# ABSTRACT


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| Title of Thesis: | ASSESSING THE SUSCEPTIBILITY TO RNA INTERFERENCE OF THE MILKWEED BUG, *ONCOPELTUS FASCIATUS* (HEMIPTERA) |
| | Ebony Michelle Argaez, Master of Science, 2023 |
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RNA interference (RNAi) is an effective method to knock down gene expression in insects and other organisms. It has been adopted for basic research, to elucidate gene function, and applied research, to control insect pests. Here, I examined parameters needed for effective RNAi in the milkweed bug, *Oncopeltus fasciatus,* an emerging insect model species. For two developmental genes, *Sex combs reduced,* and *even-skipped*, very small amounts of dsRNA trigger a robust parental RNAi response. The higher the dose of dsRNA applied, the longer the duration of embryos laid with defects. Testing length-dependence, effectiveness decreased with dsRNAs in the 150 bp to 75 bp range. These developmental genes resulted in subtle, gene-specific defects which provided a more sensitive assay than lethality. Finally, effects of RNAi were transmitted across generations through trophic interactions, the first such discovery to our knowledge. This suggests potential unanticipated environmental risk to non-target insects from RNAi-based insecticides.

# ASSESSING THE SUSCEPTIBILITY TO RNA INTERFERENCE OF THE MILKWEED BUG, *ONCOPELTUS FASCIATUS* (HEMIPTERA)

by

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Thesis submitted to the Faculty of the Graduate School of the
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# Dedication

*Para mis padres; Janet y Fernando*

*Que llegaron sin nada y me lo dieron todo.*

*For their sacrifices, love, and support for my pursuit for education.*

*To my siblings; Dito and Jae.*

*To those who believed in me.*

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The support and comfort of those around me has been instrumental throughout my journey. I could not have done this myself. I am the product of a team effort.

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# List of Abbreviations

2°RNAs: secondary RNAs

AEL: after egg laying

*C. elegans: Caenorhabditis elegans*

*D. maculatus: Dermestes maculatus*

*D. melanogaster: Drosophila melsnogaster*

dsRNA: double-stranded RNA

*eve: even-skipped*

*ftz-f1: fushi tarazu factor 1*

*O. fasciatus* or *Of: Oncopeltus fasciatus*

PCR: Polymerase Chain Reaction

pRNAi: parental RNA interference

PRG: Pair-rule gene

RNAi: RNA interference

RT-PCR: Reverse Transcription Polymerase Chain Reaction

siRNAs: small interfering RNAs

*Scr: Sex combs reduced*

SSC: Saline-Sodium Citrate

*T. castaneum or Tribolium: Tribolium castaneum*

*Vha68-1: V-ATPase subunit A*

# Chapter 1: Introduction

## 1.1 Mechanism of RNAi

RNA interference was first described in the nematode, *Caenorhabditis elegans (C. elegans),* by Fire et al. in the late 1900s who discovered that double-stranded RNA (dsRNA) can trigger gene silencing. In *C. elegans,* exogenous dsRNA is effective when introduced via ingestion or injection. When dsRNA is ingested, transporter SID-2 is required for the transport of dsRNA across the gut (Winston et al. 2007). The dsRNA enters cells throughout the body, via transporter SID-1 (Hunter et al. 2006; Feinberg and Hunter 2003). Once in the cell, DICER cleaves the dsRNA (Ketting et al. 2001; Bernstein et al. 2001), resulting in small RNAs also known as small interfering RNAs (siRNAs) (Elbashir et al. 2001). Single-stranded siRNAs are bound by Argonaute proteins, forming the RNA induced silencing complex (RISC) (Zamore et al. 2000; Bernstein et al. 2001; Hammond et al. 2000). The siRNA directs RISC to matching mRNA sequences where the mRNA is then cleaved and destroyed (Zamore et al. 2000) which results in gene silencing or "knockdown." Besides this degradation of mRNA, the RNAi effect in *C. elegans* is amplified by the production of additional antisense single-stranded RNAs, also known as secondary RNAs (Pak and Fire 2007a). RNA dependent RNA polymerase is recruited to the target mRNA, that is then used as a template to generate secondary RNAs (Pak and Fire 2007b). Generation of tertiary RNAs have also been observed in *C. elegans* and contributes to the amplification of the RNAi systemic response (Sapetschnig et al. 2015).

Both cell autonomous RNAi, restricted to cells that generate the dsRNA, and non-cell autonomous RNAi, which includes environmental and systemic RNAi, have been described in *C. elegans* (Whangbo and Hunter 2008). Systemic RNAi occurs when a cell exposed to dsRNA triggers gene silencing in other cells. Environmental RNAi involves the uptake of dsRNA, encountered in the environment, into the gut or lumen. This is followed by systemic spread of gene silencing.

Since its discovery, RNAi has been found in protozoans and metazoans (Agrawal et al. 2003) and several mechanisms of RNAi have been identified including: the small interfering RNA pathway, crucial for protection against viruses; the microRNA pathway involved in gene regulation; and the piwi-interacting pathway that provides the germline protection from transposons (Tuschl et al. 1999; Lee and Ambros 2001; Schott et al. 2005; Nishida et al. 2007). RNAi has become a powerful technique utilized to study gene function, and has been applied for medical use (Y. Zhu et al. 2022), and in the agricultural field (Das and Sherif 2020; Christiaens et al. 2020).

### 1.2 RNAi in Insects

Soon after its' discovery in *C. elegans*, researchers asked whether RNAi could be used to study gene function in other organisms, including insects. For insects, RNAi was first tested in *Drosophila*, where dsRNA targeting *frizzled* and frizzled 2 genes were injected into embryos, demonstrating their roles in the Wnt signaling pathway and potential use of RNAi to study gene function in insects.

The RNAi pathway has been found to play a role in insect immunity, protecting them from viral infection (Gammon and Mello 2015). It also plays a role in control of gene expression. For example, micro RNAs regulate the production of dopamine in

locusts (Yang et al. 2014) and the piwi-RNA pathway, first described in *Drosophila,* for its role in maintenance of germ-line stem cells (Cox et al. 1998).

Researchers quickly realized that RNAi is a useful tool for functional studies. RNAi is a post-transcriptional gene silencing tool that avoids manipulating the genome and has the ability to knock down a gene without generating a mutant line. Performing RNAi facilitates testing the role of a gene in tissue-specific or cell-specific knockdowns at specific times during development (Roignant et al. 2003; Dietzl et al. 2007). The ability to knockdown genes at any point in development, makes it useful to study the function of pleiotropic genes (Piano et al. 2002; Nybakken et al. 2005). For example, if a gene functions in early embryogenesis but also later in development, animals carrying a loss-of-function mutations will die due to the earliest defect precluding assessment of the later function. This late function can be assessed by introducing dsRNA at later stages of development ( Zhang et al. 2021). Advantages include using RNAi for a screen of multiple genes (Boutros and Ahringer 2008) and using it in insects for which genetic tools are not available. RNAi has been used for functional studies in many insects, including wasps (Rosenberg et al. 2014), beetles (Bucher, Scholten, and Klingler 2002; Shukla and Palli 2012), crickets (Mito et al. 2005), true bugs (Hughes and Kaufman 2000a; Reding et al. 2019), and others. This approach has allowed for tests of gene function in species not yet amenable to genetic analysis.

Although RNAi has been used in various insects, there is variability in RNAi response between insects. Coleopterans (beetles) are highly susceptible to RNAi, while other insect orders vary in their susceptibility to RNAi (Christiaens et al. 2020). This variation between insects is due to many factors such as degradation of dsRNA before and after exposure to the insect, the ability of an insect to process dsRNA and whether an insect has the ability to mount a systemic response (reviewed in. Cooper et al. 2019). Many components of the RNAi pathway have been identified in the model insect *Drosophila melanogaster*, although less is known about RNAi mechanisms in other insect species. Much of the RNAi machinery is shared between *D. melanogaster* and *C. elegans*, such as Dicer and Argonaute proteins (Hammond et al. 2000; Pham et al. 2004; Kim et al. 2006; Galiana-Arnoux et al. 2006). However, differences in the RNAi machinery found in different insects appear to influence variation in RNAi response. For example, SID-1 required for the uptake of dsRNA into cells in *C. elegans*, has been identified in Coleoptera and Hemiptera but is not involved in the cellular uptake in *Tribolium castaneum (Tribolium)*, where clatharin-dependent endocytosis is thought to be involved in entry of dsRNA into cells (Tomoyasu et al. 2008; Saleh et al. 2006; Zhou et al. 2008; Xu et al. 2013; Cappelle et al. 2016).Another key player involved the response to ingestion of dsRNA in *C. elegans* is SID-2; this has not been found in insects or even other Caenorhabditis species (Winston et al. 2007). Improvement in our understanding of the RNAi machinery found in insects will aid in the development of RNAi technology.

### 1.3 RNAi as a tool for pest control

Insects are the largest and most diverse phyla on earth and insect pests are responsible for crop damage worldwide of up to 40% (Douglas 2018). Global crop demand and reduction of crop damage caused by insects is of great importance in order to feed the continuously growing world population (Tilman et al. 2011; Ray et al. 2013). Therefore in order to meet these needs, increase of food production without loss to biodiversity is recommended (Crist, Mora, and Engelman 2017). Strategies to control insect pests involve the use of RNAi (Das and Sherif 2020; Christiaens et al. 2020) due to the ability to design species-specific dsRNAs, making it a potentially safe means to target pests.

Since RNAi was shown to function in insects, researchers sought to make use of it for pest control. Utilizing RNAi for control of insect pests through feeding requires efficient digestion of dsRNA by the insect. Turner et al. was able to feed dsRNA via an artificial diet to larvae of the light brown apple moth and saw RNAi effects two days after feeding (Turner et al. 2006). Baum et al, 2007 performed an RNAi screen for the Western corn rootworm (*Diabrotica virgifera virgifera)*, fed dsRNA targeting 290 genes which resulted in 14 genes being identified as being effective for gene silencing (Baum et al. 2007). The screening approached used by Baum et al. can be used to identify targets for any pest of interest. The ability to generate species-specific dsRNA is an important consideration for producing RNAi based insecticides. Overall, the use of RNAi has shown potential as a mode of action in pest management to control insect pests (Baum et al. 2007; Price and Gatehouse 2008).

As previously mentioned, insects vary in RNAi response. Other challenges to think about for RNAi use for pest control include the stability of dsRNA in the environment, how the dsRNA is delivered, and whether targeted pests synthesize secondary RNAs. Degradation of dsRNAs in the environment may impact how much dsRNA is ingested and the impact of ingested dsRNA is influenced by enzymes it is exposed to in the insects' digestive system (Thompson et al. 2012; Christiaens, Swevers, and Smagghe 2014).

To predict hazard of RNAi, sequence of dsRNA is often used to determine off-targets, as well as potential impact to non-target organisms (Heinemann, Agapito-Tenfen, and Carman 2013; Lundgren and Duan 2013; Bachman et al. 2016). An RNAi screen in *Tribolium* performed to study gene silencing effects revealed novel genes involved during embryogenesis and metamorphosis (Schmitt-Engel et al. 2015). A rescreening was performed to identify sequences of RNAi gene targets that could potentially be used for other insects and found potential off target effects caused by similarity of sequences to other insects such as *Aedes aegypti, Apis mellifera,* and *Acryrthosiphon pisum* (Ulrich et al. 2015). If secondary RNAs are generated in insects as they are in *C. elegans*, sequences that do not match the insecticidal dsRNA could potentially match genes in non-targeted organisms. Therefore, the species-specific dsRNAs designed become less specific if secondary RNAs are made and non-targeted insects are exposed to them via trophic interactions. Thus, there is an increased risk of off-target effects within the targeted pest as well as posing additional risk to other exposed insects. Therefore, there is a need to better understand RNAi mechanisms in a variety of insects in order to better predict risk

caused by RNAi insecticides. In my thesis, I used the milkweed bug *Oncopeltus fasciatus* as a model to explore RNAi mechanisms in this hemipteran model species.

