposure. To provide better visualization of fluctuations in gene expression over the time points analyzed, relative expression was calculated as the expression of *OnLg1* relative to the geometric mean of the reference genes (i.e., ΔC_T rather than Ct).

2.7 Statistical analysis

Statistical differences between treatment means were assessed in Minitab 18 or 19 (Minitab, State College, PA, USA) with either a two-sample *t*-test or a one-way ANOVA followed by the Tukey post hoc test procedure depending on the number of groups that were compared. The significance level (α) was set at 0.05 for the entire family of comparisons. All data sets were subjected to the Anderson-Darling normality test and/or the Levene's test for equal variance to verify statistical assumptions. Data that did not meet the assumptions were subjected to either a Mann-Whitney U test, or a Kruskal-Wallis one-way ANOVA on ranks test, followed by multiple Wilcoxon signed-rank tests for each desired pairwise comparison. The Bonferroni correction was used to control for type I error occurring from the use of

multiple parametric statistical tests. In all experiments, treatments were replicated at least three times.

3. Results

3.1 Putative dsRNA stability-enhancing reagents

Comparison of dsRNA stability in ECB tissue extracts, or PBS as a control, indicated that NP ds*GFP* was not significantly degraded in HE compared to the control incubations, despite significant degradation of naked ds*GFP* in HE [$F(1,20)=6.11$, $p=0.023^*$] (Fig. 1A). Conversely, NP ds*GFP* was significantly degraded in GC compared to the PBS control incubations, but NP ds*GFP* incubated in GC was still significantly more stable than ds*GFP* incubated in GC [$F(1,8)=5.66$, $p=0.045^*$] (Fig. 1B). Specifically, NP enhanced the stability of dsRNA in ECB HE and GC by 16.6- and 36.1-fold, respectively (Table 1). However, there was still 2.2-fold less dsRNA in the NP ds*GFP* in GC treatment than in the NP ds*GFP* in PBS control treatment, indicating that NP could not completely protect dsRNA from degradation in ECB GC but could completely protect dsRNA from degradation in ECB HE.

Similarly, Meta (Fig. S-2), EDTA (Fig. S-3), and Zn^{2+} (Fig. S-4A, S-5A) all significantly enhanced dsRNA stability and completely protected dsRNA from degradation in ECB tissue extracts (Table 1). However, Lipo (Fig. S-6), Mn^{2+} (Fig. S-4B, S-5B), and Co^{2+} (Fig. S-4C, S-5C) did not significantly enhance dsRNA stability in ECB GC or HE (Table 1).

3.2 RNAi efficiency in ECB

Feeding of ds*OnLgl* to neonate ECB larvae did not significantly affect the relative gene expression of *OnLgl* in four separate bioassays, even when candidate RNAi-enhancing reagents such as Lipo, 1% fluorescent brightener 28 (FB28), 50 mM Zn^{2+} , and 6 mM EDTA were employed (Table 2). However, when a similar oral RNAi experiment was performed with 0, 10, and 20 mM Zn^{2+} on second-instar larvae, there was a significant effect on the relative expression of *OnLgl* due to dsRNA after six days of dsRNA feeding (Fig S-9A). Specifically, relative expression of *OnLgl* was suppressed by 68% or 3.2-fold after six days of feeding on ds*OnLgl* compared to larvae that fed on ds*GFP*, regardless of Zn^{2+} concentrations examined [$F(2,12)=9.93$, $p=0.003^*$] (Fig. 2). No phenotypes were observed due to the suppression of *OnLgl* at any of the time points investigated (Table 2, Fig S-9B, C).

Injection of ds*OnLgl* into third-instar ECB larvae resulted in RNAi-mediated suppression of *OnLgl* in two out of three bioassays (Table 2). However, injection of ds*OnLgl* into second instar larvae, wandering fifth-instar larvae, and pupae did not significantly affect the relative expression of *OnLgl*, even when RNAi enhancing reagents such as Zn^{2+} , Meta, and Lipo were used (Table 2). In adults, the relative expression of *OnLgl* increased by 1.4-fold three days after injection [$F(1,8)=5.13$, $p=0.053^*$] (Table 2, Fig S-17).

Specifically, third-instar ECB larvae injected with ds*OnLgl* in one bioassay had 2.3-fold lower relative expression of *OnLgl*, or 57% gene suppression, by day 6 compared to larvae injected with ds*GFP*, regardless of whether dsRNA was encapsulated in Meta or not [$F(1,8)=6.16$, $p=0.038^*$] (Fig. 3A). There was no significant effect of reagent on the relative

expression of the target gene in this experiment, indicating that Meta dsRNA did not enhance RNAi efficiency when ds*OnLgl* was injected into third-instar ECB larvae (Fig S-14A). The Meta reagent significantly reduced survivorship by 1.25-fold [$F(1,8)=13.50$, $p=0.006^*$] (Fig. 3B) and body weight by 1.24-fold [$F(1,8)=11.95$, $p=0.009^*$] (Fig. 3C) during this bioassay, but had no effect in other bioassays testing Meta on other developmental stages (Table 2).

