Molecular mechanisms influencing the efficiency of RNA interference in the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae)

by

Anastasia M. W. Cooper

B.S., Mississippi State University, 2010
M.A., Arkansas State University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

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2020

Abstract

RNA interference (RNAi) pathways function in endogenous gene regulation and protection against viruses and transposons in eukaryotic organisms. The exogenous RNAi mechanism has been utilized as a powerful reverse genetics tool to analyze gene function. In addition, RNAi-based pest management strategies are now emerging; however, applications are limited due to inefficient results for some insects, especially lepidopterans. The European corn borer (ECB), *Ostrinia nubilalis,* is an agriculturally relevant lepidopteran pest that exhibits very low RNAi efficiency but has a great need for new control strategies that utilize novel modes of action. This dissertation research seeks to investigate molecular mechanisms influencing RNAi efficiency in ECB and determine if commonly used reagents and tactics can improve RNAi efficiency in this species. The specific objectives were: 1) to investigate double-stranded RNA (dsRNA) stability in larval gut contents and hemolymph in ECB, 2) to identify and characterize dsRNA-degrading nuclease genes from ECB, 3) to identify and characterize core RNAi pathway genes from ECB, and 4) to compare strategies for enhancing RNAi efficiency (nanoparticles, transfection reagents, and nuclease inhibitors) in ECB. *Ex vivo* incubation experiments showed that dsRNA was degraded in ECB gut contents and hemolymph under physiologically relevant pH conditions. Sequencing revealed transcripts for four dsRNA-degrading endonuclease (OndsRNase) genes and one RNAi efficiency-related nuclease (*OnREase)* gene in ECB. Expression analysis indicated that *OnREase*, *OndsRNase2,* and *OndsRNase4* might impact oral RNAi efficiency in ECB, whereas *OnREase* and *OndsRNase1* may impact injection RNAi efficiency. Sequencing also revealed a single transcript for Dicer 2 *(OnDcr2*), R2D2 *(OnR2D2),* and Argonaute 2 (*OnAgo2)*. Expression analysis indicated that each of these core RNAi pathway genes were expressed in all ECB developmental stages and tissues investigated. *Ex vivo* incubation

experiments showed that Metafectene Pro, chitosan-based nanoparticles, EDTA, and Zn^{2+} enhanced dsRNA stability in ECB gut contents and hemolymph, but Lipofectamine RNAiMax, Co^{2+}, and Mn^{2+} did not. Surprisingly, improving dsRNA stability did not increase RNAi efficiency in ECB *in vivo*. Ingestion, injection, and tissue culture-based RNAi assays indicated that most target genes and all ECB developmental stages investigated were highly refractory to RNAi. These findings suggest that multiple mechanisms, including instability of dsRNA, deficient core RNAi machinery, and refractory target genes, likely contribute to low RNAi efficiency in ECB. Primary outcomes of this project included an in-depth investigation of dsRNA instability under physiological conditions in ECB tissues, a preliminary identification of candidate dsRNA-degrading nuclease genes that likely contribute to dsRNA instability in ECB, a foundational characterization of core RNAi pathway genes presumed to participate in the exogenous RNAi response of ECB, and a detailed comparison of numerous strategies for enhancing dsRNA stability and potentially RNAi efficiency in ECB. Overall, results from this research support that multiple mechanisms likely contribute to RNAi inefficiency in lepidopteran insects. This investigation will help improve RNAi efficiency in lepidopteran insects, so effective RNAi-based pest management strategies can be developed.

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Major Professor
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List of Symbols

α	alpha, used in gene names and statistics
β	beta, used in gene names
χ	chi, used in statistics
Δ	delta, used to show a change in gene expression
μ	micro, 10^{-6}
±	plus or minus
&	and

List of Abbreviations

%E	percent primer efficiency
µg	microgram(s)
µl	microliter(s)
18s	18S ribosomal RNA gene
aa	amino-acid(s)
Ago	argonaute gene
aka	also known as
ANOVA	analysis of variance
AP50	adaptor protein complex 50 gene
Arf72A	ADP ribosylation factor 72A gene
b	y-intercept
BD	blue dextran
bp	base pair(s)
Bt	*Bacillus thuringiensis*
°C	temperature in degrees Celsius
cDNA	complementary deoxyribonucleic acid
Chc	clathrin heavy chain gene
Co^{2+}	cobalt
CR	carcass: integument and tissues remaining after removal of major organs
Cry	crystal proteins
Ct	cycle threshold
Dcr	dicer gene
ds	double-stranded
dsRNA	double-stranded ribonucleic acid
dsRNase	dsRNA-degrading endonuclease gene
Dv	*Diabrotica virgifera virgifera*
E	primer efficiency
ECB	European corn borer
ED1	one-day-old eggs
EDTA	ethylenediaminetetraacetic acid
Ef1α	elongation factor-1 alpha gene
F	F-statistic: test statistic distribution, used for parametric ANOVA tests
FB	fat body: insect tissue equivalent to the liver
FB28	fluorescent brightener 28: chitin synthase inhibitor
FD1	one-day-old adult females
Fig.	figure
GAPDH	glyceraldehyde 3-phosphate dehydrogenase gene
GC	gut contents

GFP	enhanced green fluorescent protein gene
GU	gut tissue
h	hour(s)
H	H-statistic: test statistic distribution, used for Kruskal Wallis H test, a nonparametric, one-way ANOVA on ranks test
HE	hemolymph: body fluid of an insect
HP	hypothetical protein
kDa	kilodaltons
KSU	Kansas State University, Manhattan, Kansas, United States of America
Lgl	lethal giant larvae gene
LI1	first-instar larvae
Lipo	Lipofectamine RNAi Max: transfection reagent
m	slope
MD1	one-day-old adult males
Meta	Metafectine Pro: transfection reagent
min	minute(s)
miRNA	micro ribonucleic acid
ml	milliliter(s)
Mn^{2+}	manganese
mM	millimolar: one millimole per liter
mRNA	messenger ribonucleic acid
MRPL28	mitochondrial ribosomal protein L28 gene
n/a	not applicable
NASL	not in any subcellular locations
NCBI	National Center for Biotechnology Information
NF	nuclease-free
ng	nanograms(s)
nl	nanoliter(s)
NP	nanoparticles
NSNuc	nonspecific nuclease gene
On	*Ostrinia nubilalis*
ORF	open reading frame
P	evolutionary distance score generated by MEGA7
p	p-value: the probability of a statistical measure
PA	protein asteroid gene
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
pH	a measure of acidity and alkalinity of a solution
pI	isoelectric point: pH at which a molecule carries no net electrical charge
piRNA	piwi-interacting ribonucleic acid

PM	peritrophic matrix: structure surrounding food bolus
PRRs	pattern recognition receptors
PU	pupae
R2D2	two dsRNA-binding domains associated with dicer 2 gene
RACE	rapid amplification of cDNA ends
REase	RNAi-efficiency related nuclease gene
RISC	RNA-induced silencing complex
RNAi	RNA interference
RPS3	ribosomal protein S3 gene
RT-qPCR	reverse transcription quantitative real-time polymerase chain reaction
s	second(s)
SE	standard error
siRNA	short interfering RNA
SXU	Shanxi University, Taiyuan, Shanxi, Peoples Republic of China
t	t-statistic: test statistic distribution, used for parametric two-sample t-tests
U	U-statistic: test statistic distribution, used for Mann Whitney U test, a nonparametric, two-sample, rank-sum test
UP	uncharacterized protein
V	V-score: pairwise variation score generated by NormFinder
Vha16	V-type proton ATPase 16 kDa proteolipid subunit gene
VhaSFD	V-type proton subunit H gene
WCR	western corn rootworm
XPG	Xeroderma Pigmentosum Complementation Group G, N-terminal PIN domain-like endonuclease
α	significance level, or probability of rejecting the null hypothesis
βActin	beta-actin gene
ΔCt	delta CT: a method for calculating fold change in gene expression relative to the reference gene(s)
ΔΔCt	delta-delta CT: a method for calculating fold change in gene expression relative to a reference gene(s) and relative to a calibrator treatment
χ^2	chi-squared statistic: test statistic distribution, used for Freidman's test, a nonparametric, two-way ANOVA by ranks test
Zn^{2+}	zinc

Nomenclature

Acyrthosiphon pisum	Pea aphid (Hemiptera: Aphididae)
Aedes aegypti	Yellow fever mosquito (Diptera: Culicidae)
Aedes albopictus	Asian tiger mosquito (Diptera: Culicidae)
Aequorea victoria	Crystal jellyfish (Leptothecata: Aequoreidae)
Aethina tumida	Small hive beetle (Coleoptera: Nitidulidae)
Amyelois transitella	Navel orangeworm (Lepidoptera: Pyralidae)
Anastrepha fraterculus	South American fruit fly (Diptera: Tephritidae)
Anopheles gambiae	African malaria mosquito (Diptera: Culicidae)
Anthonomus grandis	Boll weevil (Coleoptera: Curculionidae)
Aphis glycines	Soybean aphid (Hemiptera: Aphididae)
Apis mellifera	European honeybee (Hymenoptera: Apidae)
Bacillus thuringiensis	Bacteria (Bacillales; Bacillaceae)
Bactrocera dorsalis	Oriental fruit fly (Diptera: Tephritidae)
Blattella germanica	German cockroach (Blattodea: Ectobiidae)
Bombus terrestris	Bumblebee (Hymenoptera: Apidae)
Bombyx mori	Silkworm (Lepidoptera: Bombycidae)
Caenorhabditis elegans	Nematode (Rhabditida; Rhabditidae)
Camponotus floridanus	Bull ant (Hymenoptera: Formicidae)
Chilo suppressalis	Striped rice stem-borer (Lepidoptera: Crambidae)
Chironomus tentans	Midge (Diptera: Chironomidae)
Cinara cedri	Cedar bark aphid (Hemiptera: Aphididae)
Curtomerus puncticollis	Beetle (Coleoptera: Cerambycidae)
Diabrotica virgifera virgifera	Western corn rootworm (Coleoptera: Chrysomelidae)
Diaphorina citri	Asian citrus psyllid (Hemiptera: Liviidae)
Drosophila melanogaster	Fruit fly (Diptera: Drosophilidae)
Drosophila suzukii	Spotted wing drosophila (Diptera: Drosophilidae)
Euschistus heros	Neotropical brown stink bug (Hemiptera: Pentatomidae)
Helicoverpa armigera	Cotton bollworm (Lepidoptera: Noctuidae)
Heliothis virescens	Tobacco budworm (Lepidoptera: Noctuidae)

Holotrichia diomphalia	Korean black chafer (Coleoptera: Scarabaeidae)
Homo sapiens	Human (Primates: Hominidae)
Hyphantria cunea	Fall webworm (Lepidoptera: Erebidae)
Leptinotarsa decemlineata	Colorado potato beetle (Coleoptera: Chrysomelidae)
Locusta migratoria	Migratory locust (Orthoptera: Acrididae)
Manduca sexta	Tobacco hornworm (Lepidoptera: Sphingidae)
Mayetiola destructor	Hessian fly (Diptera: Cecidomyiidae)
Monomorium pharaonis	Pharaoh ant (Hymenoptera: Formicidae)
Nezara viridula	Southern green stink bug (Hemiptera: Pentatomidae)
Nilaparvata lugens	Brown planthopper (Hemiptera: Delphacidae)
Ostrinia furnacalis	Asian corn borer (Lepidoptera: Crambidae)
Ostrinia nubilalis	European corn borer (Lepidoptera: Crambidae)
Papilio polytes	Common Mormon swallowtail (Lepidoptera: Papilionidae)
Pectinophora gossypiella	Pink bollworm (Lepidoptera: Gelechiidae)
Penaeus vannamei	Whiteleg shrimp (Decapoda: Penaeidae)
Periplaneta americana	American cockroach (Blattodea: Blattidae)
Plutella xylostella	Diamondback moth (Lepidoptera: Plutellidae)
Schistocerca gregaria	Desert locust (Orthoptera: Acrididae)
Serratia marcescens	Bacteria (Enterobacteralis: Yersiniaceae)
Spodoptera exigua	Beet armyworm (Lepidoptera: Noctuidae)
Spodoptera frugiperda	Fall armyworm (Lepidoptera: Noctuidae)
Spodoptera litura	Tobacco cutworm (Lepidoptera: Noctuidae)
Temnothorax curvispinosus	Ant (Hymenoptera: Formicidae)
Tribolium castaneum	Red flour beetle (Coleoptera: Tenebrionidae)
Zophobas atratus	Giant mealworm (Coleoptera: Tenebrionidae)

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Dedication

This dissertation is dedicated to my husband, Clayton Cooper,
and to my mentor, Kun Yan Zhu, for keeping me going through it all.

Chapter 1 - Literature Review and Research Objectives

Molecular Mechanisms Influencing Efficiency of RNAi Interference in Insects

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Abstract

RNA interference (RNAi) is an endogenous, sequence-specific gene silencing mechanism elicited by small RNA molecules. RNAi is a powerful reverse genetic tool and is currently being utilized for managing insects and viruses. Widespread implementation of RNAi-based pest management strategies is presently hindered by inefficient and highly variable results when different insect species, strains, developmental stages, tissues, and genes are targeted. Mechanistic studies have shown that double-stranded ribonucleases (dsRNases), endosomal entrapment, deficient function of the core machinery, and inadequate immune stimulation contribute to limited RNAi efficiency. However, a comprehensive understanding of the molecular mechanisms limiting RNAi efficiency remains elusive. The recent advances in dsRNA stability in physiological tissues, dsRNA internalization into cells, the composition and function of the core RNAi machinery, as well as small-interfering RNA/double-stranded RNA amplification and spreading mechanisms are reviewed to establish a global understanding of the obstacles impeding broader understanding of RNAi mechanisms in insects.

Introduction to RNA interference

RNAi pathways and potential uses in entomology

RNA interference (RNAi) is a highly conserved, post-transcriptional gene silencing mechanism in which small RNA molecules are utilized by a set of core machinery (i.e., core RNAi enzymes) that degrade complementary RNA molecules in a sequence-specific nature (Timmons and Fire, 1998). RNAi pathways functions in endogenous gene regulation and in immune responses against viruses and transposons (Fig. 1-1). The exogenous small-interfering-RNA pathway (exo-siRNA) can be exploited by experimentally introducing double-stranded RNA (dsRNA) to silence specific genes, and elucidate their function or induce mortality (Dowling et al., 2016).

In the laboratory, RNAi is commonly used as a reverse genetic tool to elucidate gene function, especially in non-model organisms. RNAi is used to study gene function through targeting of individual genes, as well as to implicate specific genes in molecular processes by using the RNAi of the RNAi technique (Miyata et al., 2014). RNAi is also expanding as a functional genomics tool for the discovery of gene function (Downward, 2004, Perrimon et al., 2010; Armas-Tizapantzi and Montiel-González, 2016). Unfortunately, RNAi technology is currently limited by the fact that not all organisms are equally susceptible to dsRNA, so this tool cannot be applied to all systems, including model organisms such as *Drosophila melanogaster* and *Bombyx mori*.

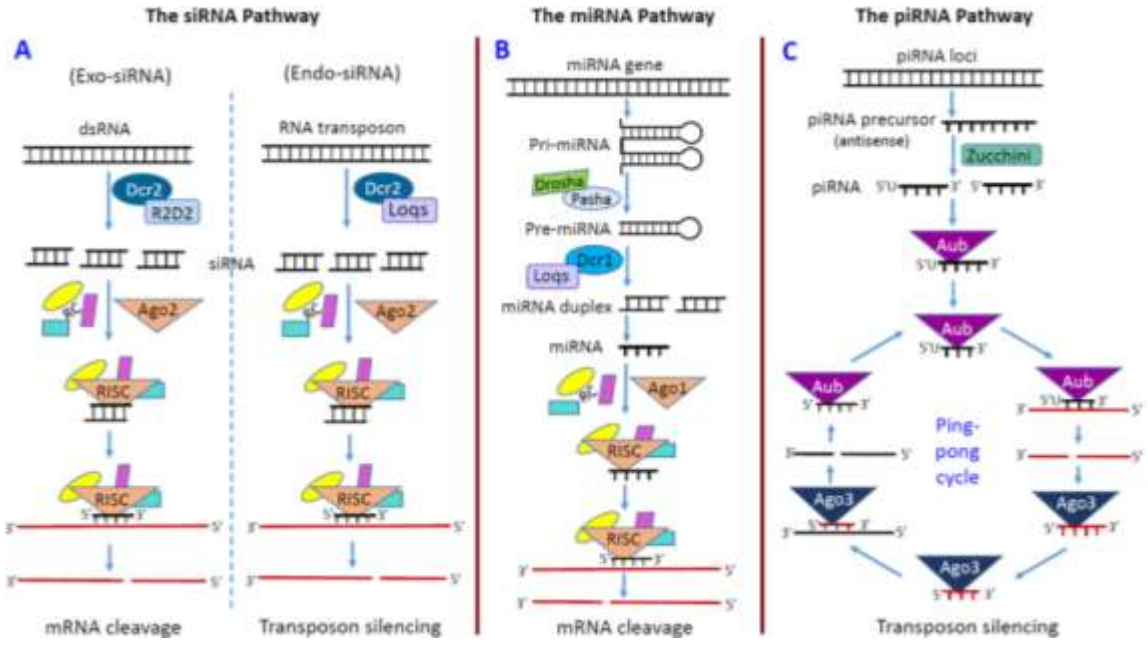

Figure 1-1. Core enzymes of three RNA interference (RNAi) pathways in insects.

A) The small interfering RNA (siRNA) pathway consists of the exogenous siRNA pathway (exo-siRNA) for viral defense and the endogenous siRNA (endo-siRNA) pathway for transposon defense in the genome. The exo-siRNA pathway is commonly exploited by experimentally introducing double-stranded RNA (dsRNA) to silence specific genes. In both the exo- and endo-siRNA pathways, long double-stranded RNA (dsRNA) is cut into 19-21 bp siRNA fragments by dicer 2 (Dcr2). With the aid of dsRNA binding proteins, double-stranded RNA-binding domains associated with dicer 2 (R2D2) or loquacious (Loqs), siRNA is loaded into argonaute 2 (Ago2). A variety of other protein components (RC) then assemble to form the RNA-induced silencing complex (RISC). The passenger strand of siRNA is eliminated, and the active Ago2 enzyme within RISC cleaves complementary transcripts, thus silencing gene expression. **B)** In the microRNA (miRNA) pathway, dsRNA-specific endoribonuclease (Drosha) and partner of drosha (Pasha) trim primary miRNA (Pri-miRNA) into hairpins as they are synthesized from miRNA genes by RNA polymerase II. Precursor miRNA (Pre-miRNA) is then cut into 21-24 bp miRNA fragments by dicer 1 (Dcr1) to yield miRNA duplex. The miRNA duplex is unwound to a single-stranded mature miRNA by an RNA helicase, and the mature miRNA is then loaded into argonaute 1 (Ago1). After RISC components (RC) have assembled, the active Ago1 enzyme within RISC cleaves complementary mRNA for endogenous gene regulation. **C)** The piwi-interacting RNA (piRNA) pathway is involved in defense against transposable elements in the germline. After antisense precursor piRNAs are synthesized from repetitive elements by RNA polymerase II, they are cut into 26-32 nucleotide piRNA fragments by zucchini. Antisense piRNA fragments are loaded into aubergine (Aub), which cleaves complementary transposon transcripts to generate sense piRNA fragments. Argonaute 3 (Ago3) uses the sense piRNA fragments to bind and cleave additional complementary antisense transposon transcripts to generate more antisense piRNAs. Aub and P-element induced wimpy testis (Piwi) trim the antisense piRNAs, which are used to cleave additional complementary sense transposon transcripts to create a feedback loop known as ping-pong cycle.

In addition to being utilized as a powerful molecular tool, RNAi is also being used for pest management. In China, transgenic papayas expressing dsRNA are being used for the control of the papaya ring spot virus (Jia et al., 2017). In addition, dsRNA baits are available for the control of the Israeli acute paralysis virus in honey bees (Hunter et al., 2010). Furthermore, transgenic corn expressing dsRNA for the control of corn rootworms is now available from Monsanto (Head et al., 2017), and a spray-able dsRNA formulation for the control of *Leptinotarsa decemlineata* will soon be available as well (Borel, 2017).

In the future, in addition to being employed for insect (Borel, 2017) and virus control (Fuentes et al. 2016), RNAi may be used to control other pests such as weeds (Shaner and Beckie, 2014), nematodes (Dutta et al., 2014), fungi (Majumdar et al., 2017), and bacteria (Katiyar-Agarwal et al., 2006). RNAi also has the potential to effectively manage insecticide resistance in both agricultural pests and disease vectors (Liu, 2015) and to disrupt vectors of

animal and plant viruses (Whitfield and Rotenberg, 2015). It could even be used for the control of invasive species (Trivedi, 2010). Thus, RNAi has many uses in entomology and agriculture.

RNAi-mediated crop protection strategies, including transgenic plants, baits, and sprays, are valuable resources for sustainable agriculture and food security because they provide new approaches to address yield loss (Godfray et al., 2010), they provide a novel mechanism of action to combat pesticide resistance (Khajuria et al., 2018), they provide greater species-specificity to reduce off-target effects (Whyard et al., 2009; Liu et al., 2019), and they provide low environmental impact as dsRNA is a natural molecule that degrades quickly (Dubelman et al., 2014). Unfortunately, RNAi technology is currently hindered by inefficient and highly variable results when different insects are targeted, especially lepidopterans (Terenius et al., 2011; Kolliopoulou and Swevers, 2014). Given that lepidopterans are the most devastating pests of crops, forests, and stored products, mechanistic research is needed to elucidate the factors influencing RNAi efficiency (i.e., susceptibility) (Khajuria et al., 2011; Smagghe and Swevers, 2014). Many hypotheses have been proposed to explain limitations in RNAi efficiency in insects; however, a comparative approach using agriculturally relevant insect pests has not yet been attempted. Without a detailed understanding of molecular mechanisms influencing RNAi efficiency in agriculturally relevant insects, the use of RNAi-based molecular techniques and RNAi-based pest management strategies will be severely limited.

Variable RNAi efficiency in insects

RNAi efficiency, or the sensitivity of an insect to gene suppression via treatment with dsRNA, varies drastically due to many factors. For example, the dsRNA delivery method employed significantly affects RNAi efficiency. High levels of gene suppression can be achieved when dsRNA is injected or fed to some insects, such as *Diabrotica virgifera virgifera* (Fishilevich et al., 2016). In contrast, suppression of transcript levels is only observed following injection, but not feeding, of dsRNA in *Locusta migratoria* (Luo et al., 2013), and *Schistocerca gregaria* (Wynant et al., 2014d). Similarly, the developmental stage can have a critical effect on RNAi efficiency. *Drosophila melanogaster* larvae are refractory to injection or feeding of naked dsRNA, but transgenic RNAi is successful in larvae as is injection into embryos and adults (Miller et al., 2008). In addition, even closely related insects have widely differing responses to RNAi. *Ostrinia nubilalis* exhibits highly inefficient RNAi responses regardless of the delivery

method (see Chapter 5). Still, a sister species, *Ostrinia furnacalis* (Wang et al., 2011), responds to dsRNA by soaking and by injection into embryos. Furthermore, insects like *S. gregaria* have tissues, such as ovaries, that are immune to dsRNA, even though other tissues are susceptible to dsRNA (Wynant et al., 2012). To make matters worse, some genes are better targets than others (Baum et al., 2007), and this is not always due to tissue-specific variations in gene expression (Luo et al., 2013). These examples demonstrate the complexity associated with understanding the molecular mechanisms that limit RNAi efficiency in insects.

Early efforts to understand mechanisms limiting RNAi largely focused on identifying trends in RNAi effects between insect orders. The more that RNAi efficiency has been explored in insects, the more exceptions and contradictions that were uncovered. Orthoptera, Blattodea, and most Coleoptera generally exhibit efficient RNAi responses with at least one dsRNA delivery method (Scott et al., 2013; Swevers et al., 2013b). Conversely, Lepidoptera and Diptera generally have much lower RNAi efficiencies, which has greatly impeded investigations of RNAi mechanisms in these organisms (Terenius et al., 2011; Swevers et al., 2013b). Hemiptera has highly variable efficiency, and mortality is hard to achieve through feeding (Yu et al., 2014; Cao et al., 2018). Due to these convoluted observations, investigations of the molecular mechanism(s) governing and development of strategies for enhancing RNAi efficiency in insects have become predominant research topics.

Review of potential mechanisms affecting RNAi efficiency

Many hypotheses have been proposed to explain the observed differences in RNAi efficiency among insects. The dsRNA instability, incomplete dsRNA internalization, deficient core RNAi machinery, impaired systemic spreading, and refractory target genes have all been proposed as factors limiting RNAi efficiency (Fig. 1-2), with varying amounts of evidence supporting them. Little is known about the factors that may influence the sensitivity of a target gene to suppression by RNAi, either due to bias in selecting genes and endpoints for analysis or due to lack of knowledge, particularly about protein stability and turnover (Table S1-1). Accordingly, discussion of target genes has been omitted. Instead, we focus on the evidence supporting each of the other mechanisms mentioned above that contribute to limiting RNAi efficiency.

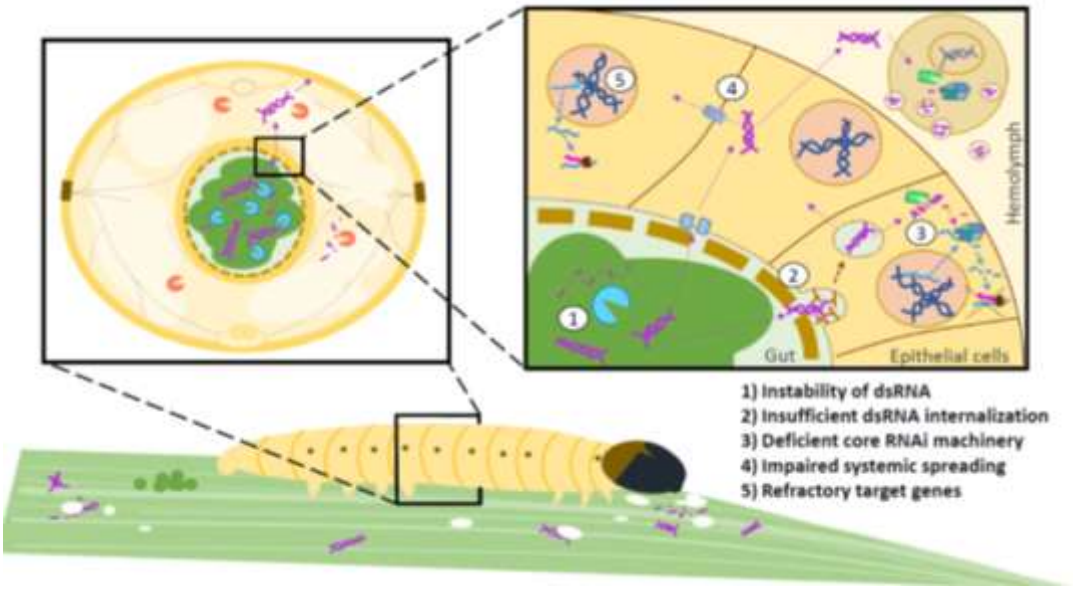

Figure 1-2. Proposed mechanisms of low RNA interference (RNAi) efficiency in insects: 1) Instability of double-stranded RNA (dsRNA) in the gut, hemolymph, and/or saliva; 2) Insufficient dsRNA internalization through endocytosis, transmembrane channels, or other mechanisms; 3) Deficient function or expression of core RNAi machinery; 4) Impaired systemic spreading of silencing signals to distant tissues; and 5) Refractory target genes.

Stability of dsRNA

In RNAi experiments, dsRNA is typically either injected into the body cavity or fed to insects. Thus, the first environment dsRNA encounters prior to cellular uptake is either hemolymph or gut contents. Degradation and/or instability of dsRNA has been documented in the body fluids of insects from many orders (Table S1-2). Recently a comparison of dsRNA stability in body fluids documented higher stability in orthopterans and coleopterans than in body fluids from hemipterans and dipterans, with degradation of dsRNA being highest among lepidopterans (Singh et al., 2017). However, considerable species-level variation in dsRNA stability was observed among the 11 coleopterans and two orthopterans investigated. Furthermore, whole-body nuclease activity may not be a reliable indicator of RNAi efficiency (Peng et al., 2018). Accumulated evidence suggests that instability of dsRNAs in insect tissues is attributable to the enzymatic activity of double-stranded ribonucleases (dsRNases) as well as to physiological pH, which affects enzymatic activity and the stability of dsRNA. DsRNA degradation due to dsRNases in the midgut has been well documented (Wynant et al., 2014d; Prentice et al., 2017; Song et al., 2017; Spit et al., 2017), but less is known about dsRNases from

hemolymph (Garbutt et al., 2013) and salivary (Allen and Walker, 2012) tissues. In addition, a new class of Lepidopteran-specific nucleases, dubbed RNAi efficiency-related nucleases (REases), were recently identified that might contribute to low RNAi efficiency in this order (Guan et al., 2018).

dsRNases

Rapid degradation of dsRNAs reduces RNAi efficiency in insects (Wang et al., 2016; Spit et al., 2017) and dsRNases have been implicated as the causative agents of dsRNA instability (Wynant et al., 2014d; Song et al., 2017; Spit et al., 2017). In nematodes, RNAi efficiency is reduced by structure-specific 3'-5' exonucleases, referred to as Eri-1 enzymes that degrade intracellular siRNA (Kennedy et al., 2004). Most insects lack true *Eri-1* homologs (Tomoyasu et al., 2008), and *Eri-1-like* genes do not appear to impact RNAi efficiency (Table S1-3). Recently, another type of exonuclease called Rrp44-like proteins have also been suggested to be responsible for degradation in *T. castaneum* (Cao et al., 2018) and *Nasonia vitripennis* (Lomate and Bonning, 2016)*;* however, the influence of Rrp44-like exonucleases on RNAi efficiency has not yet been evaluated.

Most insects investigated to date possess two to four dsRNase genes based on genome-wide BlastP searches (AMW Cooper, unpublished). Interestingly, investigations working with orthopterans have shown that *dsRNase2,* and not *dsRNase1, 3,* or *4,* is responsible for most dsRNA degradation in the gut (Wynant et al., 2014d; Song et al., 2017). In addition, suppression of dsRNase(s) with RNAi enhances RNAi efficiency in *Leptinotarsa decemlineata* (Spit et al., 2017), and *L. migratoria* (Song et al., 2017; Spit et al., 2017), supporting the idea that dsRNases limit RNAi efficiency. Degradation of dsRNA occurs in the gut contents of some blattodeans, coleopterans, orthopterans, and lepidopterans, as well as in the hemolymph of hemipterans and lepidopterans (Table S1-2). Interestingly, only hemipterans degrade dsRNA in the saliva (Allen and Walker, 2012; Christiaens et al., 2014; Lomate and Bonning, 2016), as documented in a limited number of studies, which has been shown to impact dsRNA stability in insect diet (Cao et al., 2018). Interestingly, even insects that are highly susceptible to oral RNAi, such as *L. decemlineata* (Spit et al., 2017) express dsRNases in the gut, suggesting that the presence of dsRNases is not indicative of inefficient RNAi. However, dsRNA degrading nuclease activity in the gut fluids and hemolymph are good indicators of ingestion and injection RNAi, respectively

(Peng et al., 2018). Why dsRNases negatively impact RNAi efficiency in some insects or tissues and not others may depend upon the expression and activity of nucleases, physiological pH, magnesium concentrations, and coevolutionary history with viruses (Peng et al., 2018; Swevers et al., 2013b). In addition, there is variation in domain structure among insects dsRNases that may affect their function, but the significance of these variations have yet to be investigated (Singh et al., 2017).

The expression of dsRNase genes is influenced by multiple factors. In *Bombyx mori* larvae, exposure to exogenous dsRNA upregulates dsRNase expression, suggesting that dsRNases may be a viral defense mechanism (Liu et al., 2013). In addition, dsRNase expression varies by developmental stage (Spit et al., 2017) and in response to starvation (Rodríguez-Cabrera et al., 2010). These observations suggest that variation in RNAi efficiency among developmental stages or in response to different delivery methods or experimental protocols may in part be due to dsRNases. Targeting tissues and developmental stages with the lowest expressions of nucleases could be used to overcome dsRNA stability issues in insects. Alternatively, it may be possible to use continuous exposure to dsRNA to overcome dsRNA degradation (Prentice et al., 2017).

REases genes are also upregulated following dsRNA exposure, and suppression of these enzymes in *O. furnacalis* and *H. armigera* enhanced dsRNA stability *ex vivo* (Guan et al., 2018). Furthermore, RNAi-mediated suppression of *REase* in *O. furnacalis* increased RNAi efficiency, while recombinant expression of REase in transgenic *D. melanogaster* reduced RNAi efficiency. These findings support that REases contribute to RNAi efficiency limitations in lepidopterans; however, the enzymatic activity of recombinant REases is surprisingly low (Guan et al., 2018). Therefore, further investigation of the activity of REases is needed to fully understand their influence on RNAi efficiency.

Physiological pH

While the pH of hemolymph is relatively constant among insects (pH 6.4-7.5), the pH of the gut lumen varies considerably (Harrison, 2001; Peng et al., 2018). Crop pH is generally acidic, and the hindgut neutral to acidic (Harrison, 2001). Orthopterans, dipterans, and hymenopterans typically have alkaline midguts, lepidopterans have extremely alkaline midguts (pH>8.0), whereas coleopterans and hemipterans have slightly acidic midguts (Harrison, 2001;

Swevers et al., 2013a; Garcia et al., 2017; Peng et al., 2018). Different environmental conditions in the gut have been hypothesized to affect oral RNAi efficiency in insects by directly affecting dsRNA stability and indirectly by affecting the activity of nucleases in the gut (Baum and Roberts, 2014).

RNA is most stable in solution at pH 4.0-5.0 (Järvinen et al., 1991). RNA is susceptible to hydrolysis at pH>6.0 and below pH<2.0 (Bernhardt and Tate, 2012). RNA is also vulnerable to depurination a pH<3.0 (Jobst et al., 2016). The highly alkaline environment of the lepidopteran gut is particularly concerning for dsRNA stability, but alkaline conditions in the gut of orthopterans, dipterans, and hymenopterans may also reduce RNAi efficiency by hydrolyzing dsRNA, although this has not been experimentally investigated. Furthermore, dsRNases generally have the greatest enzymatic activity in alkaline conditions of pH 9.0 or above (Baum and Roberts, 2014; Song et al., 2017; Peng et al., 2018). However, the optimal pH range for dsRNA-degrading nucleases in *Anthonomus grandis* is from pH 5.5-5.6, which matches the pH value of the anterior midgut, suggesting that the majority of dsRNA degradation is occurring in this region (Garcia et al., 2017). Interestingly, the biochemical characterization of nuclease activity from various tissues in four insect species found that the substrate affinity and maximum velocity varied among insect nucleases, and were influenced by the physiological conditions (pH, temp, and magnesium concentration) of the surrounding tissues, supporting that insects possess a variety of dsRNA enzymes with different properties and activities that are influenced by physiological conditions (Peng et al., 2018). However, the enzymatic conditions for dsRNA degrading activity were not optimal in the hemolymph or pH of any species tested, suggesting that dsRNA degradation can still occur even under suboptimal pH conditions (Peng et al., 2018). Together these results suggest that differences in gut pH contribute to low oral RNAi efficiency by influencing dsRNase activity, as well as possibly through hydrolysis of dsRNA. Additives that alter the enzymatic activity or pH in the gut or saliva might also prove useful for enhancing RNAi efficiency in insects with dsRNA stability issues (Lin et al., 2017).

Systemic spreading of RNAi effect

In some organisms, transcriptional suppression from dsRNA is observed in tissues throughout the body due to spreading of the RNAi signal between tissues. This non-cell autonomous RNAi response is referred to as systemic RNAi. In the nematode model, *C. elegans*,

dsRNA in the gut is imported by SID-2 channel transporters. Then siRNAs generated by Dicer-2 are transported from cell to cell, and their numbers amplified by an RNA-dependent RNA polymerase (RdRP) (Gordon and Waterhouse, 2007). In *C. elegans*, both the receiving and donor cells must have systemic defective-1 (SID-1) channel transporters for cell-to-cell transport to occur. However, vesicle transport and endocytosis are also involved (Sarkies and Miska, 2014). Sid-3 is a tyrosine kinase also required for the import of dsRNA into recipient cells and interacts with Sid-1 (Rocheleau, 2012). Conversely, Sid-5 is an endosome-associated protein that aids in the export of dsRNA from cells (Rocheleau, 2012). A similar mechanism was hypothesized for insects; however, all insect genomes lack RdRPs (Gordon and Waterhouse, 2007), and some lack SID homologs. Until recently, little was known about the mechanisms involved in systemic RNAi in insects (Table S1-7), even though impaired cell-to-cell transport of the silencing signal could severely limit RNAi efficiency.

Amplification mechanisms

Evidence for an amplification mechanism in insects comes from studies of viral immunity in *D. melanogaster.* Experiments utilizing a reverse transcriptase inhibitor demonstrated that viral RNA is converted into vDNA by endogenous reverse transcriptase and is then used as a template to synthesize siRNAs for immune priming (Tassetto et al., 2017). In fact, this group recently used a novel sequencing technique that can distinguish primary and secondary siRNAs, to show that viral RNA is converted into DNA inside hemocytes in an Ago2-dependent manner, which is then used as a template to synthesize secondary siRNAs *de novo*. This demonstrates, for the first time, the existence of an RNAi amplification mechanism in insects (Tassetto et al., 2017). The caveat is that this amplification mechanism is only stimulated in response to viral infections, and in the absence of a viral infection, exogenous dsRNA is only converted into primary siRNA, and not into secondary siRNAs (Tassetto et al., 2017). This suggests that exogenous dsRNA application alone may not sufficiently induce the required immune response that initiates systemic RNAi in some insects. This is consistent with a report that documented the upregulation of *Ago2* in *B. terrestris* in response to endogenous, exogenous, and viral dsRNA, but found no production of siRNA or any effect on the replication of an avirulent virus (Niu et al., 2016). Together these findings suggest that insufficient immune stimulation contributes to low RNAi efficiency in some insects by limiting systemic RNAi.