# Chapter 2: The milkweed bug *Oncopeltus fasciatus* (Hemiptera) is highly susceptible to parental RNA interference

## 2.1 Introduction

The discovery of RNA interference (RNAi) led to an explosion of investigations into applications of this gene knockdown technology. The mechanisms underlying RNAi were first described by Fire et al. in nematodes and are best characterized in this system (Fire et al. 1998). RNAi has been shown to function in a wide range of eukaryotic organisms (Agrawal et al. 2003). In *C. elegans*, RNAi is effective when dsRNA is either ingested by or injected into the worm. This initiates a process where degradation of targeted mRNAs results in gene silencing (Whangbo and Hunter 2008). dsRNAs can be designed and be adapted, for improved delivery, to target any gene as long as the sequence is known. As a result, it has become a powerful tool to study gene function and has been applied for medical use (Zhu et al. 2022) and agriculture, for crop improvement and plant protection from diseases and viruses (Das and Sherif 2020). Further, as insects are major pests of agriculture, vectors of human disease, and household nuisances, RNAi has been investigated as a method for safe pest control (Vogel et al. 2019; Cooper et al. 2019; Zhu and Palli 2020; Christiaens et al. 2020). Recently, several countries, such as Japan, Brazil, and Canada have approved the use of transgenic corn, SmartStax Pro, expressing dsRNA to target an insect pest, the western corn rootworm, *Diabrotica virgifera virgifera* (Head et al. 2017; "MON87411| GM Approval Database- ISAAA.Org" n.d.).

RNAi has also provided a means to study gene function in model insect species as well as non-model insects for which genetic tools were not available. RNAi has been exploited in the major genetic model system *Drosophila melanogaster* where the earliest experiment demonstrating gene silencing via dsRNA targeted *frizzled* and *frizzled 2* genes, documenting their roles in the Wnt signaling pathway (Kennerdell and Carthew 1998). RNAi has been performed in diverse insect species such as the flour beetle *Tribolium castaneum* (Bucher, Scholten, and Klingler 2002; Dönitz et al. 2015), the milkweed bug *Oncopeltus fasciatus* (Liu and Kaufman 2004), and many more insects.

These studies have used RNAi to study patterning in early embryogenesis (Hughes and Kaufman 2000a; Brown, Hilgenfeld, and Denell 1994; Lynch et al. 2006; Rosenberg et al. 2014; Xiang, Forrest, and Pick 2015; Wexler, Pick, and Chipman 2023), sex determination (Hasselmann et al. 2008; Shukla and Palli 2012), ecdysone signaling (Cruz et al. 2006; Zhang et al. 2018;), social behavior (Zhou, Oi, and Scharf 2006; Guidugli et al. 2005), pigmentation (Vargas-Lowman et al. 2019; Reding, Lê, and Pick 2023) and more. RNAi has also been used for large scale screens in *Drosophila* to study signaling pathways (Clemens et al. 2000), as well as in *Tribolium* (Dönitz et al. 2015; Schmitt-Engel et al. 2015; Ulrich et al. 2015).

Because of their importance as model species for basic research and their importance as pests, the utility of RNAi in insects has been examined and compared in different insect taxa, including Diptera (Kennerdell and Carthew 1998), Lepidoptera (Quan, Kanda, and Tamura 2002; Terenius et al. 2011), Coleoptera (Bucher, Scholten, and Klingler 2002; Baum et al. 2007; Xiang, Forrest, and Pick

2015), Hemiptera (Liu and Kaufman 2004), Isoptera (Zhou, Oi, and Scharf 2006; Zhou et al. 2008), Orthoptera (Mito et al. 2005), Hymenoptera (Lynch et al. 2006; Rosenberg et al. 2014), and Phthiraptera (Yoon et al. 2011), using a variety of delivery methods and parameters. RNAi response differs in insects due to the RNAi machinery found, the ability to take up dsRNA from the environment into cells and the ability to mediate a systemic response (Cooper et al. 2019; Christiaens et al. 2020). Coleopterans are generally thought to be highly sensitive to RNAi while Diptera, Lepidoptera, Orthoptera, Hymenoptera and Hemiptera vary in their response to RNAi (Christiaens et al. 2020).

Although not an approach useful for pest control, delivery of dsRNA by injection has proved an effective way to study parameters/requirements for effective RNAi in different species. Particularly useful for basic research was the finding that dsRNA can be delivered to adult female insects by injection, with genes knocked down in their offspring (parental RNAi, pRNAi) allowing study of the effects of genes with roles in early embryogenesis (Bucher, Scholten, and Klingler 2002; Liu and Kaufman 2004). The use of pRNAi obviates the need to inject individual eggs, a process that often causes damage to the egg itself which can complicate interpretation of RNAi effects. pRNAi also makes it possible to obtain many offspring displaying defects, via injection of a small number of females. For example, Liu and Kaufman (Liu and Kaufman 2004) used pRNAi to knockdown *hunchback* to determine its functional role in *Oncopeltus* embryonic development. This study, in keeping with most reports of RNAi in insects, utilized high doses of dsRNA to maximize the response– that is, to ensure that many or most animals showed defects. In a different

study, pRNAi used to knockdown *even-skipped*, was tested at a range of doses, demonstrating that and  concentrations as low as 0.002 ug/µl caused a range of defects (Liu and Kaufman 2005). It has also been suggested that longer dsRNA molecules are more effective than short dsRNAs. (Saleh et al. 2006) showed that uptake of dsRNA to *Drosophila* S2 cells was length dependent, with longer dsRNAs of 1000 bp causing silencing and shorter dsRNAs of 21 bp failing to result in silencing. One study specifically tested the importance of dsRNA length for effective RNAi in *Tribolium* (Miller et al. 2012). Here, it was shown that shorter dsRNAs of 31 bp or less did not cause efficient knockdown while fragments of 69 bp and 250 bp caused efficient knockdown. Beyond this, there are few studies that have systematically analyzed the parameters required for RNAi effectiveness in non-model insects.

*Oncopeltus* is an emerging model system for Hemiptera, largely because of genetic studies in the 1960's on segmentation and cell cycle (Lawrence 1966; P. A. Lawrence 1968; Lawrence 1973; Lawerence and Green 1975) and the pioneering work from the Kaufman lab on probing gene function with RNAi in this species (Hughes and Kaufman 2000a; Liu and Kaufman 2004; 2005). Here, we have examined the susceptibility of *Oncopeltus* to pRNAi using two well-characterized developmental regulatory genes, *even-skipped (eve)* and *Sex combs reduced (Scr)*. Knockdown of these genes produces very specific defects that show a range of severity, allowing us to assess subtle and long-lasting effects of dsRNA. Our results show that use of these developmental genes to test effectiveness of RNAi is more sensitive than scoring death due to knockdown of a housekeeping gene, *V-ATPase*.

We show that the duration of dsRNA response increases as dsRNA amounts increases, and longer dsRNAs are more effective than shorter dsRNAs. We found an RNAi effect when dsRNA is delivered to *Oncopeltus* via feeding and demonstrate a low-level transmission of RNAi effects when *Oncopeltus* females were fed eggs that resulted from *Of-eve* pRNAi. This is, to our knowledge, the first study to demonstrate dsRNA induced effects due to trophic exposure.

## 2.2 Methods

### 2.2.1 Gene Isolation

RNA was extracted from *O. fasciatus* embryos 48-72 h after egg laying (AEL) and reverse transcription was performed to prepare cDNA, using M-MuLV RT (NEB) and RNase Inhibitor (NEB). To isolate *even-skipped (eve)* and *Sex combs reduced (Scr),* gene specific primers were used with Phusion or Q5 high-fidelity DNA polymerase (NEB). To isolate V-*ATPase subunit A (Vha68-1),* gene specific primers and 5' and 3' RACE were performed using the cDNA amplification kits for 5' and 3' RACE (Takara; see Appendix III). Gene products were purified and inserted into pGEM-T Easy Vector (Promega) by TA cloning. All primer sequences used in this study are listed in Table S1.

### 2.2.2 dsRNA synthesis

Primers with T7 promoter sequence at the 5'ends were used to PCR amplify dsRNA templates for *Of-Vha68-1, Of-eve* and *Of-Scr.* The PCR products were used as templates for dsRNA synthesis using the MEGAscript T7 Transcription kit (Ambion) according to the manufacturer's protocol. After ethanol precipitation,

dsRNA was dissolved in injection buffer (0.1 mM $NaH_2PO_4$, 5mM KCl, pH 6.8). For feeding experiments, the dsRNA was dissolved in dd $H_2O$. *gfp* dsRNA, 633 bp, was used as a negative control in all experiments. For synthesis of short dsRNA fragments, Ultramer IDT DNA oligonucleotides, with the T7 promoter sequence on the 5' and 3' end, were used to produce the template for dsRNA synthesis. The oligonucleotides were diluted to 100 uM and annealed by heating at 94 °C for 2 min, followed by removal from heat and slow cooling at room temperature for 1 hr. Following dilution to 10 uM in nuclease free water duplex, the dsRNA was stored at -20 °C. A 1:100 dilution of the dsRNA was used to run on a gel to check the quality after synthesis. This dilution was quantified using a NanoDrop spectrophotometer on the RNA setting at 260 nm; the average of 3 measures was used to determine the concentration.

2.2.3 dsRNA injections

dsRNA was diluted to desired amount in injection buffer (5 mM KCl, 0.1 mM phosphate buffer, pH 6.8) with McCormick green food coloring (1:40 or 1:50 ratio) such that the desired dose could be seen and delivered in a 3 µl injection. Needles were prepared from borosilicate capillary tubes (1.0 mm OD, 0.75 mm ID, World Precision Instruments catalog # TW100-4) pulled by a program with heat at 535, pull at 90, velocity at 100, and time of 160 on the P-97 Flaming/Brown™ Micopipette Puller. The pulled needles were backloaded with the dsRNA solution using a pipette fitted with Eppendorf Microloader Tips. The needles were inserted in a Microelectrode holder attached via tubing to a 60 ml syringe.

Young adult females, 6-8 day old, were anesthetized with $CO_2$ and injected under a dissection microscope. *Oncopeltus* adults were injected horizontally using the pulled glass capillary tubes between the 4th and 5th sternites, using the pressure exerted by the syringe. Successfully injected females were placed into a small cage, containing water, sunflower seeds, paper shelter and cotton used for egg collection. One day after injection males were added to each cage at a 1:1 female to male ratio.

2.2.4 Embryo collection and analysis

0–24-hour embryos were collected, counted, and then placed in a 5 cm petri dish. Embryos were kept at 25 °C and 50% humidity, 16:8hr L:D cycle. Six days after AEL, a cotton ball soaked with water and food were added to the petri dish to reduce cannibalization, which occurred in all experiments. Eight days AEL the dish was placed in the -20° C for at least 5 mins to halt hatchlings from moving. Hatchlings and embryos were transferred to a 1.5 ml tube and 300-400 µl of Pampel's solution (Bioquip, catalog #1184C) was added. Pampel's solution was removed after 3 days and PBST was added for scoring analysis and storage.

Photographs of embryos and hatchlings were taken in a glass well plate using a Zeiss Discovery V12 stereo microscope with color image capture using an Axiocam 506 color capture device or Olympus SZX16 microscope. For photography, hatchlings and embryos were immersed in PBST. A pair of tweezers were used to remove the chorion when needed. Some wells in the plate were partially filled with resin, making a flat, less slippery surface for securing the embryos for photographing.