In another bioassay, third-instar ECB larvae injected with ds*OnLgl* in the presence of 25 mM Zn^{2+} had 1.5-fold or 32% suppression of *OnLgl* three days after injection [$t(4)=3.69$, $p=0.021^*$] (Fig. 4), which is less suppression than observed for feeding of second instars (68%; Fig. 2) and injection of third instars in the Meta bioassay (57%; Fig. 3A), suggesting that 25 mM Zn^{2+} did not enhance RNAi efficiency in ECB (Table 2). No phenotypes were observed due to the suppression of *OnLgl* at any of the time points investigated (Table 2, Fig S-19B, C).

To determine if the expression of *OnLgl* was affected at earlier time points than those investigated in the majority of these bioassays, transcriptional responses of *OnLgl* to dsRNA exposure were investigated at earlier time points after dsRNA feeding and injection in the two bioassays conducted on second instar larvae (Table 2). However, *OnLgl* expression was unaffected at 3, 6, 12, and 24 h after dsRNA feeding (Fig. S-22) or injection (Fig. S-23), even when various concentrations of the Zn^{2+} nuclease inhibitor were used.

3.3 Comparison of multiple target genes in tissue culture

When excised midguts were incubated with ds*GFP* or various concentrations of ds*OnLgl*, there were no significant differences in the relative expression of *OnLgl* between treatments, indicating that suppression of *OnLgl* did not occur at a measurable level (Fig. S-24A). Similar findings were obtained for dsRNA targeting *OnChc*, *OnArf72A*, and *OnAP50* (Fig. S-24; Table 3). Conversely, excised midguts incubated with ds*OnVha16* had significantly lower relative expression of *OnVha16* transcripts compared to midguts incubated with ds*GFP*, regardless of ds*OnVha16* concentrations tested [$F(3,17)=7.20$, $p=0.003^*$] (Fig. 5A). Similar results were observed for *OnVhaSFD* [$H(4)=17.20$, $p=0.002^*$] (Fig. 5B). Expression of *OnVha16* was suppressed by 54.8% on average, and *OnVhaSFD* was suppressed by 46.2% on average (Table 3).

When excised midguts were incubated with ds*GFP* or ds*OnARF72A* in the presence of various nuclease inhibitors (i.e., Zn^{2+} , Mn^{2+} , Co^{2+}), significant differences in target transcript levels relative to control were observed for Zn^{2+} [$U(4)=10.00$, $p=0.030^*$], but not Mn^{2+} or Co^{2+} (Fig. 6). These findings suggest that Zn^{2+} enhanced RNAi efficiency in ECB midgut cultures. However, testing of Zn^{2+} on additional target genes revealed that RNAi efficiency was only enhanced for two of the six target genes (Table 3). In the presence of Zn^{2+} , *OnARF72A*, *OnChc*, *OnVha16*, and *OnVhaSFD* were suppressed by 92, 33, 59, and 33% respectively, but no suppression was detected for *OnLgl* or *OnAP50* (Figs. 6A, S-25; Table 3). Compared to the previous results for these target genes without Zn^{2+} , it appears that Zn^{2+} enhanced the RNAi efficiency of *OnARF72A* and *OnChc*, but not of *OnVha16* or *VhaSFD*, which were suppressed at similar levels with and without Zn^{2+} (Table 3).

4. Discussion

In the present study, we developed oral and injection RNAi procedures for multiple developmental stages of ECB and used them to test seven reagents for their ability to enhance dsRNA stability in ECB tissue extracts, as well as their ability to improve RNAi efficiency in ECB. A total of 13 RNAi bioassays were performed, but RNAi-mediated suppression of *OnLgl* was only observed in one of five oral RNAi experiments, and two out of eight injection experiments (Table 2). In these experiments where suppression was observed, the relative expression of *OnLgl* was suppressed by 68, 32, and 57%, respectively. Nonetheless, the suppression of *OnLgl* was only observed in 23% of all bioassays presented here (Table 2). These findings confirm that ECB is refractory to RNAi and support the need for additional strategies and reagents that can be used to enhance RNAi efficiency in ECB.