Therefore, it might be possible to use genetically engineered viruses to deliver dsRNA and initiate a systemic RNAi response in many RNAi refractory insects, as was demonstrated in transgenic *D. melanogaster* (Saleh et al., 2009). It might even be possible to co-inject a harmless virus with dsRNA to enhance RNAi efficiency.

Interestingly, most of the work completed on systemic spreading in *D. v. virgifera* suggests that an RNAi amplification mechanism does not exist in this species, despite *D. v. virgifera* being highly amenable to RNAi (Ivashuta et al., 2014; Fishilevich et al., 2016; Li et al., 2015a). DsRNA has been detected in tissues throughout the body after oral feeding and has been visualized spreading to distant tissues, indicating that dsRNA acts as the primary silencing signal in *D. v. virgifera* (Ivashuta et al., 2014; Li et al., 2015a). Furthermore, efforts to detect secondary siRNA production (i.e., amplification) have failed (Li et al., 2018). It is important to note, however, that conventional sequencing techniques cannot detect secondary siRNAs because they have a 5' –triphosphate moiety that prevents linker ligation during sequencing (Pak et al., 2012; Tassetto et al., 2017). The novel sequencing techniques (Tassetto et al., 2017) employed in the *Drosophila* study of viral RNA replication need to be applied to *D. v. virgifera* to verify that amplification of secondary siRNAs does not occur. The possibility of an amplification mechanism for long dsRNA also needs to be investigated, if dsRNA is found to be the primary silencing signal in *D. v. virgifera*. Perhaps dsRNA can act as a spreading signal in some insects, while siRNAs amplified from dsRNA mediate spreading in other species.

Spreading mechanisms

Systemic spreading of the RNAi signal to distant tissues has been confirmed in some insects from Coleoptera (Bolognesi et al., 2012; Miller et al., 2012), Lepidoptera (Wang et al., 2011), Diptera (Tassetto et al., 2017), Hemiptera (Yu et al., 2014; Wang et al., 2015), Orthoptera (Luo et al., 2012; Wynant et al., 2012), and in some tissues of Hymenoptera (Jarosch and Moritz, 2011; Cappelle et al., 2016b). The spreading mechanism(s) mediating systemic RNAi in insects remain mostly unknown, except in *D. melanogaster*. Systemic antiviral immunity is mediated by siRNAs encased in exosome-like vesicles that originate from hemocytes and participate in immune priming (Tassetto et al., 2017). In addition, in the presence of a viral infection, nanotube-like structures transport dsRNA and components of the RNAi machinery between adjacent cells to mediate systemic RNAi (Karlikow et al., 2016). These reports indicate that

multiple spreading signals (dsRNA and siRNA) function in a single species, together with an independent spreading mechanism for the different silencing signals that respond to viral infections. These uptake mechanisms need to be explored in other insects, along with the possibility of siRNA transport that occurs in nanotube-like structures and dsRNA transport in vesicles.

Variation in the efficiencies of systemic RNAi in different insects has been well documented, so the existence of additional spreading mechanisms is likely, especially in species where Sid-like (Sil) transmembrane channels have been implicated in uptake. It might be that insects that exhibit robust RNAi responses, like *D. v. virgifera*, favor direct dsRNA spreading mechanisms for systemic RNAi, whereas insects that exhibit low RNAi efficiency evolved an amplification and spreading mechanism mediated by hemocytes (Saleh et al., 2009; Tassetto et al., 2017). The level of immune stimulation needed to elicit a systemic response may also vary between RNAi refractory and amenable species. In addition, the ability of individual tissues to mediate a systemic RNAi response may differ among insect species or lineages due to evolutionary pressures associated with viral infection (Tassetto et al., 2017). Some insects may rely on specialized immune tissues (i.e., hemocytes and fat bodies) to mediate systemic RNAi responses, whereas others may not.

Another reason that RNAi efficiency might vary among insect species is that the physiological environment within the insect or tissue may influence systemic spreading mechanisms, with more protective reagents such as extracellular vesicles or binding proteins (Jose, 2015) favored in insects that have high nuclease activity in the hemolymph. In humans, extracellular RNA is present in blood plasma bound to Ago2 proteins and high-density lipoproteins for enhanced stability (Jose, 2015), suggesting that Ago2 may be transported with dsRNA inside of nanotube-like structures that prevent degradation from nucleases. None of these hypotheses have been tested in insects, but the high variability in systemic RNAi efficiency between insects and the recent mechanistic investigations performed in the *D. melanogaster* and *D. v. virgifera* suggest that many variations in the mechanisms of systemic spreading in insects may exist. Furthermore, the study in *D. melanogaster* suggests that RNAi efficiency can be limited in insects due to inadequate immune stimulation needed to initiate systemic RNAi.

Cellular uptake of dsRNA

The internalization of dsRNA into cells is an essential step to generate high RNAi efficiency. If dsRNA is delivered orally, dsRNA must pass through the peritrophic matrix before being internalized by midgut epithelia. In the hemolymph, dsRNA binds to lipophorins, which are proposed to participate in dsRNA uptake, although this hypothesis has not been well investigated yet (Table S1-4). In insects, cellular uptake mainly occurs through receptor-mediated endocytosis; however, SSil transmembrane channels may play a role in some coleopterans, hymenopterans, and hemipterans (Table S1-5). Once inside cells, dsRNA must reach the core machinery of the RNAi pathway. The core RNAi machinery forms clusters known as GW-bodies that are present in the cytoplasm and attached to membrane compartments (Lee et al., 2010). If entering through endocytosis, dsRNA must escape the endosome to reach the core machinery in the cytoplasm to affect the targeted mRNA (Shukla et al., 2016).

Deficient dsRNA uptake limits RNAi efficiency in dipterans (Boisson et al., 2006; Miller et al., 2008; Li et al., 2015b), as well as in specific tissues of some orthopterans (Wynant et al., 2012; Ren et al., 2014) and hymenopterans (Jarosch and Moritz, 2011). In addition, impaired endosomal escape of dsRNA appears to be limiting in lepidopterans (Shukla et al., 2016), and needs to be investigated in additional insect species and orders. Lipophorins may also play a role in insect RNAi, but it is still unclear how they affect RNAi efficiency (Table S1-4). Furthermore, it is unclear if the mechanism(s) of dsRNA uptake differs for different tissues (Cappelle et al., 2016a).

Endocytosis

DsRNA uptake occurs through receptor-mediated clathrin-dependent endocytosis in dipterans (Saleh et al., 2006; Ulvila et al., 2006), coleopterans (Xiao et al., 2015; Cappelle et al., 2016a), and orthopterans (Wynant et al., 2014b). Based on work with pharmacological inhibitors, neither caveolae-dependent endocytosis nor phagocytosis, are involved in dsRNA uptake in *D. melanogaster* (Saleh et al., 2006) or *Tribolium castaneum* (Xiao et al., 2015). Macropinocytosis and phagocytosis have been implicated in the uptake of modified dsRNA particles (Gillet et al., 2017) and dsRNA-expressing bacteria (Rocha et al., 2011), respectively.

The involvement of other non-classical endocytosis mechanisms warrant investigation (Howes et al., 2010).

Scavenger receptors mediate clathrin-dependent dsRNA uptake in *D. melanogaster* (Ulvila et al., 2006), *S. gregaria* (Wynant et al., 2014b), and *L. decemlineata* (Yoon et al., 2016). Scavenger receptors are known pattern recognition receptors involved in immune responses, so their role in dsRNA uptake is not surprising. However, they are usually associated with phagocytosis of bacterial pathogens rather than clathrin-dependent endocytosis (Ulvila et al., 2006). In *T. castaneum* and *D. v. virgifera*, uptake of dsRNA through receptor-mediated endocytosis is size-dependent (a minimum of 60-100 bp required for uptake) (Bolognesi et al., 2012; Miller et al., 2012) and saturable (Miyata et al., 2014). However, dsRNA expressed in transgenic plants is converted into siRNA by the RNAi machinery in the plant, but RNAi is still initiated in *D. v. virgifera* (Baum et al., 2007; Li et al., 2015a) as well as in other insects such as *Bactericera cockerelli* (Wuriyanghan and Falk, 2013), *Helicoverpa armigera* (Mao et al., 2007), *Nilaparvata lugens* (Zha et al., 2011), and *Myzus persicae* (Pitino et al., 2011). Furthermore, some insects such as *A. mellifera* (Jarosch and Moritz, 2011), *H. armigera* (Kumar et al., 2009), *Plutella xylostella* (He et al., 2012), and *Acyrthosiphon pisum* (Mutti et al., 2006) exhibit an RNAi-mediated suppression in response to siRNA delivered through injection and/or feeding. This suggests that either the size requirements described for dsRNA uptake are variable, or additional uptake mechanisms exist for siRNA. Fundamental studies on the size-dependent nature of clathrin-mediated dsRNA-uptake are needed, as well as studies addressing how dsRNA interacts with scavenger receptors.

Repeated oral exposure to dsRNA leads to reduced RNAi efficiency for up to thirty days through downregulation of endocytosis in *Bactrocera dorsalis* (Li et al., 2015b). The duration of RNAi-refractoriness is dose-dependent as well as sequence-dependent and is mediated by the composition of polyunsaturated fatty acids (i.e., the ratio of linoleic acid to arachidonic acid) in the hemolymph (Dong et al., 2017). Lipids play an important role in endocytosis because the fluidity, flexibility, and selective permeability of membranes are controlled by the composition of fatty acids. In addition, adaptor proteins that mediate clathrin coat assembly bind to lipids in the plasma membrane (Dong et al., 2017). Reports of endocytic-downregulation in *B. dorsalis* demonstrate the existence of "RNAi blocking factors" that regulate the endocytic uptake of dsRNA and influence RNAi efficiency (Dong et al., 2017). However, repeated exposure to

dsRNA is commonly used in numerous insect species when employing the RNAi of RNAi technique (Miyata et al., 2014) with no prior reports of induced refractoriness (Kim et al., 2015), suggesting that downregulation of endocytosis after dsRNA exposure may be specific to dipterans. Impaired uptake occurs in various tissues of *L. migratoria* (Ren et al., 2014), *Apis mellifera* (Jarosch and Moritz, 2011), *D. melanogaster* (Miller et al., 2008), and *Anopheles gambiae* (Boisson et al., 2006).

Limitations in dsRNA uptake can be overcome through the use of positively charged, lipophilic transfection reagents and nanoparticles, which enhance penetration of plasma membranes by nucleic acids (Zhang et al., 2010; Taning et al., 2016b). Recombinant expression of dsRNA-transmembrane channels from *Caenorhabditis elegans*, the nematode model for RNAi, has also been used to enhance dsRNA uptake in *B. mori* (Kobayashi et al., 2012; Mon et al., 2012) and *D. melanogaster* (Feinberg and Hunter, 2003) cultured cells. Another approach is to express dsRNA in recombinant microbes for oral delivery to insects (Abrieux and Chiu, 2016). In addition, symbiotic bacteria could be genetically engineered to express and deliver dsRNA intracellularly to overcome uptake limitations (Whitten and Dyson, 2017). It might also be possible to enhance dsRNA uptake by stimulating clathrin-dependent endocytosis using arachidonic acid or hydrogen peroxide, as was done for *B. dorsalis* (Li et al., 2015b; Dong et al., 2017).

Endosomal escape

After being internalized through receptor-mediated clathrin-dependent endocytosis, the clathrin coat is removed from the vesicle, which then fuses with an early endosome. Early endosomes then fuse to form internal luminal vesicles known as multivesicular bodies or late endosomes (Fig. 1-3). Mature late endosomes eventually fuse with lysosomes, and the cargo is degraded (Lee et al., 2010). Therefore, dsRNA must escape from endosomes to reach the core machinery of the RNAi pathway in the cytoplasm (Xiao et al., 2015). Fluorescently labeled dsRNA is internalized into cells equally well in lepidopteran (Sf9 and Hv-E6) and coleopteran (Lepd-SL1 and TcA) cell lines. However, in lepidopteran cells, dsRNA is never converted to siRNAs, as occurs in coleopteran cells, indicating that dsRNA may be degraded in lysosomes and endosomal escape may be limiting RNAi efficiency in lepidopterans (Shukla et al., 2016). Experiments showing co-localization of dsRNAs with early and late endosomes, but not

lysosomes, in lepidopteran cells is consistent with this idea (Shukla et al., 2016). Further investigation showed that dsRNA is converted into siRNA after feeding and injection in 12 species of coleopterans and two species of orthopterans, but is not converted into siRNA in any of ten lepidopteran species (Singh et al., 2017). Additionally, dsRNA was converted into siRNA after injection in seven out of ten hemipteran and in two out of five dipteran species but was not detected after feeding dsRNA in any dipterans or hemipterans tested (Singh et al., 2017). This data supports the hypothesis that endosomal escape is limiting in lepidopterans and possibly in some hemipterans and dipterans, but not in coleopterans or orthopterans. Further work, however, is needed to determine if the impaired conversion of dsRNA into siRNA documented in this study is due to impaired endosomal escape, high levels of enzymatic degradation in body fluids, uptake limitations, or unresponsive core RNAi machinery.

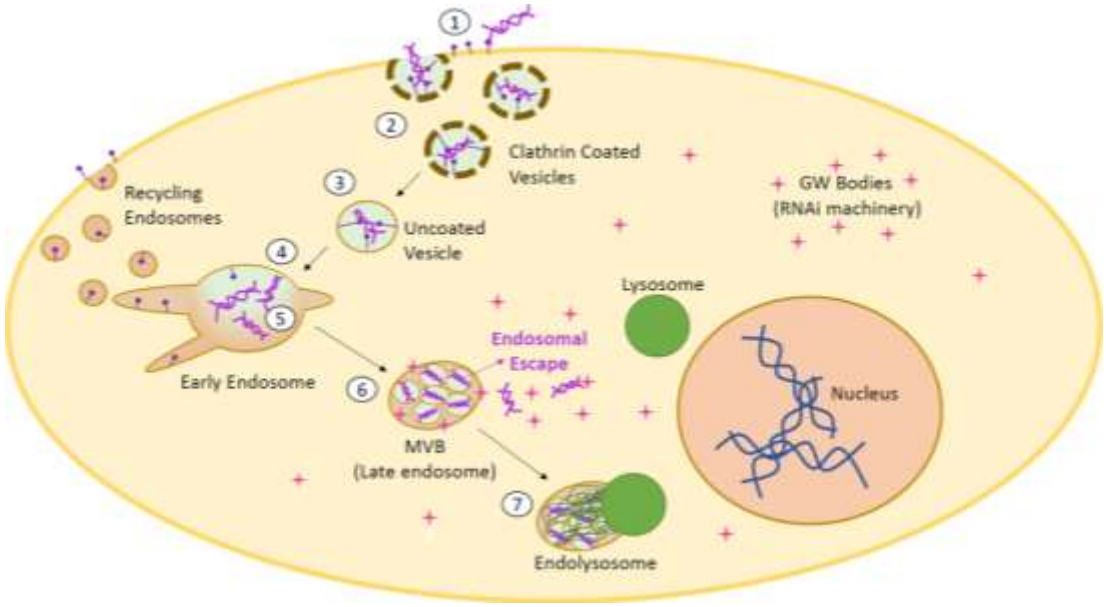

Figure 1-3. Proposed double-stranded RNA (dsRNA) internalization through receptor-mediated clathrin-dependent endocytosis in insects:

1) dsRNA binds to scavenger receptors on the plasma membrane; 2) dsRNA is internalized through clathrin-dependent endocytosis; 3) The clathrin coat is removed from the vesicle; 4) The uncoated vesicle merges with an early endosome; 5) Cargo (i.e., dsRNA) is sorted and receptors are recycled back to the plasma membrane for reuse; 6) Early endosomes fuse to form multivesicular bodies (MVB) also known as late endosomes; and 7) Late endosomes fuse with lysosomes to form endolysosomes where cargo (i.e., dsRNA) is degraded. For RNA interference-mediated gene silencing to occur, dsRNA must escape from the endosome and/or reach the core RNAi machinery located in GW bodies (pink stars) prior to formation of the endolysosome.

Interestingly, a different group reported that dsRNA is converted into siRNAs in *D. melanogaster* (Saleh et al., 2009), suggesting that endosomal escape and deficiency in dsRNA uptake may be two separate issues that contribute to RNAi efficiency limitations in insects. In *D. melanogaster*, GW-bodies containing active RNAi machinery are associated with the plasma membranes of multivesicular bodies (Lee et al., 2010), suggesting that endosomal escape may occur at this stage. When endosome maturation is blocked in transgenic *D. melanogaster* by depleting the ESCRT6 protein involved in multivesicular body formation, RNAi-mediated silencing is abolished. But when multivesicular bodies are prevented from fusing with lysosomes by depleting the HPS4 tethering factor, GW-bodies over accumulate, and RNAi efficiency is enhanced (Lee et al., 2010). Perhaps GW-bodies accumulate in different locations or compartments in different insects, making endosomal escape more important in some species than in others. Differences in endosome acidification could also alter endosome permeability to dsRNA, possibly due to variations in vacuolar H^+- ATPase (vATPase) structure or function (Yoon et al., 2016). vATPases are implicated in endocytosis-mediated uptake of dsRNA in multiple insects (Saleh et al., 2006; Xiao et al., 2015; Cappelle et al., 2016a; Yoon et al., 2016), and further investigation of this hypothesis is underway. Alternatively, the rate of endosome maturation and fusion with lysosomes could differ among insects and tissue types, altering the time window when endosomal escape could occur. In the future, transgenic manipulation of endosome maturation might provide a means to enhance RNAi efficiency by avoiding or delaying lysosomal fusion (Lee et al., 2010). In addition, endosome penetrating peptides, lipids, and nanocarriers from the medical field may aid in overcoming endosomal escape and uptake issues in insects (Wan et al., 2015).

Transmembrane Sid-like channels

In *C. elegans*, a model organism for RNAi in animals, dsRNA import is mediated by SID proteins (systemic RNAi defective proteins) (Whangbo and Hunter, 2008). Sid2, a single-pass transmembrane protein, binds to dsRNA in the gut and internalizes dsRNA (over 50 bp in length) through receptor-mediated endocytosis. Whereas Sid1, a multi-pass transmembrane protein, acts as a channel that allows dsRNA into the cell (Whangbo and Hunter, 2008). The presence of *Sid1* homologs in insects (known as *Sid-like* or *Sil* genes) has been extensively studied, but the involvement of this uptake mechanism in RNAi varies considerably. *Sil* genes are present in

most insects, but not *Sid2* homologs (Baum and Roberts, 2014). The genes identified for spreading RNAi in *C. elegans*, namely *Sid* or *RNAi spreading defective* (*Rsd*), are found to be rapidly evolving genes in insects, having only low degrees of conservation even in the most closely related nematode taxa (Dalzell et al., 2011; Mon et al., 2012). The number of *Sil* genes varies between insects as well, and *Sils* are entirely absent from Antliophora (i.e., Diptera, Mecoptera, and Siphonaptera) (Dowling et al., 2016). Unsurprisingly, the number of *Sils* was initially proposed to explain the variation in RNAi efficiency between insects (Obbard et al., 2009). However, this does not seem to hold true (Tomoyasu et al., 2008; Luo et al., 2012). Nonetheless, *Sil* is required for RNAi in *N. lugens* (Xu et al., 2013) and multiple *Sils* seems to play a minor role in dsRNA uptake in *L. decemlineata* (Cappelle et al., 2016a; Yoon et al., 2016) and *D. v. virgifera* (Miyata et al., 2014).

Tissue-specific expression of *Sils* also varies considerably among species (Bansal and Michel, 2013; Dong and Friedrich, 2005; Gong et al., 2015; Luo et al., 2012), as does domain structure (Singh et al., 2017) suggesting dissimilar roles in different insects. In *A. mellifera*, *Sil* is upregulated in response to dsRNA (Aronstein et al., 2006), yet radiolabeled dsRNA is not taken up by any tissue except the fat body (Jarosch and Moritz, 2011), supporting the idea that *Sil* may not be involved in dsRNA uptake in Hymenoptera. Intriguingly, insect *Sils* appear to be more closely related to the cholesterol uptake-associated gene of *C. elegans* than to Sid1 (Luo et al., 2012; Xu et al., 2013; Cappelle et al., 2016a; Dowling et al., 2016), indicating that *Sil* genes serve different functions in insects and nematodes. Therefore, we are only able to conclude that *Sils* likely participate in RNAi, at least in some insects, but the importance of this uptake mechanism is still unknown. In addition, the involvement of *Sils* in endocytosis is still unclear. *Sils* may facilitate the escape of dsRNA from endosomes in some species, allowing dsRNA to reach the core enzymes of the RNAi pathway, but this has not been investigated (Cappelle et al., 2016a). Furthermore, *D. v. virgifera* gut tissue culture only takes up dsRNA, but fat body tissue cultures are capable of taking up both siRNA and dsRNA, suggesting that different uptake mechanisms may be acting in different tissues (Ivashuta et al., 2014), although the effect of gene suppression in different tissues on insect mortality has not been comprehensively analyzed. As the investigation of *Sil* continues, it is essential to assess both the involvement of *Sils* and endocytosis, so we can begin to understand if these two uptake mechanisms function individually or in tandem.

Lipophorins

Lipophorins are hemolymph proteins that transport lipids between insect cells. Under laboratory conditions, lipophorins also bind to dsRNA when dsRNA is incubated *ex vivo* in *S. gregaria* or *B. mori* hemolymph (Sakashita et al., 2009; Wynant et al., 2014a). In addition, a similar electrophoretic shift was observed when dsRNA was incubated in hemolymph from *Periplaneta americana*, *Sarcophaga crassipalpis*, and *Acheta domesticus*, suggesting that the presence of dsRNA binding proteins in the hemolymph may be widespread among insect orders (Sakashita et al., 2009). Vertebrate lipophorins bind to scavenger receptors, and deliver DNA into cells (Ulvila et al., 2006; Wynant et al., 2014a), so lipophorins have been hypothesized to aid in the delivery of dsRNA by acting as dsRNA binding proteins in the hemolymph (Wynant et al., 2014c). Alternatively, lipophorins may sequester dsRNA, preventing cellular uptake (Kobayashi et al., 2012). Unfortunately, the involvement of lipophorins in the RNAi pathway has not been assessed. The RNAi of RNAi technique (Miyata et al., 2014) (where dsRNA targeting a component thought to be involved in RNAi is delivered first, followed by dsRNA targeting a reporter gene) could easily determine the impact of lipophorins on RNAi efficiency, by examining the influence suppressing lipophorin genes have on RNAi efficiency of a reporter gene. It is also possible that lipophorins could protect dsRNA from nucleases (Whitten and Dyson, 2017). However, this has not been investigated either. The role of lipophorins in dsRNA uptake in insects is still ambiguous, but if they are involved in uptake, overexpression or supplementation of lipophorins might enhance dsRNA uptake. Conversely, if lipophorins sequester dsRNA, strategies to prevent binding of lipophorins to dsRNA could be developed, such as molecular modifications or protective reagents.

Core RNAi machinery

Even if dsRNA is internalized and reaches the core machinery of the RNAi pathway in the cytoplasm (i.e., reaches the core RNAi enzymes), differences in the expression and function of the core RNAi enzymes could contribute to differences in RNAi efficiency among insects (Singh et al., 2017). In insects, there are three main RNAi pathways (Fig. 1-1). The small-interfering-RNA (siRNA) pathway (Fig. 1-1 A), which is the main focus of this review, is

naturally involved in defense against dsRNA of viral origin (exo-siRNA) as well as in protection against transposable elements in the genome (endo-siRNA) (Dowling et al., 2016). First dsRNA is fragmented by the RNase III enzyme dicer 2 (Dcr2) into 21-nt siRNA duplexes. With the help of dsRNA-binding proteins, loquacious (Loqs) and R2D2, (which are required for efficient siRNA production) siRNAs are then loaded into the argonaute 2 (Ago2) enzyme. A variety of other proteins then assemble to form an RNA-induced silencing complex (RISC) (Bronkhorst and van Rij, 2014). The passenger strand of the siRNA duplex is eliminated from RISC by Ago2, the main catalytic enzyme of RISC, as well as component 3 promoter of RISC (C3PO), an endoribonuclease activator that is comprised of two subunits: translin and trax (Liu et al., 2009). Once activated, the guide siRNA strand leads RISC to complementary mRNAs for degradation by Ago2 (Hammond, 2005; Bronkhorst and van Rij, 2014). These core enzymes of the siRNA pathway (e.g., Ago2, Dcr2, RISC) are present in all plants, animals, and fungi. However, in insects, genes in the RNAi pathways have diversified through gene duplications and deletions, as has the responsiveness of these genes to dsRNA (Table S1-5). In addition, core RNAi enzymes from two related RNAi pathways, the micro-RNA (miRNA) pathway and piwi-interacting-RNA (piRNA) pathway, participate in the exogenous RNAi response of *B. mori* (Kolliopoulou and Swevers, 2013; Nie et al., 2013; Wang et al., 2013), *L. decemlineata* (Yoon et al., 2016), and *D. melanogaster* (Zhou et al., 2008). Many viruses also produce viral suppressors of RNAi that can interfere with the function of the core RNAi enzymes and influence RNAi efficiency (Berry et al., 2009; Cappelle et al., 2016b; Swevers et al., 2016).

Gene copy number

In insects, the core enzymes of the siRNA pathway have undergone various gene duplications and deletions (Dowling et al., 2016). Extra copies of *Ago2* and *R2D2* are suspected to be responsible for the superior RNAi efficiency exhibited by *T. castaneum* compared to *D. melanogaster*, which only has a single copy of each core siRNA enzyme (Tomoyasu et al., 2008). In addition, missing core enzyme genes are proposed to contribute to low RNAi efficiency in other species (Dowling et al., 2016). However, genes encoding core enzymes for all three RNAi pathways have now been identified in a variety of species from every insect order (Table S1-5) and enzyme copy number does not clearly explain RNAi efficiency differences among insects.

Gene duplications have been documented in *Ago2*, *Dcr2*, and *R2D2* for some insects from every order investigated, except Blattodea (Table S1-5: duplicated enzymes bolded). *R2D2* was not identified in primary wingless insects (nonpterygotes) (Dowling et al., 2016) or in *Diaphorina citri* (Taning et al., 2016a), and it is proposed that *Loquacious* from the miRNA pathway compensates for lack of R2D2 in these organisms. Interestingly, R2D2 is duplicated in many coleopterans (Singh et al., 2017), but R2D2 is undetectable in the transcriptome of some lepidopterans (Ditrysia) (Dowling et al., 2016). In addition, *Spodoptera frugiperda* (Sf21 cells) is the only species reported to lack Ago2 (Ghosh et al., 2014). Therefore, the loss or low expression of core siRNA enzymes in Lepidoptera may partially explain RNAi efficiency differences between species. It might be that the expression levels of the core RNAi enzymes are more important than the number of gene copies of each core RNAi enzyme, although increased gene copies could result in greater expression of the core enzymes. Thus, some have speculated that some insects have inefficient RNAi responses due to poor upregulation of the core RNAi enzymes (Terenius et al., 2011; Kolliopoulou and Swevers, 2014).

Insufficient upregulation or expression

When dsRNA is injected into *B. germanica*, a species amenable to RNAi action by dsRNA injection, *Dcr2* is upregulated 5-fold within six hours and remains elevated for 24 h, suggesting that upregulation of the core siRNA enzymes is necessary for a robust RNAi response (Lozano et al., 2012). The upregulation of core siRNA enzymes in response to exogenous dsRNA occurs in many insect species (Table S1-5, + sign indicates upregulated enzymes). However, in *Manduca sexta*, *translin* (a member of the C3PO complex, which activates RISC) is not upregulated in response to dsRNA (Garbutt and Reynolds, 2012). In fact, this enzyme is expressed at very low levels in *M. sexta*, as well as in a *B. mori* cell line (BmN cells) (Swevers et al., 2011; Garbutt and Reynolds, 2012). *R2D2* is also deficient in another *B. mori* cell culture line (Bm5 cells), as well as *B. mori* larvae and pupae (Swevers et al., 2011), and possibly other Ditrysia (Dowling et al., 2016). However, an RNAi response was detected when a transfection reagent was used on Bm5 cells. Furthermore, recombinant expression of *T. castaneum R2D2* in Bm5 cells did not increase RNAi efficiency, suggesting that deficient expression of these enzymes does not limit RNAi efficiency.

Many groups report the basal expression of the core siRNA enzymes in different developmental stages and tissues (Table S1-5). In *L. decemlineata*, expression of the core siRNA enzymes peaks in young larvae, which is when RNAi efficiency is highest (Guo et al., 2015). However, in *B. dorsalis*, peak expression does not correlate with high RNAi efficiency (Xie et al., 2016). More studies exploring relationships between high expression of the core siRNA enzymes and high RNAi efficiency are needed, as one study did find a correlation between low RNAi efficiency in reproductive tissues of *S. gregaria* and low expression of *Ago2* and *Dcr2* (Wynant et al., 2012). In addition, RNAi-based suppression of *Ago2* and *Dcr2* in *S. gregaria* inhibited suppression of a reporter gene, supporting the idea that deficient expression of these enzymes can limit RNAi efficiency. The basal expression of core siRNA enzymes needs to be investigated in more RNAi-refractory insects, but caution is required when interpreting correlations between mRNA abundance of core RNAi enzymes and RNAi efficiency. *Ago2* and *Dcr2* were expressed in all tissues and developmental stages of *B. mori* (Swevers et al., 2011) which at first glance appears to refute the hypothesis that low basal exoression limits the effiency of RNAi, but overexpression of *B. mori Ago2* in transgenic *B. mori* larvae enhanced RNAi efficiency (Li et al., 2015c). In addition, overexpression of Dcr2 increased RNAi efficiency in transgenic *D. melanogaster* expressing hairpin RNAs (Dietzl et al., 2007), supporting the hypothesis that the expression of the core siRNA enzymes influences RNAi efficiency.

It might be possible to enhance RNAi efficiency in some insects by targeting developmental stages with the highest expression of the core siRNA enzymes (Gong et al., 2015). However, in the case of *O. furnacalis*, core RNAi enzyme induction is less important than nuclease activity. In *O. furnacalis*, *Dcr2* and *Ago2* are upregulated upon dsRNA exposure, but the dsRNA degrading enzyme *REase* is upregulated earlier and higher than Ago2 and Dcr2, which results in a lower abundance of target siRNA reads, possibly because REases outcompete Dcr2 for dsRNA (Guan et al., 2018). When *REase* was suppressed with RNAi, though, *Dcr2* and *Ago2* induction was partially suppressed, RNAi efficiency increased, and the abundance of target siRNA reads increased (Guan et al., 2018). Thus, in order to avoid misinterpreting the significance of core RNAi enzyme induction and expression on RNAi efficiency, future investigations need to consider the core RNAi enzymes in combination with other mechanisms potentially impacting RNAi efficiency.

Many factors influence the expression of the core siRNA enzymes, including repeated exposure to dsRNA, environmental factors, and pathogens, which might explain some of the variations in RNAi efficiency observed by different research groups with different strains of the same insect species. Repeated injection of dsRNA in *M. sexta* larvae elevates the expression of *Ago2* and *Dcr2* (Garbutt and Reynolds, 2012). In addition, short exposure to dsRNA (12 h) enhanced RNAi efficiency in the *L. decemlineata*, but prolonged exposure (>72 h) decreased core enzyme expression and RNAi efficiency (Guo et al., 2015). In *B. dorsalis*, exposure to stressors both increases and decreases the expression of the core siRNA enzymes in different situations (Xie et al., 2016). Exposure to bacteria (*Escherichia coli*), high (40°C) or low (0°C) temperatures, and starvation (4 h) all decreased expression of core enzymes, possibly due to the effects of these physiological stresses on insect development. However, exposure to iron or a virus increased expression through yet unknown mechanisms. Exposure of *B. mori* to a virus also increased expression of *Ago2*, as well as *Ago1*, *Ago3* and *Piwi* from the other RNAi pathways, which may also affect RNAi efficiency (Wang et al., 2013). Furthermore, in *Bombus terrestris*, *Dcr2* was upregulated in response to virulent Israeli acute paralysis virus or acute slow bee paralysis virus, but siRNAs were only produced in response to the virulent virus, not the acute infection (Niu et al., 2016). Similarly, in *Apis mellifera*, *Dcr2* and *Ago2* were upregulated in response to acute viruses (Galbraith et al., 2015), but all core siRNA enzymes were downregulated in highly infected *A. melllifera* carrying multiple viruses (De Smet et al., 2016). These studies suggest that many abiotic and biotic stresses (and experimental logistics) can affect the expression of the core RNAi enzymes and potentially change RNAi efficiency. As our understanding of molecular repressors and enhancers of the core RNAi enzymes grows, it might one day be possible to manipulate RNAi efficiency (Ghosh et al., 2014).

Another factor to consider is that the biochemical kinetics of the core RNAi enzymes could vary among insect species, as do the biochemical kinetics of Ago proteins between insects and mammals. In *D. melanogaster*, Ago2 strongly binds the guide strand siRNA to the matching complementary mRNA, leading to efficient target cleavage, while in a mouse model, Ago2 has a high dissociation rate, implying the importance of different enzyme kinetics in overall RNAi efficiency (Wee et al., 2012). Often insect Argonaute enzymes contain a unique N-terminal glutamine-rich domain (Hain et al., 2010). In *T. castaneum*, this domain is found in Ago2 and has 107 out of 213 N-terminal amino-acid residues (Y Park, unpublished), which could be

responsible for the high RNAi efficiency exhibited by this species. The function of this glutamine-rich domain remains to be investigated, but these findings indicate that comparative studies on the biochemical kinetics of core RNAi enzymes in insects are warranted. Furthermore, variations in domain structure among insects have been reported for Dcr2, Ago2, and R2D2, (Singh et al., 2017) supporting that the biochemical properties of these enzymes may differ among insects.

Overlapping RNAi pathways

In addition to the siRNA pathway, there are two additional RNAi pathways (Fig. 1-1). The miRNA pathway (Fig. 1-1 B) mainly regulates endogenous gene expression by using miRNAs derived from genomic DNA to target mRNA for degradation (Dowling et al., 2016). The piRNA pathway (Fig. 1-1 C) is involved in defense of the germline against transposable elements, and involves degradation of transposable elements to yield primary piRNAs that are then used to target complementary transposable elements for degradation and generation of secondary piRNAs (Dowling et al., 2016). If enzymes from more than one RNAi pathway are involved in the RNAi response, it could influence RNAi efficiency. Additionally, if there is overlap between RNAi pathways in some insects but not others, this could potentially contribute to differences in RNAi efficiency among insects.

Studies conducted in *B. mori* (Kolliopoulou and Swevers, 2013; Nie et al., 2013; Wang et al., 2013a), *L. decemlineata* (Yoon et al., 2016), and *D. melanogaster* (Zhou et al., 2008) indicate that argonaute enzymes from all three pathways participate in antiviral responses and processing of dsRNAs. In *B. mori,* Ago2 from the siRNA pathway participates in the miRNA and piRNA pathways in addition to the siRNA pathway (Nie et al., 2013). However, different core enzymes from the three pathways play different roles in each pathway (Zhou et al., 2008; Kolliopoulou and Swevers, 2013; Wang et al., 2013a). In *B. mori*, all of the *Ago* genes, except *Piwi*, are expressed at very low levels (Wang et al., 2013). This suggests that the expression of core RNAi enzymes could be limiting even when enzymes from multiple RNAi pathways are involved. In addition, suppression of Ago2 in *D. melanogaster* enhanced the efficiency of the miRNA pathway, supporting the idea that components shared between pathways can be limiting (Zhou et al., 2008). Moreover, recombinant expression of core RNAi enzymes (*Argo1, Ago2b, Aub, Vha16* but not *Ago2a*) from *L. decemlineata* in a *S. frugiperda* cell line (Sf9 cells) resulted in

enhanced RNAi efficiency (Yoon et al., 2016). This supports the hypothesis that RNAi efficiency is dependent upon the function and composition of the core RNAi enzymes participating in the response. However, the number of core enzymes in the miRNA and piRNA pathways also varies between species (Table S1-5). For instance, Ago3 has been lost from Dictyoptera, but the Piwi/Aub subclade expanded numerous times in Diptera, Hemiptera, Thysanoptera, and Hymeonptera (Dowling et al., 2016). This may or may not influence RNAi efficiency by increasing the expression of duplicated core miRNA and piRNA enzymes. Furthermore, in Diptera the expanded Piwi/Aub subclade has undergone functional divergence and neofunctionalization (Lewis et al., 2016), indicating that duplicated enzymes cannot necessarily compensate for each other. Extensive work is required to understand the effect(s) of overlap between the three RNAi pathways. Interestingly, viral suppressors of RNAi inhibit the siRNA pathway in *D. melanogaster*, but not the miRNA pathway, suggesting that the overlap between the three RNAi pathways may be a mechanism to overcome viral suppressors of RNAi (Berry et al., 2009).