2.2.5 Trophic exposure of dsRNA

*gfp* and *Of*-eve dsRNA were each diluted in injection buffer (5 mM KCl, 0.1 mM phosphate buffer pH 6.8) with McCormick green food coloring (1:50 ratio) such that 5000 ng of dsRNA could be delivered in a 3 µl injection. To get enough eggs for feeding, a total of 15 female adult per treatment (P1) were injected. Eggs were collected every 24 hours for 12 consecutive days. Eggs collected on the first day were discarded since RNAi effects are often not observed on day 1 after injections (based on the *Of-eve* knockdown experiment (Fig. 2-2C). Injected adults and embryos were kept at 25 °C and 50% humidity, 16:8hr Light:Day cycle. 25% of the eggs collected were aged to confirm *eve*-dsRNA defects. These were analyzed under a dissection scope 5 days AEL. 75% of the eggs collected (G1) were fed to *Oncopeltus* adults (P2). These G1 eggs were counted, colored with McCormick green food coloring, and then glued to a piece of cardboard egg carton using gluten based non-toxic wallpaper paste (Fig. 2-5Bii), The G1 eggs were given to starved P2 females 24 h later (Fig. 2-5Biii). Before adding them to the cage, the carboard was tapped to remove any loosely attached eggs. The G1 eggs on the cardboard were counted to keep track of the number of eggs fed to P2 females.

The same day P1 adults were injected, P2 females were separated from the colony to be fed. They were placed in a cage, made from a pipette box, with just females, with sunflower seeds, water, and cotton placed for egg-laying. Any eggs collected before they were exposed to dsRNA eggs (G1) were discarded. 5 P2 females were placed in a cage per treatment: 5 females for the ds-*gfp* control eggs and 5 females for the ds-*Of-eve* eggs. These P2 females were fed eggs for two days,

switched to sunflower seeds for two days, and rotated between the two for a total of 10 days. Offspring from P2 females were collected every 24 hours, G2 eggs. G2 eggs were collected first from the cage, counted under a microscope, and checked for food coloring stains. P1 and P2 females and G1 and G2 offspring were kept in different incubators in different rooms under the same conditions to avoid contamination. After 5 days, G2 eggs were placed into Pampel's solution and 3 days later switched to PBST for scoring.

Once egg collection was performed, dyed eggs on cardboard were removed, and counted to keep track of how many eggs were eaten. Cages were cleaned and any loose eggs were removed and discarded. This process continued for a total of 10 collection days (schematic shown in Fig. 2-5Bi).

## 2.3 Results

### 2.3.1 RNAi knockdown of developmental regulatory genes *Of-eve* and *Of-Scr* causes a range of specific morphological defects

Liu and Kaufman previously showed that injection of dsRNA matching the embryonically expressed gene *Of-even-skipped* (*Of-eve*) into adult females caused a range of defects in early embryonic development of their offspring (Liu and Kaufman 2005). Here, we made use of this parental RNAi (pRNAi) approach to assess susceptibility of *Oncopeltus* to dsRNA.

Adult females were injected with varying amounts of *Of-eve*-dsRNA, or control *gfp*-dsRNA and embryos were examined (Fig. 2-1). Embryos were scored as hatched or unhatched and morphology was compared. No defects were observed in

any embryo that successfully reached the hatchling stage (Fig. 2-1A). Unhatched embryos were scored as (1) no observable defect (Fig. 2-1ii); (2) Moderate defects with a range of segment deletion or fusion in either or both the thoracic and abdominal segments, corresponding to Class II and III categories of Liu and Kaufman (2005), (Fig. 2-1Bi-iv); (3) Severe defects with embryos lacking almost the entire body and are referred to here as "head-only" embryos, Class I defects observed in Liu and Kaufman (2005) (Fig. 2-1Bv-vii).

As shown previously, parental RNAi for *Of-Scr* results in homeotic transformation of the mouthparts towards leg identity (Hughes and Kaufman 2000b; Chesebro et al. 2009). To assess the sensitivity of homeotic transformation as an assay for RNAi susceptibility, we scored the frequency and extent of homeotic transformation in offspring of injected females. Hatched embryos were scored, and morphology was compared and classified as follows: (1) No observed defects, no transformation of the mouthparts (Fig. 2-1Aiii-iv). (2) Mild transformation of the mouthparts, characterized by a rounder or a small split at the end (Fig. 2-1Ci); Moderate/Severe transformation of the mouthparts, characterized by a split along the mouthpart, referred to as a leg-like or antennae-like appendage in (Hughes and Kaufman 2000b; Chesebro et al. 2009) (Fig. 2-1Cii-v). Eggs that were cannibalized, unfertilized eggs, or eggs that failed to show any developmental progression were not scored for *Of-eve, Of-Scr* and *gfp* dsRNA treatments.

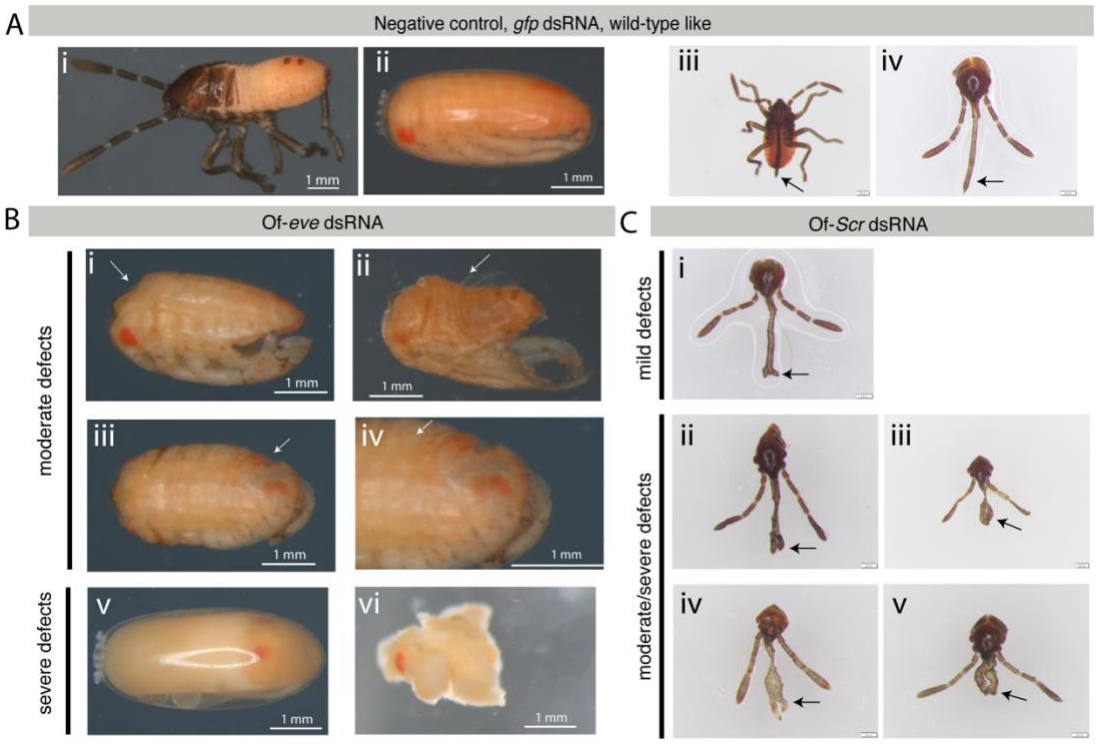



**Figure 2-1. Developmental defects associated with pRNAi knockdown of *Of-eve* and *Of-Scr*.** (Ai) Wild type-like hatchling, *gfp* control; (Aii) Wild type-like unhatched embryo *gfp* control, lateral view; (Aiii) Hatched nymph with no homeotic transformation; (Aiv) Head of hatched nymph with no homeotic transformation. (B) Offspring laid by females injected with dsRNA matching *Of-eve:* (Bi) *Of-eve* moderate defect, segmental fusion in head region, arrow; (Bii) truncation of abdomen; (Biii) partial fusion of abdominal segments, arrow; (Biv) Zoomed in partial fusion of abdominal segment; (Bv) severe defect, Undissected embryo with development of head only; (Bvi) Dissected embryo displaying head-only defect. (C) Offspring laid by females injected with dsRNA matching *Of-Scr*. Head of hatched nymphs with a homeotic transformation and categorized as mild, characterized by a rounder or a small split at the end, (Ci) or moderate/severe characterized by a split along the mouthpart or leg-like or antennae-like (Cii-iv). Ai-ii, and B were prepared by James B. Digel.

### 2.3.2 The developmental genes *Of-eve* and *Of-Scr* are sensitive indicators of RNAi susceptibility

To assess the susceptibility of *Oncopeltus* to dsRNA, increasing amounts of *Of-eve* or *Of-Scr* dsRNA were injected into females (Fig. 2-2A-B). Individual adult females were injected with 0.1 ng, 10 ng, 40 ng, 100 ng, 400 ng, or 1000 ng dsRNA. Successfully injected females were placed into a vial, containing water, sunflower

seeds, and cotton used for egg collection. One day after injection, males were added to each cage. Eggs were collected from three adult females every 24 hours for days 2-5 after injection. Eggs from the first 24 hour collection were discarded. For phenotypic analysis, eggs were placed in Pampel's solution seven days AEL for *Of-eve* pRNAi, before eggs hatched, and eight days AEL for *Of-Scr* pRNAi . As a control ds-*gfp* was injected into adults in parallel and eggs collected were placed in Pampel's solution either 7 or 8 days AEL, for comparison to *Of-eve* or -*Scr*, respectively.

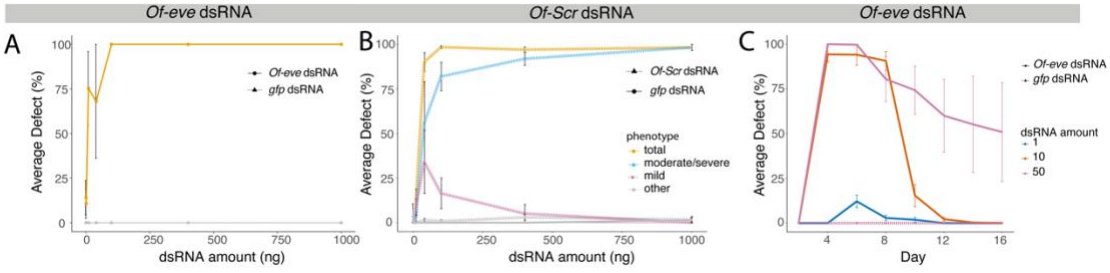



**Figure 2-2. Response and duration of developmental defects resulting from knockdown of *Of-eve* or *Of-Scr*.** Females were injected with the indicated amount of dsRNA and embryos were collected from 3 replicates. (A) *Of-eve* dsRNA (345 bp) and (B) *Of-Scr* (600 bp) and (A-B) a negative control, *gfp* dsRNA (633 bp) dsRNA was injected to *Oncopeltus* at a range of doses of 0.1 ng, 10 ng, 40 ng, 100 ng, 400 ng, and 1000 ng and embryos were collected for 5 days AEL. (C) Doses of 1 ng, 10 ng, and 50 ng were injected for *Of-eve* dsRNA and *gfp* dsRNA. Duration of RNAi was monitored for a total of 16 days. Raw data for A and C were collected by James B. Digel, under my supervision.

Injecting small amounts of *Of-eve* dsRNA into females, in the nanogram range, were sufficient to cause defects in some offspring (Fig. 2-2A).

Injection of increasing amounts of *Of-eve* dsRNA into females led to an increasing number of offspring showing defects – after injection of 0.1 ng, and 1 ng of *eve* dsRNA 14.11% (n=5), and 10.68 % (n=6) of embryos displayed moderate defects as

well as the severe, head-only, phenotype while the rest resembled wild type controls (n=51). At higher doses, defects increased to 75.29% at 10 ng (n=68) and 68.04% at 40 ng (n=57) with 100% of embryos exhibiting defects at 100 ng (n=91), 400 ng (n=102) and1000 ng (n=104). In sum, as the dose of *eve* dsRNA increased, an increasing number of embryos displayed developmental defects, reaching saturation at 100 ng injected.