Liposomal transfection reagents are cationic lipids used to facilitate the delivery of nucleic acids into cells. Lipofectamine 2000 (Invitrogen) and Metafectene Pro (Biontex) are two proprietary liposomal formulations developed for the transfection of nucleic acids into eukaryotic cells. Previously, these reagents enhanced oral RNAi efficiency in *D. sukikii* larvae and adults¹⁵ in *Euschistus heros* nymphs,²⁹ and in *Chilo suppressalis*.³⁰ Metafectene Pro (Meta) dsRNA lipoplexes were previously used to achieve successful RNAi with injection in *H. cunea* pupae,¹⁸ and *S. exigua* larvae.^{17,16} Enhanced RNAi efficiency associated with transfection reagents was originally attributed to enhanced dsRNA uptake,^{15,30} but evidence suggests that both Lipofectamine 2000 and Meta may also enhance dsRNA stability.^{29,30}

In our experiments, Lipofectamine RNAiMax (Lipo), an RNAi-specific cationic lipid formulation designed explicitly for high transfection efficiency of siRNA and miRNA into a variety of cell types, and Meta were tested on ECB. Unfortunately, encapsulation of dsRNA in Lipo or Meta did not enhance RNAi efficiency when fed or injected into any of the ECB developmental stages tested (Table 2), even though Meta increased the stability of dsRNA in ECB hemolymph *ex vivo* (Table 1). These results conflict with previous reports of enhanced dsRNA-stability and/or RNAi efficiency when transfection reagents were used in lepidopteran,^{16–18,30} hemipteran,²⁹ and dipteran¹⁵ insects, but agree with a report that Lipofectamine 2000 did not enhance oral RNAi efficiency in an orthopteran.³¹ Together these results suggest that transfection reagents, such as Lipo and Meta, are not suitable for enhancing RNAi in ECB, despite the success found in other species.

Chitosan/dsRNA nanoparticles were first developed to facilitate oral RNAi in *Anopheles gambiae* larvae.¹⁹ The nanoparticles have been used to facilitate oral RNAi in *Aedes aegypti* larvae,^{32,33} *Chilo suppressalis* larvae,³⁰ *Chironomus tentans* larvae,³⁴ *Penaeus vannamei* shrimp,³⁵ and *Caenorhabditis elegans* nematodes.³⁶ In *C. suppressalis*, chitosan/dsRNA nanoparticles enhanced dsRNA stability and uptake, resulting in enhanced RNAi efficiency.³⁰ In addition, evidence from *C. elegans* indicates that uptake of chitosan/dsRNA nanoparticles occurs through clathrin-mediated endocytosis.³⁶ Initial testing of chitosan/dsRNA nanoparticles on ECB larvae indicated that oral RNAi efficiency was not enhanced (unpublished). Therefore, a new version of proprietary chitosan-based/dsRNA nanoparticle (NP) was developed for testing on ECB. Excitingly, NP completely protected dsRNA from

degradation in ECB HE and enhanced dsRNA stability in GC (Fig. 1; Table 1). Unfortunately, the NP dsRNA formulation proved challenging to assay as it was problematic to solubilize, difficult to resuspend into a homogenous solution, and would not migrate through 1% agarose gels. Nonetheless, the *ex vivo* data presented here demonstrate that NP are promising candidates for enhancing RNAi in ECB, as a result of enhanced dsRNA stability, but more testing is needed to ascertain effectiveness *in vivo*.

EDTA is a chelating agent that sequesters di- and trivalent metal ions. EDTA is often used as a nuclease inhibitor because many enzymes use metal ions as inorganic cofactors, which are required for catalytic activity. A final concentration of 10–20 mM EDTA was previously reported to enhance dsRNA stability in tissue extracts from *Manduca sexta*,³⁷ *T. castaneum*,³⁸ *Acyrtosiphon pisum*,³⁸ and ECB.²⁰ In addition, 3% (w/v) EDTA was reported to enhance dsRNA stability in saliva from nymphs as well as oral RNAi efficiency in *Euschistus heros*.²⁹ *Ex vivo* incubation in ECB tissue extracts indicated that 6 mM EDTA (the highest concentration that was consumed without detrimental effects in optimization experiments) completely protected dsRNA in ECB tissue extracts (Table 1). Despite this, oral delivery of dsRNA with and without EDTA did not enhance RNAi efficiency in unfed neonate ECB larvae (Table 2). However, the consumption of EDTA for six days significantly reduced larval body weight.