Viral suppressors of RNAi

The upregulation of the core siRNA enzymes in response to viral infection is consistent with the role of these enzymes in viral defense. However, during the evolutionary arms race between viruses and insects, viruses evolved viral suppressors of RNAi to combat and disarm the siRNA interference pathway. Viral suppressors of RNAi, which are a variety of proteins with no conserved features or structures, target various steps in the RNAi pathway (Mongelli and Saleh, 2016). The main modes of action for viral suppressors of RNAi are 1) binding of dsRNA that prevents cleavage by Dcr2, 2) binding of siRNA that prevents loading into RISC, 3) degrading siRNA and 4) direct interaction with Dcr2 or Ago2 that prevents action (Mongelli and Saleh, 2016). Viral suppressors of RNAi can combine multiple modes of action, and individual viruses can express one or more viral suppressors of RNAi. Not all viruses express viral suppressors of RNAi or even induce upregulation of the core RNAi enzymes (Obbard et al., 2009; Mongelli and Saleh, 2016). Accordingly, the ability of viral infections to influence RNAi machinery has caused researchers to investigate the influence of common insect viral infections and viral suppressors of RNAi on RNAi efficiency.

In *A. mellifera*, infection with Israeli acute paralysis virus (IAPV) improved RNAi efficiency, whereas cricket paralysis virus (CPV), which encodes viral suppressor of RNAi 1A, had no effect (Cappelle et al., 2016b). In both infections, *Dcr2* was upregulated, indicating that the upregulation of *Dcr2* expression does not determine the outcome of dsRNA treatment during viral infection (Cappelle et al., 2016b). No effects on RNAi efficiency were documented in lepidopteran cell lines (Sf21 and Hi5-SF) in response to persistent viral infections that produce viral suppressors of RNAi (Swevers et al., 2016). The authors suggested that viral infection levels may be too low to impact RNAi efficiency and that viral suppressors of RNAi may act locally on core RNAi enzymes near mitochondrial membranes where viral replication occurs, but do not affect core RNAi enzymes located throughout the cytoplasm (Swevers et al., 2016). In contrast, in *D. melanogaster*, expression of two insect viral suppressors of RNAi and three out of six plant viral suppressors of RNAi inhibited siRNA responses associated with viral RNA and injected dsRNA, suggesting that some viral suppressors of RNAi can negatively impact RNAi efficiency in some systems (Berry et al., 2009). Together these studies hint at the diverse ways that viral infections can impact RNAi efficiency in insects. Work is needed to determine how commonly RNAi efficiency is impacted by viruses, and what parameters determine if the effect on RNAi efficiency is positive or negative so that RNAi efficiency issues associated with viral infections or other stressors can be overcome.

Interestingly, RNAi efficiency among insect orders is hypothesized to correlate with the prevalence of viral infections in each order (Swevers et al., 2013b). Most insect viruses attack lepidopterans, and to some lesser extent dipterans, which are both refractory to RNAi. Whereas coleopterans, hymenopterans, orthopterans, and blattodeans have very low levels of viral infections (Swevers et al., 2013b). It is possible that a functional RNAi response may have been selected against in lineages of insects characterized by high viral loads in favor of alternative viral defense mechanisms that cannot be overcome by viral suppressors of RNAi, such as overexpression of dsRNases or downregulation of viral entry pathways (Swevers et al., 2013b). In addition, functional redundancy among the core enzymes and crosstalk between RNAi pathways could be mechanisms that evolved in insects to overcome viral suppressors of RNAi (Obbard et al., 2009). The core RNAi enzymes are among the fastest evolving genes in the *D. melanogaster* genome (Obbard et al., 2006) and overlap among the three RNAi pathways occurs

in many species (Zhou et al., 2008; Berry et al., 2009; Kolliopoulou and Swevers, 2013; Nie et al., 2013, Wang et al., 2013; Yoon et al., 2016)

The previous subsection demonstrated the diverse ways that overlap between the three RNAi pathways occurs, and suggested functional redundancy among Ago proteins in multiple insect lineages. The effects of overlap between the RNAi pathways on RNAi efficiency are not clear, however, and functional redundancy among Dicer proteins has not been well investigated. We speculate that having multiple core enzymes from multiple RNAi pathways participate in the siRNA response might enhance RNAi efficiency in the presence of viral suppressors of RNAi, but decrease RNAi efficiency in their absence due to competition between pathways. Nonetheless, there is evidence to conclude that the expression of the core enzymes does influence RNAi efficiency in insects, as does the presence of some, but not all, viral infections. In addition, all these investigations underscore the variation there is in the composition, upregulation, expression, and function of the core RNAi machinery among insects.

Concluding remarks on mechanisms of RNAi in insects literature review

RNAi efficiency in insects can be limited by dsRNA instability, incomplete dsRNA internalization, deficient core RNAi machinery, and impaired systemic spreading. Instability of dsRNA is attributed to dsRNases and physiological pH in the gut, as well as in salivary secretions and hemolymph (Table S1-2 and references within). Incomplete dsRNA internalization is a complex issue that can be due to insufficient endosomal escape (Shukla et al., 2016), lipid-based RNAi blocking factors (Li et al., 2015b; Dong et al., 2017), or possibly different internalization mechanisms (endocytosis (Saleh et al., 2006; Ulvila et al., 2006; Xiao et al., 2015) vs transmembrane channels (Xu et al., 2013; Miyata et al., 2014; Cappelle et al., 2016a; Yoon et al., 2016). Efforts to determine the role of insect *Sils* in endocytosis (Cappelle et al., 2016a), and the mechanism(s) of dsRNA uptake from the gut and hemolymph are badly needed. It is unclear if lipophorins are involved in uptake (Sakashita et al., 2009; Wynant et al., 2014a) or sequestration (Kobayashi et al., 2012) of dsRNA, but their influence on RNAi efficiency also requires investigation.

The composition, upregulation, expression, and function of the core RNAi enzymes varies considerably among insect species, and expression appears to influence RNAi efficiency more than gene copy number (Table S1-5). Another reason that RNAi efficiency might vary

among insect species is that the physiological environment within the insect or tissue may influence systemic spreading mechanisms, with more protective reagents such as extracellular vesicles or binding proteins (Jose, 2015) favored in insects that have high nuclease activity in the hemolymph. However, the effects of having enzymes from multiple RNAi pathways participate in the endogenous RNAi response in insects are still ambiguous (Table S1-5 and references within) Comparisons between the biochemical kinetics of core RNAi enzymes of different insect species are encouraged. In *D. melanogaster*, systemic RNAi is mediated by 1) nanotube-like structures that transport dsRNA and components of the RNAi machinery between adjacent cells (Karlikow et al., 2016), and 2) by siRNAs encased in exosome-like vesicles that originate from hemocytes after siRNA amplification via reverse transcriptase (Saleh et al., 2009; Tassetto et al., 2017).

The development of a novel sequencing technique (Tassetto et al., 2017) for assessing whether secondary siRNAs are synthesized will undoubtedly facilitate the investigation of amplification and systemic mechanisms in other insects. This is important because achieving lethal RNAi effects for pest control, midgut-specific target genes will need to be utilized for insects lacking systemic RNAi mechanisms, whereas genes expressed in any tissue can be targeted in insects with systemic spreading mechanisms. Furthermore, it may be possible to use lower doses of dsRNA in insects that amplify siRNA. Unfortunately, the application of dsRNA alone (in the absence of an active viral infection) may not sufficiently induce the required immune response necessary to initiate systemic RNAi in some insects (Tassetto et al., 2017). Therefore, future efforts will likely focus on determining the extent to which insufficient immune stimulation contributes to low RNAi efficiency in insects. In addition, attempts to use viral components to overcome this limitation are likely to follow.

Currently, transgenic platforms and genome-wide RNAi screens are used to select target genes (Table S1-1). In the future, identifying common characteristics of responsive target genes in insects will aid in our selection of ideal target genes. While further study is still needed to fully elucidate the physiological basis of all mechanisms affecting RNAi efficiency in each insect species, progress is being made towards the implementation of RNAi technology for multiple insect pests, and RNAi-based techniques are being applied to entomological research worldwide (Zhu, 2013). A better understanding of mechanisms influencing RNAi efficiency will allow the development of RNAi-based pest management strategies for a broader range of insect

species, as well as identify potential mechanisms of resistance. Given the rate at which molecular mechanisms influencing RNAi are being explored, the applications of RNAi in applied entomology are expanding rapidly.

Dissertation Objectives

The overall goal of this project is to use the European corn borer (ECB), *Ostrinia nubilalis* (Hübner), as a representative species to study mechanisms limiting RNAi efficiency in lepidopterans. I hypothesize that one or more of the following mechanisms are lowering RNAi efficiency in lepidopterans (Fig. 1-2):

i) Instability of double-stranded RNA (dsRNA) in the gut, hemolymph, and/or saliva.

ii) Insufficient dsRNA internalization through endocytosis, transmembrane channels, or other mechanisms.

iii) Deficient function or expression of core RNAi machinery.

iv) Impaired systemic spreading of silencing signals to distant tissues.

v) Refractory target genes.

Thus, my dissertation focuses on investigating the role some of these mechanisms play in governing RNAi efficiency in ECB. My specific objectives are to:

1. Compare dsRNA stability in larval gut contents and hemolymph in ECB.
2. Identify and characterize dsRNA-degrading nuclease genes (OndsRNases, *OnREase*) from ECB.
3. Identify and characterize core RNAi pathway genes (*OnDcr2, OnR2D2, OnAgo2*) from ECB.
4. Compare strategies for enhancing RNAi efficiency (nanoparticles, transfection reagents, and nuclease inhibitors) in ECB.

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Chapter 2 - Stability of dsRNA in Gut Contents and Hemolymph of *Ostrinia nubilalis* Larvae

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Abstract

RNA interference (RNAi) is a revolutionary technique for silencing gene expression, but the success of this technique is dependent upon the stability of double-stranded RNA (dsRNA) molecules. In many insects, especially lepidopteran species, RNAi efficiency is limited by high instability of dsRNA in the gut and/or hemolymph, preventing the development of RNAi-based strategies for many serious pests. Previous attempts to perform RNAi on *Ostrinia nubilalis* (ECB, Lepidoptera: Crambidae) indicate low RNAi efficiency with both dsRNA injection and feeding. To investigate the contribution of dsRNA instability to low RNAi efficiency in ECB, a serious of *ex vivo* incubation experiments were performed where dsRNA integrity was assessed following incubation in larval gut continents and hemolymph using gel electrophoresis or RT-qPCR. DsRNA was less stable in the gut contents from ECB than in gut contents from *Diabrotica virgifera virgifera*, a coleopteran exhibiting high RNAi efficiency. Furthermore, characterization of dsRNA stability in ECB gut contents and hemolymph revealed that dsRNA was rapidly degraded under physiologically relevant conditions as a result of enzymatic activity that was neither size- nor sequence-dependent. These findings suggest that instability of dsRNA in ECB tissues is a contributing factor to the poor efficiency of RNAi in this pest. This work advances our understanding of mechanisms impacting RNAi efficiency in ECB and related lepidopteran insects for which novel pest management strategies are needed, and may facilitate the development of strategies for enhancing dsRNA stability in ECB tissues.

Background

RNA interference (RNAi) is a highly conserved, post-transcriptional gene silencing mechanism in which small RNA molecules are utilized by a set of core machinery to degrade complementary RNA molecules in a sequence-specific manner (Timmons and Fire, 1998). In addition to being utilized as a powerful molecular tool to elucidate gene function, RNAi is also being used for pest management. RNAi-mediated crop protection strategies, including transgenic plants, baits, and sprays, are valuable resources for sustainable agriculture and food security because they provide new strategies to address yield loss (Godfray et al., 2010), combat pesticide resistance (Khajuria et al., 2018), and provide greater species-specificity to reduce off-target environmental effects (Whyard et al., 2009). Unfortunately, RNAi technology is currently hindered by inefficient and highly variable results when different insects are targeted, especially lepidopterans (Terenius et al., 2011; Kolliopoulou and Swevers, 2014), such as the European corn borer (ECB), *Ostrinia nubilalis* (Khajuria et al., 2010; Khajuria et al., 2011).

The ECB is one of the most damaging pests to corn in North American and Europe (Thieme et al., 2018). The larvae of this Crambid moth tunnel into corn stalks, causing lodging, ear droppage, kernel damage, and secondary infections (Whitworth et al., 2014). In the US, there are one to four generations of ECB per year (Whitworth et al., 2010), which cost over one billion dollars in yield losses and control efforts annually (Bohnenblust et al., 2014). In addition, ECB can feed on over 200 species of plants, and is pest of many other vegtables and crops as well (Capinera, 2000). Management of ECB usually includes monitoring, cultural practices, resistant host plants, and insecticide applications (Capinera, 2000; Whitworth et al., 2014). Currently, Bt-cry toxins are the most effective tool for controlling ECB, but the development of Bt resistance in the field has always been a serious concern (Hutchison et al., 2010; Siegfried and Hellmich, 2012; Bohnenblust et al., 2014; Thieme et al., 2018). Therefore, novel pest management strategies for ECB that utilize a novel mode of action are needed to combat resistance and to alleviate our reliance on chemical pesticides, which can have harmful environmental effects. RNAi-based pest management strategies could be an excellent option for the management of ECB if the molecular mechanisms limiting RNAi efficiency can be understood and overcome.

Instability of dsRNA in insect tissues is one of the five mechanisms known to limit RNAi efficiency in insects, especially lepidopterans (Cooper et al., 2019). DsRNA is actually more unstable in lepidopteran tissues than in tissues from other insects, which may contribute to the

extremely low RNAi efficiency exhibited by this insect order (Garbutt et al., 2013; Shukla et al., 2016; Wang et al., 2016; Singh et al., 2017; Mogilicherla et al., 2018). Prior investigations of dsRNA stability in nine species of lepidopteran insects demonstrated that dsRNA was rapidly degraded in gut contents (Arimatsu et al., 2007; Rodríguez-Cabrera et al., 2010; Liu et al., 2013; Baum and Roberts, 2014; Yang and Han, 2014; Wang et al., 2016; Vatanparast and Kim, 2017; Guan et al., 2018; Peng et al., 2018), and usually to a lesser extent in hemolymph (Garbutt et al., 2013; Liu et al., 2013; Yang and Han, 2014; Shukla et al., 2016; Wang et al., 2016; Vatanparast and Kim, 2017; Peng et al., 2018). Instability of dsRNA observed in lepidopteran gut contents (GC) and hemolymph (HE) has been attributed to nuclease activity (Arimatsu et al., 2007, Liu et al., 2013, Garbutt et al., 2013, Vantaparast and Kim, 2017; Guan et al., 2018, Guan et al., 2019) as well as physiological pH (Baum and Roberts, 2014; Song et al., 2017). Therefore, instability of dsRNA in GC and HE from ECB may contribute to the low RNAi efficiency observed in this species (Khajuria et al., 2010; Khajuria et al., 2011). However, the stability of dsRNA in ECB tissues had not been investigated.

In the present study, we investigated the stability of dsRNA in larval ECB GC and HE to determine if instability of dsRNA in these environments contributes to low RNAi efficiency in ECB. A series of incubation experiments were also performed to characterize the specificity and physiological factors contributing to dsRNA degradation in ECB GC. The results suggest that the instability of dsRNA in ECB is likely reducing RNAi efficiency in ECB.

Methods

Insect rearing

The ECB used in this study originated from French Agricultural Research (Lumberton, MN), and were continuously reared in the laboratory at Kansas State University (Manhattan, KS) at 25°C and ≥60% humidity under a 16:8 h photoperiod. Larvae were mass-reared on an ECB artificial diet in 18-cm plastic dishes equipped with wax-covered cardboard pupation rings (French Agricultural Research). Adult moths were maintained on 20% sucrose in 2-L beakers and allowed to oviposit on wax paper.

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* at different developmental stages were purchased from Crop Characteristics (Farmington, MN) as needed, and briefly maintained in the laboratory at Kansas State University. In the laboratory, larvae

were maintained in 25°C and ≥ 60% humidity under a 16:8 h photoperiod in 4.5 cm plastic dishes and mass-fed a semi-artificial diet, modified from southern corn rootworm diet (Frontier Scientific Services, Newark, DE) and optimized for WCR by Monsanto researchers (Pleau et al., 2002; Bolognesi et al., 2012). Adults were housed in 30 X 30 X 30 cm polypropylene insect rearing cages at 22°C with ambient humidity under a 16:8 h photoperiod and provided with artificial adult WCR diet containing pollen substitute (Frontier Scientific Services).

Determination of physiological pH

To determine the physiological pH of ECB HE and GC, tissue extracts were diluted and assayed with Whatman pH indicator paper (Type CF, GE Healthcare Life Sciences, Marlborough, MA). HE was collected with a pipette from five unstarved fifth-instar larval after severing their prolegs. The HE was transferred to a tube containing a small amount (about the size of a grain of rice) of phenylthiourea (PTU) to prevent melanization and then diluted 4-fold with ultrapure water (pH 7.0). GC were collected by dissecting the midgut from five unstarved fifth-instar individuals and agitating the excised guts in 30 µl of ultrapure water (pH 7.0) to release the contents. Ten µl of each sample was then applied to each square of the pH indicator paper strip, and immediately scored based on comparison to the provided color key. Three experimental replicateswere performed, each containing tissue from five individuals. Buffer Solutions pH 7.00 and pH 4.00 (Fisher Scientific, Pittsburgh, PA) were used for method validation.

Preparation of insect extracts

To obtain GC for *ex vivo* incubation assays, last-instar larvae were dissected in ice-cold PBS (phosphate-buffered saline, pH 7.4), and whole guts transferred to ice-cold tubes containing 15 µl of PBS. Fifteen guts were collected in each 0.5 ml tube and then cut repeatedly with dissection scissors to release GC. Shredded tissue samples were then centrifuged twice at 17,000 x g for 20 min at 4°C, and each supernatant was transferred to a fresh, ice-cold tube, after each spin. The supernatant of each sample was then aliquoted into ice-cold tubes and stored at -80°C until use.

To obtain HE for *ex vivo* incubation assays, the abdominal prolegs of last-instar larvae were excised, and the larvae allowed to bleed out on ice-cold Parafilm M. HE was then

transferred with a pipette to an ice-cold 1.5 ml tube containing PTU dissolved in 15 µl PBS (Garbutt et al., 2013). HE from fifteen individuals was collected per tube and then centrifuged twice at 17,000 x g for 20 min each time at 4°C. After each spin, the supernatant was transferred to a fresh, ice-cold tube. The supernatant of each sample was then aliquoted into ice-cold tubes and stored at -80°C until use.

Immediately prior to performing each *ex vivo* incubation assay, the frozen samples were thawed on ice and diluted with PBS (pH 7.0 for HE and pH 8.0 for GC) for protein quantifications using Bradford Reagent and bovine serum albumin as protein standard (Sigma-Aldrich, St. Louis, MO) according to Bonjoch and Tamayo (2001). The HE and GC samples containing specific amounts of total protein were used in the incubation assays.

Synthesis of dsRNA

To obtain dsRNA for the ex vivo incubation experiments, dsRNA was synthesized using purified PCR product which was amplified from a PCR II vector (Invitrogen, Carlsbad, CA) containing either the full length enhanced green fluorescent protein (GFP) cDNA sequence (LC336974.1) or an 800-bp cDNA fragment of the ECB or WCR lethal giant larvae gene (OnLgl or DvLgl) (GenBank accession MT467568 or MT467569). The cDNA template was purified using the QIAquick PCR Purification kit (Qiagen, Germantown, MD). Primers for amplifying dsDNA templates were designed using the E-RNAi Web service (https://www.dkfz.de/signaling/e-rnai3/). The primer sequences for dsRNA synthesis are listed in Table S2-1, and diagrams depicting the location of each dsRNA are shown in Figures S2-1 and S2-2. The HiScribe T7 High Yield RNA synthesis kit (New England Biolabs, Ipswich, MA) was used to transcribe dsRNA from DNA template following the manufacturer's instructions. Forward and reverse dsRNA strands were annealed together after synthesis by heating to 70°C for 10 min and then cooling at room temp for 20 min. Prior to ethanol washing and precipitation, dsRNA was treated with DNase 1 (Thermo Fisher Scientific, Walthan, MA) to remove DNA template. Pelleted dsRNA was resuspended in nuclease-free water and quantified using a Nanophotometer (Implen, Westlake Village, CA). The size and integrity of the dsRNA were verified on a 1.5% agarose gel. After purification, dsRNA was stored at -20°C until further use.

Incubations of dsRNA in insect tissue extracts

To investigate the relative stability of dsRNA in GC, various sizes of dsRNA were incubated in tissue extracts for multiple periods of time, and then visualized with gel electrophoresis. Specifically, 4 µl (250 ng) of dsRNA targeting either *OnLgl* or *DvLgl* was combined with 4 µl (3.5 µg total protein) of insect extract supernatant or PBS (buffer only control) and then allowed to incubate at room temperature for various periods of time (10, 30, 60 min). At the end of the incubation periods, reactions were quenched with 2 µl of EDTA (final concentration 20 mM) and immediately visualized with gel electrophoresis. To determine if nuclease activity was impacting stability of dsRNA, samples of insect GC and HE supernatants were heated (80°C for 10 min) or treated with EDTA (final concentration 20 mM) to inhibit enzymatic activity prior to incubation (Garbutt et al., 2013).

To determine the percent of dsRNA that was degraded in ECB GC and HE under various pH conditions, multiple *ex vivo* incubation experiments were performed. Each experiment consisted of dsRNA incubated in heated and unheated ECB GC or HE adjusted to a specific pH using Britton-Robinson buffers. Specifically, 1 µl (1 µg) of dsRNA targeting *GFP* was combined with 2.7 µl of normalized insect extract supernatant and either PBS or Britton-Robinson buffer (Reynolds et al., 2013) to reach a final volume of 14 µl, and allowed to incubate at room temperature for 30 min. Incubations for each tissue extract at every pH level were performed in triplicate. The total protein content of each tissue extract was adjusted so that 13.5 µg of GC extract and 75.6 µg of HE extract were used in every experiment. PBS with pH 7.0 was used to adjust the final concentration of HE extract, and PBS (pH 8.0) was used to adjust GC samples. Following incubation, reactions were quenched by heating to 65°C for 10 min. DsRNAs persisting in each sample after the incubations were quantified with reverse transcription quantitative PCR (RT-qPCR). Heated tissue extracts were used as control incubations (instead of PBS buffer) to account for bias associated with the binding of dsRNA to congealed HE.

Visualization of dsRNA stability with electrophoresis

To visualize dsRNA after incubation, 2 µl of 6X Gel Loading Buffer (Lamda Biotech, St. Louis, MO) was added to each incubation reaction and 12 µl of the mixture were loaded on a 2% (w/v) agarose gel containing 0.005% (v/v) SaveView Classic DNA Stain (Applied Biological Materials, Richmond, Canada). 100 bp DNA Ladder (Lamda Biotech) (5 µl) was also loaded on

each gel. Loaded agarose gels were then run at 100 V for 45 min in 0.5 X Tris/Borate/EDTA (TBE) buffer. DsRNA bands were visualized and photographed using a UVP high-performance UV transilluminator.

Quantification of dsRNA stability with RT-qPCR

To quantify dsRNA after incubation, half of each incubation reaction (7 µl) was combined with 2 µl of 5X PrimeScript Buffer (Takara, Mountain View, CA), 0.5 µl of PrimeScript Reverse Transcriptase (Takara), and 0.5 µl of gene-specific primer (2 µm; Table 2-2). To convert dsRNA to cDNA, these 10 µl reverse transcription reactions were incubated in a thermal cycler at 42°C for 15 min, followed by 85°C for 15 sec. The cDNA was diluted 100-fold, and 2 µl used for RT-qPCR.

RT-qPCR was performed on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA) using BrightGreen 2x qPCR Master Mix (Applied Biological Materials) in a 20 µl reaction with the following program: 95°C for 3 min, 49 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by a melt curve analysis to determine amplicon specificity using a temperature range from 65 to 95°C. Primer sequences for quantifying ds*GFP* abundance are included in Table S2-1. All qPCR reactions were performed using two technical replicates of each of at least three biological 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. The primer efficiency [$E=10^{(-1/slope)}$] and percent primer efficiency [%E=(E-1)*100%] for each primer pair were calculated using the standard curve method for each primer pair.

Serial dilutions of ds*GFP* were used to construct standard curves for quantification of dsRNA with RT-qPCR. Ct values corresponding to five known dilutions of ds*GFP* were plotted against the log of ds*GFP* quantity (4, 0.4, 0.04, 0.004, 0.0004 ng) and analyzed by linear regression to obtain a linear equation representing the relationship between Ct values and ds*GFP* quantity in Log of nanograms (Li et al., 2005). The slope (m) and y-intercept (b) values from the linear equation were used to calculate the amount of the remaining ds*GFP* following *ex vivo* incubation with GC or HE from Ct values obtained from RT-qPCR using the equation: [ng of dsRNA=10^((Ct-b)/m)]. Heated tissue extracts were used for control incubations to account for the binding of dsRNA to HE or GC. Percent degradation of dsRNA was then calculated based on the ratio of the ng of dsRNA that persisted in the unheated vs. heated tissue extracts at each pH

using [% degradation=((heated-unheated)/heated)*100%]. Percent degradations of dsRNA in ECB HE and GC at various pH conditions between pH 3.0 and pH 10.0 were then plotted on a single graph for comparison.

Statistical analyses

Statistical differences between treatment means for each incubation experiment were assessed in Minitab 19 software with a two-tailed t-test. Differences between treatment means at each pH level for both tissue extracts were assessed with a one-way analysis of variance (ANOVA) followed by the Tukey procedure depending on the number of groups that were compared. Significance levels (α) were set at $p \leq 0.05$ 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 were met. In all experiments, treatments were replicated at least three times.

Results

Comparison of dsRNA stability in ECB and WCR gut contents

Bands from dsRNA incubated in heated or unheated WCR GC were comparable to the bands produced by dsRNAs incubated in PBS only for all time points and dsRNA lengths (Fig. 2-1) suggesting that dsRNA is stable in WCR GC for at least one hour. Conversely, incubation of dsRNA in unheated GC from ECB caused gel bands to fade substantially when compared to heated GC or PBS after 10 min. In the case of the 500 and 800 bp dsRNAs, no band was visible after 10 min in ECB GC, but significant streaking was observed on the gel. Similarly, after 30- and 60-min incubations in ECB GC, the dsRNA bands appeared as faint, low-molecular-weight streaks, or the bands were completely absent.

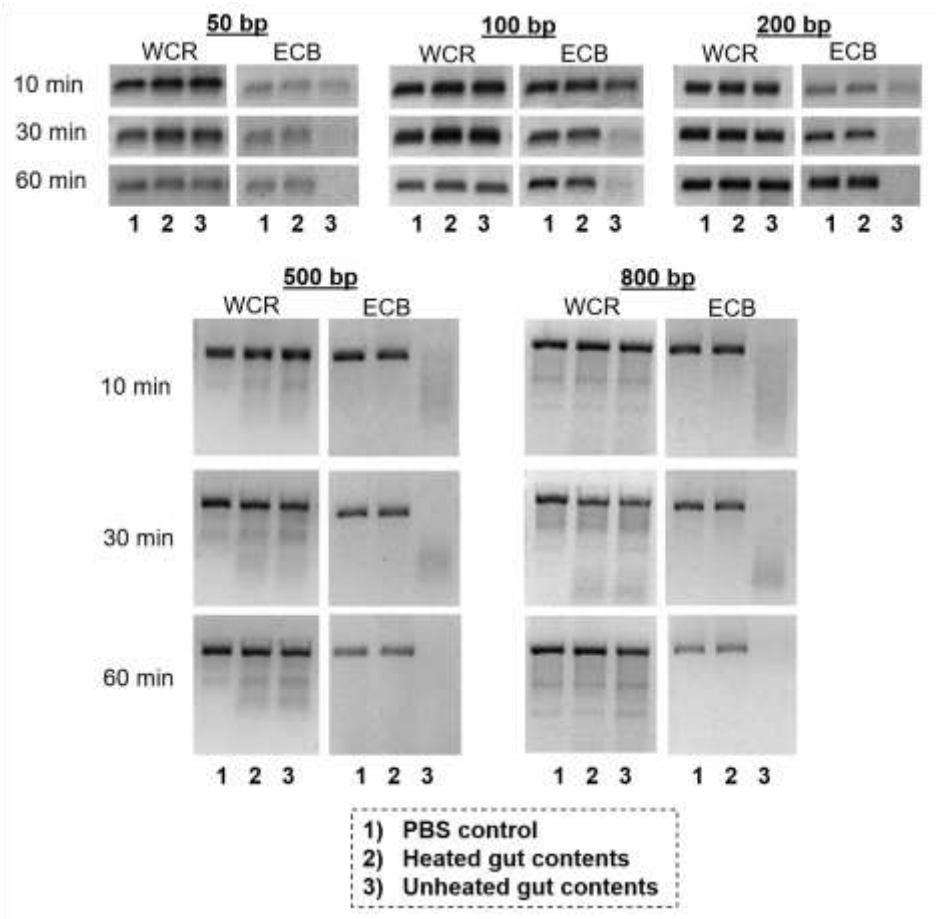

Figure 2-1. Representative gel images showing that dsRNA was rapidly degraded in ECB gut contents but not WCR gut contents.

The relative stability of 250 ng of A) 50 bp, B) 100 bp, C) 200 bp, D) 500 bp, and E) 800 bp, conspecific dsRNA targeting the *Lgl* gene of each species, after incubation for 10 min, 30 min, or 60 min in 1) PBS control buffer, 2) 3.5 µg of heated gut contents, or 3) 3.5 µg of unheated gut content collected from thirty ECB or WCR larvae. Intact dsRNA appeared as dark bands, whereas degraded dsRNA appeared as lighter bands or a faint streak.

Effects of dsRNA size on stability of dsRNA in ECB gut contents

Comparison of the loss of dsRNA bands for the different sizes of dsRNAs after incubation in ECB GC for 10 min suggested that dsRNA stability may be size-dependent (Fig. 2-2 left panel). Shorter bands (50, 100, or 200 bp) appeared as intact bands, comparable to the control incubation bands, though fainter, whereas longer dsRNAs (500, or 800 bp) appeared as streaks on the gel. To determine if shorter dsRNAs were more stable in ECB GC than longer dsRNAs, another incubation experiment was performed in which dsRNA amounts between different sizes were normalized by molar concentration instead of molecular mass. As in our

earlier experiments, heated GC had no effect on the appearance of dsRNA bands when compared to PBS for either the 100 or 500 bp dsRNA (Fig. 2-2, right panel). After incubation of ds*OnLgl* for 10 min in unheated ECB GC, the 100 bp band was completely absent on the gel, whereas the 500 bp band was visible as a faint band with a dark streak below it (Fig. 2-2, right panel). These results indicate that dsRNA stability in ECB GC is not size-dependent.

To generate additional support that dsRNA degradation in ECB GC was enzymatic in nature, a fourth treatment was also added to this experiment, in which 20 mM EDTA was incubated with unheated GC and dsRNA. The addition of 20 mM EDTA in the *ex vivo* incubation resulted in a dark dsRNA band comparable to dsRNA incubated in the PBS control or in heated ECB GC (Fig. 2-2, right).

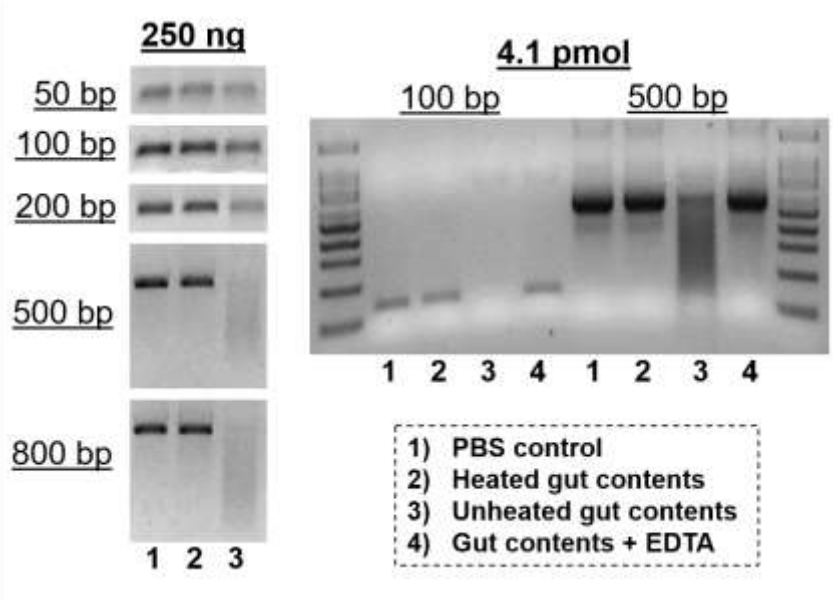

Figure 2-2. Representative gel images showing that degradation of dsRNA in ECB gut contents is not size-dependent.

The relative stability of A) 250 nanograms or B) 4.1 picomoles of various lengths of conspecific dsRNA targeting *OnLgl*, after a 10 min incubation in 1) PBS control buffer, 2) 3.5 µg of heated gut contents, 3) 3.5 µg of unheated gut contents, or 4) 20 mM EDTA with 3.5 µg of unheated gut contents, collected from thirty ECB larvae.

Effects of dsRNA sequence on dsRNA stability in ECB gut contents

To determine if dsRNA degradation in ECB GC is sequence- or species-specific, four additional 50 bp dsRNA constructs targeting various regions of *Lgl* from ECB and WCR were

designed (Fig. S2-2). When the four conspecific and four heterospecific dsRNAs were incubated for 10 or 30 min in unheated ECB GC, all eight dsRNAs appeared to degrade at similar rates (Fig. 2-3). Therefore, the dsRNA sequence difference does not seem to affect dsRNA stability when incubated with ECB GC.

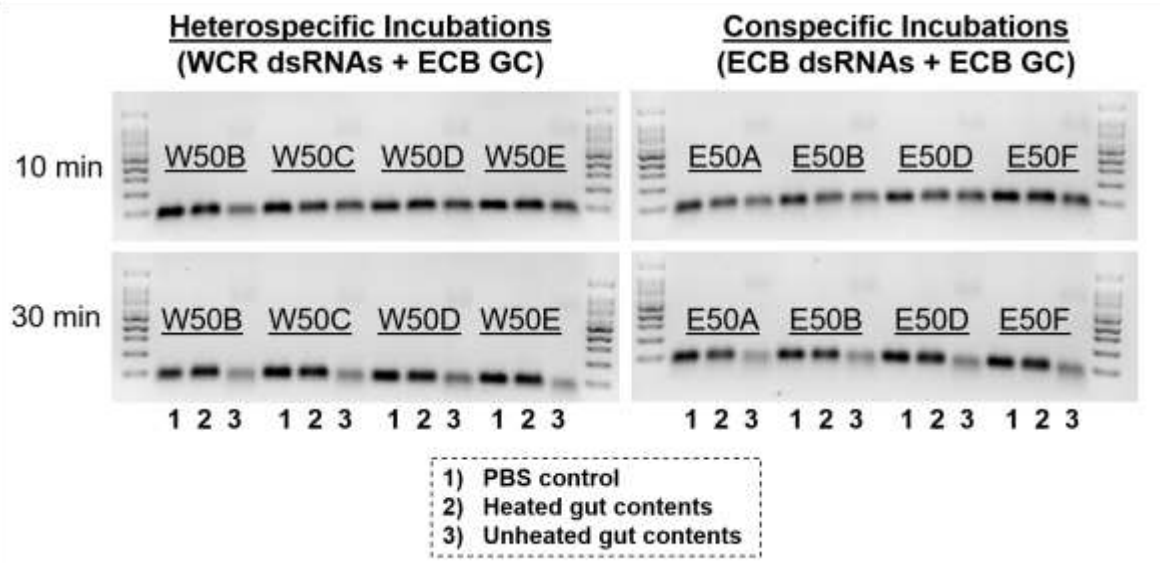

Figure 2-3. Representative gel images showing that degradation of dsRNA in ECB gut contents is not sequence- or species-specific.

Representative gel images of four 50 bp dsRNA constructs targeting different regions of *DvLgl* or *OnLgl* after incubation for 10 min and 30 min in 1) PBS control buffer, 2) 3.5 µg of heated gut contents, or 3) 3.5 µg of unheated gut content from thirty ECB larvae. In heterospecific incubation, W50B, W50C, W50D, and W50E represent ds*DvLgl* (50 bp) from the western corn rootworm (W) that were incubated with the gut contents (GC) from ECB larvae. In conspecific incubation, E50A, E50B, E50D, and E50F represent ds*OnLgl* (50 bp) from ECB (E) that were incubated with the gut contents from ECB larvae.

Comparison of dsRNA stability in ECB gut contents and hemolymph

To further examine if *ex vivo* degradation of dsRNA in ECB HE and GC could be influenced by the different amounts of total protein in the incubation, we compared dsRNA band integrity after incubation of dsRNA in equal amounts of total protein from each tissue extract. DsRNA incubated in 3.5 µg of unheated ECB GC resulted in a low-molecular-weight smear instead of a 500 bp band after only 10 min, a low molecular weight smear after 30 min, and no detectible nucleic acids after 60 min. In contrast, incubation of 500 bp dsRNA in 3.5 µg of

unheated ECB HE resulted in a weak band with a high molecular weight smear (compared to heated-HE and buffer-only controls) even after a 60 min incubation (Fig. 2-4).

Experimentation with incubations of dsRNA in ECB HE, and particularly undiluted HE had a tendency to mask the presence of dsRNA on a gel, producing very dark, high molecular weight bands with or without exogenous dsRNA (Fig. S2-3). Serial dilutions of HE revealed that the 500 bp dsRNA band was masked in ECB HE that was diluted less than one hundredfold (Fig. S2-3B, C), a concentration that may not yield biologically relevant data. Accordingly, we developed a RT-qPCR method for assessing dsRNA stability in undiluted ECB HE for subsequent experiments.