Injecting small amounts of *Of-Scr* dsRNA into females, in the nanogram range, was also sufficient to cause defects in offspring (Fig. 2-2B). Injection of increasing amounts of *Of-Scr* dsRNA into females led to an increasing number of offspring showing moderate and severe defects – injection of 0.1 ng *Of-Scr* dsRNA did not produce detectable homeotic transformation. Defects were first detected after injection of 1 ng (0.78%, n=1) and increased to an average of 11.66% at 10 ng (n=15) and 90.03% at 40 ng (n=112). Injection of dsRNA of 100 ng, 400 ng, and 1000 ng exhibited more than 97% defects (n=120, n=69, n=102 respectively). *Of-Scr* resulted in 0.83 – 3 % defects at 40 ng, 100 ng, 400 ng, or 1000 ng (n=7). As dsRNA dose increased, mild defects decreased, and severe defects increased. Mild defects increased from 0.78 % at 1 ng (n=1) and 8.7 % at 10 ng (n=11) to 34.13 % at 40 ng (n=1). Moderate and severe defects were first observed at 2.96 % at 10 ng (n=4) and increased to 55.9 % at 40 ng (n=100). At higher doses, mild defects decreased to 16.47 % (n=20) at 100 ng, 5.13 % (n=4) at 400 ng and no mild defects were observed at 1000 ng while a majority of embryos, 81.94 - 98.25 %, displayed moderate and severe defects at 100 ng, 400 ng, and 1000 ng (n= 100, 65, 102, respectively). In sum,

response to dsRNA increased as dose increased. Saturation was achieved with 100 ng, 400 ng and 1000 ng, with almost all embryos showing defects.

No defects were observed for *gfp* dsRNA control treatment at 0.1 ng, 10 ng, 40 ng, 100 ng, 400 ng, or 1000 ng (n=48, 53, 54, 51, 56, 58, 54, respectively). These experiments show that *Oncopeltus* are susceptible to low doses of injected dsRNA, that they respond in a dose-dependent fashion and that the genes *Of-eve* and *Of-Scr* are useful for assessing parameters for RNAi in this species.

2.3.3 Duration of dsRNA response is dose-dependent

To determine whether amount of dsRNA affects duration of dsRNA-induced defects, we injected a *Of-eve* dsRNA at a range of doses (1 ng, 10 ng and 50 ng). As shown in Figure 2-2C, response and duration were both dose-dependent. At 1 ng, defects were first observed at 12.15 % (n= 28) on day 6 AEL and decreased to 1.87 % (n=2) by day 10 AEL with no defects observed before day 6 or after day 10. At 10 ng, defects ranged between 90.67 – 94.3 % between days 4-8 and decreased to 15.41 % (n=44) by day 10; on days 12-14 defects ranged between 0.34 – 2.19 % with no defects observed before day 4 or after day 14. At 50 ng, defects were first observed on day 4 at 100 % (n=96) and defects started to decrease from 99.71 % (n = 199) on day 6 to 50.88 % (n-44) by day 16. Defects lasted for 3 days at 1 ng, 6 days at 10 ng and defects persisted until day 16, at 50 ng. In sum, we found that duration of *Of-eve* dsRNA induced effects was proportional to the dose administered with 50 ng effects lasting longer than 1 ng and 10 ng.

2.3.4 Longer dsRNA is more effective than short dsRNA in *Oncopeltus*

It has been reported that longer dsRNA is more effective than short dsRNAs (Saleh et al. 2006; Miller et al. 2012). We used the specific *Of-Scr p*RNAi-associated defects to test the impact of length of dsRNA on effectiveness in this species. Our strategy to test varying lengths of dsRNA was to generate a 600 bp dsRNA targeting *Of-Scr* and deliver it by injection at a range of doses (Fig. 2-3A). To then compare effects of length while retaining the same sequences, we divided the 600 bp fragment into two 300 bp fragments. The two fragments were injected together and separately, at comparable doses. If the 600 bp dsRNA was more effective at causing defects than the same amount of nucleic acid delivered as 300 bp and 300bp, this would indicate that 600 bp is a more effective length than 300 bp. If effects are similar, this would indicate that there is no significant effect of length at this size of dsRNA. Using the same design, we then subdivided the 300 bp dsRNA into two 150 bp dsRNA, the 150 bp into two 75 bp dsRNAs and finally the 75 bp dsRNA into two 38 bp dsRNAs.

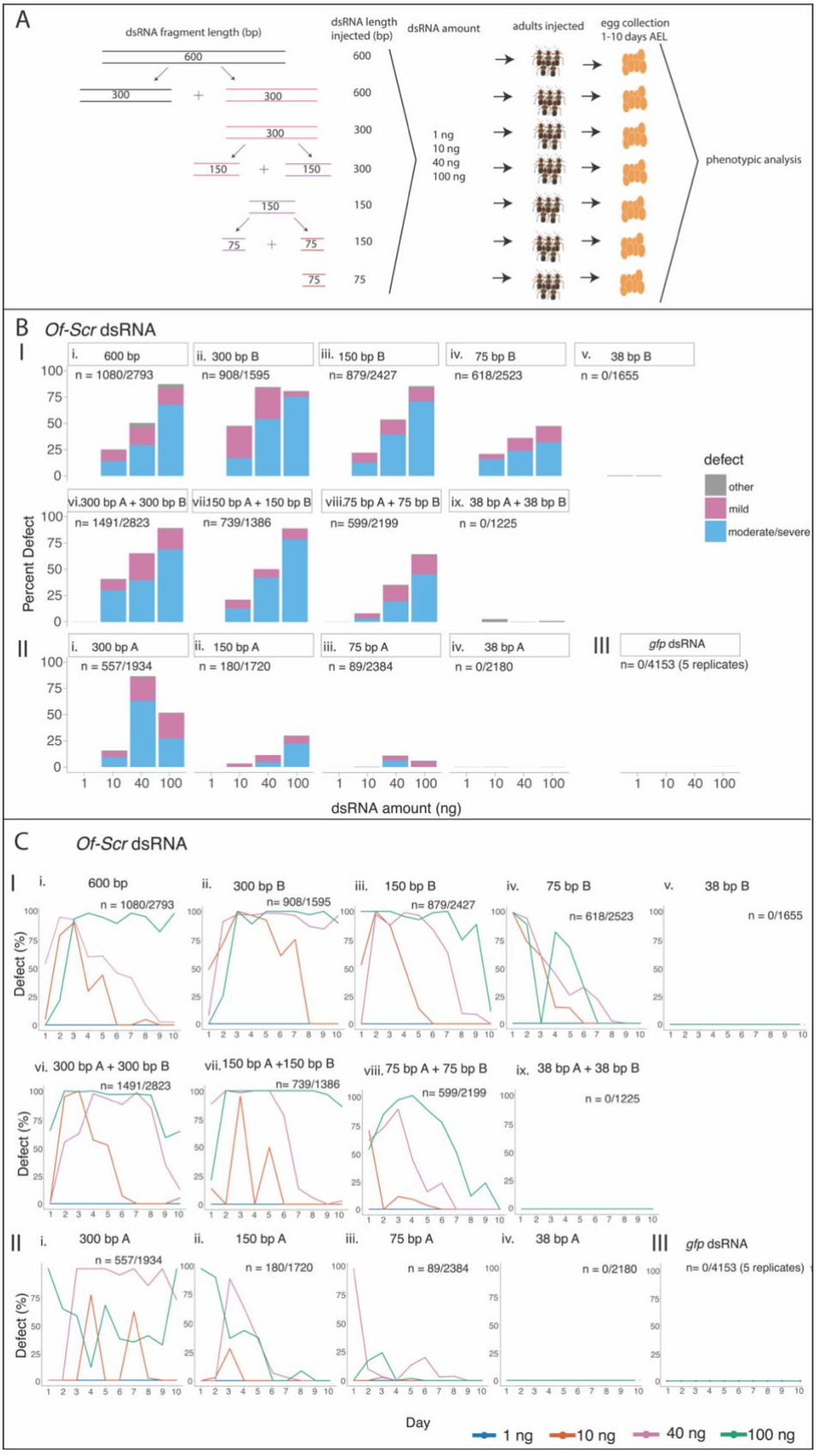

A
dsRNA fragment length (bp)
600
300
300
300
150
150
150
75
75
75
dsRNA length injected (bp)
dsRNA amount
adults injected
egg collection 1-10 days AEL
600
600
300
300
150
150
75
1 ng
10 ng
40 ng
100 ng
phenotypic analysis
B
Of-Scr dsRNA
I
i. 600 bp
n = 1080/2793
ii. 300 bp B
n= 908/1595
iii. 150 bp B
n= 879/2427
iv. 75 bp B
n= 618/2523
v. 38 bp B
n = 0/1655
vi.300 bp A + 300 bp B
n= 1491/2823
vii.150 bp A + 150 bp B
n= 739/1386
viii.75 bp A + 75 bp B
n= 599/2199
ix. 38 bp A + 38 bp B
n = 0/1225
Percent Defect
defect
other
mild
moderate/severe
II
i. 300 bp A
n = 557/1934
ii. 150 bp A
n = 180/1720
iii. 75 bp A
n = 89/2384
iv. 38 bp A
n = 0/2180
III
gfp dsRNA
n= 0/4153 (5 replicates)
dsRNA amount (ng)
C
Of-Scr dsRNA
Defect (%)
Day
1 ng
10 ng
40 ng
100 ng

**Figure 2-3. RNAi effectiveness decreases with short dsRNAs.** (A) Schematic of experimental design used to test effectiveness of different dsRNA lengths. (B) Graphs showing the percent defect of *Of-Scr* dsRNA at different lengths, (BI) i. 600bp B, ii. 300 bp B, iii, 150 bp B, iv. 75 bp B, v. 38 bp B, vi. 300 + 300 bp, vii. 150+150 bp, viii. 75 + 75 bp, viii. 38 + 38 bp; (BII) i. 300 bp A, ii, 150 bp A, iv. 75 bp A, v. 38 bp A; (BIII) negative control, *gfp* dsRNA treatment (633 bp); at 1 ng, 10 ng, 40 ng, or 100. ng. Blue bars, moderate/severe defects; pink bars, mild defects; gray bars, nonspecific defects. Number of embryos displaying defects shown for mild and moderate/severe defects. (C) Duration response of developmental defects resulting from knockdown of *Of-Scr* and the negative control, *gfp* dsRNA (633 bp) at a range of doses across 10 days. Graphs showing the percent defect of *Of-Scr* dsRNA at different lengths, (CI) i. 600bp B, ii. 300 bp B, iii, 150 bp B, iv. 75 bp B, v. 38 bp B, vi. 300 + 300 bp, vii. 150+150 bp, viii. 75 + 75 bp, viii. 38 + 38 bp; (CII) i. 300 bp A, ii, 150 bp A, iv. 75 bp A, v. 38 bp A; by amount of dsRNA at 1 ng, 10 ng, 40 ng, and 100. ng. (B-C vi-ix.) Graphs showing the percent defect of subdivided *Of-Scr* dsRNA, where two fragments were injected together at different lengths: vi. 300 bp A and 300 bp B (600bp total), vii. 150 bpA and 150 bpB (300 bp total), viii. 75 bpA and 75bpB (150 bp total), ix. 38 bpA and 38bpB (75 bp total). (B-C) dsRNA B lengths were generally more effective than dsRNA A lengths therefore we continued to test dsRNA B lengths. Numbers display total defects in offspring out of total offspring scored and numbers. Note that B and C are representations of the same data set in two different ways.

For each *Of-Scr* dsRNA length, 5 young female adults were injected with 1 ng, 10 ng, 40 ng, or 100 ng dsRNA. A negative control, *gfp* dsRNA (633 bp) was injected alongside each length. Embryos were collected for the span of 10 days after their first egg laying (AEL). Embryos were analyzed and scored as described above. The sum of the defects over 10 days was divided by the total eggs scored to determine the total percent defect (Fig. 2-3).