Zn²⁺, Mn²⁺, and Co²⁺ are three divalent ions that inhibited the activity of purified BmdsRNase from *Bombyx mori*.³⁹ *Ex vivo* incubation assays indicated that 10–20 mM Zn²⁺ was able to completely protect dsRNA from degradation in ECB HE and GC, whereas Mn²⁺ and Co²⁺ did not (Table 1). Injection of 25 mM Zn²⁺ in third instar ECB larvae resulted in 32% suppression of the *OnLgl* target gene (Table 2, Fig. 4). Two additional bioassays were then performed on second instar larvae using feeding and injection of dsRNA in combination with 0, 10, and 20 mM Zn²⁺ so the effects of dsRNA and Zn²⁺ on relative gene expression could be investigated for the target gene. Relative expression of *OnLgl* was not significantly affected at any of the early time points examined after dsRNA feeding or injection (Figs. S-22, S-23). In addition, significant suppression was only observed at the 6-d time point of the oral RNAi bioassay, but not at the 3-d time point for either delivery method (Table 2). In *Ostrinia furnacalis*, the transcriptional response of numerous genes was evaluated after dsRNA exposure, and most of the transcripts that were unaffected or upregulated six hours after dsRNA exposure were shown to be refractory to RNAi, whereas genes that were downregulated within six hours were effective RNAi targets.²² Based on this report, and the short-term transcriptional response observed for *OnLgl* after dsRNA exposure, there may be other target genes that are more sensitive to RNAi in ECB than *OnLgl*. Further testing of Zn²⁺ is needed to understand how it enhances RNAi efficiency *ex vivo*, and testing on additional insects is recommended.

To determine how sensitivity of *OnLgl* to RNAi compares to other target genes in ECB, a midgut tissue culture system was used to compare RNAi efficiency between six target genes (Table 3). This investigation was performed using ECB tissue cultures to reduce the assay time, as well as the amount of dsRNA required to screen multiple target genes. Only two of the six target genes (*OnVhal6* and *OnSFD*) exhibited RNAi-mediated suppression after 24 h of incubation in tissue culture media containing dsRNA (Table 3; Figs. 5, S-24).

Surprisingly, suppression in ECB midgut cultures did not appear to be dose-dependent (Fig. 5). However, RNAi-mediated suppression was also not dose-dependent in *A. aegypti*,⁴⁰ and *A. pisum*⁴¹ possibly due to insufficient activation of core RNAi pathway components.

Comparison of Zn²⁺, Mn²⁺ and Co²⁺ on RNAi efficiency in ECB midgut tissue cultures indicated that 20 mM Zn²⁺ significantly enhanced RNAi efficiency of the *OnArf72A* target gene (Fig. 6). Therefore, tissue culture assays using 20 mM Zn²⁺ were performed on the other five target genes. Surprisingly, RNAi efficiency was only enhanced by Zn²⁺ in two of the six genes assayed (*OnArf72A* and *OnChc*) (Table 3). Based on these results, it appears that nucleases may impact RNAi efficiency differently for different genes in ECB, possibly suggesting that nuclease activity is sequence-specific, or another unknown mechanism is present. However, our previous investigation of dsRNA stability in ECB found no evidence for sequence-specific degradation of dsRNA in ECB GC.²⁰ Therefore, additional research is needed to understand why inhibition of nuclease activity enhances RNAi efficiency for some but not all genes in ECB midgut tissue cultures.

Taken together, the results presented here indicate that instability of dsRNA due to nuclease activity does contribute to low RNAi efficiency in ECB (at least for some target genes in ECB midgut cultures), but enhancing dsRNA stability alone may not improve RNAi efficiency in ECB. Further, RNAi efficiency in ECB did not appear to be higher in early developmental stages or non-feeding stages of ECB (Table 2), as previously observed in other insects, including many lepidopterans.^{5,42} Therefore, RNAi efficiency in ECB appears to be more refractory than in other species, perhaps due to the involvement of multiple mechanisms. Instability of dsRNA²⁰ and refractory target genes (Table 3) contribute to limited RNAi efficiency in ECB, and there is evidence that deficient activation of the core RNAi pathway components may limit RNAi efficiency in ECB as well (unpublished). While incomplete internalization of dsRNA and systemic spreading have not been investigated in ECB yet, these mechanisms are known to contribute to low RNAi efficiency in other lepidopterans and may be at play in ECB. Thus, multiple reagents and strategies may need to be combined in order to enhance RNAi efficiency in ECB. In addition, it is important to select a wide range of candidate genes for screening both *ex vivo* and *in vivo*, as not all genes exhibiting specific transcript depletion will necessarily cause mortality or phenotypic defects, as was the case here for *OnLgl* (Table 2).

Despite the complex and challenging nature of enhancing RNAi efficiency in lepidopterans, RNAi represents a novel mode of action to combat insecticide resistance in many devastating pests, such as ECB. The reagents evaluated here that enhance dsRNA stability in ECB HE and GC are good candidates for testing in other insect species, including orthopterans and hemipterans, which often exhibit low RNAi efficiency due to rapid degradation of dsRNA.²⁰ Furthermore, enhancing dsRNA stability could possibly reduce the amount of dsRNA needed for pest control by improving the environmental stability of dsRNA and/or the susceptibility of the pest to dsRNA (data not shown). Enhanced stability and lower doses would be especially beneficial, given the high price of producing dsRNA for pest control.⁴³