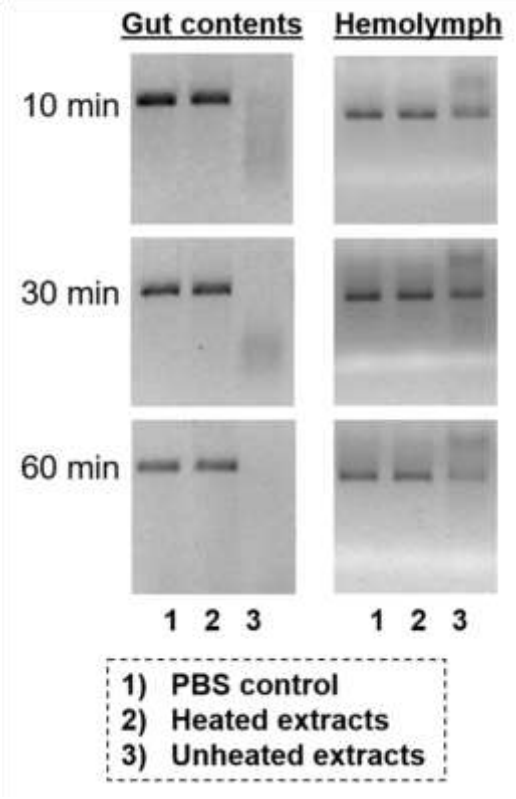

Figure 2-4. Representative gel images showing greater stability of dsRNA in ECB hemolymph than gut contents when the same amount of total protein from both tissue extracts were used.

The relative stability of 250 ng of 500 bp dsRNA targeting *OnLgl*, after incubation for 10 min, 30 min, or 60 min in 1) PBS control buffer, 2) 3.5 µg of heated insect extracts or 3) 3.5 µg of unheated insect extracts from thirty ECB larvae.

Effects of pH on dsRNA stability in ECB gut contents and hemolymph

To assess dsRNA stability in ECB HE, an assay using RT-qPCR was developed, similar to the protocol described by Wang et al. (2016). In brief, after *ex vivo* incubation of dsRNA in ECB HE, the remaining dsRNA was used as a template for reverse transcription into cDNA for subsequent quantification with standard qPCR. Ct values obtained from RT-qPCR for each sample were used to calculate the amount of the remaining ds*GFP* based on a standard curve generated with known amounts of ds*GFP* (Fig. S2-4). DsRNA targeting *GFP* was used instead of *Lgl* to ensure that only cDNA generated from exogenous dsRNA was amplified during RT-qPCR and not from endogenous mRNA. In addition, specific primers for ds*GFP* were used to synthesize cDNA to ensure that the efficiency of reverse transcription was not biased by the presence of mRNA in reactions containing insect extracts allowing for unbiased quantification of dsRNA stability in insect extracts including HE or GC.

Comparison of dsRNA stability in ECB tissue extracts adjusted to various pH levels indicated that ds*GFP* was significantly degraded under all pH conditions in unheated GC compared to heated GC, and under all pH conditions in unheated HE compared to heated HE, except for pH 3.0 (Table 2-1, Table S2-2). Measurements of the physiological pH of ECB HE and GC showed they were close to pH 7.0 and 8.0, respectively. At pH 7.0, dsRNA was degraded 99.5% on average in ECB HE, and at pH 8.0 dsRNA was degraded 97.9% on average (Fig. 2-5). However, the dsRNA degrading activity of these extracts showed a wide pH range (Table 2-1, Fig. 2-5). More than 95% of dsRNA was degraded in ECB HE at pH 5.0 and above, as well as in ECB GC at pH 6.0 and above. Even at pH 4.0 in ECB HE, more than 78% of the dsRNA was degraded in 30 min, although this was significantly less degradation than occurred in HE at higher pH conditions [$F(7,16)=62.08$, $p<0.0001$*]. Similarly, in ECB GC, more than 70% degradation of dsRNA occurred at pH 3.0, 4.0, and 5.0 [$F(7,16)=3.82$, $p<0.013$*].

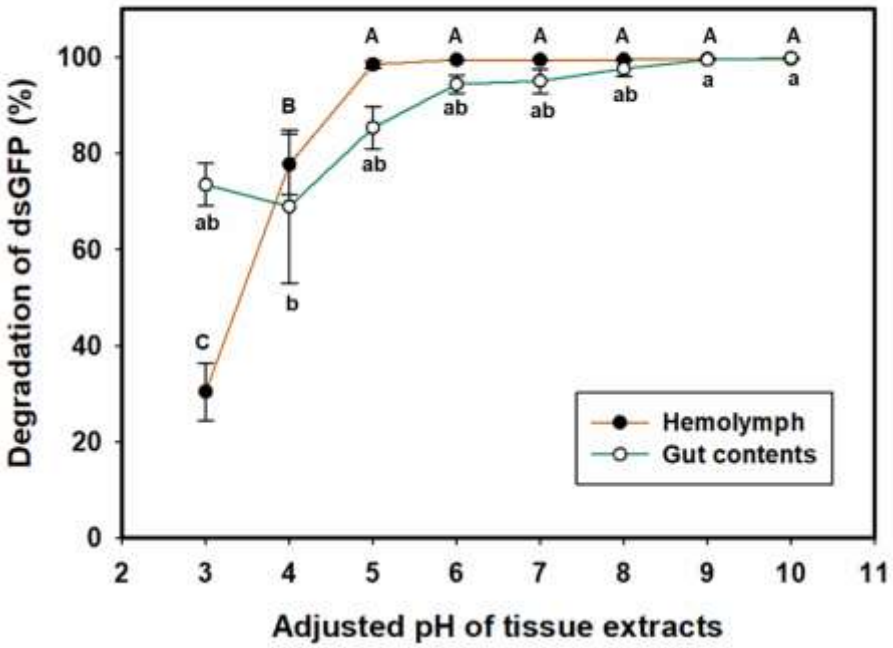

Figure 2-5. Graph showing the degradation of dsRNA after 30 min in ECB hemolymph and gut contents under physiologically relevant pH conditions.

The percent of ds*GFP* molecules in each incubation that was degraded in unheated tissue extracts relative to heated tissue extracts collected from 15 fifth-instar ECB larvae, and adjusted to various pH levels. Means that do not share a letter are significantly different; upper case letters refer to HE and lower case letters to GC.

Table 2-1. Recovery of ds*GFP* after *ex vivo* incubation in ECB tissue extracts.

The mean nanograms (plus and minus standard error) of 300 bp ds*GFP* that was recovered after a 30 min incubation in heated (i.e., heat denatured) and unheated ECB tissue extracts adjusted to various pH levels are presented. Significant differences between treatments are indicated with an asterisk.

	Hemolymph (HE)		**Gut Contents (GC)**	
pH	Heated-HE (ng) [control]	Unheated-HE (ng)	Heated-GC (ng) [control]	Unheated-GC (ng)
3.0	2.16±0.310	1.47±0.113	135±15.4	35.8±8.15*
4.0	101±11.8	21.2±4.62*	145±14.0	41.1±17.3*
5.0	148±12.8	2.11±0.995*	128±9.42	19.3±6.28*
6.0	199±34.3	1.10±0.212*	172±19.7	8.90±2.07*
7.0	148±21.7	0.744±0.169*	189±34.0	7.74±3.07*
8.0	90.6±9.90	0.438±0.100*	313±30.4	6.58±3.83*
9.0	160±36.8	0.521±0.080*	271±37.2	1.17±0.844*
10.0	208±50.8	0.808±0.370*	287±45.2	0.433±0.222*

Discussion

In the present study, we demonstrate that dsRNA is highly degraded in both ECB GC and HE (Fig. s. 2-1, 2-5), but is stable in WCR GC (Fig. 2-1). This is consistent with prior reports of dsRNA stability in other lepidopterans and coleopterans. Interestingly, dsRNA stability in coleopteran GC is reported to be more variable between species than dsRNA stability in lepidopteran species (Singh et al., 2017). In coleopteran GC from *Cylas puncticollis* (Prentice et al., 2017), *Leptinotarsa decemlineata* (Prentice et al., 2017; Spit, 2017), *Anthonomus grandis* (Garcia et al., 2017), and *Cylas brunneus* (Prentice et al., 2017) dsRNA is degraded after 5 min, 10 min/1h, 30 min, and 1 h , respectively. However, in *Zoophobas atratus* (Wang et al., 2016), *Holotrichia diomphalia* (Guan et al., 2018), and *Aethina tumida* (Powell et al., 2017), dsRNA is stable for over one hour, if not much longer. WCR appears to fit into this second group of beetles that exhibit high stability of dsRNA in the GC. In contrast to Coleoptera, in most lepidopterans studied, including *Bombyx mori* (Liu et al., 2013), *Manduca sexta* (Garbutt et al., 2013), *Spodoptera frugiperda* (Rodríguez-Cabrera et al., 2010; Baum and Roberts, 2014), *Heliothis virescens* (Shukla et al., 2016), and *Helicoverpa armigera* (Yang and Han, 2014), dsRNA is completely degraded in GC in <10 min. However, in the GC of *Ostrinia furnacalis* (Guan et al., 2018), *Spodoptera litura* (Wang et al., 2016; Peng et al., 2018), and *Spodoptera exigua* (Vatanparast and Kim, 2017), dsRNA is stable for nearly an hour, so dsRNA stability in ECB appears to be somewhere between these two groups of lepidopterans.

Multiple groups have previously reported a correlation between dsRNA degradation and low RNAi efficiency after using a comparative approach with various insect species exhibiting high and low RNAi efficiency (Garbutt et al., 2013; Wang et al., 2016; Prentice et al., 2017; Singh et al., 2017; Cao et al., 2018; Peng et al., 2018). In addition, pre-digestion of dsRNA in *Schistocerca gregaria* GC lowered oral RNAi efficiency in *L. decemlineata* larvae (Spit et al., 2017). Our results show that dsRNA is quickly degraded in both HE and GC of a pest known to exhibit low RNAi efficiency. Taken together, these results suggest that the degradation of dsRNA contributes to low RNAi efficiency in insects, including ECB.

Peng et al. (2018) reported that the dsRNA degrading activity in GC was over 100-fold higher than in other tissues for *S. litura*, *Locusta migratoria*, *Periplaneta americana*, and *Z. atratus*, which differs from our findings that dsRNA is highly degraded in both the HE and GC of ECB. This discrepancy could relate to differences in how dsRNA degradation was measured

in these studies (endpoint vs. kinetic assays). Alternatively, dsRNA may be more degraded in ECB HE than in HE from other insects, which could explain why ECB is so highly refractory to both oral and injected RNAi (Chapter 5). Further investigation is needed to determine how dsRNA stability in ECB compares to stability in other insects exhibiting low RNAi efficiency. Unfortunately, variation in the methods used to investigate dsRNA stability in insects (i.e., gel electrophoresis, RT-qPCR, and microplate enzymatic assays) and in the units used to report dsRNA persistence (see Table S1-2 from Cooper et al., 2019 for examples), severely limits comparisons of dsRNA stability and persistence among studies. Direct quantification of dsRNA with RT-qPCR based on a dsRNA standard curve, as described in this study and previously used by Wang et al. (2016), is advantageous because it allows for accurate measurement of dsRNA target molecules that persist in undiluted insect tissues both *in vivo* and *ex vivo,* that can be easily compared between different insects, tissues, and laboratory groups. The use of biologically relevant concentrations of insect tissue extracts is vitally important when assessing dsRNA stability. DsRNA initially appeared more stable in ECB HE than in ECB GC when incubated in the same amount of total protein (Fig. 2-4). However, the total protein content of ECB HE was roughly tenfold higher than total protein content of the GC *in vivo* (data not shown), and when this ratio was maintained and dsRNA stability measured with RT-qPCR it became apparent that dsRNA is highly degraded in both ECB GC and HE (Fig. 2-5). In addition, protein concentration is known to vary largely between individuals of the same species, so pooling multiple individuals per biological replicate is important to capture this variation, as well as to provide sufficient material for analysis.

Degradation of dsRNA in insect tissues is primarily attributed to nuclease activity as well as physiological pH (Baum and Roberts, 2014). Heating can denature enzymatic activity by causing conformational changes in protein structure. In addition, many nucleases require divalent cations for enzymatic activity, and EDTA forms bonds with metal ions, depriving enzymes of these cofactors (Arimatsu et al., 2007). In the present study, we show that the degradation of dsRNA was inhibited by heat denaturing and by EDTA treatment, supporting the hypothesis that nuclease activity contributes to the instability of dsRNA in ECB (Fig. 2-2). These results are consistent with a similar dsRNA stability experiment that used heating and EDTA to limit dsRNA degradation in insect tissues (Garbutt et al., 2013; Cao et al., 2018).

Interestingly, when dsRNA stability was compared between *Tribolium castaneum* and *Acyrthosiphon pisum*, the authors noted differences in the appearance of the degradation products (Cao et al., 2018). DsRNA incubated in *T. castaneum* extracts appeared as a smaller band that decreased in size over time, whereas dsRNA incubated in *A. pisum* extracts appeared as a faint smear that rapidly disappeared. The authors claim these patterns are indicative of exonuclease and endonuclease respectively and suggest that rapid degradation in *A. pisum* is due to exo- and endo-nucleases, whereas slow degradation in *T. castaneum* is due to endo-nucleases only. A similar phenomenon as that described in *A. pisum* occurred for 500 and 800 bp dsRNA in ECB (Fig. 2-1), suggesting that the nature of the ribonucleases acting in ECB and *A. pisum* may be similar. However, this hypothesis requires further study. The stability of dsRNA in insect tissues may also vary among developmental stages (Spit et al., 2017; Vantanparsest and Kim, 2017) and in response to starvation (Rodriguez-Cabrera et al., 2010). These variables may impact dsRNA stability in ECB, but were also outside the scope of the current investigation.

We also showed that dsRNA stability is pH-dependent in ECB HE and GC (Fig. 2-5). However, the pH range over which dsRNA degradation occurred in ECB was wider than for other lepidopterans such as *B. mori* (Arimastu et al., 2007a), *S. exigua* (Baum and Roberst, 2014), and *S. litura* (Peng et al., 2018), which were shown to exhibit maximum dsRNA degradation under highly alkaline pH conditions only. High activity under a broader range of physiological conditions may be one factor that makes dsRNA less stable in ECB than in other insects.

The pH conditions in the midgut are known to vary greatly within the gut as well as among insects, (reviewed in Cooper et al., 2019), so the physiological pH of ECB extracts was measured to determine which of the tested pH conditions were physiologically relevant. Various methods were tested to measure the pH of the gut and HE in ECB, including paper pH indicator strips, universal pH indicator dies, and a micro pH probe. Ideally, pH is measured *in vivo* so the pH gradient that exists in the midgut can be visualized without interrupting cellular vATPase function (Harrison, 2001). Unfortunately, *in vivo* measurements failed because the micro pH probe and universal indicator dye both yielded inconsistent readings. Ultimately, Whatman pH Indicator Paper (Type CF) proved most effective. The pH conditions measured in ECB were fairly similar to the pH conditions reported in *S. litura* HE (pH 6.69) and midgut (pH 8.72) (Peng et al., 2018).

Unlike previous studies, we investigated how characteristics of dsRNA molecules themselves impact dsRNA stability. Our results indicate that dsRNA stability in ECB GC is size-independent (Fig. 2-2) and sequence-independent (Fig. 2-3). Shorter lengths of dsRNA initially appeared more stable in ECB GC than shorter dsRNAs when loaded by mass, because shorter dsRNA molecules have less mass than longer dsRNA molecules. For example, five times as many 100-bp dsRNA molecules as 500-bp dsRNA molecules were needed to equal the same mass (i.e., 250 ng of 100-bp dsRNA is 4.105 pmol, whereas, 250 ng of 500-bp dsRNA is 0.823 pmol). Therefore, the differing ratios of dsRNA-degrading enzymes to dsRNA molecules in the 100-bp incubations versus the 500-bp incubations were likely responsible for the size-dependent pattern observed when the gel was loaded according to dsRNA mass (Fig. 2-2). Thus degradation of dsRNA was not size-dependent in the sense that a certain length would prevent degradation. However, it is possible that there could still be differences in the efficiency of nucleases to degrade molecules of different sizes, and this possibility deserves further investigation.

Our finding that all eight dsRNAs appear to deteriorate at the same rate indicates that dsRNA degradation in ECB GC is likely sequence- and species-independent. If dsRNA degradation was sequence-specific, dsRNAs of different sequences would be expected to degrade at different rates because they would each presumably contain different numbers of the target sequence(s). However, if dsRNA degradation was sequence-independent, then all the 50-bp dsRNAs would be expected to be degraded at the same rate. Therefore, our finding that dsRNA stability is sequence-independent indicates that dsRNA molecules targeting different sequences, genes, or species all degrade at similar rates in ECB. For our investigation, we used dsRNA targeting various regions of *Lgl* from ECB and from WCR, but these results likely apply to other target genes and sequences as well. Nonetheless, further testing is required to confirm that dsRNA degradation is not size-, sequence-, or species-specific in other insects.

Taken together, the findings presented in this study support the hypothesis that instability of dsRNA is a common issue among insects, especially lepidopterans, which must be overcome in order to enhance RNAi efficiency and facilitate the development of RNAi-based pest management strategies. Strategies to enhance dsRNA stability in insect tissues are needed. Reagents such as nuclease inhibitors (Castellanos et al., 2018), nanoparticles (Zhang et al., 2010; Christiaens et al., 2018), and transfection reagents (Lin et al., 2017; Castellanos et al., 2018) can likely be used to protect dsRNA. Using transgenic microbes (Yang and Han, 2014), yeast

(Abrieux and Chiu, 2016), baculoviruses (Liu et al., 2019), and endosymbionts (Whitten et al., 2016) expressing dsRNA as delivery systems for RNAi may also help prevent dsRNA degradation. Large doses of dsRNA and continuous feeding of dsRNA may overwhelm nucleases (Prentace et al., 2017). Additionally, it may be possible to avoid dsRNA degradation by targeting developmental stages and tissues with low nuclease expression/activity. Together, these reagents and strategies may provide solutions for enhancing dsRNA stability in insects. However, RNAi efficiency in lepidopterans could also be limited by additional mechanisms of low RNAi efficiency, such as insufficient internalization of dsRNA, inadequate endosomal escape, the insufficient response of the core RNAi pathway genes, and/or refractory target genes. Therefore, additional effort is needed to understand the contribution of multiple mechanisms to low RNAi efficiency in agriculturally relevant lepidopteran pests, such as ECB.

Conclusion

This investigation demonstrated that dsRNA was less stable in the GC from ECB than in GC from WCR, a coleopteran exhibiting high RNAi efficiency. Characterization of dsRNA stability in ECB GC and HE revealed that dsRNA was rapidly degraded under physiologically relevant conditions, dsRNA degradation was mainly due to enzymatic activity, and dsRNA degradation was not size or sequence-dependent. Together, these findings indicate that instability of dsRNA in ECB tissues is likely contributing to low RNAi efficiency in ECB, as was demonstrated in other insects (Wang et al., 2011; Spit et al., 2017). These findings are significant because they suggest that enhancing dsRNA stability in ECB tissues may improve RNAi efficiency. This knowledge advances our understanding of mechanisms impacting RNAi efficiency in ECB and may facilitate the development of strategies for enhancing dsRNA stability in ECB tissues and related lepidopteran insects for which novel pest management strategies are needed.

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Chapter 3 - Molecular Characterizations of Double-Stranded RNA Degrading Nuclease Genes from *Ostrinia nubilalis*

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Abstract

Variable RNA interference (RNAi) efficiencies limit RNAi-based pest management strategies for many pests. Previous efforts to understand mechanisms contributing to low RNAi efficiency indicate that double-stranded RNA (dsRNA) is degraded in the European corn borer (ECB), *Ostrinia nubilalis*, due to nuclease activity. To investigate the contribution of dsRNA-degrading endonucleases (dsRNases) and lepidopteran-specific RNAi efficiency-related nucleases (REases) to dsRNA instability and low RNAi efficiency in ECB, five complementary DNAs putatively encoding four dsRNases (*OndsRNase1*, *2*, *3*, and *4*) and one REase (*OnREase*) were sequenced. Characterization of these transcripts revealed that substrate specificity might vary among the four dsRNases due to different amino-acid combinations in the substrate-binding sites. Gene expression analysis indicated that *OndsRNase2* and *OnREase* were highly expressed in the larval gut, and *OndsRNase1* showed the highest expression in hemolymph, especially in older developmental stages. Transcript level analysis after dsRNA exposure revealed that expression of *OnREase* rapidly increased upon dsRNA ingestion or injection, whereas *OndsRNase4* expression only increased after long-term ingestion of dsRNA. While the biological function of these nucleases remains to be verified, our results suggest that OnREase and OndsRNase2, and OndsRNase1 and OndsRNase4 may be responsible for degradation of dsRNAs in the ECB gut and hemolymph, respectively, thereby contributing to low RNAi efficiency.

Background

RNA interference (RNAi)-mediated gene suppression uses double-stranded RNA (dsRNA) molecules to induce degradation of specific messenger RNAs before translation into proteins. RNAi pathways can be exploited by experimentally introducing dsRNA to silence specific genes, and elucidate their function or induce mortality (Dowling et al., 2016). As a result, RNAi is commonly used in the laboratory to analyze gene function, and RNAi-based pest management strategies are now being developed (Borel, 2017; Head et al., 2017). Unfortunately, RNAi is not equally effective in all insect taxonomic groups, limiting the ability of RNAi to control some insects, especially lepidopterans (Terenious et al., 2011; Kolliopoulou and Swevers, 2014).

The European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is one of the most destructive pests of corn in the US and Europe (Thieme et al., 2018). *Bacillus thuringiensis* (Bt) Cry toxins and conventional insecticides are the most effective tools for controlling ECB, but the development of resistance in the field is compromising the effectiveness of traditional control methods (Siegwart et al., 2017; Yu et al., 2018), highlighting the need for developing new control strategies that utilize novel modes of action, such as RNAi. Unfortunately, ECB is one of the lepidopteran pests that exhibit very low RNAi efficiency (Khajuria et al., 2010; Khajuria et al., 2011), preventing the application of this promising new technology to the control of this notorious pest. Therefore, mechanistic research aimed at elucidating the factors limiting RNAi efficiency in ECB is needed to facilitate the development of RNAi-based pest management strategies in ECB and similar lepidopterans (Kolliopoulou and Swevers, 2014).

RNAi efficiency in insects can be limited by dsRNA instability, poor dsRNA internalization, lack of endosomal escape, deficient core RNAi machinery, and impaired systemic spreading of the silencing RNA molecules (Cooper et al., 2019). DsRNA-degrading endonucleases (aka dsRNases) degrade dsRNA in various lepidopterans, orthopterans, and coleopterans (reviewed in Cooper et al., 2019). DsRNases likely play important roles in limiting RNAi efficiency because they degrade dsRNA before dsRNA can be internalized into cells where the RNAi mechanism takes place (Cooper et al., 2019). In addition, lepidopteran-specific RNAi efficiency-related nucleases (REase) have been reported in *Ostrinia furnacalis* (Guan et al., 2018) and *Helicoverpa armigera* (Guan et al., 2019) and are alleged to contribute to the extremely low RNAi response of lepidopterans. Investigation of dsRNA stability in ECB

demonstrated that dsRNA was rapidly degraded by enzymatic activity in larval ECB gut contents and hemolymph at physiologically relevant pH conditions (Cooper et al., 2020). However, the genes encoding nucleases that degrade dsRNA have not been identified or characterized in ECB and have been characterized only in a few insect species. Accordingly, we identified five complementary DNAs (cDNAs) putatively encoding four dsRNases and one REase from ECB. In addition, we characterized gene expression profiles from ECB to determine the likelihood of these nucleases contributing to the instability of dsRNA in hemolymph and gut contents from ECB. The results support the hypothesis that dsRNA-degrading nucleases contribute to low dsRNA stability in ECB, and thus low RNAi efficiency in ECB.

Methods

Insect rearing

The ECB used in this study originated from French Agricultural Research (Lumberton, MN), and were continuously reared in the laboratory at Kansas State University (Manhattan, KS) as described in Cooper et al. 2020.

Sequencing

To amplify cDNA derived from putative ECB dsRNase and REase transcripts, specific primers were designed based on transcriptome data from *O. furnacalis*. Primer pairs (Table S-1) were designed manually or using the National Center for Biotechnology Information's (NCBI's) Primer-BLAST web tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and then ordered from Invitrogen (Carlsbad, CA, USA). Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions and resuspended in DEPC-treated water. The quality and quantity of total RNA were assessed using a NanoPhotometer (Implen, Westlake Village, CA, USA). One microgram of total RNA from each sample was then treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) to remove genomic DNA and converted to cDNA using the Prime-Script RT Reagent Kit (Takara, Mountain View, CA) using both random hexamers and oligo(dT) primers supplied in the kit. Overlapping PCR products of the expected sizes were amplified from cDNA obtained from pooled last-instar larvae. Complementary DNA fragments of the genes were amplified by Advantage 2 polymerase

(Takara, Mountain View, CA, USA). PCR products were separated by electrophoresis on 1% agarose gels. Target bands were purified using the Gel/PCR DNA fragments extraction kit (IBI Scientific, Road Dubuque, IA, USA) and directly ligated into the pCR-II vector (Invitogen) for transformation into DH5α competent *Escherichia coli* cells using heat shock. Positive plasmids containing the target gene fragment were identified by colony PCR using universal M13 primers. Purified plasmids were sent to Genewiz LLC (South Plainfield, NJ, USA) for Sanger sequencing. Overlapping sequenced fragments spanning the length of the transcripts were aligned with Sequencher 5.0 DNA Sequence Analysis software (Gene Codes Corporation, Ann Arbor, MI, USA) and used to design specific primers for 5'- and 3'- rapid amplification of cDNA ends (RACE) using a Smarter RACE Amplification kit (Takara) according to the manufacture's protocol. Finally, specific primers designed near the start and stop codon were used to amplify the entire open reading frame (ORF) and confirm the full-length sequences (Table S-1). Putative nucleotide sequences were translated using Gene Runner ver. 3.01 software (Hasting Software Inc., Las Vegas, NV, USA), and finally submitted to GenBank. The molecular mass and isoelectric points were predicted with the isoelectric.org web tool.

Phylogenetic, domain, and peptide analyses of predicted proteins

To verify the identity of the putative dsRNA-degrading nucleases, multiple sequence alignments, phylogenetic analysis, domain comparisons, and subcellular localization predictions were performed. Amino-acid residues in the active site were also compared to investigate possible variation in substrate specificity among OndsRNase proteins. Amino-acid sequences were obtained from NCBI, the National Agricultural Library (NAL) i5k Workspace, the supplementary information from Swevers et al., (2013) and Guan et al. (2018), or predicted based on the cDNA sequenced by our group at either Kansas State University (KSU) in the USA or at Shanxi University (SXU) in China as described above. Amino-acid sequences for REases were obtained by pBLAST of OnREase against the NCBI database for each insect order.

Multiple sequence alignments were performed with the MUSCLE method using MEGA7 software (Kumar et al., 2016) and visualized with GeneDoc 2.7 (https://genedoc.software.informer.com/). Phylogenetic analyses were also constructed using MEGA7 following the Maximum Likelihood procedure described by Hall (2013). Bootstrap consensus trees inferred from 500 replicates are shown, with branches corresponding to

partitions reproduced in less than 50% bootstrap replicates collapsed. Full-length amino-acid sequences were used for the REase tree, whereas only the endonuclease domain and surrounding 20 amino-acids were used for the dsRNase tree to achieve better resolution among clades.

Protein domains were predicted with the Pfam 32.0 web tool (http://pfam.xfam.org/search/sequence) (El-gebali et al., 2019). Signal peptides were predicted with the SignalP-5.0 Server web tool (http://www.cbs.dtu.dk/services/SignalP/) (Armenteros et al., 2019a), and the Target P-2.0 Server web tool. (http://www.cbs.dtu.dk/services/TargetP/) (Armenteros et al., 2019b). Subcellular localization was predicted with the WoLF PSORT computational web tool (https://www.genscript.com/wolf-psort.html) (Horton et al., 2007), the Euk-mPLoc 2.0 server (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/) (Chou et al., 2007), and the iLoc-Animal server (http://jci-bioinfo.cn/iLoc-Animal) (Lin et al., 2013).

Stage- and tissue- specific expression profiles

To examine where and when transcripts encoding putative nucleases were expressed in ECB, tissue-specific and developmental stage-specific expression profiles were generated for each nuclease gene. Whole-body and tissue samples were collected from laboratory-reared ECB in TRIzol reagent (Invitrogen) for the extraction of total RNA. At least three individuals were used per replicate, and three biological replicates per treatment. The developmental stages analyzed included: one-day-old eggs, four-day-old eggs, first, second, third, fourth, and fifth instar larvae, pupae, one-day old male moths, three-day-old male moths, one-day-old female moths, and three-day-old female moths. The tissues that were analyzed from fith instar larvae included: hemolymph, foregut, midgut, hindgut, Malpighian tubules, silk glands, fat bodies, and carcass (i.e., what was left after the other tissues were removed).

Tissue and whole-body samples were homogenized in Trizol reagent (Invitrogen) and frozen at -80°C until further processing. Total RNA was extracted following the manufacturer's instructions and resuspended in DEPC-treated water. The quality and quantity of total RNA were assessed using a spectrophotometer (Implen). One microgram of total RNA from each sample was treated with DNase I (Thermo Fisher Scientific) to remove genomic DNA, and then converted to cDNA using the EasyScript cDNA synthesis kit (Applied Biological Materials, Richmond, Canada) following the manufacturer's instructions. Obtained cDNA was diluted 5-fold with nuclease-free water for use as template for expression analysis with RT-qPCR.

RT-qPCR

Reverse transcription quantitative PCR (RT-qPCR) was used to examine tissue and developmental stage-specific expression of nuclease genes in ECB. RT-qPCR was performed in accordance with the minimum information necessary for RT-qPCR (MIQE) guidelines (Bustin et al., 2009) as described in Cooper et al. (2020).

To provide better visualization of fluctuations in gene expression over the time points analyzed, relative expression was calculated as the expression of the target gene relative to the geometric mean of the reference genes (i.e., ΔCt rather than ΔΔCt (Livak et al., 2001)). 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 ΔCt. 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 (Anderson et al., 2004), GeNorm (Vandersompele et al., 2002), BestKeper (Pfaffl et al., 2004), and RefFinder (http://150.216.56.64/referencegene.php) were used to verify the stability of reference genes across ECB tissues and developmental stages. Percent suppression of the target gene was calculated as [(control-target)/control)x100%].

DsRNA synthesis

Long dsRNAs (500 bp) 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 for feeding and injection experiments in ECB larvae to assess the transcriptional responses of nuclease genes to dsRNA treatment. DsRNA was synthesized using purified as described in Cooper et al. (2020).

Transcriptional response of nuclease genes to dsRNA exposure

To investigate short- and long-term transcriptional responses of nuclease genes from ECB to dsRNA exposure, three individuals per replicate of each treatment were collected at various time points after ingestion or injection of dsRNA into second-instar larvae for expression

analysis with RT-qPCR. Second instar larvae were selected for this investigation because the dsRNA feeding assay was optimized for young larvae and microinjection is less harmful to second instars than neonate larvae. Experiments were 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), nuclease inhibitor (e.g., 0, 10, 20 mM Zn^{2+}), or interaction between both factors could be investigated.

Injection of dsRNA was performed 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 phosphate buffered saline (PBS; pH 7.0) was used for control injections. Twenty to twenty-five individuals were injected per replicate and placed on artificial diet inside of 37-ml clear plastic cups sealed with oversnap caps (Frontier Agricultural Sciences, Newark, DE, USA). Larvae were maintained under rearing conditions until sampling.

Ingestion of dsRNA was performed based on Khajuria et al. (2010, 2011). Ten micrograms of dsRNA with and without each reagent were applied to artificial diet. An equal volume of nuclease-free water or phosphate buffered PBS (pH 8.0) was used for ds*GFP* controls. 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). Larvae were then 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, and then allowed to feed for another three days (until all diet was again consumed). Thus, an estimated 20 µg of dsRNA was fed to each larva over a period of six days. Larvae were maintained under rearing conditions until sampling.

Sampling for expression analysis was performed at 3, 6, 12, and 24 h after the start of dsRNA exposure. 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 for RT-qPCR. The effects on transcript levels were calculated as described above.

Statistical analyses

Statistical differences between treatment means were assessed in Minitab 18 with either a one- or two-way ANOVA followed by a Tukey HSD test. Significance levels (α) were set at 0.05 for the entire family of comparisons and significant p-values indicated with an asterisk. All data sets were subjected to the Anderson-Darling normality test and Levene's test for equal variance to verify statistical assumptions were met. Data that did not meet the assumptions were subjected to either a Kruskal-Wallis one-way ANOVA on ranks test, or to dual Friedman's two-way analysis by ranks tests 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 nonparametric statistical tests. In all experiments, treatments were replicated at least three times.

Results

Sequencing and characterization

Sequencing revealed four dsRNase transcripts and a single REase transcript expressed in ECB (Table 3-1). All four predicted OndsRNase proteins were similar in length (Table 3-1), contained an extracellular secretion peptide (Table 3-1, Fig S3-1) and single DNA/RNA non-specific endonuclease (IPR001604) domain (Fig. 3-S1), and clustered in the main lepidopteran clade on a phylogenetic tree of insect dsRNase proteins (Fig. 3-1). Conversely, OnREase was predicted to be intracellular (Table 3-1) and included an XPG N-terminal domain (PF00752) (Fig. 3-2) as well as three conserved residues common to PIN-family domains (Fig. S3-2). Phylogenetic analysis indicated that OnREase clustered with other REase and uncharacterized/hypothetical proteins from lepidopterans, but separately from protein asteroid proteins, protein asteroid-like proteins, and uncharacterized/hypothetical proteins from other insects (Fig. 3-3). Comparison of domain architecture indicated that most proteins from the REase cluster are predicted to have an XPG N-terminal domain (PF00752), whereas all proteins from the asteroid cluster are predicted to have an XPG domain-containing (PF12813) region (Fig. 3-2). The XPG N-terminal domain was not identified in HaREase, but was identified in OnREase and OfREase.

Multiple sequence alignments of predicted dsRNase proteins revealed that the first and eighth key residue of the predicted dsRNase active site were variable among dsRNases from ECB as well as from other insects (Fig. 3-4). OndsRNase1 and OndsRNase2 both have an alanine (A) and an arginine (R) at positions 1 and 2 in the active site. Conversely, OndsRNase3 and OndsRNase4 both have a serine (S) and arginine (R) at positions 1 and 2 in the active site.

Assessment of developmental stage-specific expression profiles (Fig. 3-5) indicated that *OndsRNase1* expression was lowest in eggs and larvae but increased throughout the rest of the developmental cycle, peaking in adult males [$F(11,24)=8.76$, P<0.0001*]. *OndsRNase2* was detectable in all ECB developmental stages except one-day-old eggs, but expression was highest in larval stages [$F(11,23)=45.83$, $p<0.0001$*]. *OndsRNase3* expression was only detectable in four-day-old eggs. *OndsRNase4* was highly expressed in pupae, and adult males and expression of *OndsRNase4* was highest in adult males [$F(11.,24)=12.49$, $p<0.0001$*].

Tissue-specific expression profiles from fifth-instar larvae (Fig. 3-6) indicated that *OndsRNase1* was detectable in all tissues investigated, with the highest expression in hemolymph [$F(3,8)=15.09$, $p=0.001$*]. *OndsRNase2* was highly expressed in the gut and to a much lesser extent in the carcass, but not detectable in hemolymph or fat bodies [$F(3,8)=209.26$, $p<0.0001$*]. *OndsRNase3* expression was only detectable in the hemolymph. *OndsRNase4* was expressed in carcass and fat bodies, but not detectable in gut and hemolymph [$F(3,8)=1.62$, $p=0.260$].

OnREase was detected in all developmental stages and tissues investigated (Fig. 3-7). Developmental stage-specific expression of *OnREase* was highest in third and four larval instars and lowest in four-day-old eggs, though these were not significantly different from the other developmental stages examined [$F(11, 23)=3.74$, $p=0.004$*]. Tissue-specific expression of *OnREase* was highest in the gut of fifth-instar larvae, compared to other tissues investigated [$F(3,8)=40.85$, $p<0.0001$*].

Table 3-1. Characteristics of five nuclease transcripts and their deduced proteins from ECB.

The likelihood and/or cleavage site of the Sec signal peptide (Sec/SPI) signal peptide based on the SignalP-5.0 server is listed first, and then the likelihood/cleavage site based on the Target-P-2.0 server. The localization predictions with the highest probabilities according to WoLF PSORT, Euk-mLoc 2.0, and iLoc-Animal are listed.

Attribute	***OndsRNase1***	***OndsRNase2***	***OndsRNase3***	***OndsRNase4***	***OnREase***
GenBank accession number	MT524715	MT524712	MT524713	MT524714	MT524716
ORF (bp)	1,341	1,347	1,182	1,212	1,866
Protein (aa)	446	448	393	403	621
Mass (kDa)	50.66	50.01	44.13	46.14	71.99
Isoelectric point (pI)	5.67	7.17	7.95	6.26	5.64
Sec/SPI likelihood	0.9956, 0.999	0.9978, 1.000	0.9948, 0.997	0.7565, 0.9536	0.0013, 0.0005
Sec/SPI cleavage site (aa)	18-19	16-17	20-21	22-23, 23-24	n/a
Localization predictions	Extracellular, NASL	Extracellular, Mitochondria	Extracellular, NASL	Extracellular, NASL, Mitochondria	Cytoplasm, Nucleus

Abbreviations: dsRNase, double-stranded ribonuclease; ORF, open reading frame; bp, base pair; kDa, kilodalton, aa, amino-acid, m, molecule mass; NASL, not in any subcellular locations; Sec/SPI, Sec signal peptide.