As the dose of *Of-Scr* dsRNA increased at each length, an increasing number of embryos displayed developmental defects (Fig. 2-3B). Knockdown of *Of*-Scr at 1 ng did not result in detectable defects. As the dsRNA dose increased -- 10 ng, 40 ng, and 100 ng, the effectiveness of dsRNA also increases for every dsRNA length. However, the sensitivity of the defects differed for lengths below 300 bp. As shown in Figure 2-3BI iii and viii, there is a difference of effectiveness when *Of-Scr* dsRNA of 150 bp was injected as one fragment versus the two 75 bp *Of-Scr* dsRNA fragments together, specifically at the 10 ng and 40 ng amounts. Also, the

effectiveness of 75 bp dsRNA was lower when two fragments are injected together compared to one single 75 bp dsRNA fragment. Therefore, effectiveness of the dsRNA decreased for the 150 - 75 bp fragment. At lengths greater than 150 bp, severity of defects differs across the various doses of dsRNA specifically at 10 ng and 40 ng, the non-saturating doses.

When determining at which length to continue, for example 300 bp A or 300 bp B, we chose the most effective dsRNA length out of the two, to continue to test for length. In general, the dsRNA B fragments were more effective than dsRNA A fragments therefore we continued to test dsRNA B fragments (Fig. 2-3BI-BII). The 38 bp dsRNA lengths did not result in detectable defects (Fig. 2-3BIv,BIix,BIIiv).

To determine whether length of dsRNA affects RNAi duration we looked at the total defect percent across 10 days (Fig. 2-3C). Response and duration of RNAi effect were dose dependent as seen previously with knockdown *Of-eve* RNAi. At each length of dsRNA, we observed it takes about 2-3 days AEL for dsRNA effects to be observed. In general, for the dsRNA B fragments, defects lasted longer than the dsRNA A fragments (Fig. 2-3CI-CII). The 38 bp dsRNA did not result in detectable defects (Fig. 2-3CIv,CIix,CIIiv).

For the dsRNA B fragments, at 10 ng, defects were observed for 6 days for the 600 bp fragment (Fig. 2-3CIi) and the two 300 bp fragments (Fig. 2-3CIvi) while the effect of the single 300 bp dsRNA, defects were observed for 7 days (Fig. 2-3CIii,CIIi). Defects for the 150 bp fragment (Fig. 2-3CIiii) and 75 bp (Fig. 2-3CIiv) lasted for 5 days, 3 days for the two 150 bp fragments (Fig. 2-3CIvii), 4 days for the two 75 bp fragments (Fig. 2-3CIviii). At 40 ng, the duration of defects ranged across

these lengths of dsRNA. At 100 ng, the 600 bp, 300 bp, two 300 bp fragments, and the two 150 bp fragments, 150 bp defects lasted throughout the 10 days when defects were first observed, with the 300 bp fragment exhibiting defects above ~85 % for 9 out of the 10 days (Fig. 2-3CIi,ii,vi,vii). Defects lasted for 9 days for the two 75 bp fragments, and 5 days for 75 bp. For each length we found that duration of *Of-Scr* dsRNA induced effects was proportional to the dose administered with 100 ng lasting longer than 10 ng. At 10 ng and 40 ng, there was variability of effectiveness at each length of dsRNA applied. At the highest amount of dsRNA applied, 100 ng, each length of dsRNA remained effective from 150 bp to 600 bp and declined in effectiveness when one or two 75 bp dsRNA's were injected separately or together. In sum, we found that effectiveness decreased with dsRNAs in the 150 bp to 75 bp range.

### 2.3.5 Death due to knockdown of *V-ATPase* is less sensitive to dsRNA than developmental genes

Tests of RNAi often rely on genes that cause death of the organism to which dsRNA is applied, as this is often the main purpose of pest management. To determine if this type of assay is as sensitive as scoring subtle developmental defects, we tested *Of-V-ATPase subunit A* (*Vha68-1*) at doses of dsRNA similar to those used for *Of-eve* and *Of-Scr*. V-ATPase are proton pumps (Nishi and Forgac 2002) that play an essential role in nutrient uptake in insects (Klein 1992). Knockdown results in mortality (Liu et al. 2022) and this gene is commonly used for RNAi-based pest management experiments (Zhang, Li, and Miao 2013; Baum and Roberts 2014). We

injected 5 females per replicate with *Of-Vha68-1* dsRNA or *gfp* dsRNA control, at a range of doses (0.1 ng, 1 ng, 10 ng, 40 ng, 100 ng, 400 ng, 1000 ng).

Mortality was monitored for 35 days (Fig. 2-4A-J). Survival of control adults injected with *gfp* dsRNA over the assay period of 35 days ranged between 10 % and 60 % across dose of dsRNA, 60 % survival was observed at 1 ng (n=6) and 10 % survival was observed at 1000 ng (n=1) (Fig. 2-4A,B,D-J). Survival of adults injected with *Of-vha68-1* dsRNA over the assay period of 35 days was 10% at 0.1 ng (n=1) and 1 ng (n=1) (Fig. 2-4A,C,D-J). Adults injected with *Of-vha68-1* dsRNA experienced 100 % mortality on day 31 at 40 ng, day 21 at 100 ng, day 19 at 400 ng and 1000 ng (Fig. 2-4A,C,D-J). At day 16, approximately halfway through the assay period, survival of control adults injected with *gfp* dsRNA was 80% at 0.1 ng (n=8), 90 % at 1 ng (n=9), 60 % at 10 ng (n=6), 90 % at 40 ng (n=9), 70 % at 100 ng (n=7) and 400 ng (n=7) and 50 % at 1000 ng (n=5) (Fig. 2-4A,B,D-J). Adults injected with *Of-Vha68-1* dsRNA survival was 50% at 0.1 ng (n=5), 80 % at 1 ng (n=8), 70 % at 10 ng (n=7), 50 % at 40 ng (n=5), 20 % at 100 ng (n=2), 40 % at 400 ng (n=4) and 20 % at 1000 ng (n=2) (Fig. 2-4A,C,D-J). While *Of-Vha68-1* knockdown did increase death compared to controls by day 35, the end of the assay period, those injected with the control were also dying.

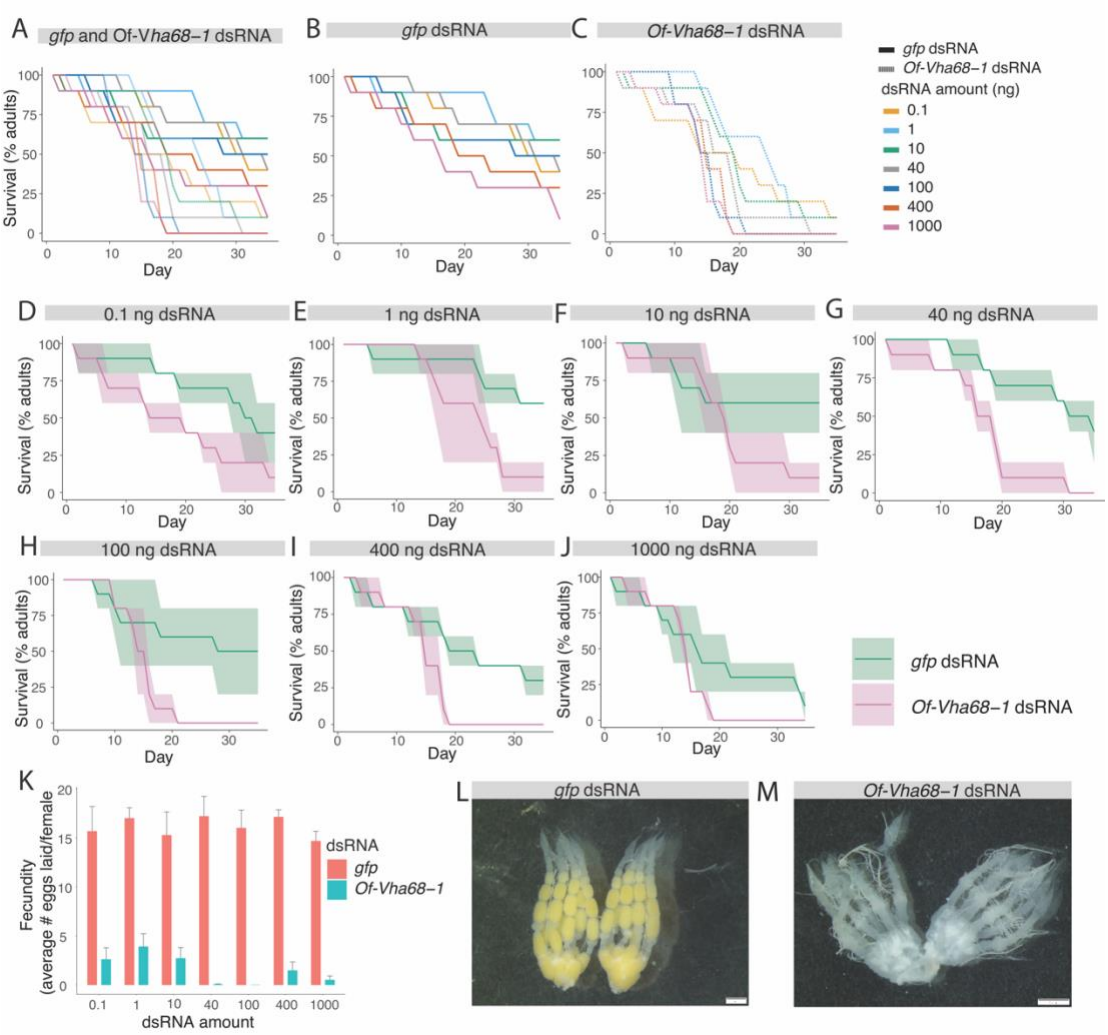



**Figure 2-4. Effects of *Of-Vha68-1* dsRNA in adult *Oncopeltus.*** Graphs represent data collected from 3 replicates of *Oncopeltus* injected with *Of-Vha68-1* dsRNA. Survival was monitored for 35 days. (A-C) Effect of *gfp* dsRNA and *Of-Vha68-1* on adult survival at a range of doses: 1 ng, 10 ng, 40 ng, and 100. ng. (D-J) Replotting of data from A-C showing percent mortality at a range of dsRNA amounts (D) 0.1 ng (E) 1 ng (F) 10 ng (G) 40 ng (H) 100 ng (I) 400 ng (J) 1000 ng. *gfp* dsRNA is represented by the green line and *gfp* dsRNA standard error is represented by green shaded region and *Of-Vha68-1* dsRNA is represented by the purple line and *Of-Vha68-1* dsRNA standard error represented by purple shaded region. (K) *Of-Vha68-1* dsRNA blocked oogenesis and resulted in failure of ovarioles to develop. Eggs laid by females injected with varying doses of dsRNA is shown. Pink bars, ds-*gfp*; Green bars, ds-*Of-Vha68-1.* Bars represent standard error. (L) negative control, *gfp* dsRNA, wild type-like ovarioles that are yellow in color and oval shaped eggs. (M) *Of-Vha68-1* dsRNA, no eggs laid by female, and failure of ovarian development. Raw data for A-J were collected by James B. Digel.

We observed that females injected with *Of-vha68* dsRNA ceased egg laying, as reported in the corn planthopper (Yao et al. 2013). Fecundity was measured alongside mortality, shown as average number of eggs laid per female per day (Fig. 2-4K). For *Of-Vha68-1,* we collected eggs across 9 days. For those injected with *gfp* dsRNA we collected eggs for 6 days after egg laying, and discarded day 1 of egg collection, so the data shows 2-6 days after egg laying. Those injected with *Of-vha68* dsRNA laid eggs 2-4 days after injection and stopped laying eggs entirely 1-2 days after egg laying. In contrast, those injected with *gfp* dsRNA, continued to lay eggs throughout the assay. On average, those injected with *Of-Vha68-1* dsRNA, laid 0-3.9 eggs per female while those injected with *gfp* dsRNA laid an average of ~14-17 eggs per female (Fig. 2-4K). To determine why eggs were not laid after *Of- Vha68-1* dsRNA, ovaries were dissected. In animals injected with *gfp* dsRNA, wild type-like ovarioles that are yellow in color and containing oval shaped eggs, were observed (Fig. 2-4L). In contrast, oogenesis was blocked in animals injected with *Of- Vha68-1* dsRNA: ovarioles were not pigmented, appeared translucent and no developed eggs observed (Fig. 2-M) This sublethal sterility effect was observed within a week of injection. We conclude that the sublethal sterility effect caused by knockdown of *Of-Vha68-1* is a more sensitive assay than death for this gene.