5. Conclusion

This investigation demonstrated that RNAi efficiency in ECB was very low, and highly variable, with both feeding and injection of dsRNA targeting *OnLgl*, as well as incubation of midgut tissue cultures with dsRNA targeting six genes. Of the 13 RNAi bioassays and 12 tissue culture assays, gene suppression was only observed in 23 and 50% of the assays, respectively. In addition, four of the six stabilizing reagents tested enhanced the stability of dsRNA in ECB tissue extracts, *ex vivo*, but this was insufficient to improve RNAi efficiency in ECB, *in vivo*. However, enhanced dsRNA stability translated to improved RNAi efficiency in tissue culture for two of the four refractory target genes tested. These findings indicate that multiple mechanisms are contributing to low RNAi efficiency in ECB, suggesting that conventional strategies for enhancing RNAi efficiency in insects may be insufficient to increase RNAi efficiency in ECB. This knowledge will facilitate the development of novel approaches that simultaneously overcome multiple RNAi efficiency limitations in lepidopteran insects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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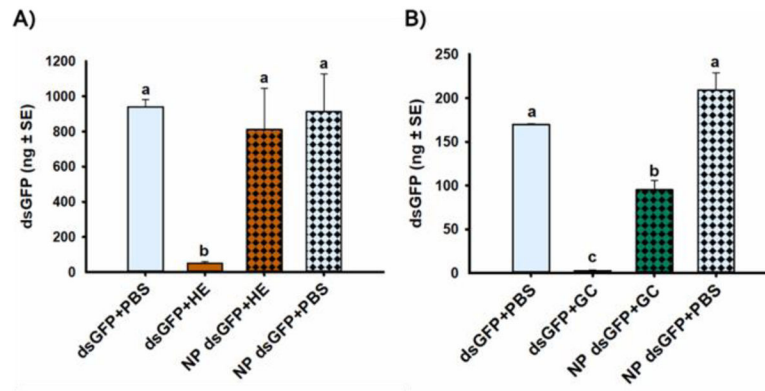


Figure 1. Enhancement of dsRNA stability in A) hemolymph (HE) and B) gut contents (GC) due to chitosan-based dsRNA nanoparticles (NP).

The mean amount of ds*GFP* (in ng) recovered after 30 min incubation in PBS buffer (control) or tissue extracts harvested from 15 fifth-instar ECB larvae. Means that do not share a letter are significantly different.

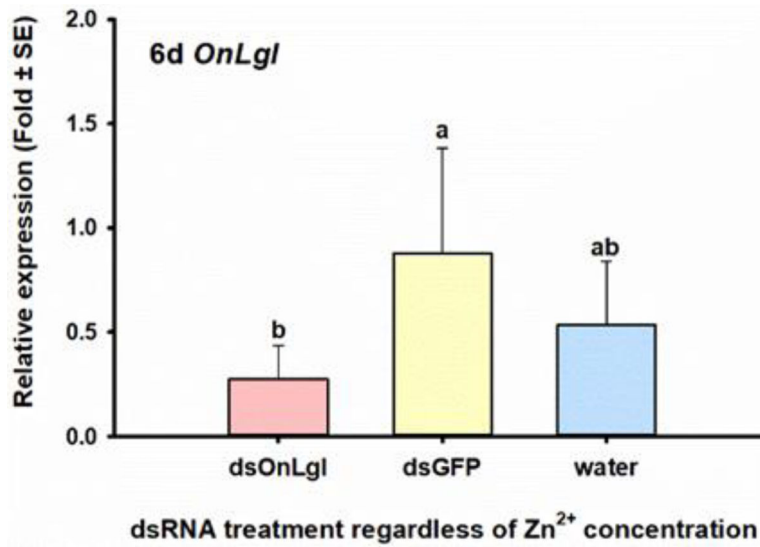


Figure 2. Significant effects on RNAi efficiency in 48-h old ECB larvae six days after oral delivery of dsRNA, regardless of nuclease inhibitor (Zn^{2+}) concentration. Relative expression of the *OnLgl* six days after feeding of ds*OnLgl* or ds*GFP* incorporated into diet, with 10 or 20 mM Zn^{2+} . Means that do not share a letter are significantly different.

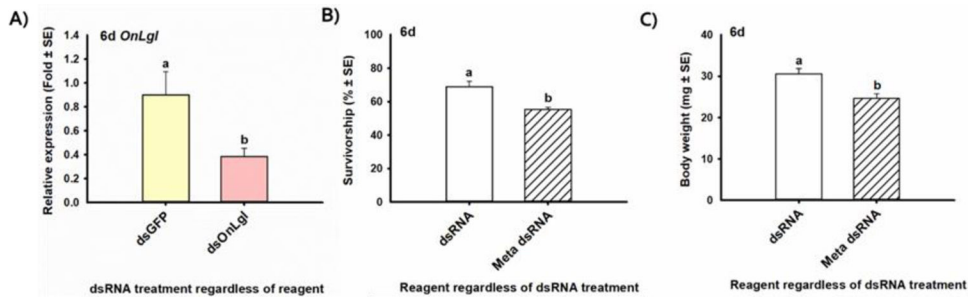


Figure 3. Significant effects on RNAi efficiency in third-instar ECB larvae after injection of dsRNA with and without Metafectene Pro (Meta) lipoplexes. The mean A) relative expression of the *OnLgl* target gene, B) percent of injected larvae to survive, and C) body weight, six days after microinjection of dsRNA targeting either *OnLgl* or *GFP*, with and without encapsulation of dsRNA in Meta lipoplexes. Means that do not share a letter are significantly different.