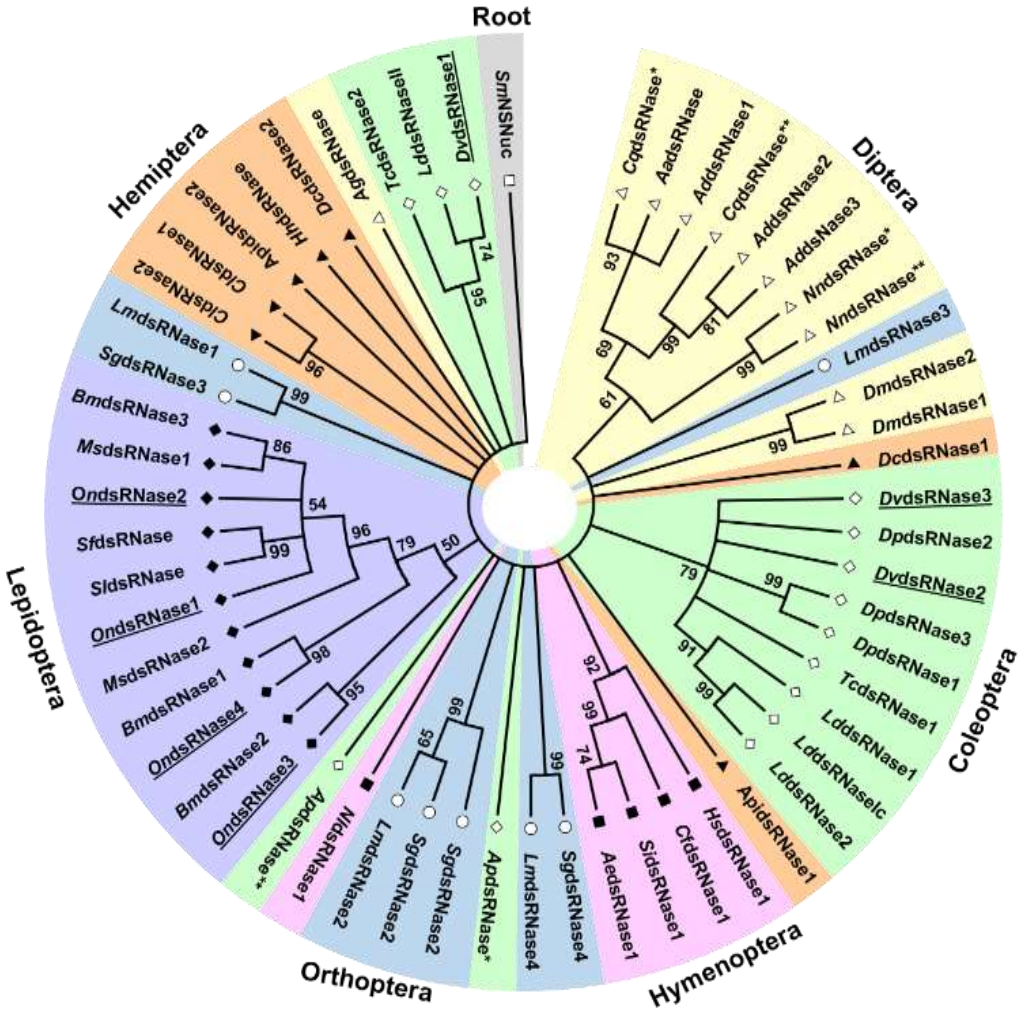

Figure 3-1. Phylogenetic tree showing the relationship of insect dsRNase proteins and bacterial nonspecific nuclease.

Bootstrap support is indicated at internal nodes. Different shapes and shading denote different insect orders. The species and gene accession numbers corresponding to each sequence label are as follows for each order. Asterisks (*,**) differentiate unnumbered dsRNases from the same species. Diptera: AadsRNase, *Aedes aegypti* (EAT42072); CqdsRNase*, *Culex quinquefasciatus* (EDS34867.1); AddsRNase1, *Anopheles darling* (ETN62076.1); CqdsRNase**, *C. quinquefasciatus* (EDS38458.1); AddsRNase2, *A. darling* (ETN61460.1); AddsRNase3, *A. darling* (ETN61459.1); NndsRNase*, *Nyssomyia neivai* (JAV11177.1); NndsRNase**, *N. neivai* (JAV11176.1); DmdsRNase1, *Drosophila melanogaster* (AAF49206.1). DmdsRNase2, *D. melanogaster* (AAF49208.1); AgdsRNase, *Anopheles gambiae* (XP_320813.4). Coleoptera: DvdsRNase2, *Diabrotica virgifera virgifera* (MT653319); DvdsRNase3, *D. v. virgifera* (MT653320); DpdsRNase2, *Dendroctonus ponderosae* (ERL84039.1); TcdsRNase1, *Tribolium castaneum* (XP_970494.1); DpdsRNase3, *D. ponderosae* (AEE63490.1); DpdsRNase1, *D. ponderosae* (ENN82866.1); LddsRNase1/Ib, *Leptinotarsa decemlineata* (APF31792.1); LddsRNase2/Ia, *L. decemlineata* (APF31793.1); LdsRNaseIc, *L. decemlineata* (Swevers et al., 2013); ApdsRNase*, *Agrilus planipennis* (XP_018334885.1); ApdsRNase**, *A. planipennis*

(XP_018323185.1); TcdsRNase2, *T. castaneum* (XP_015840884.1); DvdsRNase1, *D. v. virgifera* (MT653318); LddsRNaseII, *L. decemlineata* (Swevers et al., 2013). Hymenoptera: NldsRNase1, *Neodiprion lecontei* (XP_015515106.1); AedsRNase1, *Acromyrmex echinatior* (XP_011064189.1); SidsRNase1, *Solenopsis invicta* (XP_011156845.1); CfdsRNase1, *Camponotus floridanus* (XP_011263277.1); HsdsRNase1, *Harpegnathos saltator* (XP_011148137.2). Orthoptera: LmdsRNase3, *Locusta migratoria* (KY386893); SgdsRNase2, *Schistocerca gregaria* (AHN55089.1/APF31794.1); LmdsRNase2, *L. migratoria* (ARW74135.1); SgdsRNase1, *S. gregaria* (AHN55088.1); SgdsRNase4, *S. gregaria* (AHN55091.1); LmdsRNase4, *L. migratoria* (KY386894); SgdsRNase3, *S. gregaria* (AHN55090.1); LmdsRNase1, *L. migratoria* (ARW74134.1). Lepidoptera: BmdsRNase2, *Bombyx mori* (NP_001091744.1); OndsRNase1, *Ostrinia nubilalis* (MT524713); OndsRNase4, *O. nubilalis* (MT524714); BmdsRNase1, *B. mori* (XP_004922835.1); OndsRNase*1*, *O. nubilalis* (MT524715); OndsRNase2, *O. nubilalis* (MT524712); SldsRNase, *Spodoptera litura* (CAR92522.1); SfdsRNase, *Spodoptera frugiperda* (CAR92521.1); MsdsRNase2, *M. sexta (*Msex2.04564); *Bm*dsRNase3/AlkNuc, *B. mori* (BAF33251.1); MsdsRNase1, *M. sexta* (Msex2.04563). Hemiptera: DcdsRNase1, *Diaphorina citri* (XP_017297751.1); ApidsRNase1, *A. pisum* (XP_003242652.1); CldsRNase1, *Cimex lectularius* (XP_014241898.1); CldsRNase2, *C. lectularius* (XP_014241376.1); HhdsRNase, *Halyomorpha halys* (XP_014282547.1); DcdsRNase2, *D. citri* (XP_008483858.1); ApidsRNase2, *Acyrthosiphon pisum* (XP_003248225.1). Root: Enterobacteriales: SmNSNuc, *Serratia marcescens* (AAA26560.1)

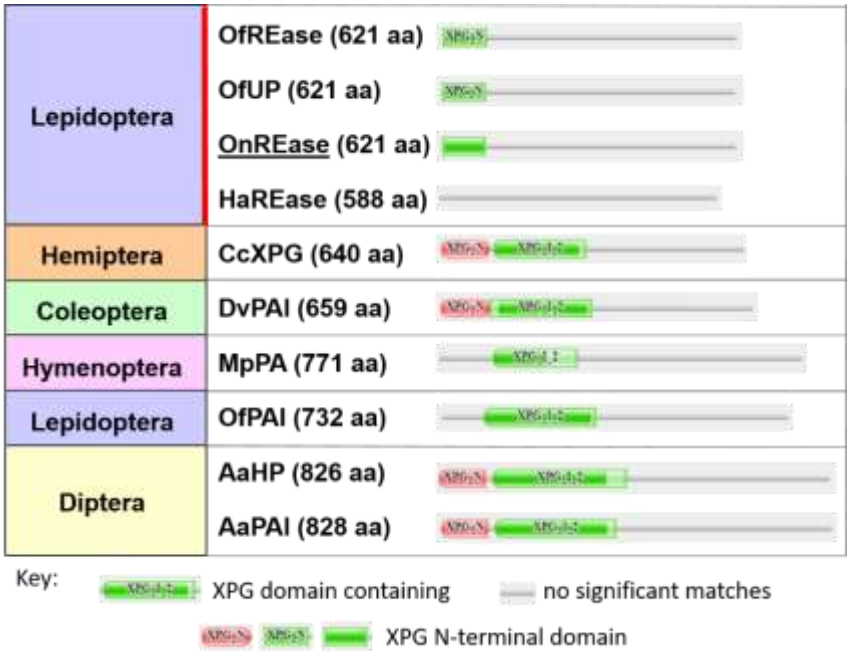

Figure 3-2. Comparison of domain architecture showing the difference between lepidopteran REase (red line) and insect protein asteroids.

The species and gene accession numbers corresponding to each sequence label are as follows for each order. Lepidoptera: OfREase, *Ostrinia furnacalis* (XP_028162616.1); OfUP, *O. furnacalis* (XP_028162616.1); OnREase, *Ostrinia nubialis* (MT524716); HaREase, *Helicoverpa armigera*

(XP_021192733.1); OfPAI, *O. furnacalis* (XP_028160864.1). Hemiptera: CcXPG, *Cinara cedri* (VVC40419.1)/ Coleoptera: DvPAl, *Diabrotica virgifera virgifera* (XP_028153404.1). Hymentoptera: MpPA, *Monomorium pharaonis* (XP_012539364.1). Diptera: AaHP, *Aedes albopictus* (KXJ83147.1); AaPAl, *A. albopictus* (XP_019538095.2); *Abbreviations: RNAi-efficiency related nuclease, REase; uncharacterized protein, UP; hypothetical protein, HP; XPG N-terminal PIN domain-like endonuclease, XPG; protein asteroid, PA; protein asteroid-like, PAl.*

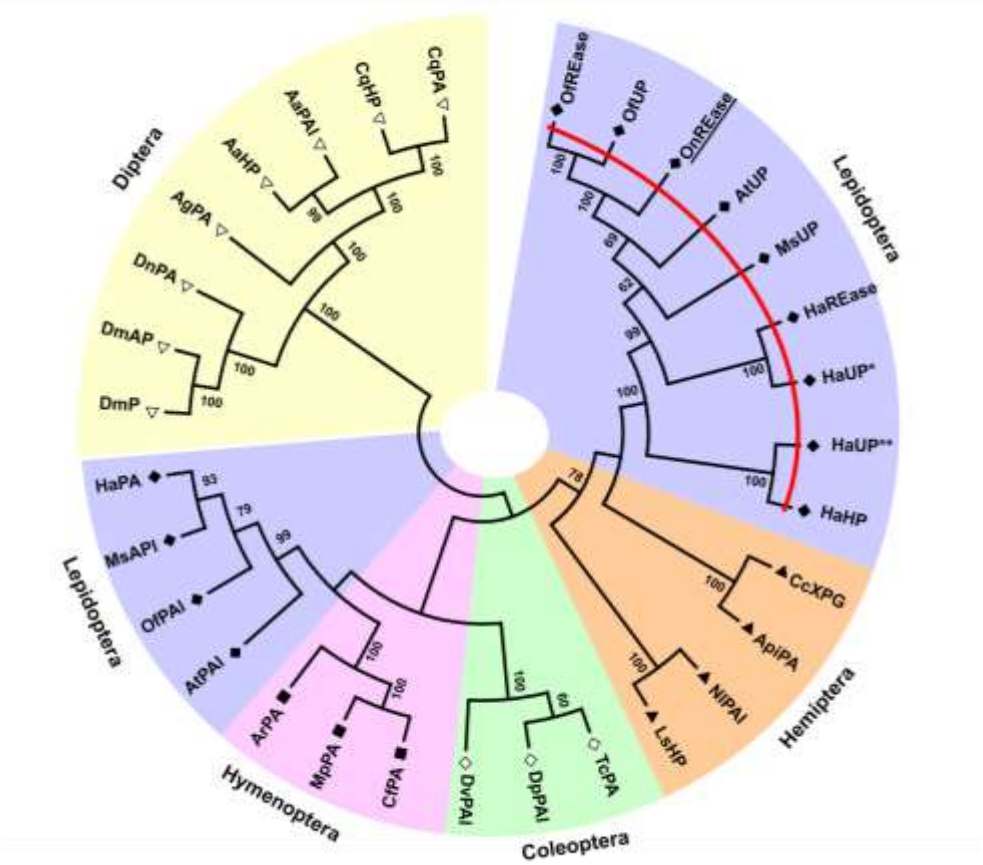

Figure 3-3. Phylogenetic tree showing the relationship of REase proteins (red line), protein asteroids, and unknown/hypothetical proteins from insects.

Bootstrap support is indicated at internal nodes. Different shapes and shading denote different insect orders. The species and gene accession numbers corresponding to each sequence label are as follows for each order. Asterisks (*,**) differentiate unnumbered proteins from the same species. Lepidoptera: OfREase, *Ostrinia furnacalis* (XP_028162616.1); OfUP, *O. furnacalis* (XP_028162616.1); OnREase, *Ostrinia nubilalis* (MT524716); AtUP, *Amyelois transitella* (XP_013194003.1); MsUP, *Manduca sexta* (XP_030022308.1); HaREase, *Helicoverpa armigera* (XP_021192733.1); HaUP*, *H. armigera* (XP_021192733.1); HaUP**, *H. armigera* (XP_021195627.1); HaHP, *H. armigera* (PZC74001.1); AtPAI, *A. transitella*

(XP_013200440.1); OfPAI, *O. furnacalis* (XP_028160864.1), MsPAI, *M. sexta* (XP_030035285.1); HaPA, *H. armigera* (XP_021187181.1). Hemiptera: CcXPG, *Cinara cedri* (VVC40419.1); ApiPA, *Acyrthosiphon pisum* (XP_008180179.1); NlPAl, *Nilaparvata lugens* (XP_022192084.1); LsHP, *Laodelphax striatellus* (RZF34787.1). Coleoptera: TcPA, *Tribolium castaneum* (XP_975212.1); DpPAl, *Dendroctonus ponderosae* (XP_019754476.1); DvPAl, *Diabrotica virgifera virgifera* (XP_028153404.1). Hymentoptera: CfPA, *Camponotus floridanus* (XP_011264114.1); MpPA, *Monomorium pharaonis* (XP_012539364.1); ArPA, *Athalia rosae* (XP_012251909.1). Diptera: DmP, *Drosophila melanogaster* (AAK93494.1); DmAP, *D. melanogaster* (NP_523451.2); DnPA, *Drosophila navojoa* (XP_030240503.1); AgPA, *Anopheles gambiae* (XP_318635.3); AaHP, *Aedes albopictus* (KXJ83147.1); AaPAl, *A. albopictus* (XP_019538095.2); CqHP, *Culex quinquefasciatus* (XP_001870537.1); CqPA, *C. quinquefasciatus* (XP_001846107.1). No blast hits were found for Orthoptera. *Abbreviations: RNAi- efficiency related nuclease, REase; uncharacterized protein, UP; hypothetical protein, HP; XPG N-terminal PIN domain-like endonuclease, XPG; protein asteroid, PA; protein asteroid-like, PAl.*

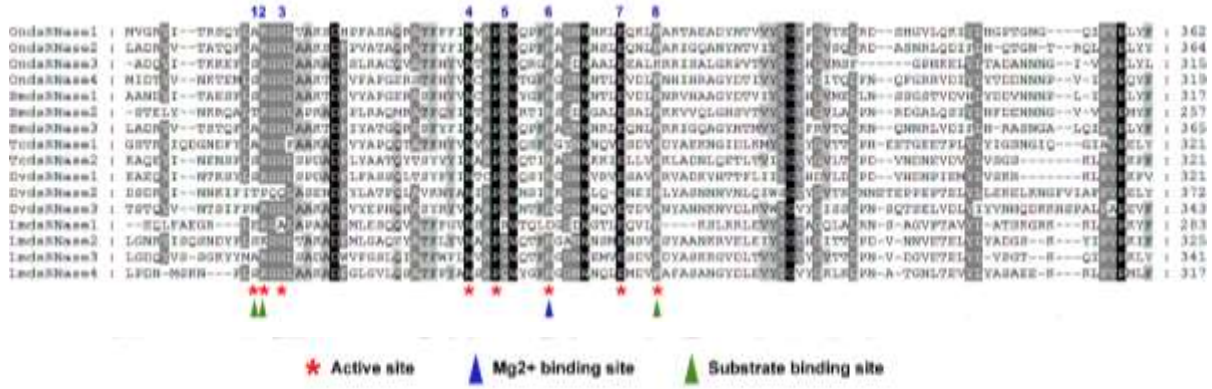

Figure 3-4. Multiple sequence alignment showing conserved residues in the active site of insect dsRNases.

The eight amino-acid residues that form the active site are indicated by a red asterisk and numbered along the top. Amino-acid residues that participate in the substrate-binding site and Mg^{2+} binding site are indicated with green and blue triangles, respectively. Black shading indicates 100% identity, dark-grey shading indicates 80–99% identity and light-grey shading indicates 60–79% identity. The species and gene accession number corresponding to each sequence label is as follows: OndsRNase1, *O. nubilalis* (MT524715); OndsRNase2, *Ostrinia nubilalis* (MT524712); OndsRNase3, *O. nubilalis* (MT524713); OndsRNase4, *O. nubilalis* (MT524714); BmdsRNase1, *B. mori* (XP_004922835.1); BmdsRNase2, *Bombyx mori* (NP_001091744.1); BmdsRNase3/AlkNuc, *B. mori* (BAF33251.1); TcdsRNase1, *Tribolium castaneum* (XP_970494.1); TcdsRNase2, *T. castaneum* (XP_015840884.1); DvdsRNase1, *Diabrotica virgifera virgifera* (MT653318); DvdsRNase2, *D. v. virgifera* (MT653319); DvdsRNase3, *D. v. virgifera* (MT653320); LmdsRNase1, *L. migratoria* (ARW74134.1); LmdsRNase2, *L. migratoria* (ARW74135.1); LmdsRNase3, *Locusta migratoria* (KY386893); LmdsRNase4, *L. migratoria* (KY386894).

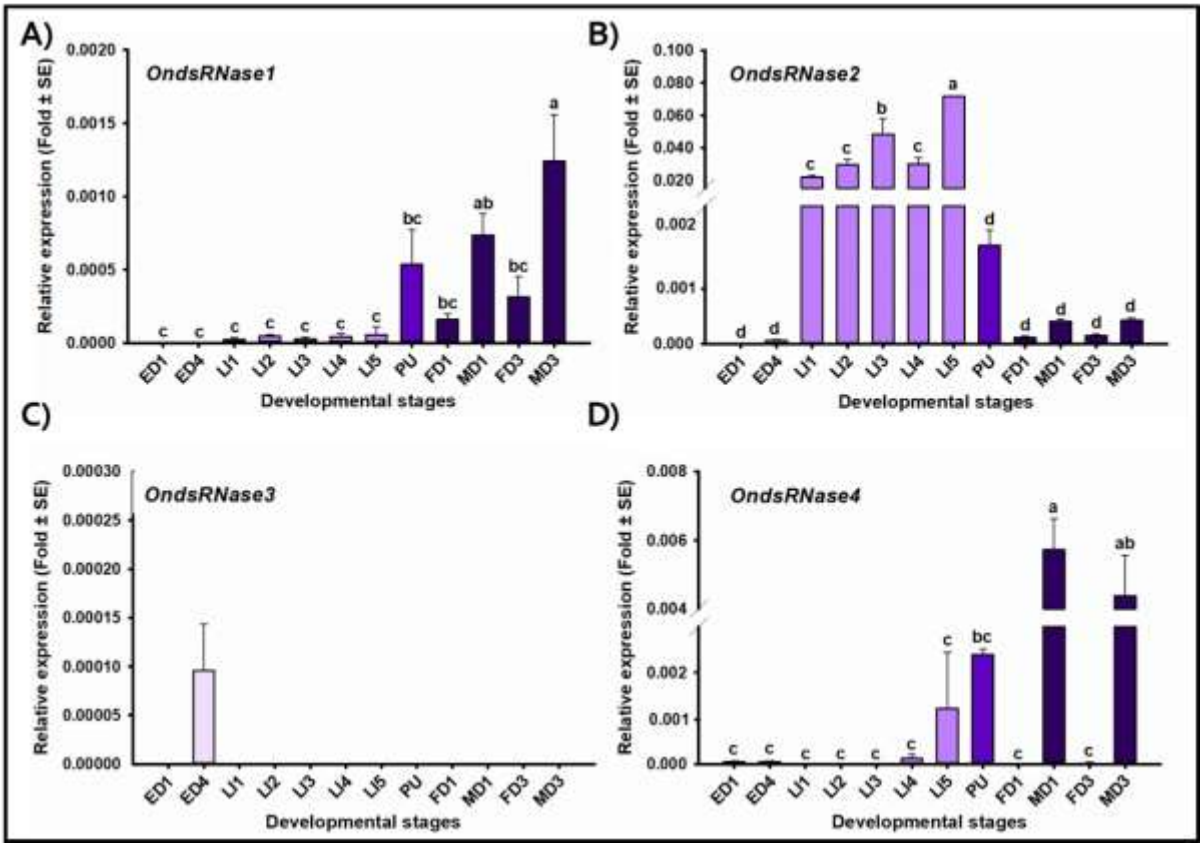

Figure 3-5. Expression profiles showing relative expression of OndsRNase transcripts across developmental stages of ECB.

Relative expression level (fold plus or minus standard error) of A) *OndsRNase1*, B) *OndsRNase2*, C) *OndsRNase3*, and D) *OndsRNase4* in one-day-old eggs (ED1) four-day-old eggs (ED4), first larval instar (LI1), second larval instar (LI2), third larval instar (LI3), fourth larval instar (LI4), fifth larval instar (LI5), pupae (PU), one-day-old adult females (FD1), one-day-old adult males (MD1), three-day-old adult females (FD3), and three-day-old adult males (MD3). Fold change is relative to the expression of reference genes only (ΔCt). Significant differences among treatments are indicated by different letters.

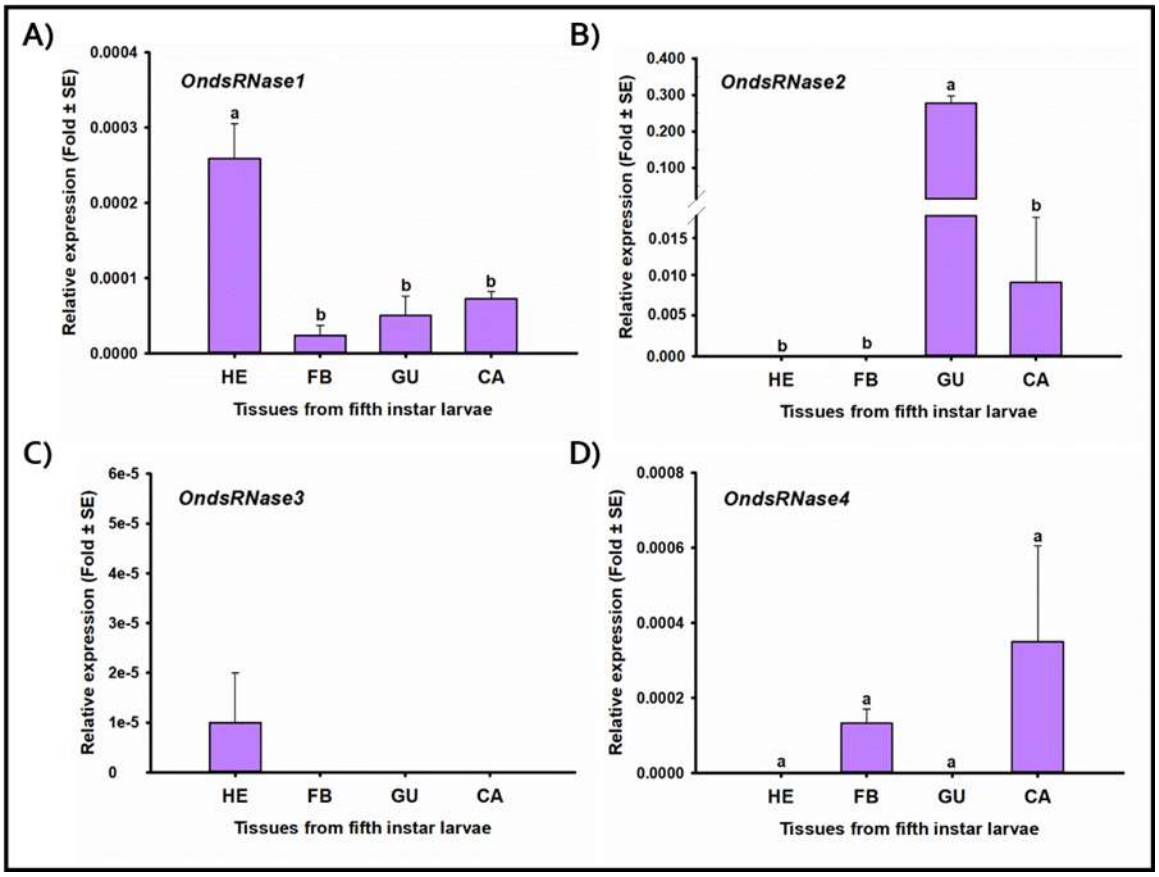

Figure 3-6. Expression profiles showing relative expression of OndsRNase transcripts across ECB tissues.

Relative expression level (fold plus or minus standard error) of A) *OndsRNase1*, B) *OndsRNase2*, C) *OndsRNase3*, and D) *OndsRNase4* in hemolymph (HE), fat body (FB); gut (GU), and carcass (CA). Fold change is relative to the expression of reference genes only (ΔCt). Significant differences among treatments are indicated by different letters.

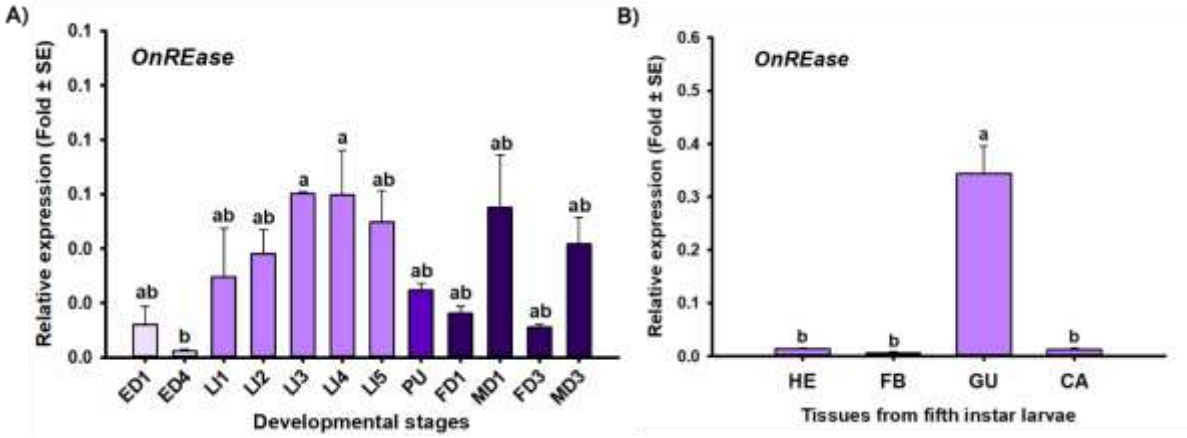

Figure 3-7. Expression profiles showing relative expression of *OnREase* across ECB A) developmental stages and B) tissues.

Relative expression level (fold plus or minus standard error) of *OnREase* in one-day-old eggs (ED1) four-day-old eggs (ED4), first-instar larvae (LI1), second-instar larvae (LI2), third-instar larvae (LI3), fourth-instar larvae (LI4), fifth-instar larvae (LI5), pupae (PU), one-day-old adult females (FD1), one-day-old adult males (MD1), three-day-old adult females (FD3), and three-day-old adult males (MD3), as well as in hemolymph (HE), fat body (FB); gut (GU), and carcass (CA). Fold change is relative to the expression of reference genes only (ΔCt). Significant differences among treatments are indicated by different letters.

Transcriptional responses to dsRNA

None of the OndsRNase genes were significantly upregulated after dsRNA feeding or injection at any of the short-term time points investigated. Transcript levels of *OndsRNase2* and *OndsRNase3* were unaffected by dsRNA/Zn^{2+} injection and feeding at all of the time points investigated (data not shown). In addition, the expression of *OndsRNase4* was unaffected by dsRNA/Zn^{2+} injection (data not shown). However, three days after the start of dsRNA feeding, expression of *OndsRNase4* increased by 5.3-fold in the ds*OnLgl* fed treatment group compared to the ds*GFP* fed treatment group [$F(2,12)=3.50$, $p=0.064$] (Fig. 3-8). Conversely, *OndsRNase4* expression was unaffected by dsRNA/Zn^{2+} feeding (data not shown), but decreased by 2.8-fold on average six hours after Zn^{2+} injection [$F(2,17)=5.24$, $p=0.017$*] (Fig. 3-9A, B), and by 2.1-fold twelve hours after Zn^{2+} injection [$F(2,18)=5.03$, $p=0.018$*] (Fig. 3-9C, D).

Transcript levels of *OnREase* were significantly upregulated after both dsRNA ingestion and injection. *OnREase* expression significantly increased by 4.7-fold six hours after injection of ds*GFP* compared to water injection, regardless of Zn^{2+} concentration [$F(2,17)=6.67$, $p=0.007$*]; however, there was no significant difference between *OnREase* expression in the ds*OnLgl* injected treatment group versus the water-only treatment group at six hours after injection (Fig. 3-10B). Transcript levels of *OnREase* were also upregulated 22.0-fold three hours after the start of dsRNA feeding, regardless of Zn^{2+} concentration [$F(2,18)=3.10$, $p=0.070$] (Fig. 3-10C, D), but not at any of the other time points investigated.

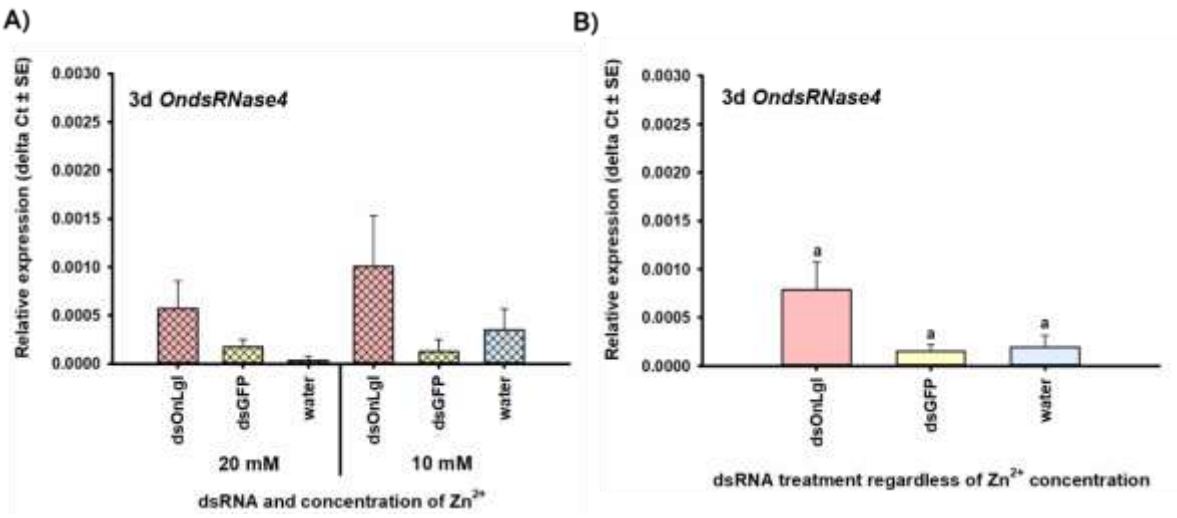

Figure 3-8. Relative expression of *OndsRNase4* after three days of feeding on dsRNA/nuclease inhibitor for A) all treatment groups, and B) for main effects due to dsRNA treatment.

Relative expression of *OndsRNase4* in second instar ECB larvae, 3 days after the start of feeding on artificial diet treated with various combinations of dsRNA and nuclease inhibitor (Zn^{2+}). Expression levels are relative to reference genes only (ΔCt).

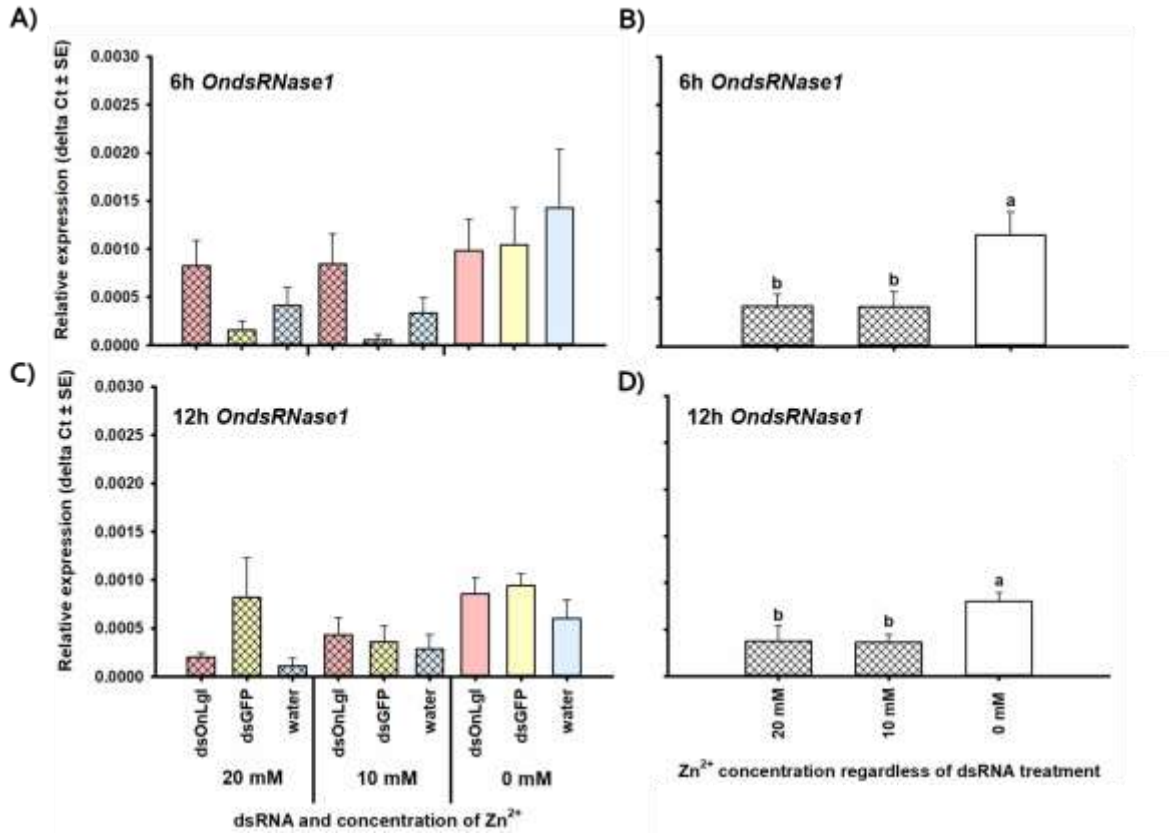

Figure 3-9. Relative expression of *OndsRNase1* six (A and B) and twelve h (C and D) after injection of dsRNA/nuclease inhibitor for all treatment groups (A and C) and for main effects due to Zn^{2+} concentration (B and D).

Relative expression of *OndsRNase1* 6 and 12 h after injection of various combinations of dsRNA and Zn^{2+} into second-instar larvae. Expression levels are relative to reference genes only (ΔCt). Significant differences among treatments are indicated by different letters.