### 2.3.6 *Oncopeltus* is susceptible to dsRNA via feeding

To test whether *Oncopeltus* are susceptible to dsRNA via feeding, we fed similar amounts of *Of-eve* dsRNA to adult females as were used in the injection experiments (Fig. 2-5A). Five female adults were separated one day before feeding and were starved of water and food overnight. Adults were fed the dsRNA via a

droplet of water watched individually for 10 minutes to ensure consumption of the droplet of water. If adults did not drink within the allotted time, a new female was used. Eggs were collected for 1-7 days after injection. Moderate and severe *eve*-specific defects were observed in offspring of females fed *Of-eve* dsRNA but not in control females fed *gfp* dsRNA. Both moderate and severe defects were observed after feeding of 0.1 ng or more *Of-eve* dsRNA, with more than 10% of offspring showing defects after feeding of 400 or 1000 ng of dsRNA. At similar levels, 100% of offspring showed defects after injection of dsRNA, showing that injection is much more efficient as a delivery method. Non-specific defects seen for both *gfp* dsRNA and *Of-eve* dsRNA could not be scored for *eve*-specific abnormalities and include failure of dorsal closure. These experiments demonstrate that *Oncopeltus* are susceptible to RNAi via feeding and these effects can be passed from mothers to offspring, the first demonstration of this for *Oncopeltus* to our knowledge.

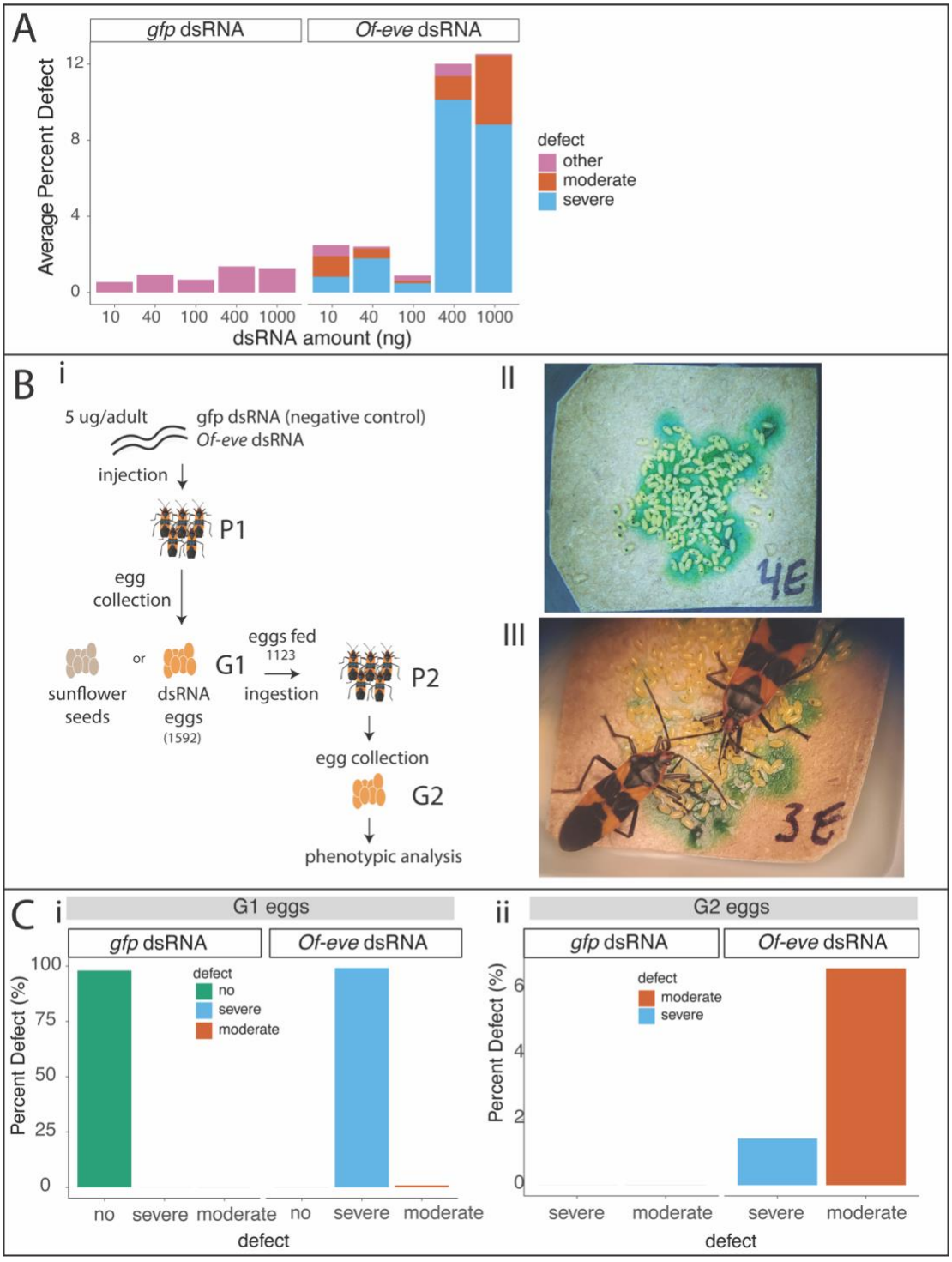

A
gfp dsRNA
Of-eve dsRNA
Average Percent Defect
12
8
4
0
10 40 100 400 1000 10 40 100 400 1000
dsRNA amount (ng)
defect
other
moderate
severe
B i
5 ug/adult
gfp dsRNA (negative control)
Of-eve dsRNA
injection
P1
egg collection
or
sunflower seeds
dsRNA eggs (1592)
G1
eggs fed 1123
ingestion
P2
egg collection
G2
phenotypic analysis
II
4E
III
3E
C i
G1 eggs
gfp dsRNA
Of-eve dsRNA
Percent Defect (%)
100
75
50
25
0
defect
no
severe
moderate
no severe moderate no severe moderate
defect
ii
G2 eggs
gfp dsRNA
Of-eve dsRNA
Percent Defect (%)
6
4
2
0
defect
moderate
severe
severe moderate severe moderate
defect

**Figure 2-5. RNAi defects of *Of-eve* dsRNA in adult *Oncopeltus* via ingestion and trophic exposure of dsRNA.** (A) Ingestion of dsRNA. Graphs showing the percent defect for eggs from female adults that ingested *gfp* dsRNA or *Of-eve* dsRNA at a range of doses (10 ng, 40 ng, 100 ng, 400 ng, and 1000 ng). Embryos were collected for 7 days AEL from 3 replicates. Blue bars, severe defects; orange bars, moderate defects; pink bars, nonspecific defects. (B) Trophic exposure experimental design (i) Schematic. Adults (P1) were injected with 5 ug of *gfp* dsRNA, negative control, or 5 ug of *Of-eve* dsRNA. Eggs (G1) were collected from P1 adults and fed to unexposed *Oncopeltus* female adults (P2). Eggs (G2) were collected from P2 females and analyzed for a total of 10 days. (ii) G1 eggs collected from P1 *Oncopeltus* females were glued to a piece of cardboard and dyed with green food coloring. (iii) *Oncopeltus* P2 adults were seen ingesting the dyed G2 eggs. (C) Trophic exposure results (i) Defects displayed by G1 eggs (percent) after injection of *gfp* or *Of-eve* into P1 females, as indicated. Green bars, no defect; orange bars, moderate defects; blue bars, severe defects. (ii) Trophic response in G2 eggs after feeding on G1 eggs from P1 females injected with *gfp* dsRNA or *Of*-eve dsRNA; orange bars, moderate defects; blue bars, severe defects. Embryos with non-specific defects (*gfp* dsRNA 2.71%, *Of-eve* dsRNA 3.78%) were not scored for *eve*-specific abnormalities. Non-specific defects include failure of dorsal closure. James B. Digel fed dsRNA to adults for A.

### 2.3.7 Trophic exposure results in low level transmission of RNAi effects

Given that *Oncopeltus* are susceptible to dsRNA via ingestion, we next asked whether RNAi effects can be passed from one insect to another via trophic exposure. We made use of the fact that *Oncopeltus* cannibalize eggs if sufficient food is not present (Root and Chaplin 1976), which we also observed throughout the course of our injection experiments. Figure 2-5Bi outlines our experimental design: Adult females (P1) were injected with *Of-eve* dsRNA at levels that produce severe *eve*-specific defects in 100% of offspring, or the same amount of control *gfp* dsRNA. Eggs (G1) were collected from these females and fed to other females (P2) who had never been exposed to dsRNA. Offspring of those fed females (G2) were scored for *eve*-specific defects. Surprisingly, in a preliminary experiment, effects of *eve* dsRNA were observed in these G2 eggs laid by P2 females. To rule out the possibility of bias in this experiment, a blinded experiment was carried out next, following the experimental design in Reding et al. (Reding, Lê, and Pick 2023). One researcher performed the P1 injections and the other researcher with no previous knowledge of

the treatment - “the analyzer” - scored the defects. In both the preliminary experiment and the blinded experiment, the G1 all displayed severe, head-only defects but G2 eggs included both severe and moderate defects, strongly suggesting they were not G1 contaminants. Finally, this experiment was repeated a third time by one of the same researchers and a different second researcher, reported here. In this third experiment, to further rule out the possibility of contamination of the G2 population with G1 eggs or due to a rare P1 female entering the G2 cages and laying eggs there, G1 eggs were marked with green dye (Fig. 2-5Bii) and counted before and after feeding. As shown in Figure 2-5Biv, females actively fed on the eggs provided. In this experiment, 8.03 % (n=51) of G2 eggs displayed *eve*-specific defects that included 6.61% moderate and 1.42% severe defects (Fig. 2-5C). For all three experiments, no *eve*-specific defect was ever observed G1 or G2 eggs resulting from P1 females injected with *gfp* dsRNA. In sum these experiments demonstrate that effects of dsRNA can be transmitted to untreated insects by feeding on other insects displaying RNAi-induced defects. The rate of transmission was in the range of 10% in this within-species experimental design, which represents a worst-case scenario in that the dsRNA injected into P1 females exactly matches the mRNA in the G2 embryos.

**2.4 Discussion**

Here we tested susceptibility to pRNAi in *Oncopeltus* using two well-characterized development regulatory genes, *eve* and *Scr*. Knockdown of these genes resulted in specific morphological defects that allowed us to assess subtle and long-lasting effects due to RNAi. We found that duration of *Of-eve* dsRNA induced effects

was proportional to the dose administered, with duration of dsRNA response increasing as dsRNA amounts increase. We saw similar results for duration response for knockdown of *Of-Scr*. We also observed that longer dsRNAs are more effective than shorter dsRNAs with effectiveness decreasing at the 150 bp to 75 bp range. When comparing RNAi effectiveness of developmental genes to *V-ATPase*, we found that developmental genes *eve* and *Scr* are better. We also found that the sublethal effect of oogenesis being blocked due to *V-ATPase* is specific and can be quantified to test RNAi effectiveness.

We were the first to demonstrate, to our knowledge, *Oncopeltus'* susceptibility to ingestion of dsRNA, with effects passed from mothers to offspring. We also discovered dsRNA-induced effects through trophic exposure. I did a very rough estimate of amounts of dsRNA that G2 eggs could have been exposed to. This estimate would represent the maximum possible exposure of G2 eggs to dsRNA because it assumes that the dsRNA injected into P1 females was 100% stable, without degradation of dsRNA due to exposure to the environment and digestive system of the insect, and that all the dsRNA was directly transported into G1 eggs during the day examined in our experiment. We estimate that each P2 female was exposed to a maximum of 656 ng of *Of-eve* dsRNA via feeding on eggs. We injected a total of 25 ug in 15 females. If all the dsRNA was transported into the eggs we collected, each G1 egg would contain ~16 ng. As previously mentioned, G1 eggs were counted before and after feeding. A total of 1123 G1 eggs were fed to P2 adults across 11 days and a total of 712 eggs remained. From this we estimate a total of 411 eggs were eaten by 10 adults, males and females, therefore each female ate ~41.1 eggs and if

each G1 egg contained 16 ng then each P2 female were exposed to ~656 ng. This is a maximum-exposure estimate because this does not include degradation due to factors affecting the stability of dsRNA caused by the environment, such as temperature, and after delivery to the *Oncopeltus*, including passing through the digestive system, and it assumes (incorrectly) that all of the injected dsRNA entered the eggs used for feeding, while our duration experiments suggest that dsRNA would still be present in the females for many days.