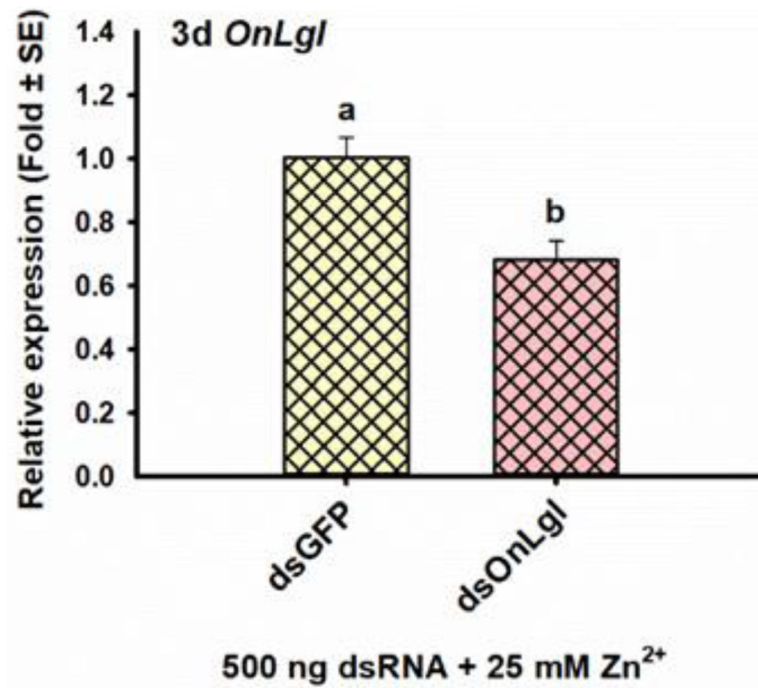


Figure 4. Significant effects on RNAi efficiency following injection of third-instar ECB larvae with dsRNA and 25 mM nuclease inhibitor (Zn²⁺).

Relative expression of the *OnLgl* target three days after injection of ds*OnLgl* or ds*GFP* in conjunction with 25 mM Zn²⁺. Means that do not share a letter are significantly different.

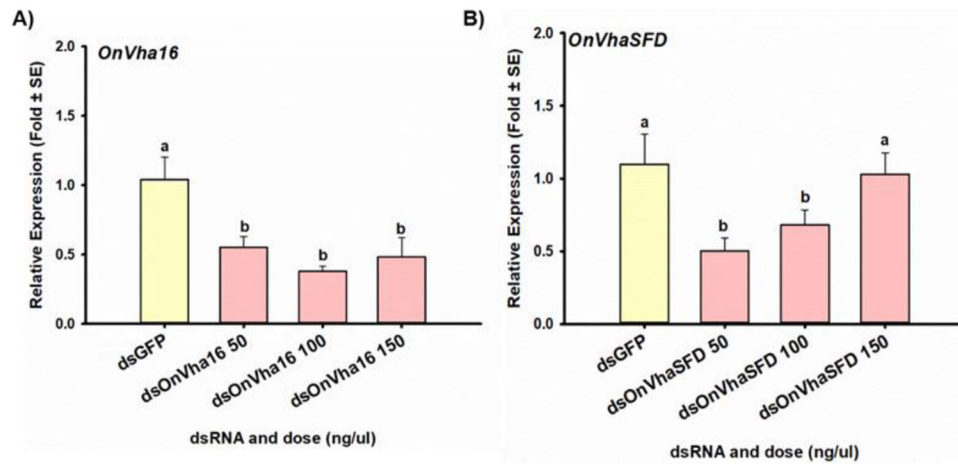


Figure 5. RNAi efficiency of two alternative target genes in ECB midgut tissue cultures. The mean relative expression of A) the V-type proton ATPase 16 kD proteolipid subunit (*OnVha16*), and B) the V-type proton ATPase subunit H (*OnVhaSFD*) target genes after incubation of excised midguts from fifth instar ECB larvae in media containing various concentrations (50, 100, and 150 ng/μl) of either ds*OnVha16*, ds*OnVhaSFD*, or ds*GFP*. Means that do not share a letter are significantly different.