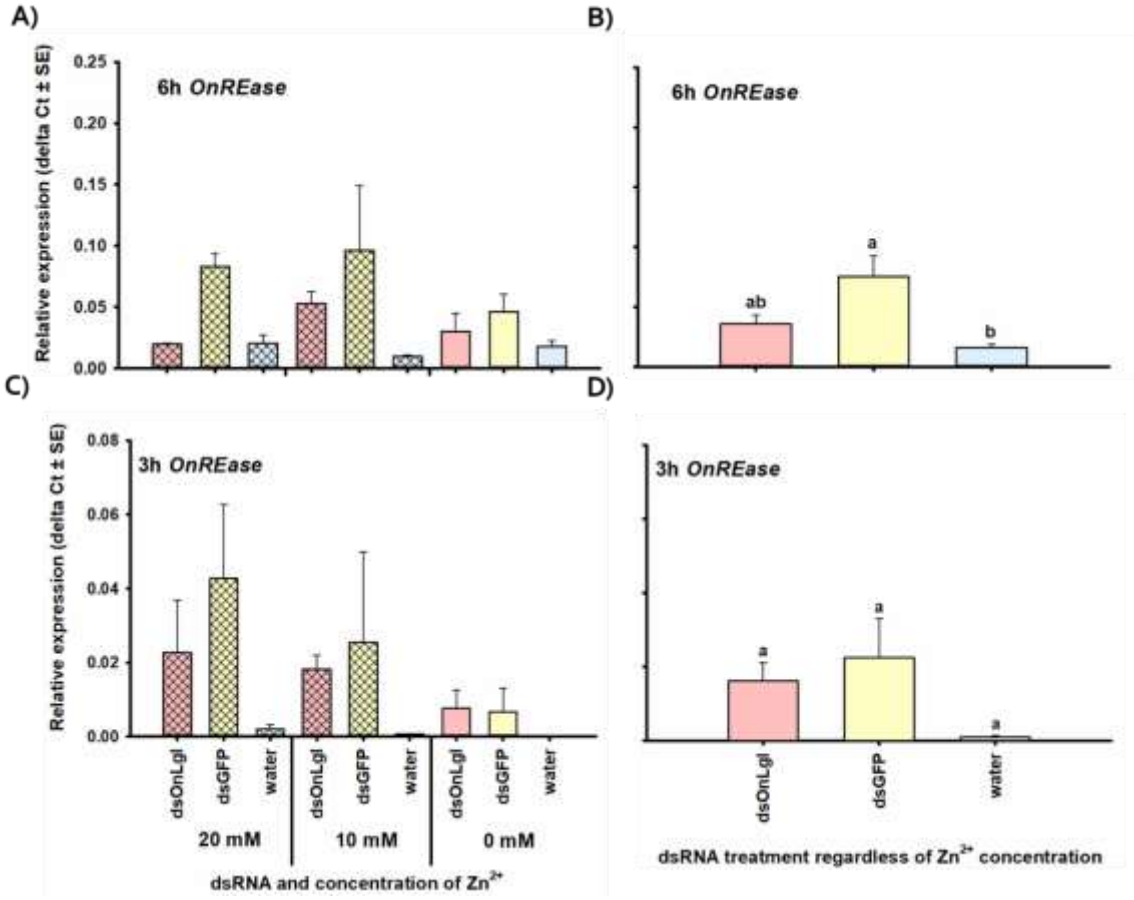

Figure 3-10. Relative expression of *OnREase* six h after injection (A and B) and three h after feeding (C and D) of dsRNA/ inhibitor for all treatment groups (A and C) and for main effects due to dsRNA treatment (B and D). Note that scales differ.

Mean fold change in the relative expression of *OndsREase* 6 h after injection and 3 h after feeding of various combinations of dsRNA and Zn^{2+} into second instar ECB larvae. Fold change is relative to the expression of reference genes only. Significant differences among treatments are indicated by different letters (ΔCt).

Discussion

Previously, we showed that dsRNA was highly unstable in ECB gut contents and hemolymph under physiological conditions, and the degradation of dsRNA is likely enzymatic in nature (Cooper et al., 2020). In the present study, we identified and characterized four dsRNase transcripts and one REase transcript in ECB that are likely associated with those previous observations. In other insects, dsRNA-degrading nucleases, such as dsRNases and REases, have been implicated in limiting RNAi efficiency by degrading dsRNA in *Bombyx mori* (Arimatsu et al., 2007a, 2007b, Liu et al., 2013), *Schistocerca gregaria* (Wynant et al., 2014), *Leptinotarsa decemlineata* (Spit et al., 2017), *Locusta migratoria* (Song et al., 2017, 2019), *O. furnacalis* (Guan et al., 2018), and *H. armigera* (Guan et al., 2019) and *Aedes aegypti* (Gieshbrecht et al., 2020). Most lepidopteran insects appear to contain three to four dsRNase transcripts (Fig. 3-1) and one to three REase transcripts (Fig. 3-3). Insects from other orders, lack REases, and contain only one to three dsRNases, supporting the hypothesis that differences in dsRNA-degrading nuclease activity may contribute to the low RNAi efficiency exhibited by many lepidopterans, including ECB.

Comparisons of domain architectures for predicted REase proteins indicated that OnREase contains an XPG N-terminal domain (a single stranded, structure-specific DNA endonuclease domain) belonging to the PIN-domain nuclease family, similar to OfREase and OfUP from *O. furnacalis* (Fig. 3- 2). However, no significant domain matches were identified in HaREase from *H. armigera* (Fig. 3-2), despite the presence of the three conserved residues at the N-terminus, which are common to all PIN-domain family members (Fig. S3-1). Perhaps these differences in domain architecture can help explain why RNAi-mediated suppression of OfREase resulted in enhanced RNAi efficiency in *O. furnacalis* (Guan et al., 2018), but knockout of HaREase with CRISPR-Cas9 did not improve RNAi efficiency in *H. armigera* (Guan et al., 2019). Similarities in domain structure between OnREase and OfREase (Fig. 3-2), their close phylogenetic relationship (Fig. 3-4), and high expression in the gut (Fig. 3-8B) suggest that OnREase may play a similar role in dsRNA degradation as OfREase (Guan et al., 2018). In addition, subcellular localization predictions and signal peptide predictions indicate that OnREase is an intracellular nuclease located in the cytoplasm. If so, OnREase and other REases likely degrade dsRNA after it is internalized into cells.

Unlike REases, all insect dsRNases were predicted to have similar domain structures and contain a signal peptide for extracellular secretion (Table 3-1), as previously described for insect dsRNases (Singh et al., 2017). Interestingly, a comparison of conserved residues in the active site of insect dsRNases and bacterial NsNuc (Fig. 3-4) suggests that there may be differences in substrate specificity among the dsRNases. Based on the crystal structure obtained for a bacterial nonspecific nuclease from *Serratia marcescens*, the first, second, and eighth key residues in the dsRNase active site are likely involved in substrate binding (Lunin et al., 1997). Variability at these residues may indicate differences among OndsRNases and DvdsRNases in substrate specificity and activity under physiological conditions, as described for LmdsRNases in *L. migratoria* (Song et al., 2017; Song et al., 2019).

OndsRNase1 and OndsRNase2 both have an alanine (A) and an arginine (R) at positions 1 and 2 in the active site similar to LmdsRNase3 (Fig. 3-4), which was shown to actively degrade both dsRNA and dsDNA under physiologically relevant conditions in *L. migratoria* hemolymph (Song et al., 2019). Interestingly, BmdsRNase3 from *B. mori*, and TcdsRNase1 from *Tribolium castaneum* also have an alanine and arginine at position 1 and 2 (Fig. 3-4). *BmdsRNase3* was first reported to have high activity against dsRNA and to a lesser extent siRNA under physiologically relevant conditions in *B. mori* gut contents (Arimatsu et al., 2007a), but later reported to have activity against dsRNA and DNA in lepidopteran HiF tissue culture cells (Liu et al., 2012). These reports from *B. mori* offer further support that alanine and arginine at these positions in the dsRNase binding site is associated with dsRNA specificity of dsRNase enzymes. The substrate specificity of individual TcdsRNases from *T. castaneum* has not been investigated yet, but dsRNA and dsDNA have been reported to be relatively stable in *T. castaneum* gut contents and hemolymph (Cao et al., 2018). Perhaps, the physiological pH in *T. castaneum* tissues is not suitable for dsRNase activity in this species, as was shown for LmdsRNase1 and 4 in hemolymph (Song et al., 2019) and LmdsRNase3 in gut contents (Song et al., 2017). Based on the data presented in Fig. 3-5 and this body of literature, it is reasonable to predict that OndsRNases1 and OndsRNase2 may have substrate specificity for dsRNA and dsDNA in ECB.

Conversely, OndsRNase3, OndsRNase4 and DvdsRNase1 all have a serine (S) and arginine (R) at positions 1 and 2 in the active site, like LmdsRNase4 (Fig. 3-5), which was shown to slightly degrade siRNA and dsRNA *in vitro*, but not at physiologically relevant

conditions in hemolymph (Song et al., 2019). DsRNase1 from *B. mori* and TcdsRNase2 from *T. castaneum*, also have a serine and arginine at position 1 and 2. The contribution of BmdsRNase1 to dsRNA instability in *B. mori* has not been investigated, but dsRNA is relatively stable in tissue extracts from *D. v. virgifera* (Cooper et al., 2020) and *T. castaneum* (Cao et al., 2018). Based on dsRNA stability data and the literature discussed here, it is reasonable to speculate that OndsRNases3 and OndsRNase4 may have a slight substrate specificity for dsRNA and siRNA, but likely are not contributing greatly to dsRNA instability in ECB. LmdsRNase2, the enzyme responsible for dsRNA degradation in gut contents and for lowering oral RNAi efficiency in *L. migratoria* (Song et al., 2018), has a serine (S) and a lysine (K) at positions 1 and 2 of its active site, which may be unique to orthopterans (Fig. 3-5; Song et al., 2019), but more investigation is necessary to be sure.

These hypotheses regarding OndsRNase specificity and activity are further supported by the developmental stage (Fig. 3-5) and tissue (Fig. 3-6) specific expression profiles generated for dsRNase transcripts in ECB, which showed very high expression of *OndsRNase2* in the larval gut, very low expression of *OndsRNase3* and *OndsRNase4* in most stages and tissues, and high expression on *OndsRNase1* in all tissues, mainly in older developmental stages. Together these findings indicate *OndsRNase1* and *OndsRNase2* are the most likely dsRNase genes to contribute to low dsRNA stability in ECB.

Given that *OndsRNase 3* expression was nearly undetectable in all developmental stages and tissues investigated (data not shown), and the protein contains a highly abnormal lysine (K) at position 8 in the substrate-binding pocket (Fig. 3-5), this nuclease may be nonfunctional (although lysine and arginine are both positively charged amino-acids, so it is possible that the protein could be functional), and is unlikely to contribute to low RNAi efficiency in ECB; however, testing of activity and substrate specificity with recombinantly expressed enzymes, or incubation of various substrates in extracts from transgenic ECB lacking specific dsRNases is needed to confirm these hypotheses. In addition, a variety of other obscure nuclease genes may contribute to dsRNA degradation, as shown in *Nezara viridula* (Lomate and Bonning, 2016) and *H. armigera* (Guan et al., 2019). Information about nucleases will aid in the development of strategies for enhancing dsRNA stability in insect tissues, with the ultimate goal being to enhance RNAi efficiency and reduce the dose of costly dsRNA needed to induce phenotypical changes associated with suppression of the target gene.

The low transcript levels of dsRNase genes in early instar larvae and adult female ECB suggests that these developmental stages may be the most amenable to RNAi, as dsRNA is likely the most stable in these developmental stages (Cooper et al., 2019). However, this hypothesis relies on the assumption that dsRNase expression is indeed correlated with dsRNA stability *in vivo*. A comparison of RNAi efficiency and/or dsRNA stability in tissues from various developmental stages of ECB is needed to confirm this. Additionally, the elevated transcript levels of *OndsRNase4*, *OndsRNase1*, and *OnREase* in adult males is also noteworthy (Figs. 3-5, 3-7A) and deserves investigation.

OndsRNase2 was not upregulated at any of the time points investigated (data not shown). This result is surprising given the high basal expression in the larval gut (Figs. 3-6A, 3-7A) and leads to questions about the importance of gene inducible vs. constitutive expression. Lack of upregulation does not necessarily indicate that nucleases such as *OndsRNase2* are not involved in dsRNA degradation or defense against invading dsRNA. In *B. mori* larvae, *BmdsRNase* was upregulated three and six hours after dsRNA injection, but not ingestion (Liu et al., 2013), despite high expression and activity of this nuclease in the midgut (Arimatsu et al., 2007a, 2007b). However, upregulation of genes such as *OndsRNase4* (Fig. 3-8) and *OnREase* (Fig. 3-10) in response to dsRNA injection may indicate that these enzymes act as pattern recognition receptors, similar to *BmdsRNase,* which was shown to mimic the response of Toll receptors and core RNAi pathway components to pathogen-associated molecular patterns such as dsRNA (Liu et al., 2013).

The transcription response of REases to exogenous dsRNA has been the subject of much interest because induction of this nuclease occurs sooner and higher than upregulation of the core enzymes in response to dsRNA in *O. furnicalis*, thus limiting the amount of dsRNA that reaches the core enzymes of the RNAi pathway and thus reducing RNAi efficiency (Guan et al., 2018). While dramatic upregulation of *Of-* and *HaREase* in response to orally delivered dsRNA is well documented (Guan et al., 2018, Guan et al., 2019), this is the first report of upregulation of a REase gene in response to injected dsRNA (Fig. 3-10A, B) This finding may explain why *O. furnicalis* and *H. armigera* larvae are generally amenable to RNAi via injection and soaking (Wang et al., 2011, Zhang et al., 2015, Guan et al., 2018), whereas ECB is much less susceptible (Chapter 5).

The downregulation of *OndsRNase1* in response to the Zn^{2+} nuclease inhibitor (Fig. 3-9) supports the existence of a feedback loop between perturbations in nuclease activity and expression of nuclease genes. In ECB, injection of a nuclease inhibitor resulted in downregulation of *OndsRNase1*, but in *H. armigera* knockout of *HaREase* resulted in upregulation of 14 other nuclease genes (Guan et al., 2019). Therefore, protective coatings such as nanoparticles and transfection reagents may be better candidates for combating dsRNA-degradation and enhancing RNAi efficiency in insects because they are unlikely to impact nuclease activity or expression.

Taken together, the results presented in this investigation are consistent with studies that characterize nuclease genes from other insects. There is a high probability that instability of dsRNA in ECB could be due to rapid degradation of dsRNA by at least some of the identified dsRNases and REase in the gut and hemolymph. Nuclease inhibitors (Garbutt et al., 2013; Cao et al., 2018; Castellanos et al., 2019), transfection reagents (Lin et al., 2017; Castellanos et al., 2019); dsRNA-guanylated polymer complexes (Christiaens et al., 2018), dsRNA-expressing bacteria (Yang and Han, 2014) and ribonucleoprotein-dsRNA (Gillet et al., 2017) can protect dsRNA from nuclease activity in insects such as *Blattella germanica*, *Euschistus heros*, *Anthonomus grandis*, *Acyrthosiphon pisum*, and *H. armigera*. These reagents and approaches are good candidates for enhancing dsRNA stability in ECB, and hopefully RNAi efficiency as well, so that RNAi-based approaches can be utilized to study and control ECB in the future. However, it might prove challenging to inhibit intracellular nucleases such as OnREase without affecting the function of core RNAi pathway genes, such as Dicer 2 or Argonature 2. There could also be additional mechanisms contributing to low RNAi efficiency in ECB (reviewed in Cooper et al., 2019) that will need to be understood and overcome before RNAi efficiency can be employed in control strategies against lepidopteran insects like ECB.

Conclusion

This investigation identified transcripts for four dsRNase genes and one REase gene expressed in ECB. Comparison of key amino-acid residues in the predicted active site of the nonspecific endonuclease domains of the dsRNases also suggested that OndsRNase1 and OndsRNase2 likely have substrate specificity for dsRNA and dsDNA, whereas OndsRNase3, OndsRNase4, and DvdsRNase1 likely are specific to dsRNA and siRNA. In addition, expression

profiles indicated that *OnREas*e, *OndsRNase2* are most likely to contribute to dsRNA instability in the larval gut, whereas *OnREase* and *OndsRNase1* are most likely to contribute to dsRNA instability in the hemolymph. However, only OnREase and OndsRNase4 were upregulated in response to dsRNA exposure, indicating that only a subset of these genes may act as pattern recognition receptors for dsRNA Together, these findings support the idea that dsRNA degradation in ECB is likely due to the enzymatic activity of dsRNA-degrading nucleases in ECB. Further testing, however, is necessary to experimentally confirm the involvement of specific nuclease genes. These findings are significant because they provide information about the mechanism(s) influencing dsRNA instability in insects. This knowledge is useful for devising strategies to enhance dsRNA stability, and possibly RNAi efficiency in ECB. Thus, the knowledge generated by this study will facilitate the development of strategies for enhancing dsRNA efficiency insects.

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Chapter 4 - Characterization, Expression Patterns, and Transcriptional Responses of Major Core RNA Interference Pathway Genes from *Ostrinia nubilalis*

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Abstract

RNA interference (RNAi) is commonly used in the laboratory to analyze gene function, and RNAi-based pest management strategies are now being employed. Unfortunately, RNAi is hindered by inefficient and highly variable results when different insects are targeted, especially lepidopterans, such as *Ostrinia nubilalis* (ECB, Lepidoptera: Crambidae). Previous efforts to achieve RNAi-mediated gene suppression in ECB revealed low RNAi efficiency with both double-stranded RNA (dsRNA) injection and feeding. One mechanism that can affect RNAi efficiency in insects is the expression and function of core genes of short interfering RNA pathway, such as Argonaute 2 (*Ago2*), Dicer 2 (*Dcr2*), and *R2D2*. To determine if deficiencies in these core RNAi pathway genes contribute to low RNAi efficiency in ECB, full-length cDNAs encoding each of these genes were sequenced and characterized, providing identification of a single transcript for each gene, *OnAgo2*, *OnDcr2*, and *OnR2D2*. A comparison of domain architecture suggested that all three predicted proteins contained the necessary domains to function. However, a comparison of evolutionary distances for each domain of the protein alignments revealed potentially important variations in the first RNase III domain of OnDcr2, both dsRBD of OnR2D2, and both the PAZ and PIWI domains of OnAgo2, which may indicate functional differences in enzymatic activity between species. Expression analysis indicated that transcripts for all three genes were expressed in all developmental stages and tissues investigated. In addition, oral delivery of dsRNA caused upregulation and downregulation of *OnDcr2* and *OnR2D2* transcript levels, respectively, whereas injection of dsRNA had no effect on the expression of any of the genes examined. The lack of transcriptional responses of the core

machinery as well as the divergence in amino-acid sequence between specific domains in each core RNAi protein may each contribute to low RNAi efficiency in ECB. Understanding the contributions of different components of the RNAi pathway is critical to adapting this technology for use in controlling lepidopteran pests that exhibit low RNAi efficiency.

Background

RNA interference (RNAi) is a post-translational gene silencing mechanism in which double-stranded RNA (dsRNA) molecules trigger the degradation of complementary RNA molecules to regulate endogenous gene expression and to defend against viruses and transposons (Dowling et al., 2016). Since the discovery that exogenously supplied dsRNA can be used to silence specific genes by triggering the core enzymes of the RNAi pathway, RNAi-mediated gene silencing has been exploited by entomologists to elucidate gene function (Downward, 2004; Miyata et al., 2014; Perrimon et al., 2010) and to alter insect phenotypes for pest and vector control (Borel, 2017; Liu, 2015; Whitfield and Rotenberg, 2015). Unfortunately, the applications of RNAi in entomology and agriculture are currently limited by low RNAi efficiency exhibited by some insects, including lepidopterans such as the European corn borer (ECB), *Ostrinia nubilalis* (Hübner) (Cooper et al., 2019; Kolliopoulou and Swevers, 2014).

The ECB is a polyphagous insect feeding on over 200 plant species, most notably corn. Despite the management strategies already in existence (Capinera, 2000; Whitworth et al., 2014), ECB is a challenging pest to control because larvae escape chemical insecticides by boring into corn stalks, and the development of resistance is well documented (Siegwart et al., 2017; Yu et al., 2018). Pest management strategies that utilize a novel mode of action, such as RNAi, are needed to combat pesticide resistance and to alleviate our reliance on chemical pesticides. However, RNAi-based pest management strategies would prove useful for the management of ECB only if the molecular mechanisms limiting RNAi efficiency can be understood and overcome.

In many insects, core RNAi pathway genes, such as Dicer 2 (*Dcr2*) and Argonaute 2 (*Ago2*), are upregulated in response to exogenous dsRNA (reviewed in Cooper et al., 2019), which results in enhanced RNAi efficiency (Ye et al., 2019). Conversely, insufficient expression and/or function of core RNAi pathway genes, including *Dcr2*, *R2D2*, *Ago2*, Translin (*Trsn*), and Translin-associated factor X (*Trax*), are suspected of limiting RNAi efficiency in some insects

(reviewed in Cooper et al., 2019). Thus, deficiencies in gene structures and transcriptional responses of the core RNAi pathway genes to dsRNA may contribute to low RNAi efficiency in ECB. However, core RNAi pathway genes from ECB had not been identified or characterized.

To that end, we cloned, sequenced, and characterized three major RNAi pathway genes from ECB, *Dcr2, R2D2,* and *Ago2*, and assessed each for changes in expression levels after microinjection or ingestion of non-target dsRNAs. In addition we generated multiple sequence alignments for each gene using the deduced amino-acid sequence and estimated the evolutionary distance of conserved domains. Our data reveal potentially important changes in expression profiles and gene structures that may contribute to low RNAi efficiency in ECB. Moreover, our results support our hypothesis that alterations in gene structures and transcriptional responses of *Ago2, R2D2,* and *Dcr2* may contribute to low RNAi efficiency in ECB.

Methods

Insect rearing

The ECB used in this study originated from French Agricultural Research, Inc. (Lumberton, MN, USA) and were continuously reared in the laboratory at Kansas State University, Manhattan, KS, as described in Cooper et al. (2020a).

Sequencing

To amplify complementary DNAs (DNAs) derived from three major ECB RNAi pathway genes (*OnDCR2*, *OnR2D2*, and *OnAgo2*), specific primers were designed based on transcriptome data from *O. furnacalis* and overlapping PCR products were then amplified Sanger sequencing as described in Cooper et al. (2020b). Finally, specific primers designed near the start and stop codon were used to amplify the entire coding sequence of each and confirm the full-length sequences (Table S-1). Deduced amino-acid sequences were obtained by translating with Gene Runner ver. 3.01 software (Hasting Software Inc., Las Vegas, NV, USA). The molecular mass and isoelectric points of the deduced proteins were predicted with the isoelectric.org web tool (Kozlowski, 2016).

Phylogenetic, domain, and peptide analyses of predicted proteins

To verify the identity of the putative core RNAi pathway transcripts, multiple sequence alignments, phylogenetic analyses, domain comparisons, and subcellular localization predictions were performed as described in Cooper et al. (2020b).

To identify potentially important variations within each of the predicted proteins, Evolutionary distance (P) was estimated for each domain based on the corresponding multiple sequence alignment. P is a ratio of the number of amino-acid substitutions per site (d) divided by the number of sites compared (L), and was computed in MEGA7 software (Kumar et al., 2016) using default settings (i.e., the Poisson model assuming uniform rates among sites, homogeneous pattern lineages, and complete deletion of gaps/missing data). The position of each domain identified in OnDcr2, OnAgo2, and OnR2D2 were located in the corresponding alignment, sequences trimmed around those positions to isolate each domain for computation of both the overall mean P (i.e., the arithmetic mean of all individual pairwise distances between taxa) for the entire population of amino-acid sequences and for the pairwise P for ECB and *Diabrotica virgifera virgifera,* a species exhibiting high RNAi efficiency (Baum et al., 2007; Cooper et al., 2019).

Stage- and tissue-specific expression profiles

Tissue-specific and developmental stage-specific expression profiles of the core RNAi pathway genes in ECB were generated as described in Cooper et al. (2020b). The developmental stages analyzed included: one-day-old eggs, four-day-old eggs, first-, second-, third-, fourth-, and fifth-instar larvae, pupae, one-day-old male moths, three-day-old male moths, one-day-old female moths, and three-day-old female moths. The tissues that were analyzed from fifth-instar larvae included: hemolymph, foregut, midgut, hindgut, Malpighian tubules, silk glands, fat bodies, and carcass (i.e., what was left after the other tissues were removed).

RT-qPCR

RT-qPCR was used to examine tissue and developmental stage-specific expression of nuclease genes in ECB. RT-qPCR was performed in accordance with the minimum information necessary for RT-qPCR (MIQE) guidelines (Bustin et al., 2009) as described in Cooper et al.

(2020a, 2020b). Percent suppression of the target gene was calculated as [(control-target)/control)x100%].

Synthesis of dsRNA

Long dsRNAs (500 bp) targeting either an endogenous gene, *lethal giant larvae* (*OnLgl;* MT467568) from ECB, or an exogenous gene, *enhanced green fluorescent protein* (*GFP*; LC336974.1), were synthesized for feeding and injection experiments in ECB larvae to assess the transcriptional responses of core RNAi pathway genes to dsRNA treatment as described in Cooper et al. (2020a).

Transcriptional response of core RNAi pathway genes to dsRNA exposure

To investigate short- and long-term transcriptional responses of the core RNAi pathway genes from ECB to dsRNA exposure, three individuals were collected at various time points after feeding or injection of dsRNA into second-instar larvae for expression analysis with RT-qPCR. Experiments were designed in a two-way factorial treatment structure so significant effects on relative gene expression, weight, and survivorship due to introduction of dsRNA (e.g., ds*OnLgl*, ds*GFP*, water), nuclease inhibitor (e.g., 0, 10, 20 mM Zn^{2+}), or interaction between both factors could be investigated. Injection and ingestion of dsRNA and sampling for expression analysis was performed as described in Cooper et al. (2020b).

Statistical analyses

Statistical differences between treatment means were assessed in Minitab 18 with either a one- or two-way ANOVA followed by a Tukey HSD test as described in Cooper et al. (2020b).

Results

Sequencing and characterization

Sequence analyses revealed one transcript for each of three core siRNA pathway genes (*Dcr2, R2D2,* and *Ago2)* from ECB (Table 4-1). The predicted proteins were predicted to

localize in the cytoplasm as well as in a few other organelles (Table 4-1). The OnDcr2 (Fig. S4-1) and OnAgo2 (Fig. S4-2) proteins clustered monophyletically in phylogenetic trees, while the predicted OnR2D2 protein clustered together with lepidopteran, orthopteran, and some hemipteran R2D2 proteins (Fig. 4-1). All insects included in the phylogenetic analysis contained a single isoform for each core siRNA pathway component, except for *Leptinotarsa decemlineata* which has two Dcr2 isoforms (Fig. S4-1); *Spodoptera frugiperda, Nilaparvata lugens, Locusta migratoria, Tribolium castaneum,* and *Mayetiola destructor* which has two Ago2 isoforms (Fig. S4-2); and *D. v. virgifera* and *M. destructor* which both have two R2D2 isoforms (Fig. 4-1). Multiple sequence alignments revealed high conservation among key residues in R2D2 (Fig. S4-3) and in the active site of Ago2 proteins (Fig. S4-4), whereas little conservation was apparent in the phosphate binding pocket of Dcr2 proteins (Figs. 4-2, S4-5).

Table 4-1. Characteristics of core RNAi pathway transcripts and their deduced proteins from ECB.

Attribute	**OnDcr2**	**OnR2D2**	**OnAgo2**
GenBank accession no.	MT921812	MT981255	MT524717
ORF (bp)	5,073	933	3,150
Protein (aa)	1,690	310	1,049
Mass (kDa)	193.14	34.63	117.42
Isoelectric point (pI)	6.19	8.26	8.76
Localization predictions	cytoplasm, plasma membranes, cortex, nucleus	cytoplasm	cytoplasm, cytoskeleton, nucleus

Abbreviations: Dcr2, dicer 2; R2D2, Two binding domains associated with Dicer 2; Ago2, Argonaute 2; ORF, open reading frame; bp, base pair; kDa, kilodalton; aa, amino-acid.

Figure 4-1. Phylogenetic tree showing the relationships among insect R2D2 proteins.

Bootstrap support is indicated at internal nodes. Different shapes and shading denote different insect orders. The species and gene accession number corresponding to each sequence label are as follows for each order. Coleoptera: DvR2D2a, *D. v. virgifera* (XP_028140225.1); DvR2D2b, *D. v. virgifera* (XP_028148994.1); LdR2D2a, *L. decemlineata* (XP_023028942.1); AgR2D2, *Anoplophora glabripennis* (XP_018571941.1); TcR2D2, *T. castaneum* (NP_001128425.1); DpR2D2, *Dendroctonus ponderosae* (XP_019759956.1); ApR2D2, *Agrilus planipennis* (AJF15707.1). Diptera: MdR2D2b, *Mayetiola destructor* (AFX89027.1); MdR2D2a, *M. destructor* (AFX89026.1); BdR2D2, *Bactrocera dorsalis (*AHI44611.1); DmR2D2, *Drosophila melanogaster* (FBpp0290544); AdR2D2, *Anopheles darling* (ETN67610.1); AaR2D2, *Aedes aegypti* (AJF11544.1): CqR2D2, *Culex quinquefasciatus* (EDS36285.1). Hymenoptera: BpR2D2, *Bombus pascuorum* (ANC73693.1); AmR2D2, *Apis mellifera* (XP_006560091.1); AfR2D2, *Apis florea* (XP_003698324.1); HsR2D2, *Harpegnathos saltator* (XP_011153401.1); NleR2D2, *Neodiprion lecontei* (XP_015523716.1); NvR2D2, *Nasonia vitripennis* (XP_008214047.1). Hemiptera: AglR2D2, *Aphis glycines* (AFZ74932.1); ApiR2D2, *Acyrthosiphon pisum* (NP_001155644.1); BtR2D2b, *Bemisia tabaci* (AIC07486.1); NilR2D2, *N. lugens* (AGH30333.1); BtR2D2a, *B. tabaci* (AHY18682.1); HhR2D2, *Halyomorpha halys* (XP_014274312.1); ClR2D2, *Cimex lectularius* (XP_024085947.1). Lepidoptera: OnR2D2, *O. nubilalis* (MT981255); OfR2D2, *O. furnacalis* (SXU); SlR2D2, *Spodoptera litura*

(AHC98011.1); BmR2D2, *Bombyx mori* (NP_001182007.1). Orthoptera: LmR2D2, *L. migratoria* (SXU).

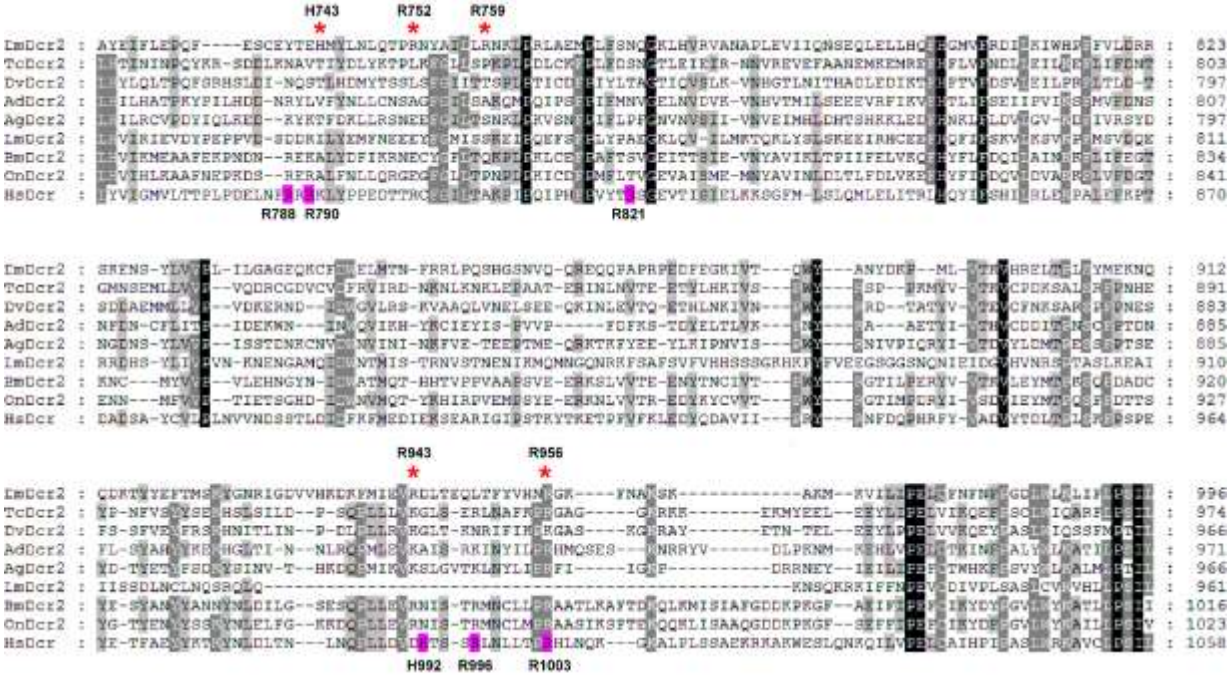

Figure 4-2. Multiple sequence alignment showing important residues in the phosphate binding pocket of Dcr2 proteins from insects as well as Dicer from human.

Amino-acid residues that form the phosphate binding pocket in *Drosophila melanogaster* Dcr2 are indicated by a red asterisk and numbered along the top. Amino-acid residues that form the phosphate binding in human Dcr are indicated in pink and numbered along the bottom. Black shading indicates 100% identity, dark-grey shading indicates 75–99% identity, and light-grey shading indicates 55–74% identity. The species and gene accession number corresponding to each sequence label is as follows: DmDcr2, *Drosophila melanogaster* (AAF57830.2); TcDcr2, *Tribolium castaneum* (NP_001107840.1); DvDcr2, *Diabrotica virgifera virgifera* (AUM60046.1); AdDcr2, *Apis dorsata* (XP_006623214.1); AgDcr2, *Aphis glycines* (AFZ74931.1); LmDcr2, *Locusta migratoria* (BAW35365.1); BmDcr2, *Bombyx mori* (NP_001180543.1); OnDcr2, *Ostrinia nubilalis* (MT921812); HsDcr, *Homo sapiens* (XP_016876610.1).

Comparisons of domain architecture indicated that Dcr2 was more variable among insects than either Ago2 or R2D2 proteins. Dcr2 from orthopterans lacks a PAZ domain (PS50821), and many insects lack a terminal double-stranded RNA binding domain (PS50137; dsRBD) (Fig. 4-3). However, OnDcr2 was of similar length and domain structure as Dcr2 from *D. v. virgifera* (DvDcr2) and *Tribolium castaneum* (Fig. 4-3), two coleopterans that exhibit high RNAi efficiency. Domain predictions indicated that OnR2D2 contained two dsRBDs, as do the majority of R2D2 proteins, and was similar in length to all other R2D2 (Fig S4-3). OnAgo2

contained two domains, including a PAZ domain and a PIWI domain (PS50822). Comparisons of domain architecture indicated that Ago2 proteins from lepidopterans are slightly larger than Ago2 from coleopterans (Fig. 4-4). In addition, the length of the PAZ domain varied among insects, but otherwise domain architecture of Ago2 proteins appeared fairly similar between insect species (Fig. 4-4).

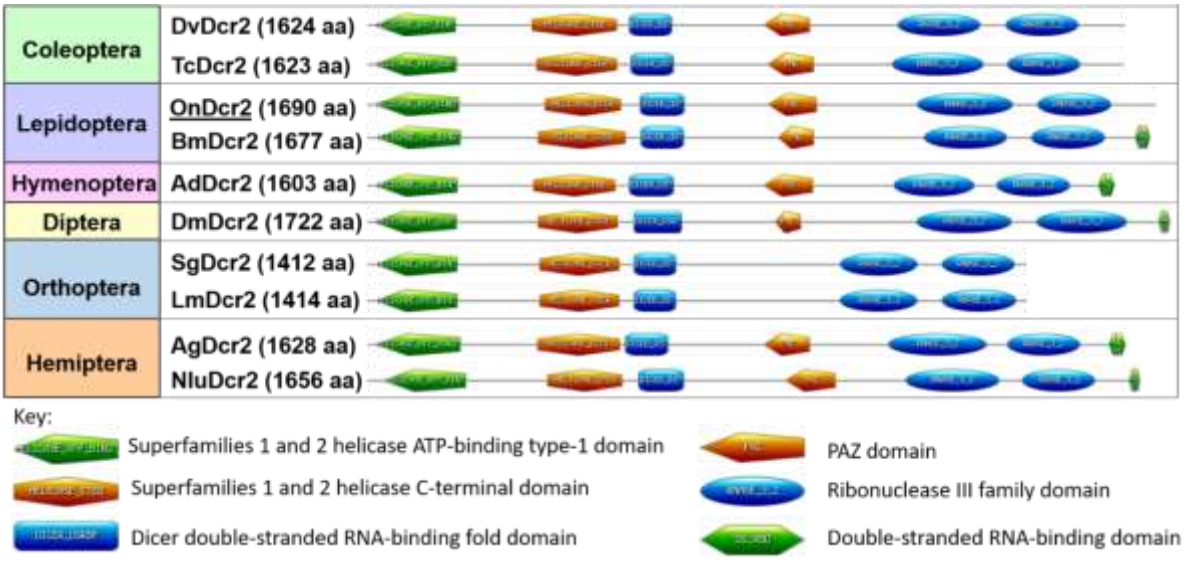

Figure 4-3. Comparison of domain architecture showing the differences in domain structure of insect Dcr2 proteins.

The species and gene accession number corresponding to each sequence label is as follows for each order. Coleoptera: DvDcr2, *Diabrotica virgifera virgifera* (AUM60046.1); TcDcr2, *Tribolium castaneum* (NP_001107840.1). Lepidoptera: OnDcr2, *Ostrinia nubilalis* (KSU); BmDcr2, *Bombyx mori* (NP_001180543.1). Hymenoptera: AdDcr2, *Apis dorsata* (XP_006623214.1). Diptera: DmDcr2, *Drosophila melanogaster* (AAF57830.2). Orthoptera: SgDcr2, *Schistocerca gregaria* (BAX36478.1); LmDcr2, *Locusta migratoria* (BAW35365.1). Hemiptera: AgDcr2, *Aphis glycines* (AFZ74931.1); NluDcr2, *Nilaparvata lugens* (AGH30333.1).