Our discovery of transmission of dsRNA induced effects across generations to untreated animals via feeding and trophic interactions suggests that there is a potential risk to non-target insects in field settings. We observed that defects from as little as 1 ng of dsRNA caused defects in offspring via pRNAi which suggests that small amounts of dsRNA exposure in agricultural settings may pose a threat to non-target insects. Our work suggests that insects may have the capacity to amplify effects caused by dsRNA and trophic interactions by RNAi insecticides may pose a threat. It is therefore necessary to continue tests of sensitivity to RNAi before use of RNAi in field settings to targeted and non-targeted insects. Improvement in the understanding of RNAi mechanisms and interaction between insects will better aid use of RNAi for pest management.

# Chapter 3: Determining if *Oncopeltus* generates gene silencing molecules, via secondary RNA's

## 3.1 Introduction

Ever since the discovery of RNA interference (RNAi) (Napoli, Lemieux, and Jorgensen 1990; Fire et al. 1998), this gene silencing tool has been used to knockdown gene expression in various species(H. Zhang, Li, and Miao 2013; Douglas 2018; Christiaens et al. 2020). In *C. elegans,* when dsRNAs are cleaved by DICER, (Ketting et al. 2001; Bernstein et al. 2001), small RNAs are generated, also known as small interfering RNA's (siRNA's) (Elbashir et al. 2001). RISC, the RNA-induced silencing complex, will use the siRNAs produced as guides, to matching mRNAs resulting in gene silencing (Zamore et al. 2000; Bernstein et al. 2001).

Secondary RNA's ($2^o$ RNAs) generated by RNA dependent RNA polymerase (RdRP) result in amplification of the RNAi response (Smardon et al. 2000; Sijen et al. 2001; Maniar and Fire 2011; Sapetschnig et al. 2015). The $2^o$ RNAs, generated by RdRP, result in antisense RNAs that may or may not match the sequence of the introduced dsRNA. In *C. elegans,* the introduced dsRNA has also been shown to pass down across generations resulting in a systemic response in progeny (Marré, Traver, and Jose 2016). Although insects are able to generate a systemic response to RNAi there is variation of RNAi susceptibility across insect orders (Vélez and Fishilevich 2018). The cellular mechanisms involved in the uptake and spread of dsRNA from extracellular dsRNA is yet to be fully understood. Although insects are able to

generate a systemic response, they do not appear to have RdRP homologs (Gordon and Waterhouse 2007), and there is no evidence of production of 2° RNAs (Li et al. 2018).

The use of dsRNA as a pesticide has shown great potential due to the ability to design species-specific dsRNAs to target genes of interest (Gordon and Waterhouse 2007). We have previously shown that *Oncopeltus* is highly susceptible to RNAi (Chapter 2) and even low amounts of dsRNA result in gene silencing and can be passed across generations via ingestion of dsRNA. This suggests that there is amplification of the RNAi effect in insects. If this amplification includes the generation of new 2° RNAs, this would increase risk to non-target insects from RNAi based pesticides because it would be virtually impossible to design species-specific dsRNAs.

Here, we use dsRNA targeting the *Oncopeltus* gene *fushi tarazu-factor 1 (Of-ftz-f1)* which is a key player in the ecdysone signaling pathway, is involved in oogenesis (Reding et al. 2019), segmentation (Yu et al. 1997; Yussa et al. 2001; Heffer et al. 2013), molting and metamorphosis (Broadus et al., n.d.; Heffer et al. 2013) to determine if the milkweed bug *Oncopeltus fasciatus* generates 2° RNAs that match new regions of the dsRNA injected. I determined that *Of-ftz-f1* plays a role in ovary development due to its expression throughout oogenesis. Knockdown of *Of-ftz-f1,* resulted in oogenesis being blocked. We designed experiments to determine whether 2° RNAs are produced in *Oncopeltus*. I successfully isolated small RNAs from large RNA but northern blotting posed many technical challenges that remain to be resolved.

## 3.2 Methods

### 3.2.1 Isolation of *Of-ftz-f1*

RNA was extracted using Trizol from *O. fasciatus* embryos 48-72 h after egg laying (AEL) and reverse transcription was performed to prepare cDNA. To isolate the full gene *Of-ftz-f1,* gene specific primers (see Fig. 3-3A), based on the *O. fasciatus* genome (Panfilio et al. 2019), were used. Gene products were purified and inserted into pGEM-T Easy Vector (Promega) by TA cloning. This plasmid (pGEM-*Of-ftz-f1*) was used for double-stranded RNA (dsRNA) and RNA probe synthesis.

### 3.2.2 *Oncopeltus* ovary dissections

To dissect *Oncopeltus* ovaries, females were placed in the freezer at -20° C for about 5 mins. Once asleep, a blade was used to remove legs and head. On a dissecting dish, on ice, the abdomen was pinned down at the outer four corners. Small dissecting scissors were then used to make an incision in the abdomen, the exoskeleton pinned with needles and ovaries were removed by cutting below the oviduct and the tracheal tubes. Ovaries were placed in a 1.5 ml Eppendorf tube with RNAzol for RNA extraction.

### 3.2.3 RT-PCR for *Of-ftz-f1*

*Oncopeltus* adults were collected every 24 h for 264 h. For each time point, 10 adults were dissected to isolate ovaries, and RNA was extracted using TRIzol (Invitrogen). cDNA was generated with reverse transcription using M-MuLV RT (NEB) and RNase Inhibitor (NEB). cDNA was pooled to generate 0-48 h, 48-96 h, 96-144h, 144-192h, 192-240h, 240-264h, 0-264 h, 48-72 h ovarian cDNA. PCR

amplification was performed for 30 cycles. Primers spanned exon boundaries, and controls without RNA as well as without reverse transcriptase were used.

### 3.2.4 dsRNA synthesis

Primers with T7 promoter sequence at the 5'ends were used to amplify fragments from *Of-ftz-f1*. The PCR products were used as templates for dsRNA synthesis. To make dsRNA, MEGAscript T7 Transcription kit (Ambion) was used according to manufacturer's protocol. For *Of-ftz-f1* dsRNA, the length of the dsRNA was 600 bp. *gfp* dsRNA, 663 bp was used as a negative control in all experiments. A 1:100 dilution of the dsRNA was used to check the quality of the dsRNA on a 1% agarose gel. This dilution was quantified using a NanoDrop spectrophotometer on the RNA setting at 260 nm; the average of 3 measures was used to determine the concentration.

### 3.2.5 dsRNA injections

dsRNA was diluted to the desired amount in injection buffer (5 mM KCl, 0.1 mM phosphate buffer pH 6.8) with McCormick green food coloring (1:40 or 1:50 ratio) such that the desired dose could be delivered in a 3 µl injection. Needles were prepared from borosilicate capillary tubes (1.0 mm OD, 0.75 mm ID, World Precision Instruments catalog # TW100-4 pulled by a program with heat at 535, pull at 90, velocity at 100, and time of 160 on the P-97 Flaming/Brown™ Micopipette Puller). The pulled needles were backloaded with the dsRNA solution using a pipette fitted with Eppendorf Microloader Tips. The needles were inserted in a microelectrode holder attached via tubing to a 60 ml syringe.

Adult females were anesthetized with $CO_2$ and injected under a dissection microscope. *Oncopeltus* adults were injected horizontally in the abdomen using the pulled glass capillary tubes between the 4th and 5th sternites, using the pressure exerted by the syringe. Successfully injected females were placed into a small cage, containing water, sunflower seeds, paper shelter and cotton used for egg collection. Males were added immediately to each cage for a 1:1 female to male ratio. Ovary dissections followed as described previously.

### 3.2.6 RNA extraction

The following RNAzol RNA extraction is the complete small RNAseq protocol from the Jose lab modified by me for use with *Oncopeltus*. RNAzol, 250 µl, was added to dissected ovaries, tubes were spun for 3 min at 13,000 rpm to pellet the ovaries which were then ground with an RNAse-free pestle. This was repeated a total of three times, 750 µl of RNAzol was added, and tubes were stored at -80 °C until ready for RNA extraction. Once ready for RNA extraction, samples were thawed on ice for a total of 5 mins with periodic mixing. 1 ml of RNAzol was used as a negative control. DNA and proteins were precipitated by adding 400 µl of nuclease-free water, spinning at 12,000 g for 15 min, and removing as much supernatant volume as possible. The DNA/Protein pellet was stored in -80 °C for future use. To further purify the supernatant and separate DNA, RNA, and protein, a phenol chloroform extraction was performed. For this, the supernatant was added in a 1.5 ml tube, 200 µl phenol – chloroform mix was added (100 µl phenol, 100 µl of chloroform), mixed and spun for 2 min at 13,000 rpm at 4° C. The aqueous layer was removed, and the phenol-chloroform extraction was repeated until the interface between the aqueous

and non-aqueous layer was minimal. 200 µl of chloroform was added to the aqueous layer of the last phenol-chloroform extraction. A final spin of 1 min at 13,000 rpm, 4° C was performed and up to 1 ml of the aqueous layer was kept, which contained the RNA. Following the phenol-chloroform extraction, the small RNA and mRNA isolation followed: 3 µ1 of Precipitation Carrier was added to 1 ml of the supernatant, mixed, and 0.4 ml of 75% ethanol (v/v) was added, mixed, and incubated at room temperature for 10 min. To separate the mRNA and the small RNA fractions, this was centrifuged at 12,000 g for 10 mins. The mRNA fraction (>200 nt) was precipitated and stored at -20 °C until it was ready to wash. To precipitate the small RNA fraction, the supernatant was split equally and 2 µl of Polyacryl Carrier, and 0.8 volumes of pure isopropanol was added to each tube and stored at 4° C for 30 minutes. This solution was spun at 12,000 g for 25 min, the supernatant was removed. The RNA precipitate contained RNA <200 nt to 10 nt. The RNA pellets were washed twice by adding 0.5 ml of 75% cold ethanol to the large RNA pellet, and adding 0.5 ml of 70% cold isopropanol to the small RNA pellet, and centrifuging at 8,000 g for 3 min. The alcohol was removed (as much as possible) before dissolving the RNA pellets in nuclease-free water. Solutions were held at room temperature for 5 minutes and spun down briefly. To each RNA sample, a DNase treatment was performed by adding DNase I reaction buffer (10x), 2 units of DNase I (RNase-free), and nuclease-free water to the RNA. This mix was incubated at 37°C for 10 minutes. To stop the reaction, 1 µl of 0.5 M EDTA was added, and the tube heat at 75°C for 10 minutes to inactivate the DNase. A 1:10 dilution of the RNA was used to measure the concentration and run on a 1% agarose gel to check the quality of the RNA. This

RNA dilution was quantified using a NanoVue Spectrophotometer on the RNA setting.

### 3.2.7 RNA probe synthesis

Sense and antisense RNA probes were synthesized using digoxigenin labeling mix (Roche). DNA templates were generated by PCR amplification from *Of- ftz-f1* plasmids. The T7 promoter sequence was added to either the forward or reverse primer. A T7 RNA transcription reaction was performed at 37°C for 2 h, followed by precipitation in ice-cold ethanol (75 µl) and 6M LiCl (1.7 µl). These digoxigenin labeled probes were then used for northern blotting. A 1:10 dilution of the RNA probe was used to measure the concentration and run on an 1% agarose gel to check the quality of the RNA.