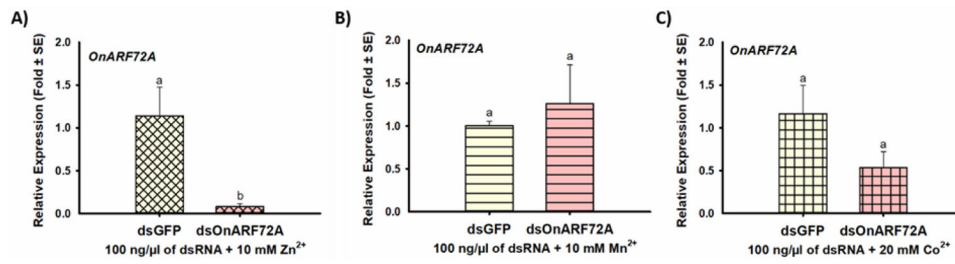


Figure 6. Enhancement of RNAi efficiency in ECB midgut tissue cultures due to inclusion of nuclease inhibitor Zn²⁺, but not Mn²⁺ and Co²⁺.

The mean relative expression of the ADP ribosylation factor 72A (*OnArf72A*) target gene after incubation of excised midguts from fifth instar ECB larvae in media containing either ds*OnARF72A* or ds*GFP* along with A) 10 mM Zn²⁺, B) 10 mM Mn²⁺, or C) 20 mM Co²⁺. Means that do not share a letter are significantly different.

Table 1. Comparison of fold-increase in stability of dsRNA in ECB tissue extracts due to candidate RNAi-enhancing reagents.

The mean amounts (nanograms plus and minus standard error) of 300 bp dsGFP that was recovered after incubation in ECB tissue extracts with and without each reagent are presented. Significant values are indicated with an asterisk.

| Reagent | Enhancement of dsRNA stability in ECB | | | | | |
|------------------|---------------------------------------|--------------------|-------------------|---------------------|-------------------|-----------------|
| | Hemolymph (HE) | | Gut contents (GC) | | | |
| | dsGFP +HE [control] | Reagent dsGFP + HE | Fold increase | dsGFP +GC [control] | Reagent dsGFP +GC | Fold increase |
| Lipo | 30.2±11.0 | 24.1±8.8 | 0.8 | 0.0013±0.0011 | 0.005±0.002 | 3.7 |
| Meta | 128.5±54.0 | 717.9±166.1* | 5.6 | -- | -- | n/a |
| NP | 49.0±10.3 | 811.7±234.3* | 16.6 | 2.6±0.9 | 95.0±10.6* | 36.1 |
| EDTA | 0.079±0.007 | 148.9±20.9* | 6 mM: 1.874.8 | 0.0013± 0.0011 | 182.7±27.9* | 6 mM: 135,947.2 |
| Zn ²⁺ | 38.0±12.3 | 165.4±12.3* | 10 mM: 4.4 | 0.098±0.038 | 77.1±26.5 | 10 mM: 790.6 |
| | | 132.3±23.8* | 20 mM: 3.5 | | 129.1±22.3* | 20 mM: 1,324.0 |
| | | 102.3±12.3 | 40 mM: 2.7 | | 57.8±22.3 | 40 mM: 592.6 |
| | | 111.3±7.9 | 60 mM: 2.9 | | 84.1±11.2 | 60 mM: 862.9 |
| Mn ²⁺ | 38.0±16.6 | 96.8±20.6 | 10 mM: 2.5 | 0.40±0.29 | 3.3±1.8 | 10 mM: 8.2 |
| | | 72.4±13.9 | 20 mM: 1.9 | | 8.6±0.8 | 20 mM: 21.5 |
| | | 52.6±11.1 | 40 mM: 1.4 | | 2.4±0.6 | 40 mM: 5.9 |
| | | 627.9±254.8 | 60 mM: 16.5 | | 229.8±105.7 | 60 mM: 573.7 |
| Co ²⁺ | 18.7±8.2 | 61.7±23.2 | 10 mM: 3.3 | 0.093±0.048 | 8.9±1.8 | 10 mM: 95.6 |
| | | 43.8±16.2 | 20 mM: 2.3 | | 27.8±7.8 | 20 mM: 300.6 |
| | | 21.0±6.2 | 40 mM: 1.1 | | 18.7±9.2 | 40 mM: 201.5 |
| | | 47.7±20.3 | 60 mM: 2.5 | | 22.5±9.5 | 60 mM: 243.3 |

Abbreviations: mM, millimolar, Lipo, Lipofectamine RNAiMax; Meta, Metafectene Pro; EDTA, ethylenediaminetetraacetic acid.

Table 2.
Comparison of RNAi efficiency among ECB developmental stages when various delivery methods and RNAi-enhancing reagents were employed.