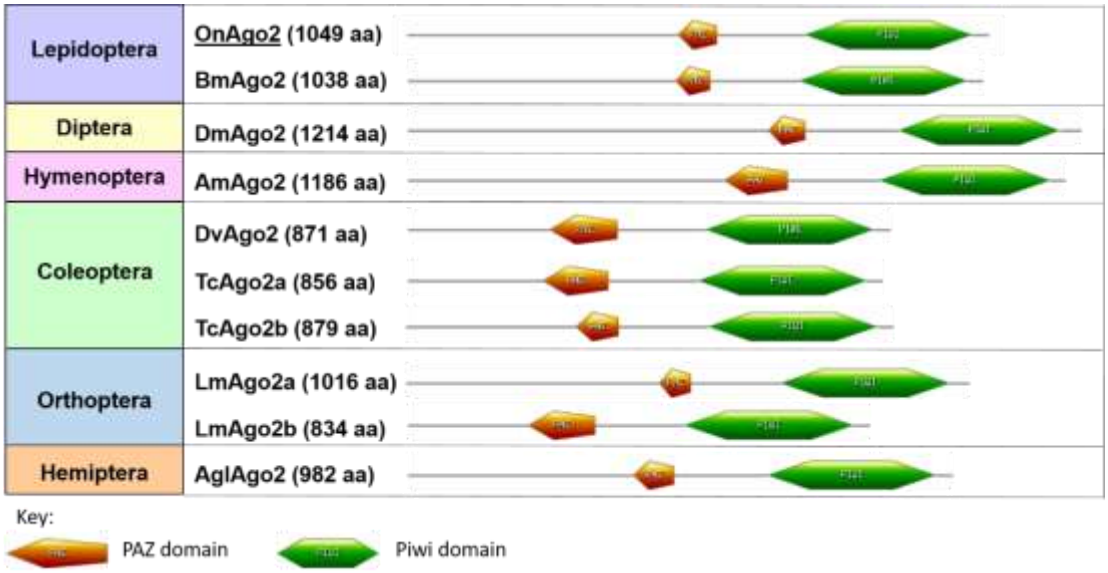

Figure 4-4. Comparison of domain architecture showing the differences in domain structures of insect Ago2 proteins.

The species and gene accession number corresponding to each sequence label is as follows for each order. Lepidoptera: OnAgo2, *Ostrinia nubilalis* (KSU); BmAgo2, *Bombyx mori* (NP_001036995.2). Diptera: DmAgo2, *Drosophila melanogaster* (NP_648775.1). Hymenoptera: AmAgo2, *Apis mellifera* (XP_395048.4). Coleoptera: DvAgo2, *Diabrotica virgifera virgifera* (AUM60042.1); TcAgo2a, *Tribolium castaneum* (NP_001107842.1); TcAgo2b, *T. castaneum* (NP_001107828.1). Orthoptera: LmAgo2a, *Locusta migratoria* (SXU); LmAgo2b, *L. migratoria* (SXU). Hemiptera: AglAgo2, *Aphis glycines* (AFZ74933.1).

Comparison of evolutionary distances (P) for each domain in each core pathway component indicated which domains had undergone the most divergence among all insects investigated, as well as between ECB and *D. v. virgifera*, a species that is highly amenable to RNAi. In Dcr2, the PAZ domain was the most variable domain among the 31 insects investigated (Fig. 4-3; Table 4-2). In addition, there was more variation in the amino-acid sequence of the first RNase III domain between OnDcr2 and DvDcr2 (P=3.091) than on average among all 30 insect Dcr2 proteins analyzed (P=1.997) (Table 4-2).

Comparison of overall mean P indicated that the first dsRBD of OnR2D2 was more conserved than the second among all 31 insects investigated (Table 3). Pairwise comparisons indicated higher divergence at the amino-acid level between OnR2D2 and DvR2D2a/DvR2D2b for the first (P=1.964, 1.750) and second (P=3.127, 2.613) dsRBDs, than on average among all 30 insect Dcr2 proteins analyzed (P=1.673, 1.966) (Table 3).

Comparison of overall mean P for each region of the amino-acid alignment corresponding to each predicted domain of OnAgo2 indicated that the PIWI domain was highly conserved among all insects, and to a lesser extent, so was the PAZ domain (Fig. 4-4; Table 4-4). However, there was more divergence between both these domains in DvAgo2 and OnAgo2 than between all other insect Ago2 proteins on average.

Table 4-2. **Position and evolutionary distance (P) of amino-acid residues corresponding to each predicted domain of OnDcr2.**

Identical sequences have a P=0. Thus, larger P values indicate greater variability or divergence within that domain. *Abbreviations:* Dcr2, dicer 2; aa, amino-acid; *On, Ostrinia nubilalis; Dv, Diabrotica virgifera virgifera.*

Dicer 2 Domains	aa start position on OnDcr2	aa end position on OnDcr2	Overall mean P among all insects	Pairwise P between OnDcr2 & DvDcr2
Superfamily 1 & 2 helicase ATP-binding type-1 domain	19	198	1.394	1.328
Superfamily 1 and 2 helicase C-terminal domain	381	544	1.738	1.500
Dicer double-stranded RNA binding fold domain	585	680	2.127	2.232
PAZ domain	860	964	3.079	2.673
Ribonuclease III family domain (first)	1178	1386	1.997	3.091
Ribonuclease III family domain (second)	1436	1595	0.945	1.266

Table 4-3. Position and evolutionary distance (P) of amino-acid residues corresponding to each predicted domain of OnR2D2.

Identical sequences have a P=0. Thus, larger P values indicate greater variability or divergence in that sequence. *Abbreviations:* R2D2, Two binding domains associated with Dicer 2; aa, amino-acid; *On, Ostrinia nubilalis; Dv, Diabrotica virgifera virgifera.*

R2D2 Domains	aa start position on OnR2D2	aa end position on OnR2D2	Overall mean P among all insects	Pairwise P between OnR2D2 & DvR2D2a	Pairwise P between OnR2D2 & DvR2D2b
double-stranded RNA-binding domain (first)	8	75	1.673	1.964	1.750
double-stranded RNA-binding domain (second)	104	176	1.966	3.127	2.613

Table 4-4. **Position and evolutionary distance (P) of amino-acid residues corresponding to each predicted domain of OnAgo2.**

Identical sequences have a P=0. Thus, larger P values indicate greater variability or divergence in that sequence. *Abbreviations:* Ago2, Argonaute 2; aa, amino-acid; *On, Ostrinia nubilalis; Dv, Diabrotica virgifera virgifera.*

Domains within OnAgo2	aa start position on OnAgo2	aa end position on OnAgo2	Overall mean P among all insects	Pairwise P between OnAgo2 & DvAgo2
PAZ domain	490	559	1.891	2.343
PIWI domain	719	1,018	0.813	1.089

Expression profiles indicated that *OnDcr2*, *OnR2D2*, and *OnAgo2* transcripts were detected in all ECB developmental stages and tissues. *OnDcr2* transcript levels were highest in egg (1-day post-oviposition) and adult males (1-day post-eclosion, Fig. 4-4A, $F(11,24)=7.50$, $p<0.0001$*), with highest transcript levels in the gut when compared to other tissues from fifth-instar larvae (Fig. 4-5B, $F(3,8)=6.45$, $p=0.016$*). Expression of *OnR2D2* transcripts was highest in adult females (3-day post-eclosion, Fig. 4-5C, $F(11,24)=11.94$, $p<0.0001$) in the gut and hemolymph when compared to expression in other tissues from fifth instar larvae (Fig. 4-5D, $F(3,8)=14.51$, $p=0.001$*). *OnAgo2* transcripts were highly expressed in adult males (Fig. 4-5E, $F(11,24)=16.61$, $p<0.0001$*) and in the gut when compared to expression in other tissues from fifth-instar larvae (Fig. 4-5F, $F(3,8)=7.56$, $p=0.010$*).

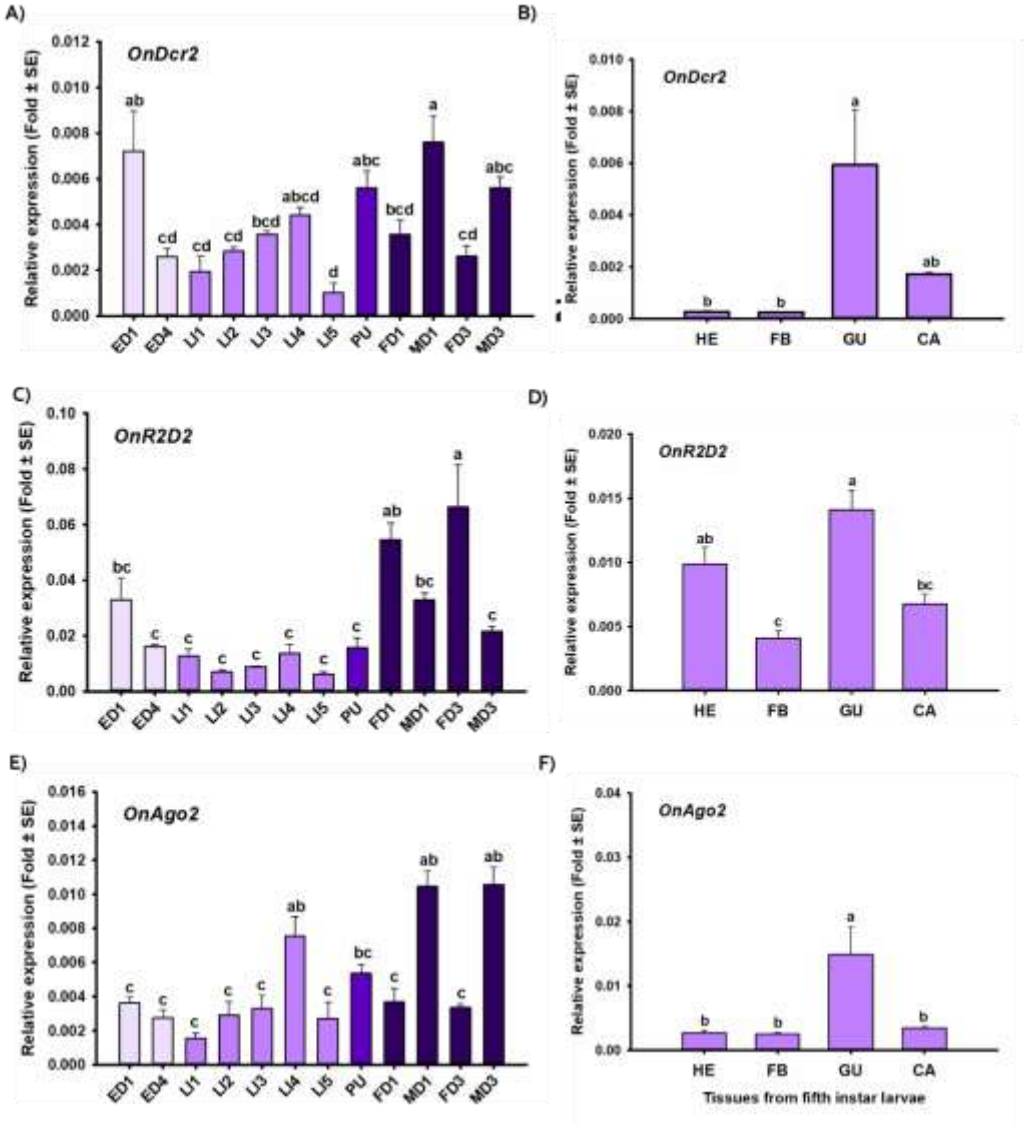

Figure 4-5. Expression profiles of *OnDcr2* (A, B), *OnR2D2* (C, D) and *OnAgo2* (E, F) across ECB developmental stages (A, C, E) and tissues (B, D, F).

Mean fold change in the relative expression of *OnAgo2 OnR2D2* and *OnAgo2* in 1-d-old eggs (ED1) 4-d-old eggs (ED4), first-instar larvae (LI1), second-instar larvae (LI2), third-instar larvae (LI3), fourth-instar larvae (LI4), fifth-instar larvae (LI5), pupae (PU), 1-d-old adult females (FD1), 1-d-old adult males (MD1), 3-d-old adult females (FD3), and 3-d-old adult males (MD3), as well as in hemolymph (HE), fat body (FB); gut (GU), and carcass (CA). Fold change is relative to the expression of reference genes only. Significant differences among treatments are indicated by different letters.

Transcriptional responses to dsRNA

OnDcr2 expression was unaffected by dsRNA injection at all the time points investigated, even when a nuclease inhibitor (Zn^{2+}) was used (data not shown). However, *OnDcr2* was upregulated at 2 and 12 h after the start of feeding dsRNA (Figs. 4-6, 4-7). At the 3 h time point, *OnDcr2* was upregulated 2.83-fold in the ds*OnLgl* fed treatment group compared to the water fed treatment group, regardless of Zn^{2+} concentration [$F(2,17)=3.13$, $p=0.070$] (Fig. 4-6B), and 2.32-fold in the 20 mM Zn^{2+} fed groups compared to the 0 mM Zn^{2+} fed group, regardless of dsRNA treatment [$F(2,17)=2.79$, $p=0.089$] (Fig. 4-6C). Similarly, at the 12 h time point, *OnDcr2* was upregulated 2.01-fold in the 10 and 20 mM Zn^{2+} injected groups on average compared to the 0 mM Zn^{2+} fed treatment group, regardless of dsRNA treatment [$F(2,18)=2.70$, $p=0.094$] (Fig. 4-7). However, these trends toward upregulation were not statistically significant. Expression of *OnDcr2* was unaffected by feeding at the other time points investigated (data not shown).

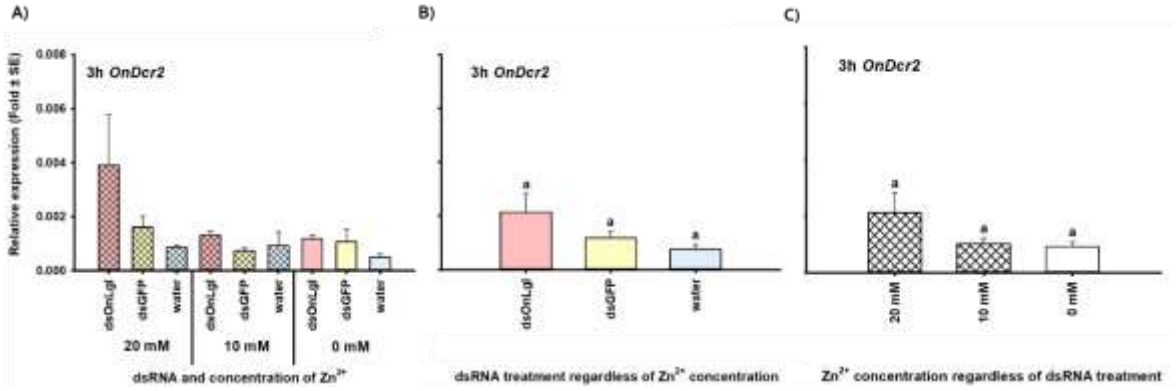

Figure 4-6. Relative expression of *OnDcr2* after 3 hours of feeding on dsRNA/nuclease inhibitor for A) all treatment groups, B) for main effects due to dsRNA treatment and C) for main effects due to nuclease inhibitor concentration.

Mean fold change in the relative expression of *OnDcr2* in second instar ECB larvae, 3 h after the start of feeding on artificial diet treated with various combinations of dsRNA and nuclease inhibitor (Zn^{2+}). Fold change is relative to the expression of reference genes only**.** Significant differences among treatments are indicated by different letters.

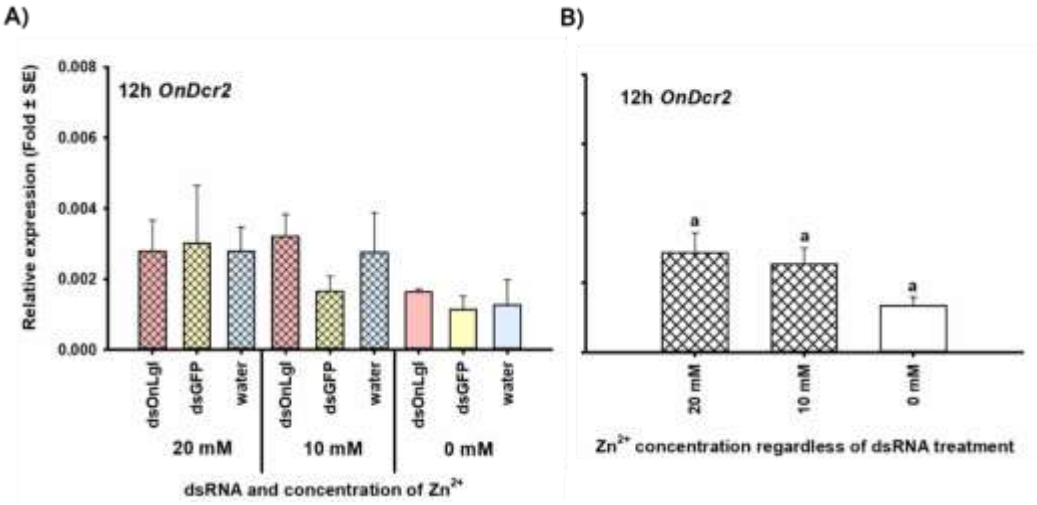

Figure 4-7. Relative expression of *OnDcr2* after 12 hours of feeding on dsRNA/nuclease inhibitor for A) all treatment groups, and B) for main effects due to nuclease inhibitor concentration.

Mean fold change in the relative expression of *OnDcr2* in second instar ECB larvae, 12 h after the start of feeding on artificial diet treated with various combinations of dsRNA and nuclease inhibitor (Zn^{2+}). Fold change is relative to the expression of reference genes only. Significant differences among treatments are indicated by different letters.

Expression of *OnR2D2* was unaffected by dsRNA injection (data not shown) at all time points investigated, even when Zn^{2+} was used. Surprisingly, *OnR2D2* expression significantly decreased 0.58-fold three h after the start of feeding in the ds*OnLgl* fed treatment group compared to the ds*GFP* fed treatment group, regardless of Zn^{2+} concentration, [F(2,18,)=3.68, p=0.046*] (Fig. 4-8). At 6-days, *OnDcr2* was significantly increased 7.37-fold in the 20 mM Zn^{2+} ds*GFP* fed treatment group compared to the 20 mM Zn^{2+} water fed treatment group [F(2,12)=3.83, p=0.052*] (Fig. 4-9). Expression of *OnR2D2* was unaffected by feeding at the other time points investigated (data not shown).

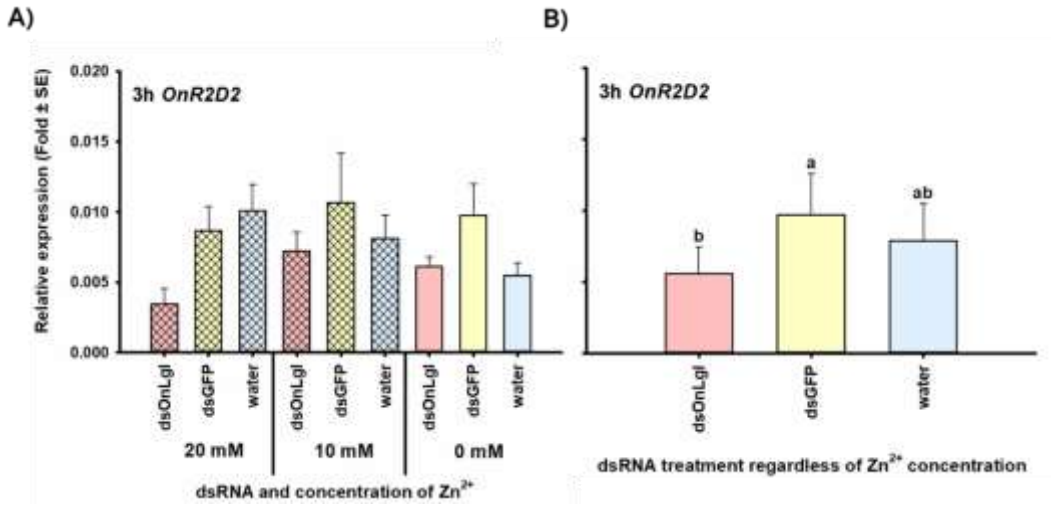

Figure 4-8. Relative expression of *OnR2D2* after 3 hours of feeding on dsRNA/nuclease inhibitor for A) all treatment groups, and B) for main effects due to dsRNA treatment.

Mean fold change in the relative expression of *OnR2D2* in second-instar ECB larvae, 3 h after the start of feeding on artificial diet treated with various combinations of dsRNA and nuclease inhibitor (Zn^{2+}). Fold change is relative to the expression of reference genes only. Significant differences among treatments are indicated by different letters.

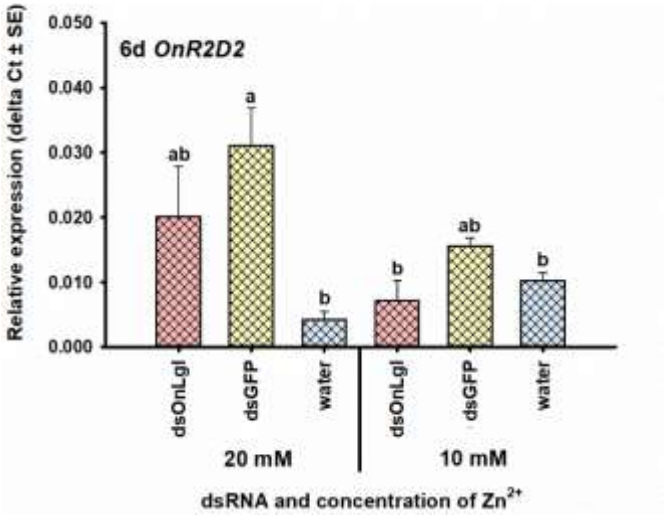

Figure 4-9. Relative expression of *OnR2D2* after 6 days of feeding on dsRNA/nuclease inhibitor for all treatment groups.

Mean fold change in the relative expression of *OnR2D2* in second-instar ECB larvae, 6 d after the start of feeding on artificial diet treated with various combinations of dsRNA and nuclease inhibitor (Zn^{2+}). Fold change is relative to the expression of reference genes only. Significant differences among treatments are indicated by different letters.

Expression of *OnAgo2* was unaffected by dsRNA injection and feeding at most time points investigated, even when Zn^{2+} was used (data not shown). There were significant differences in *OnAgo2* expression among some treatments 3 days after injection [$F(2,11)=3.95$, $p=0.051$*], but these differences could not be interpreted meaningfully (data not shown).

Discussion

The use of RNAi as a versatile research tool to study gene function in insects and as a new mode of action for insect pest management requires a better understanding of major RNAi pathway genes from agricultural pests, as well as a better understanding of mechanisms that contribute to low RNAi efficiency in representative lepidopteran pests, such as ECB. Evidence suggests that differences in the function and response of core RNAi pathway components, such as Dcr2, Ago2, R2D2, Trsn, and Trax, can influence RNAi efficiency, and may contribute to low RNAi efficiency in lepidopterans, hemipterans, dipterans, and orthopterans (reviewed in Cooper et al., 2019; Davis-Vogel et al., 2018; Ye et al., 2019). Differences in gene copy number, domain architecture, basal expression levels, response to dsRNA, and activity levels of these core RNAi pathway genes may contribute to deficiencies in the core RNAi machinery, and thus reduce RNAi efficiency in some insect taxa.

In the present study we investigated if evolutionary changes in gene structure or differences in transcriptional response of three core genes from the RNAi pathway contribute to low RNAi efficiency in ECB. One of these, *Dcr2* encodes an RNase III enzyme that initiates an RNAi response by binding to dsRNA in the cytoplasm and processing it into small interfering RNA (siRNA). *R2D2* encodes a dsRNA binding protein that facilitates the transfer of siRNA from Dcr2 to the RNAi-induced silencing complex (RISC). Lastly, *Ago2* encodes an Argonaute family protein that acts as the catalytic center of RISC by binding to siRNA and cleaving complementary messenger RNA (mRNA).

Research suggests that differences in expression of core RNAi genes in insects contributes to the variability in RNAi efficiency among agriculturally important pests (Davis-Vogel et al., 2018). In the present study, a single transcript for each of the core RNAi pathway genes, *OnDcr2, OnR2D2,* and *OnAgo2,* were identified in ECB. A single transcript per gene indicates that low expression of major RNAi pathway genes could be a concern for RNAi

efficiency in this species. However, expression of *OnDcr2, OnR2D2,* and *OnAgo2* were detected in all developmental stages and tissues investigated in ECB (Fig. 4-5) suggesting that ECB has the capacity to produce an RNAi response in all of these cases. The expression patterns for *OnDcr2, OnR2D2,* and *OnAgo2* were similar to the expression patterns shown for core RNAi components in *Nezara viridula* and *S. frugiperda* (Davis-Vogel et al., 2018). In particular, fourth-instar larvae, pupae, and adult ECB and gut tissues had highest transcript levels of these core RNAi pathway genes and are likely to exhibit the highest RNAi efficiency (Gong et al., 2015). (Fig. 4-5B, D, F).

In many insects, expression of R2D2 is undetectable, including *Diaphorina citri* (Taning et al., 2016), *L. decemlineata* (at least based on the gut transcriptome) (Swevers et al., 2013), apterygotes (primary wingless insects) (Dowling et al., 2016), and lepidopterans in the group Ditrysia (Dowling et al., 2016). Interestingly, while ECB is a member of Ditrysia, basal expression of R2D2 was detected in all stages and tissues of ECB (Fig. 4-5C,D). Nonetheless, in some species, such as *D. melanogaster* (Dietzl et al., 2007), *B. mori* (Swevers, 2011; Li et al., 2015), and *P. xylostella* (Hameed et al., 2019) expression of the core RNAi pathway genes is enough to mediate an RNAi response, but RNAi efficiency was still enhanced by overexpression of specific core genes. These studies demonstrate the important relationship between RNAi efficiency and expression of core RNAi pathway genes.

Other studies have demonstrated that upregulation of the core RNAi pathway genes in response to dsRNA is also an important predictor of RNAi efficiency, and indicate that instability of dsRNA and insufficient internalization of dsRNA can impact induction of the core RNAi pathway genes (reviewed in Cooper et al., 2019). Furthermore, upregulation of the core RNAi pathway genes has been shown to enhance RNAi efficiency in insects (Garbutt and Reynolds et al., 2012; Guo et al., 2015; Ye et al., 2019). In ECB, no significant differences in relative gene expression of *OnDcr2, OnR2D2,* or *OnAgo2* were found at any time after dsRNA injection (data not shown), but differences were observed after continuous feeding of dsRNA in artificial diet. Importantly, our injection protocol introduced dsRNA only once, while feeding enabled persistent introduction of dsRNAs. Therefore it is possible that the differences observed between injection and ingestion of dsRNA were, at least in part, due to duration of dsRNA exposure. Strangely, expression of *OnDcr2* increased 2.8-fold 3 h after continuous feeding of dsRNA (Fig. 4-6B) while expression of *OnR2D2* decreased by 0.6-fold (Fig. 4-8B) and

expression of *OnAgo2* was unaffected (data not shown). This is the first report of a core RNAi pathway gene being downregulated in response to short-term, exogenous dsRNA exposure. Continuous exposure to dsRNA for 72 h resulted in downregulation of *LdAgo2A* and *LdAgo2B* in early larval instars of *L. decemlineata*, and downregulation of *LdAgo2A*, *LdAgo2B*, *LdDcr2A*, and *LdDcr2D* in last-instar larvae (Guo et al., 2015). In addition, exposure to bacteria, temperature extremes and starvation decreased expression of core RNAi enzymes in *B. dorsalis* (Xie et al., 2016). However, these are very different circumstances than described in this study.

Upregulation of *OnDcr2* in ECB upon continuous feeding of dsRNA is comparable to upregulation of *ApDcr2* observed in *A. pisum* 12 h after a single injection of dsRNA (Ye et al., 2019). Interestingly, in other species such as *Blatella germanica*, *Manduca sexta*, and *L. decemlineata*, upregulation of *Dcr2* and *Ago2* was much higher and faster (Garbutt and Reynolds, 2012; Guo et al., 2015; Lozano et al., 2012). However, in ECB, only *OnDcr2* was upregulated, supporting the hypothesis that the core enzymes in ECB may be insufficiently upregulated to generate a robust RNAi response.

It is possible that high levels of dsRNA degradation in the ECB gut and hemolymph (data not shown) prevented the majority of dsRNA from reaching the core enzymes of the RNAi pathway thus limiting activation of the RNAi machinery, as suggested in other insects (Christianes et al. 2014; Guan et al., 2018a; Guan et al., 2019; Liu et al., 2013). If the core RNAi enzymes in ECB were not upregulated due to rapid degradation of dsRNA, then we would expect to see upregulation of core enzymes in treatments where dsRNA was delivered in combination with a nuclease inhibitor, but not for naked dsRNA. However, injection and ingestion of dsRNA in combination with the nuclease inhibitor (Zn^{2+}) resulted in significantly increased expression of *OnR2D2* only after 6 days of continuous feeding on diet containing ds*GFP* and 20 mM Zn^{2+} compared to continuous feeding on diet containing only 20 mM Zn^{2+} (Fig. 4-9). Therefore, additional mechanisms of low RNAi efficiency are likely present in ECB, such as incomplete dsRNA internalization or deficiencies of the core RNAi machinery.

The nuclease inhibitor also had a significant effect on *OnDcr2* expression 3 and 12 h after initiation of dsRNA feeding, resulting in a 2.3- and 2.0-fold increase in expression of *OnDcr2*, relative to the 0 mM Zn^{2+} treatment groups, regardless of dsRNA treatment (Fig. 4-6B, 4-7B). This finding could suggest that oral delivery of 10 and 20 mM Zn^{2+} inhibits intracellular activity of OnDcr2, resulting in upregulation of *OnDcr2* to compensate for decreased enzymatic

activity. However, many other explanations are also possible. Nonetheless, expression of the *OnLg*l target gene was suppressed by 68% 6 days after continuous feeding of ds*OnLgl* in artificial diet, compared to larvae fed on ds*EGFP* in diet, regardless of Zn^{2+} concentration (Cooper et al., 2020c). This result suggests that the RNAi pathway is functional in ECB despite treatment with Zn^{2+}, and/or downregulation of *OnR2D2* in response to oral delivery of dsRNA. RNAi-mediated suppression was not observed after injection-based RNAi assays that introduced dsRNA into second-instar larvae presented here (Cooper et al., 2020c).

It is also possible that a single injected dose of dsRNA may be insufficient to activate the core RNAi machinery in ECB, whereas long oral exposure is. In the future, it would be interesting to see if repeated injections can prime the RNAi pathway and enhance RNAi efficiency in ECB by upregulating expression of core RNAi pathway components, as described in *A. pisum*, *M. sexta*, and *L. decemlineata* (Garbutt and Reynolds, 2012; Guo et al., 2015; Ye et al., 2019). Expression of core RNAi pathway genes can also be influenced by environmental factors such as viral infections, temperature extremes, and other stressors (reviewed in Cooper et al., 2019), and the injection bioassay in second-instar larvae was performed immediately prior to complete decimation of our ECB colony by an unknown pathogen. Therefore, additional efforts are required to confirm the results presented here.

Functional differences in the catalytic activity of the core RNAi proteins are also likely to contribute to the variability in RNAi efficiency among insects (Singh et al., 2017; Davis-Vogel et al., 2018). In ECB, OnAgo2 and OnDcr2 clustered monophyletically (Figs. S4-1, S4-2), but OnR2D2 clustered with R2D2 proteins from hemipterans and a few other lepidopterans (Fig. 4-1), supporting the idea that the low and variable RNAi efficiency among lepidopterans and hemipterans could in part be due to differences associated with R2D2 in these insect lineages.

Comparison of the evolutionary distances corresponding to each domain of the protein alignments for each core RNAi component revealed potentially important variations in the first RNase III domain of OnDcr2 (Table 4-2), both dsRBDs of OnR2D2 (Table 4-3), and both the PAZ and PIWI domains of OnAgo2 (Table 4-4). Variations in the first RNase III domain may impact the catalytic activity of OnDcr2 because the two RNase III domains form a pseudo-dimer, and each domain hydrolyzes one strand of the dsRNA (Hammond, 2005). Moreover, multiple sequence alignments of the PAZ domain from insect Dcr2 proteins revealed that all five of the "conserved" residues in the phosphate-binding pocket of Dcr2 (Kandasamy and Fukunaga 2016)

were actually highly variable among insects (Fig. 4-2). These five residues in the phosphate-binding pocket (H743, R752, R759, R943, R956) are crucial for high-fidelity production of 21-nt siRNAs, which in turn is important for efficient RNAi-mediated silencing in *D. melanogaster* (Kandasamy and Fukunaga 2016). Variations at these five residues among insects further indicates that inefficient production of siRNAs may limit RNAi efficiency in some species.

The two dsRBDs of R2D2 bind dsRNA asymmetrically to favor loading of the guide strand of the siRNA into RISC (Hamond, 2005), indicating that variations in this region may impact loading of dsRNA into RISC. In addition, variations in the PAZ and PIWI domains of Ago2 may impact binding of siRNA as well as the catalytic activity of Ago2 in mRNA degradation. However, multiple sequence alignment revealed clear conservation among insects and humans in the 5' phosphate-anchoring region of the PAZ domain and the Ago Asp-Asp-His motif (Fig. S4-4), which are required for catalytic activity (Tolia and Joshua-Tor, 2007; Schirle and MacRae, 2012; Hameed et al., 2019). Thus the function of OnAgo2 is less suspect than the function of OnDcr2 and OnR2D2. Together, these variations in specific functional domains of all three major core RNAi pathway components may contribute to differences in RNAi efficiency between insects, as well as possibly provide insight into how dsRNA processing into siRNA occurs at different sequence-specific sites in different insects (Guan et al., 2018b).

Taken together, the results of this study support the idea that the function of the core RNAi enzymes could contribute to low RNAi efficiency in ECB. Specifically, the results presented here indicate that *OnR2D2* and *OnAgo2* are not upregulated in response to dsRNA. In addition, there are variations in important domains within the predicted protein sequences that may impact the function or activity of OnAgo2, OnR2D2, and OnDcr2. Efforts to characterize and compare enzyme kinetics of OnAgo2 and OnDcr2 in insects are badly needed. In addition, a greater understanding of the importance of upregulation of the core enzymes for activation of the RNAi response is needed, as upregulation of *Dcr2* appears to have occurred faster, longer, and at a greater magnitude in *A. pisum* (Ye et al., 2019) than in ECB. Core RNAi components from the miRNA pathway and piRNA pathways also need to be investigated to determine if components from these other pathways contribute to the exogenous RNA response in ECB, as has been shown in other insects (reviewed in Cooper et al., 2019). Furthermore, greater effort needs to be placed on a holistic understanding of how multiple mechanisms of low RNAi efficiency interact

and influence RNAi efficiency at the organismal and population levels, as RNAi efficiency under field conditions will undoubtedly be more variable than under controlled lab conditions.

Conclusion

This investigation identified and characterized the transcripts of three major RNAi pathway genes (*Dcr2, Ago2*, and *R2D2*), all of which are expressed in all developmental stages and tissues of ECB investigated. Their deduced amino-acid sequences contain all the necessary domains apparently needed for an efficient RNAi response; however, the activity of these proteins may differ between ECB and other insects due to variations in amino-acid sequence within key domains. Activation of *OnDcr2, OnR2D2,* and *OnAgo2* was not observed after dsRNA injection, rather was only observed after ingestion of dsRNA, and only for one of the three core pathways genes (*OnDcr2*). Together, these findings indicate that insufficient activation of the core RNAi pathway genes in response to introduction of dsRNA may contribute to low RNAi efficiency in ECB. However, further investigation of enzyme activity is necessary to confirm this. These findings are significant because they indicate that ECB can mount an RNAi response if dsRNA can reach the core RNAi machinery located within cells; however, deficiencies in the response of the core RNAi pathway genes may limit RNAi efficiency in ECB. This knowledge will facilitate the development of strategies for enhancing dsRNA stability in ECB tissues, and advance our understanding of mechanisms impacting RNAi efficiency in ECB and related lepidopterans.

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Chapter 5 - Comparison of Strategies for Enhancing RNA Interference Efficiency in *Ostrinia nubilalis*

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Abstract

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 the European corn borer (ECB), *Ostrinia nubilalis*. 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. *Ex vivo* incubation experiments revealed that Metafectene Pro, EDTA, chitosan-based dsRNA nanoparticles, and Zn^{2+} 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*. 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.

Background

Targeting insect-specific genes through post-transcriptional gene silencing with RNA interference (RNAi) is a new strategy for insect pest management (Zhu and Palli, 2020). RNAi-mediated crop protection strategies, including transgenic plants, baits, and sprays, are valuable resources for sustainable agriculture and food security, because they provide new strategies to address yield loss (Godfray et al., 2010), they provide a novel mechanism of action to combat pesticide resistance (Khajuria et al., 2018), and they provide greater species-specificity to reduce off-target environmental effects (Whyard et al., 2009). Unfortunately, RNAi technology is currently hindered by inefficient and highly variable results when different insects are targeted, especially lepidopterans (Kolliopoulou and Swevers, 2014; Terenius et al., 2011). Given that lepidopterans are 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 European corn borer (ECB), *Ostrinia nubilalis* (Hübner) (Khajuria et al., 2011; Smagghe and Swevers, 2014).

The ECB is a small, light brown, crambid moth native to Europe that was first introduced into the United States in the early 1900's and is now established in North America (Thieme et al., 2018; Capinera, 2000). ECB is one of the most damaging pests of corn in North America and Europe (Thieme et al., 2018), 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* (Capinera, 2000; Whitworth et al., 2014). Unfortunately, resistance to these methods of control has already been documented (Siegwart et al., 2017; Yu et al., 2018). Therefore, new plant-incorporated proteins exploiting a novel mode 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 mechanism limiting RNAi efficiency in ECB and other lepidopteran pests must be understood and overcome.

Investigations of molecular mechanisms influencing RNAi efficiency in insects reveals five factors possibly 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 (Cooper et al., 2019; Zhu and Palli, 2020). 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, such as *Drosopila suzukii* (Taning et al., 2016), *Spodoptera exigua* (Choi et al., 2014; Park and Kim, 2013), *Hyphantria cunea* (Yang et al., 2016), and *Anopheles gambiae* (Zhang et al., 2010). These strategies include large doses of double-stranded RNA (dsRNA), repeated exposure to dsRNA, cationic liposomes, and dsRNA-nanoparticles, among others (Cooper et al., 2019; Zhu and Palli, 2020). 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 compared.

In this chapter, 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 could enhance 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 can 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.

Methods

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. (2020).

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. (2020). The primer sequences for dsRNA synthesis are listed in Table S5-1. *OnLgl* was selected as the target

gene because it is a single copy gene with an essential role in cell proliferation (Xiao et al., 2014), 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 (Xiao et al., 2014). GFP was selected as a non-target control to account for non-specific changes in gene expression associated with dsRNA exposure (Guan et al., 2018).

To form chitosan-based dsRNA nanoparticles (NP dsRNA), ethanol precipitated dsRNA was first purified with the MEGAclear Transcription Clean-Up Kit (Invitrogen, Carlsbad, CA) and eluted in nuclease-free water. Eight micrograms of dsRNA (in 8 µl water) were then combined with 2 µl (8 ug/ul) proprietary chitosan-based polymer in 1% acetic acid solution, and 198 µl 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 x 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 (Taning et al., 2016). 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), Zn^{2+}, Mn^{2+}, or Co^{2+} in 1 X phosphate-buffered saline (PBS) to create 25-100 mM (w/v) stock solutions for use in subsequent assays.

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. (2020). 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., dsRNA, Lipo dsRNA, Meta dsRNA, or NP dsRNA) 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, Zn^{2+}, Mn^{2+}, or Co^{2+}) 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 S5-1 for primers used). Cycle threshold (Ct) values were converted to the amount of ds*GFP* in nanograms based on standard curves (Fig. S5-1) as previously described (Cooper et al., 2020). 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.

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 Zn^{2+}), or interaction between both factors could be investigated. Optimization experiments (data not shown) were preformed 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 x15 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., (2010, 2011) 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.

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),* adaptor protein complex 50 *(AP50),* ADP ribosylation factor 72A *(ARF72A),* V-type proton ATPase 16 kD proteolipid subunit *(Vha16),* and V-type proton 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. 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/ml) targeting either the target gene or the *GFP*-control, all diluted with 1 X 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).

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 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 manufacturer's 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 guidelines (Bustin et al., 2009) on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) using BrightGreen 2x qPCR master mix (Applied Biological Materials) in a 20-µl 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 S5-1. The primer efficiency [E=10^(-1/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 ΔΔCT method (Livak et al., 2001). 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 ΔCt. Then ΔCt 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 (Anderson et al., 2004), GeNorm (Vandersompele et al., 2002), BestKeper (Pfaffl et al., 2004), and RefFinder (http://150.216.56.64/referencegene.php) were used to verify the stability of reference genes across ECB tissues and developmental stages. 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 *OnLgl* relative to the geometric mean of the reference genes (i.e., ΔCt rather than ΔΔCt).

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.

Results

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. 5-1A). 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. 5-1B). Specifically, NP enhanced the stability of dsRNA in ECB HE and GC by 16.6- and 36.1-fold, respectively (Table 5-1). 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. S5-2), EDTA (Fig. S5-3), and Zn^{2+} (Figs. S5-4A, S5-5B) all significantly enhanced dsRNA stability and completely protected dsRNA from degradation in ECB tissue extracts (Table 5-1). However, Lipo (Fig. S5-6), Mn^{2+} (Figs. S5-4B, S5-5B), and Co^{2+} (Figs. S5-4C, S5-5C) did not significantly enhance dsRNA stability in ECB GC or HE (Table 5-1).

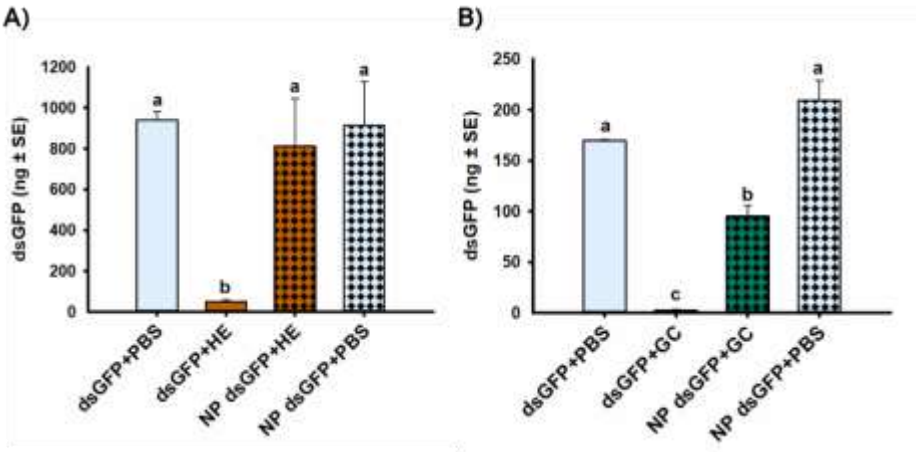

Figure 5-1. Enhancement of dsRNA stability in A) hemolymph (HE) and B) gut contents (GC) due to chitosan-based dsRNA nanoparticles (NP).

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.

Table 5-1. **Comparison of fold-increase in stability of dsRNA in ECB tissue extracts due to candidate RNAi-enhancing reagents.**

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

	Hemolymph (HE)			**Gut contents (GC)**		
Reagent	**ds*GFP* +HE [control]**	**Reagent ds*GFP* + HE**	**Fold increase**	**ds*GFP* +GC [control]**	**Reagent ds*GFP* +GC**	**Fold increase**
Lipo	30.2±11.0	24.1±8.8	0.8	0.0013±0.0011	5.005 ±0.002	3.7
Meta	128±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* 132.3±23.8* 102.3±12.3 111.3±7.9	10 mM: 4.4 20 mM: 3.5 40 mM: 2.7 60 mM: 2.9	0.098±0.038	77.1±26.5 129.1±22.3* 57.8±22.3 84.1±11.2	10 mM: 790.6 20 mM: 1,324.0 40 mM: 592.6 60 mM: 862.9
Mn^{2+}	38.0±16.6	96.8±20.6 72.4±13.9 52.6±11.1 627.5±254.8	96.8±20.6 72.4±13.9 52.6±11.1 627.9±254.8	0.40±0.29	3.3±1.8 8.6±0.8 2.4±0.6 229.8±105.7	10 mM: 2.5 20 mM: 1.9 40 mM: 1.4 60 mM: 16.5
Co^{2+}	18.7±8.18	61.7±23.2 43.8±16.2 21.0±6.2 47.7±20.3	10 mM: 3.3 20 mM: 2.3 40 mM: 1.1 60 mM: 2.5	0.093±0.048	8.9±1.8 27.8±7.8 18.7±9.2 22.5±9.5	10 mM: 95.6 20 mM: 300.6 40 mM: 201.5 60 mM: 243.3

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

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, fluorescent brightener 28 (FB28), 50 mM Zn^{2+}, and 6 mM EDTA were employed (Table 5-2). 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. S5-9A). 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. 5-2). No phenotypes were observed due to the suppression of *OnLgl* at any of the time points investigated (Fig. S5-9B, C; Table 5-2).

Injection of ds*OnLgl* into third-instar ECB larvae resulted in RNAi mediated suppression of *OnLgl* in two out of three bioassays (Table 5-2). 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 5-2). In adults, the relative expression of *OnLgl* increased by 1.2-fold three days after injection [$F(1,8)=5.13$, $p=0.053$*] (Table 5-2, Fig. S5-17).

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. 5-3A). 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 S5-14A). The Meta reagent significantly reduced survivorship by 1.25-fold [$F(1,8)=13.50$, $p=0.006$*] (Fig. 5-3B) and body weight by 1.24-fold [$F(1,8)=11.95$, $p=0.009$*] (Fig. 5-3C) during this bioassay, but had no effect in other bioassays testing Meta on other developmental stages (Table 5-2).

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. 5-4), which is less suppression than observed for feeding of second instars (68%; Fig. 5-2) and injection of third instars in the Meta bioassay (57%; Fig. 5-3A), suggesting that 25 mM Zn^{2+} did not enhance RNAi efficiency in ECB (Table 5-2). No phenotypes were observed

due to the suppression of *OnLgl* at any of the time points investigated (Figs. S5-19B, C; Table 5-2).

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 5-2). However, *OnLgl* expression was unaffected 3, 6, 12, and 24 h after dsRNA feeding (Fig. S5-22) or injection (Fig. S5-23), even when various concentrations of the Zn^{2+} nuclease inhibitor were used.

Table 5-2. 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.

Delivery method	**Stage**	**Feeding status**	**Reagent**	**Suppression of *OnLgl***	**Phenotype(s)**	**Results**
Ingestion	First-instar larvae	Unfed	50 mM Zn^{2+}	No	None	Fig. S5-10
			6 mM EDTA	No	EDTA reduced weight by 1.7-fold at 6 days (6 mM vs 0 mM)	Figs. S5-7, S5-8
			1% FB28	No	FB28 reduced weight by 2.7-fold at 6 days (1% vs 0%)	Figs. S5-12, S5-13
			Lipo	No	None	Fig. S5-11
	Second-instar larvae	Fed	10 & 20 mM Zn^{2+}	**68%,** or 3.2-fold decrease at 6 days (ds*OnLgl* vs ds*GFP*)	None	**Figs. 5-2,** S5-9
Injection	Second-instar larvae	Fed	10 & 20 mM Zn^{2+}	No	None	Fig. S5-21
	Third-instar larvae	Fed	25 mM Zn^{2+}	**32%,** or 1.5-fold decrease at 3 days (Zn^{2+} ds*OnLgl* vs Zn^{2+} ds*GFP*)	None	**Fig. 5-4,** S5-19
			50 mM Zn^{2+}	No	None	Fig. S5-20
			Meta	**57%,** or 2.3-fold decrease at 6 days (naked ds*OnLgl* vs naked ds*GFP*)	Meta decrease survivorship by 1.25-fold & weight by 1.24-fold at 6 days (Meta dsRNA vs naked dsRNA)	**Figs. 5-3,** S5-14

	Wandering (fifth-instar) larvae	Non-feeding	Meta	No*	None	Fig. S5-15
	Pupae	Non-feeding	Lipo	No	None	Fig. S5-18
			Meta	No	None	Fig. S5-16
	Adults	Unfed	Meta	No, 1.4-fold increase at 3 days (ds*OnLgl* vs ds*GFP*)	None	Fig. S5-17

*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; Zn^{2+}, nuclease inhibitor; FB28, fluorescent brightener 28; h, hour.

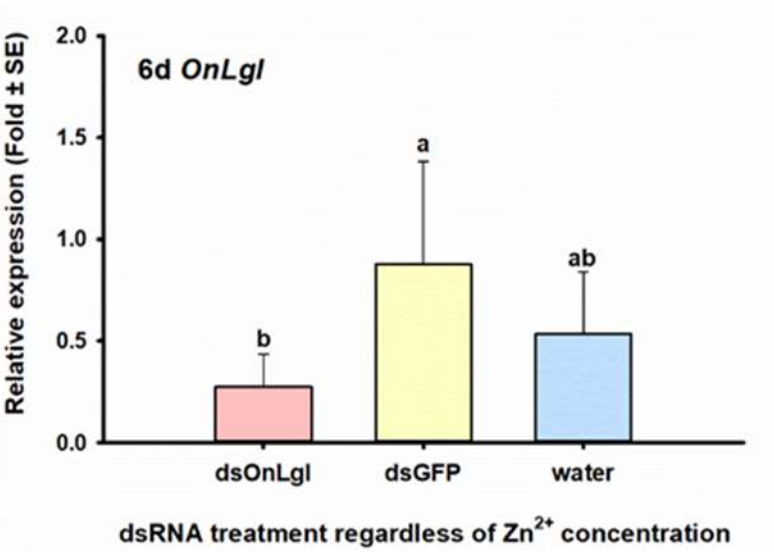

Figure 5-2. Significant effects on RNAi efficiency in 48-h old ECB larvae six days after oral delivery of dsRNA, regardless of nuclease inhibitor (Zn^{2+}) concentration.

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.

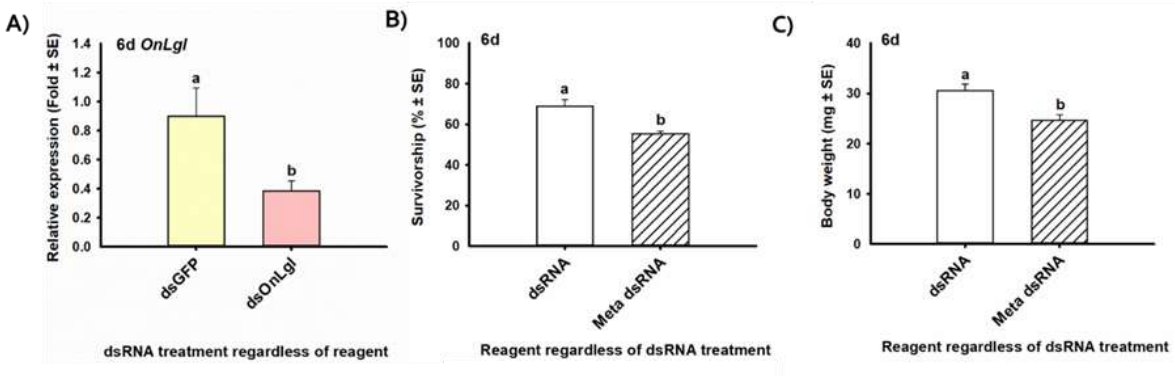

Figure 5-3. Significant effects on RNAi efficiency in third-instar ECB larvae after injection of dsRNA with and without Metafectene Pro (Meta) lipoplexes.

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.

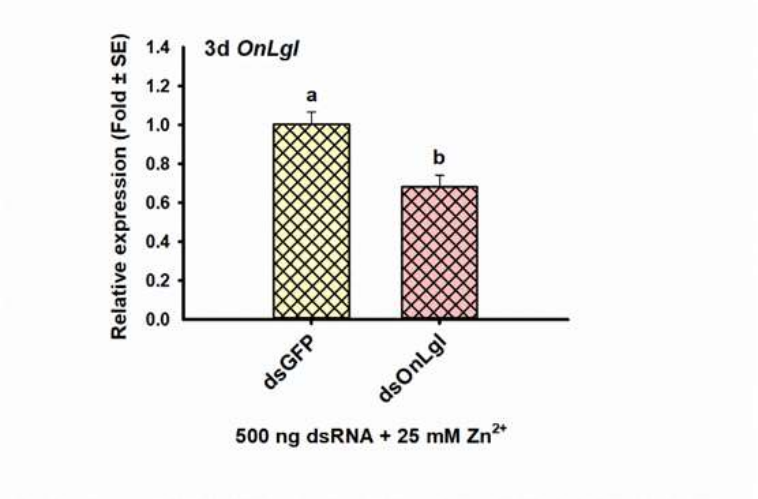

Figure 5-4. Significant effects on RNAi efficiency following injection of third-instar ECB larvae with dsRNA and 25 mM nuclease inhibitor (Zn^{2+}).

Relative expression of the *OnLgl* target three days after injection of ds*OnLgl* or ds*GFP* in conjunction with 25 mM Zn^{2+}. Means that do not share a letter are significantly different.

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. S5-24A). Similar findings were obtained for dsRNA targeting *OnChc, OnArf72A,* and *OnAP50* (Fig. S5-24; Table 5-3). 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. 5-5A). Similar results were observed for *OnVhaSFD [*$H(4)=17.20$, $p=0.002$*] (Fig. 5-5B). Expression of *OnVha16* was suppressed by 54.8% on average, and *OnVhaSFD* was suppressed by 46.2% on average (Table 5-3).

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. 5-6). 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 5-3). 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. 5-6A, S5-25; Table 5-3). 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 5-3).

Table 5-3. Comparison of enhancement of RNAi efficiency in ECB midgut culture for multiple target genes due to nuclease inhibitor Zn^{2+}.

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.

	Without Zn^{2+}			**With Zn^{2+}**		
Target gene	**ds*GFP***	**dsTarget**	**Suppression**	**ds*GFP***	**dsTarget**	**Suppression**
OnLgl	1.00±0.06	0.83±0.20	No	1.01±0.10	0.95±0.21	No
OnArf72A	1.08±0.23	0.78±0.17	No	1.14±0.33	0.08±0.03	92%
OnChc	1.03±0.13	1.42±0.10	No	1.04±0.16	0.69±0.07	33%
OnAP50	1.02±0.11	1.19±0.29	No	1.14±0.33	0.08±0.03	No
OnVha16	1.04±0.16	0.38±0.04	64%	1.06±0.20	0.44±0.14	59%
OnVhaSFD	1.10±0.21	0.68±0.10	38%	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 heavy chain; *AP50*, adaptor protein complex 50; *Arf72A*, ADP ribosylation factor 72A; *Vha16*, V-type proton ATPase 16 kD proteolipid subunit; *VhaSFD*, V-type proton subunit H.

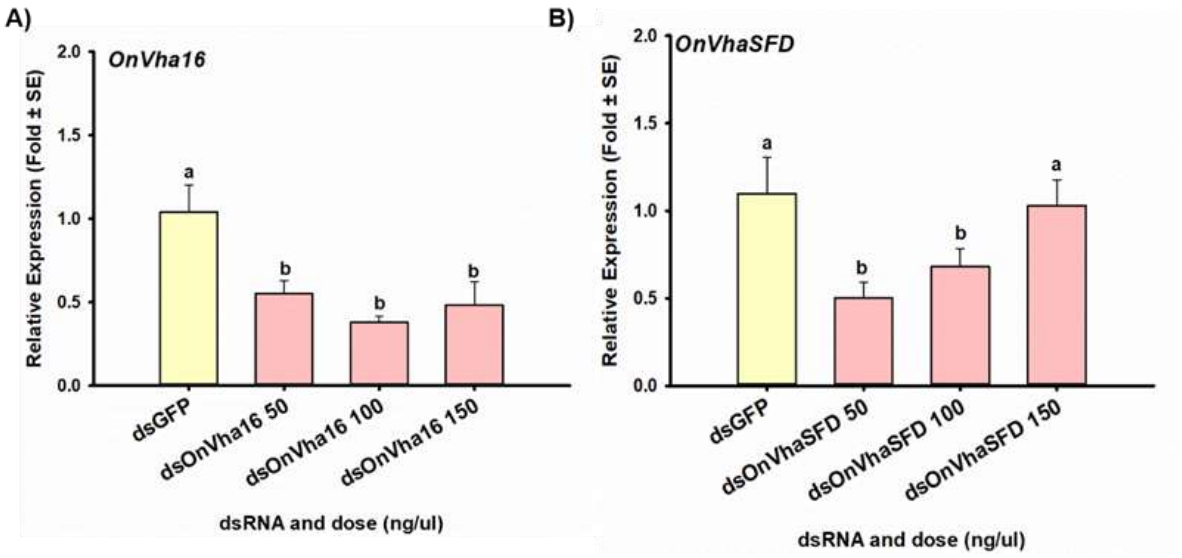

Figure 5-5. RNAi efficiency of two alternative target genes in ECB midgut tissue cultures.

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 (*OnVhaSDF*) 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.

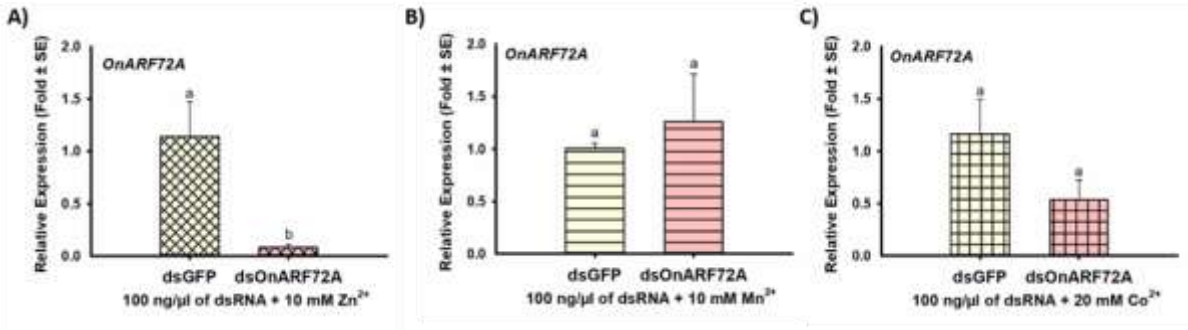

Figure 5-6. Enhancement of RNAi efficiency in ECB midgut tissue cultures due to inclusion of nuclease inhibitor Zn^{2+}, but not Mn^{2+} and Co^{2+}.

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 Zn^{2+}, B) 10 mM Mn^{2+}, or C) 20 mM Co^{2+}. Means that do not share a letter are significantly different.

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 in this study, but RNAi-mediated suppression of *OnLgl* was only observed in one of five oral RNAi experiments, and two out of eight injection experiments (Table 5-2). In the three 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 5-2). 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 (Invitogen) 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. suzukii* larvae and adults (Taning et al., 2016), in *Euschistus heros* nymphs (Castellanos et al., 2019), and in *Chilo suppressalis* (Wang et al., 2019). Metafectene Pro (Meta) dsRNA lipoplexes were previously used to achieve successful RNAi with injection in *H. cunea* pupae (Yang et al., 2016), and *S. exigua* larvae (Park and Kim, 2013; Choi et al., 2014). Enhanced RNAi efficiency associated with transfection reagents was originally attributed to enhanced dsRNA uptake (Taning et al., 2016; Wang et al., 2019), but evidence suggests that both Lipofectamine 2000 and Meta may also enhance dsRNA stability (Castellanos et al., 2018; Wang et al., 2019).

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 5-2), even though Meta increased the stability of dsRNA in ECB hemolymph *ex vivo* (Table 5-1). These results conflict with previous reports of enhanced dsRNA-stability and/or RNAi efficiency when transfection reagents were used in lepidopteran (Choi et al., 2014; Park and Kim, 2013; Yang et al., 2016; Wang et al., 2019), hemipteran (Castellanos et al., 2018), and dipteran (Taning et al., 2016) insects, but agree with a report that

Lipofectamine 2000 did not enhance oral RNAi efficiency in an orthopteran (Luo et al., 2013). 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 (Zhang et al., 2010). The nanoparticles have been used to facilitate oral RNAi in *Aedes aegypti* larvae (Kumar et al., 2016; Das 2015), *Chilio suppressalis* larvae (Wang et al., 2019), *Chironomus tentans* larvae (Tang et al., 2017), *Penaeus vannamei* shrimp (Ufaz et al., 2018), and *Caenorhabditis elegans* nematodes (Lichtenberg et al., 2019). In *C. suppressalis*, chitosan/dsRNA nanoparticles enhanced dsRNA stability and uptake, resulting in enhanced RNAi efficiency (Wang et al., 2019). In addition, evidence from *C. elegans* indicates that uptake of chitosan/dsRNA nanoparticles occurs through clathrin-mediated endocytosis (Lichtenberg et al., 2019). Initial testing of chitosan/dsRNA nanoparticles on ECB larvae indicated that oral RNAi efficiency was not enhanced (W. Cai and K.Y. Zhu, pers. comm.). 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. 5-1; Table 5-1). Unfortunately, the new 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* (Garubtt and Reynolds 2013), *T. castaneum* (Cao et al., 2018), *Acyrthosiphon pisum* (Cao et al., 2018), and ECB (Cooper et al., 2020). 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* (Castellanos et al., 2018). *Ex vivo* incubation in ECB tissue extracts indicated that 6 mM EDTA (the highest concentration that was consumed without detrimental effects) completely protected dsRNA in ECB tissue extracts (Table 5-1). Despite this, oral delivery of dsRNA with and without EDTA

did not enhance RNAi efficiency in unfed neonate ECB larvae (Table 5-2). However, the consumption of EDTA for six days significantly reduced larval body weight.

Zn^{2+}, Mn^{2+}, and Co^{2+} are three divalent ions that inhibited the activity of purified BmdsRNase from *Bombyx mori* (Arimatsu et al., 2017). *Ex vivo* incubation assays indicated that 10-20 mM Zn^{2+} was able to completely protect dsRNA from degradation in ECB HE and GC, whereas Mn^{2+} and Co^{2+} did not (Table 5-1). Injection of 25 mM Zn^{2+} in third instar ECB larvae resulted in 32% suppression of the *OnLgl* target gene (Table 5-2, Fig. 5-4). Two additional bioassays were then performed on second-instar larvae using feeding and injection of dsRNA in combination with 0, 10, and 20 mM Zn^{2+} so the effects of dsRNA and Zn^{2+} 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. S5-22, S5-23). In addition, significant suppression was only observed at the 6-day time point of the oral RNAi bioassay, but not at the 3-day time point for either delivery method (Table 5-2). 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 (Guan et al., 2018). 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 Zn^{2+} 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 (Fig 5-3). 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 (*OnVha*16 and *OnSFD*) exhibited RNAi-mediated suppression after 24 h of incubation in tissue culture media containing dsRNA (Table 5-3; Figs. 5-5, S5-24). Surprisingly, suppression in ECB midgut cultures did not appear to be dose-dependent (Figs. 5-5). However, RNAi-mediated suppression was also not dose-dependent in *A. aegypti* (Lopez et al., 2019), and *A. pisum* (Ye et al., 2019) possibly due to insufficient activation of core RNAi pathway components.

Comparison of Zn^{2+}, Mn^{2+} and Co^{2+} on RNAi efficiency in ECB midgut tissue cultures indicated that 20 mM Zn^{2+} significantly enhanced RNAi efficiency of the *OnArf72A* target gene (Fig. 5-7). Therefore, tissue culture assays using 20 mM Zn^{2+} were performed on the other five target genes. Surprisingly, RNAi efficiency was only enhanced by Zn^{2+} in two of the six genes assayed (*OnArf72A* and *OnChc*) (Table 5-3). 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 (Cooper et al., 2020). 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 5-2), as previously observed in other insects, including many lepidopterans (Bellus et al., 2010; Terenious et al., 2011). Therefore, RNAi efficiency in ECB appears to be more refractory than in other species, perhaps due to the involvement of multiple mechanisms. Instability of dsRNA (Cooper et al., 2020) and refractory target genes (Table 5-3) contribute to limited RNAi efficiency in ECB, and there is evidence that deficient activation of the core RNAi pathway components (Chapter 4) may limit RNAi efficiency in ECB as well. 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 5-2).

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 (Cooper et al., 2020). 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 (Mitter et al., 2017).

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.

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Chapter 6 - Research Summary and Future Directions to Improve RNAi Efficiency in Insects

Variable RNAi efficiency severely limits the applications of RNA interference (RNAi) in many lepidopteran insects, and strategies to enhance RNAi efficiency in agriculturally important pests are badly needed (Cooper et al., 2019; Zhu and Palli, 2020). Four reagents were identified that enhanced double-stranded RNA (dsRNA) stability in ECB tissue extracts (Table 6-1), but RNAi efficiency in ECB remained low and highly variable regardless of dsRNA delivery method, developmental stage, feeding status (i.e., fed, unfed/starved, or nonfeeding), or reagents used (Cooper et al., 2020c). RNAi-mediated suppression was only achieved in one out of five oral RNAi bioassays and two out of eight injection RNAi bioassays (Table 6-2). High variability between individuals was also reported for oral RNAi in neonate ECB larvae by Khajuria et al. (2010; 2011), which likely explains why there are so few published reports of RNAi-mediated suppression in ECB. Low RNAi efficiency in ECB is clearly a significant obstacle to the development of RNAi-based pest management strategies and research tools for this insect and other lepidopteran pests.

At least three of the five mechanisms known to limit RNAi efficiency in insects (Fig. 2-1) (Cooper et al., 2019) appeared to contribute to low RNAi efficiency in ECB: (1) dsRNA was rapidly degraded in larval ECB gut contents and hemolymph (Table 6-1) (Cooper et al., 2020a); (2) insufficient transcriptional responses of all three core RNAi pathway genes may contribute to low RNAi efficiency in ECB and the corresponding proteins may have low activity due to divergence in amino-acid sequence between specific domains in each core RNAi protein (Table 6-2) (Cooper et al., 2020d); and (3) some of the target genes investigated were refractory or resistant to RNAi even when nuclease inhibitors were used to enhance dsRNA stability (Table 5-3) (Cooper et al., 2020c). Instability of dsRNA in ECB appears to be due to *OnREase*, *OndsRNase2* and *OndsRNase4* in the gut and *OnREase* and *OndsRNase1* in the hemolymph (Table 6-1) (Cooper et al., 2020b). Instability of dsRNA in physiological environments is very concerning because it can affect dsRNA uptake as well as the response of the core RNAi pathway genes. Uptake of dsRNA in insects such as *Diabrotica virgifera virgifera* and *Tribolium castaneum* is size dependent, and anything under 60-100 bp is not internalized (reviewed in Cooper et al., 2019). If degraded dsRNA is too small to be internalized into cells it will never

reach the siRNA pathway to trigger an RNAi response. Given that RNAi mediated suppression of the target gene was observed in ECB larvae in the same oral RNAi experiment in which regulation of the core enzymes was investigated (Cooper et al., 2020c,d), it appears that at least some dsRNA can be internalized into cells and trigger a measurable RNAi response. Therefore, it seems that key RNAi pathway genes really are not upregulated in ECB in response to exogenous dsRNA exposure at any of the time points investigated, raising questions about the importance of upregulation of the core enzyme for a robust RNAi response (Table 6-2).

Possible deficiencies in the core RNAi machinery may explain why enhancing dsRNA stability was insufficient to enhance RNAi efficiency in ECB. Additionally, differences in enzymatic activity of the core RNAi machinery may contribute to the low RNAi efficiency (Cooper et al., 2020d), but this hypothesis requires further investigation. Efforts to explore factors influencing the susceptibility of individual target genes to RNAi are needed to facilitate the selection of appropriate and effective target genes (Cooper et al., 2020c). Tissue culture systems, like the one described in Cooper et al. (2020c), can facilitate the rapid screening of multiple target genes in various tissues, as well as facilitate RNAi of RNAi assays (Miyata, et al. 2014) on species exhibiting low or variable RNAi efficiency, such as ECB or *Ostrinia furnicalis* (J. Zhang and K. Zhu, pers. comm). In addition, the RT-qPCR method described in Cooper et al. (2020a) allows for quantitative measurement of dsRNA stability in challenging samples that are difficult to investigate with gel electrophoresis (e.g., hemolymph, nanoparticles, soil), as well as for quantitative comparison of dsRNA stability enhancement between candidate reagents.

While insufficient dsRNA internalization and impaired systemic spreading were not directly investigated in ECB, these factors have been implicated in low RNAi efficiency in other insects (reviewed by Cooper et al., 2019). Further research is needed to verify mechanism(s) of dsRNA uptake in ECB and screen for endosomal entrapment (Shukla et al., 2016), as it is unclear if insufficient dsRNA internalization is a common source of low RNAi efficiency in lepidopterans. In the future, it might be possible to overcome uptake/internalization issues using endosome escape-enhancing reagents or additional types of nanoparticles (Cooper et al., 2019; Zhu and Palli, 2020). Alternatively, transgenic microbes-, yeasts-, baculoviruses-, and endosymbionts-expressing dsRNA could be used to facilitate dsRNA delivery (Zhu and Palli, 2020). However, multiple RNAi-enhancing reagents and delivery methods may need to be combined in order to overcome the multiple factors limiting RNAi efficiency in ECB.

Additional efforts are also badly needed to elucidate the mechanism(s) of systemic RNAi in insects, as very little is known about dsRNA uptake, export, or spreading (reviewed in Cooper et al., 2019). In *Drosophila melanogaster*, systemic RNAi appears to be mediated by exosome-like vesicles (Tassetto et al., 2017). Exosomes are an important area of investigation that could be explored to improve RNAi efficiency, as they might protect against dsRNA from degradation and facilitate uptake (K. Silver, pers. comm.).

Given the multiple benefits of RNAi-based pest management strategies (Price and Gatehouse, 2008; Whyard et al., 2009; Godfray et al., 2010; Dubelman et al., 2014, Khajuria et al., 2018; Liu et al., 2019;), basic studies such as the current investigation are key to elucidate mechanisms that contribute to low RNAi efficiency in lepidopterans and other insects (Cooper et al., 2019). More publically available genomic and transcriptomic resources for agriculturally relevant pests would greatly facilitate this endeavor. Comparative studies using closely-related species that exhibit differential RNAi responses, such as ECB and *O. furnacalis*, or *Aedes aegypti* and *Aedes albopictus* would also help elucidate mechanisms of low RNAi efficiency in insects (K. Silver, pers. comm.). While the current investigation did not definitively implicate specific genes or factors as contributing to low RNAi in ECB, it does provide the foundation for understanding mechanisms influencing RNAi efficiency in ECB and other species, and to identify stable reference genes (*OnRPS3* and *OnEF1α*) that can confidently be used to normalize RT-qPCR data in ECB for a wide range of applications (Cooper et al., unpublished). This information will facilitate the development of RNAi-based pest management strategies and research tools for notorious pests such as ECB, as well as provide insight into potential mechanisms of resistance.

Reagents (Cooper et al., 2020c), such as chitosan-based nanoparticles and Zn^{2+}, that enhanced dsRNA stability in ECB tissues (Table 5-1) and exhibited low toxicity in RNAi assays (Table 5-2) are good candidates for enhancing RNAi efficiency in species that are mainly limited by dsRNA instability, such as *Locusta migratoria* (Song et al., 2017), *Leptinotarsa decemlineata* (Spit et al., 2017), *Euschistus heros* (Castellanos et al., 2018), and *O. furnacalis* (Guan et al. 2018). However, the applications of nuclease inhibitors for enhancing RNAi efficiency may be limited in some species, such as ECB and *O. furnacalis*, due to changes in nuclease expression in response to disruption of nuclease activity (Guan et al., 2019; Cooper et al., 2020b). Therefore, identification of alternative reagents and strategies for enhancing dsRNA stability in insect

tissues without altering nuclease expression and/or activity is needed to facilitate applications of RNAi for study and control of insects in the laboratory and field.

Table 6-1. Summary of factors possibly influencing dsRNA stability in larval ECB tissues.

Stability of dsRNA in ECB		
Factor	**Hemolymph**	**Gut**
degradation of dsRNA in 30 min	•99.5%	•97.9%
physiological pH	•7.0	•8.0
OndsRNase1	•Moderate expression in hemolymph •Detectable in all samples after injection •2.75-fold decrease at 6 h (Zn^{2+} vs. 0 mM) •2.12-fold decrease at 12 h (Zn^{2+} vs. 0 mM)	•Low expression in gut •Undetectable in a few samples after feeding
OndsRNase2	•Undetectable in hemolymph •Detectable in all samples after injection	•High expression in gut •Detectable in all samples after feeding
OndsRNase3	•Low expression in hemolymph •Detectable in all samples after injection	•Undetectable in gut •Undetectable in some or all treatments at all time points after feeding
OndsRNase4	•Undetectable in hemolymph •Undetectable in some treatments at all time points	•Undetectable in gut •Undetectable in some or all treatments at most time points after feeding *•5.25-fold increase at 3 days (ds*OnLgl *vs. ds*GFP*)*
OnREase	•Moderate expression in hemolymph •Detectable in all samples after injection •4.73-fold increase 6 h (ds*GFP* vs. water)	•High expression in gut •Undetectable in a few samples after feeding *•22.0-fold increase at 3 h (dsRNA vs. water)*

Italicized averages are trends *(not statistically significant).*
Abbreviations: GU, gut; HE hemolymph; dsRNase, double-stranded ribonuclease; REase, RNAi-efficiency related nuclease; h, hour; min, minute.

Table 6-2. Summary of transcriptional responses of key genes of interest to exogenous dsRNA in second-instar ECB larvae.

Transcriptional Responses to dsRNA		
Gene	**500 ng of injected dsRNA**	**20 µg of oral dsRNA**
OnLgl	•no changes	•3.2-fold decrease at 6 days (ds*OnLgl* vs. ds*GFP*)
OnDcr2	•no changes	*•2.8-fold increase at 3 h (ds*OnLgl *vs. water)* *•2.3-fold increase at 3 h (20 mM vs. 0 mM)* *•2.0-fold increase at 12 h (Zn^{2+} vs. 0 mM)*
OnR2D2	•no changes	•0.6-fold decrease at 3 h (ds*OnLgl* vs. ds*GFP*) •7.4-fold increase at 6 days (20 mM ds*GFP* vs. 20 mM water)
OnAgo2	•no changes*	•no changes
OndsRNase1	•2.8-fold decrease at 6 h (Zn^{2+} vs. 0 mM) •2.1-fold decrease at 12 h (Zn^{2+} vs. 0 mM)	•no changes*
OndsRNase2	•no changes	•no changes*
OndsRNase3	•no changes	•no changes
OndsRNase4	•no changes	*•5.3-fold increase at 3 days (ds*OnLgl *vs. ds*GFP*)*
OnREase	•4.7-fold increase 6 h (ds*GFP* vs. water)	*•22.0-fold increase at 3 h (dsRNA vs. water)*

*Significant differences were detected between some treatment groups but could not be meaningfully interoperated. Italicized averages are trends (*not statistically significant).*
Abbreviations: Lgl, lethal giant larvae, Dcr, Dicer; Ago, Argonaute; dsRNase, double-stranded ribonuclease; REase, RNAi-efficiency related nuclease; h, hour; ng, nanograms; µg, micrograms.

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