Unfed larvae were starved for 24 h prior to dsRNA delivery. Fed larvae were allowed to feed normally (i.e., not starved) prior to dsRNA delivery. Non-feeding stages do not feed as part of their natural developmental cycle.

| RNAi Bioassays | | | | | | | |
|-----------------|---------------------------------|----------------|-----------------------------|--|--|------------------|-----------|
| Delivery method | Stage | Feeding status | Reagent | Significant suppression of <i>OnLgI</i> | Phenotype(s) | Results | |
| Oral | First-instar larvae | Unfed | 50 mM Zn ²⁺ | No | None | Fig. S-10 | |
| | | | 6 mM EDTA | No | EDTA reduced weight by 1.7-fold at 6 d (6 mM vs 0 mM) | Figs. S-7, S-8 | |
| | | | 1% FB28 | No | FB28 reduced weight by 2.7-fold at 6 d (1% vs 0%) | Figs. S-12, S-13 | |
| | | | Lipo | No | None | Fig. S-11 | |
| Injection | Second-instar larvae | Fed | 10 & 20 mM Zn ²⁺ | 68%, or 3.2-fold decrease at 6 d (ds <i>OnLgI</i> vs ds <i>GFP</i>) | None | Figs. 2, S-9 | |
| | Second-instar larvae | Fed | 10 & 20 mM Zn ²⁺ | No | None | Fig. S-21 | |
| | Third-instar larvae | Fed | 25 mM Zn ²⁺ | 32%, or 1.5-fold decrease at 3 d (Zn ²⁺ ds <i>OnLgI</i> vs Zn ²⁺ ds <i>GFP</i>) | None | Fig. 4, S-19 | |
| | Wandering (fifth-instar) larvae | Nonfeeding | 50 mM Zn ²⁺ | No | None | None | Fig. S-20 |
| | | | Meta | 57%, or 2.3-fold decrease at 6 d (naked ds <i>OnLgI</i> vs naked ds <i>GFP</i>) | Meta decrease survivorship by 1.25-fold & weight by 1.24-fold at 6 d (Meta dsRNA vs naked dsRNA) | Figs. 3, S-14 | |
| | | | Meta | No* | None | None | Fig. S-15 |
| | Pupae | Nonfeeding | Lipo | No | None | None | Fig. S-18 |
| Meta | | | No | None | None | Fig. S-16 | |
| Adults | Unfed | Unfed | Meta | No, 1.4-fold increase at 3 d (ds <i>OnLgI</i> vs ds <i>GFP</i>) | None | Fig. S-17 | |

* Significant differences were detected between some treatment groups but could not be meaningfully interpreted. *Abbreviations*: mM, millimolar; Lipo, Lipofectamine RNAiMax; Meta, Metafectene Pro; EDTA; ethylenediaminetetraacetic acid; Zn²⁺, nuclease inhibitor; FB28, fluorescent brightener 28; h, hour; d, day.

Table 3.

Comparison of enhancement of RNAi efficiency in ECB tissue culture for multiple target genes due to nuclease inhibitor Zn²⁺.

The relative expression (in fold change plus and minus standard error) of each target gene after incubation of excised midguts in media containing 100 ng/ml of ds*GFP* or dsRNA specific to each target gene are presented.

| Target gene | RNAi Efficiency in ECB Midgut Cultures | | | |
|-----------------|--|-----------|-----------------------|-----------|
| | Without Zn ²⁺ | | With Zn ²⁺ | |
| | ds <i>GFP</i> | dsTarget | ds <i>GFP</i> | dsTarget |
| <i>OnLgl</i> | 1.00±0.06 | 0.83±0.20 | 1.01±0.10 | 0.95±0.21 |
| <i>OnArl72A</i> | 1.08±0.23 | 0.78±0.17 | 1.14±0.33 | 0.08±0.03 |
| <i>OnChc</i> | 1.03±0.13 | 1.42±0.10 | 1.04±0.16 | 0.69±0.07 |
| <i>OnAP50</i> | 1.02±0.11 | 1.19±0.29 | 1.14±0.33 | 0.08±0.03 |
| <i>OnVha16</i> | 1.04±0.16 | 0.38±0.04 | 1.06±0.20 | 0.44±0.14 |
| <i>OnVhaSFD</i> | 1.10±0.21 | 0.68±0.10 | 1.04±0.16 | 0.69±0.07 |
| | | | | 33% * |

* Italicized averages are trends (not statistically significant). Abbreviations: *On*, *Ostrinia nubilalis*; *Lgl*, lethal giant larvae; *Chc*, clathrin coat adaptor protein AP50; *Arl72A*, ADP ribosylation factor 72A; *Vha16*, V-type proton ATPase 16 kD proteolipid subunit; *VhaSFD*, V-type proton ATPase subunit H.