### 3.2.8 Northern Blotting

A 4% denaturing formaldehyde polyacrylamide gel (FDF-PAGE) was prepared (Harris & Molnar et al., 2015). RNA was heated with formaldehyde to break up dsRNA duplexes and run on a gel with 1-kb and 100 bp DNA ladders (NEB). The DNA ladder lanes were separated from the RNA lanes and stained with ethidium bromide for 10 minutes and imaged. The RNA was transferred (25 V, 1.0 A, 30 min, BioRad Transblot-Turbo) to a positively charged nylon membrane (11209299001, Roche) using Trans-Blot Turbo Transfer System (Bio-RAD). The RNA was cross linked with UV radiation at 1200 µJ/$m^2$ using the VWR UV Crosslinker for 3 minutes. Digoxigenin labeled RNA probes were denatured at 95 °C. The nylon membrane with the crosslinked RNA was placed inside a hybridization bottle and 5

ml of ULTRAhyb Buffer (Ambion LifeTechnologies) was added and rotated at desired temperature for 30 mins. 2.5 pMol of RNA probe was added to the ULTRAhyb buffer and hybridized overnight at 37°C, 42°C or 60°C in a VWR Hybridization Oven. After hybridization, the membranes were washed to remove excess probe and washed twice with low stringency buffer, high stringency buffer, 1X SSC for 5 min each and blocked with 1X DIG Blocking buffer. The membrane was then incubated with DIG antibody (Roche), washed with 1X DIG-Wash buffer (Ambion Life Technologies), and developed with CSPD, a chemiluminescent substrate, (Roche) at 37°C for 15 minutes. Blots were imaged using Fujifilm LAS-3000. The blots were stripped by washing twice in 5.0 % SDS at 80 °C for 5 minutes and rinsing twice with 2X SCC at 80°C for 5 minutes to be ready for the next probe. The previous steps were repeated for each new probe to be used on the same membrane. To determine RNA fragment size, the northern blot image was aligned to the ladders, stained with ethidium bromide, using Illustrator (Adobe). This northern blotting protocol was based on Choi and Shugarts' protocol (Choi et al. 2017).

## 3.3 Results

### 3.3.1 RNAi knockdown of *Of-ftz-f1* results in oogenesis being blocked

Yong Lu previously showed that that injection of *Of-ftz-f1* dsRNA blocked oogenesis in *Oncopeltus* with oocytes failing to mature (Lu Thesis, Reding et al. 2019). To determine when during oogenesis *Of-ftz-f1* is required, I first did a time course of wild type ovarian development in *Oncopeltus* females (Appendix IV). Ovaries were dissected for 16 days after eclosion. From days 1 to 6, there were few

developed oocytes. As the days progressed, the number of developing oocytes increased (Appendix IV A1-6). The first evidence of mature oocytes was on day 6; from day 9 (192 – 216 h) until day 16 (336 – 360) fully mature oocytes were apparent, marked by their yellow color. The sizes of oocytes increased over time, corresponding to different stages of development (Appendix IV A6-16).

I next determined when *Of-ftz-f1* is expressed in *Oncopeltus* ovaries, using RT-PCR on staged ovaries collected from 0 – 264 h after eclosion. These experiments showed that *Of-ftz-f1* is expressed at all time points throughout oogenesis (Fig. 3-1A; Appendix V). Although it is expressed throughout this time period, this does not necessarily mean that its function is required throughout oogenesis. To determine when *Of-ftz-f1* is functioning, I carried out a time course of dsRNA delivery over the span of 10 days, from 0 – 24 h after eclosion until 240 – 264 h, when I saw mature oocytes (Appendix IV). 10ug of *Of-ftzf1* dsRNA was injected into different aged female *Oncopeltus* adults ranging from 0-24h, 24-48h, 48-72h, 72-96h, 96-120h, 120-144h, 144-168h, 168-192h, 192-216h, and 216-240h after eclosion. For each time point, ovaries were dissected every 24 h after injection until 240 – 264 h (10 days post injection) was reached (Appendix V). A negative control injection was performed by injecting 10 ug of *gfp* dsRNA into 1 day old female *Oncopeltus* adults. This resulted in wild-type-like ovaries, as expected (Appendix V). The knockdown of *Of-ftz-f1* resulted in a range of defects including shape and color changes, severe ovarian defects include round shaped oocytes, and translucent ovaries (Fig. 3-1Bb,ci), mild and moderate defects include oval shaped oocytes and pale yellow in color (Fig. 3-1Bcii-iv). Adults injected 0 – 24 h after injection, displayed severe defects, with

non-mature oocytes throughout (Fig. 3-1B). Adults injected 24 – 48 h and dissected at 192 – 240 h had square shaped and light yellow oocytes. These oocytes were large enough to yield a high amount of RNA for RNA extraction (Fig. 3-1Bc). Figure 3-2 only shows two time points, chosen because they show the range of defects caused by *Of-ftz-f1* dsRNA knockdown and the time frame of adults needed to be dissected from for RNA extraction.

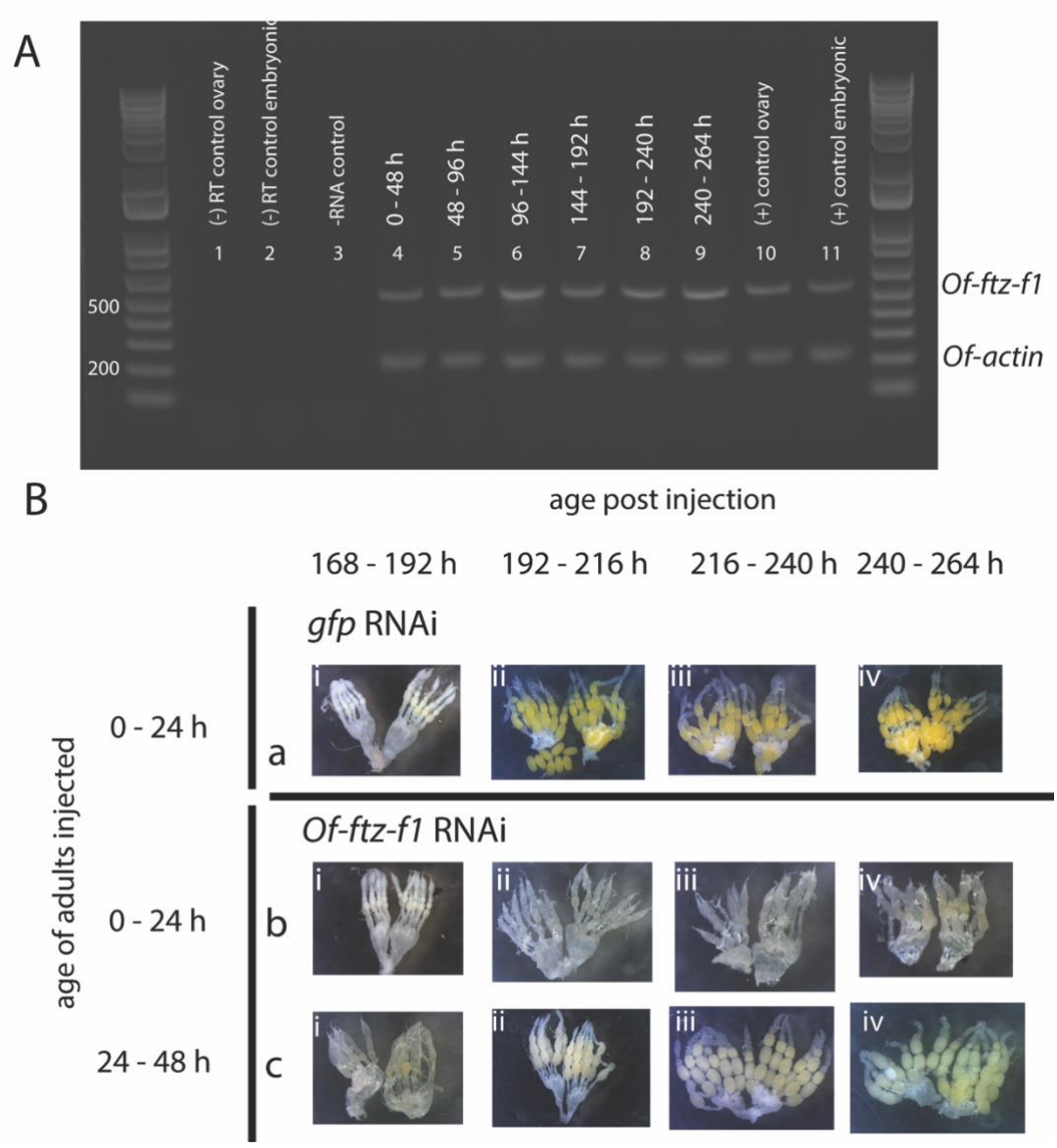


**Figure 3-1. *Of-ftz-f1* is required for oogenesis.** (A) Time of *ftz-f1* expression during oogenesis was determined by RT-PCR (30 cycles of PCR amplification). Photo of a 1% agarose gel is shown. Lanes: 1) minus RT control, ovary RNA, 2) minus RT control, embryonic RNA, 3) minus RNA negative control. Lanes 4-9, RNA was collected from *Oncopeltus* ovaries from: lane 4) 0-48 h, 5) 48-96 h, 6) 96-144 h, 7) 144-192 h, 8) 192-240 h and 9) 240-264 h. Lane 10) positive control using 0-264 h ovaries, 11) positive control using 48-72 h embryonic RNA. A separate PCR for actin was performed, in conjunction to *Of-ftz-f1* reactions, and run on a gel with the *Of-ftz-f1* reactions, amplifying a ~200 bp product as an internal positive control. DNA ladder was included on either side of the samples. *Of-ftz-f1* was first detected at 0-48 h and remained stable until 240-264 h. (B) *Oncopeltus* ovaries dissected from females injected with (a) negative control *gfp* dsRNA or (b-c) *Of-ftz-f1*dsRNA. (a) Adults injected with negative control *gfp* dsRNA 0-24 h after eclosion and dissected at (ai) 168 – 192 h, showing wild type-like developing oocytes, *gfp* control; (a ii) 192 – 216 h, (a iii) 216 – 240 h, (a iv) 240 – 264 h; with wild-type like oocytes that are oval shaped and yellow in color. (b)Adults injected with *Of-ftz- f* dsRNA 0-24 h after eclosion and dissected at (b i) 168 – 192 h, (b ii) 192 – 216 h, (b iii) 216 – 240 h, (b iv) 240 – 264 h; and showing severe ovarian defects with non-mature oocytes that are round shaped and translucent. (C)Adults injected with *Of-ftz- f* dsRNA 0-24 h after eclosion and dissected at (c i) 168 – 192 h, defects include non-mature oocytes that are round shaped and translucent; (c ii) 192 – 216 h, (c iii) 216 – 240 h, (c iv) 240 – 264 h defects include square shaped and light yellow oocytes. Figure simplified from Appendix V.

Although we had expected to find a discrete time point(s) when *Of-ftz-f1* was required for oogenesis, we found that knockdown of *Of-ftz-f1* in adult females at any day from eclosion to when eggs are laid was effective in blocking oocyte maturation. This suggests that *Of-ftz-f1* plays a role throughout the entire time course of oogenesis. Thus, introduction of *Of-ftz-f1* dsRNA on a specific day is not required because it will eventually affect oogenesis, no matter when it is delivered. Based on this, if 2°RNAs are generated in response to dsRNA injections, we would expect to detect 2°RNAs within 6 days of dsRNA injection (the time at which we detect defects), irrespective of when the dsRNA is injected into adult females.

### 3.3.2 Isolation of RNA after injection of dsRNA

Based on the RNAi time course described above, I injected adult females with *Of-ftz-f1* dsRNA 24-48 h after eclosion and isolated ovaries at 192 – 240 h (Fig. 3-2). This time point was designed to fall after the time when phenotypic defects were

observed, ensuring that the RNAi pathway is active, but before tissue degradation occurs, which could result in degradation of small RNAs. RNA was prepared to separate small RNAs from total RNA (Methods).

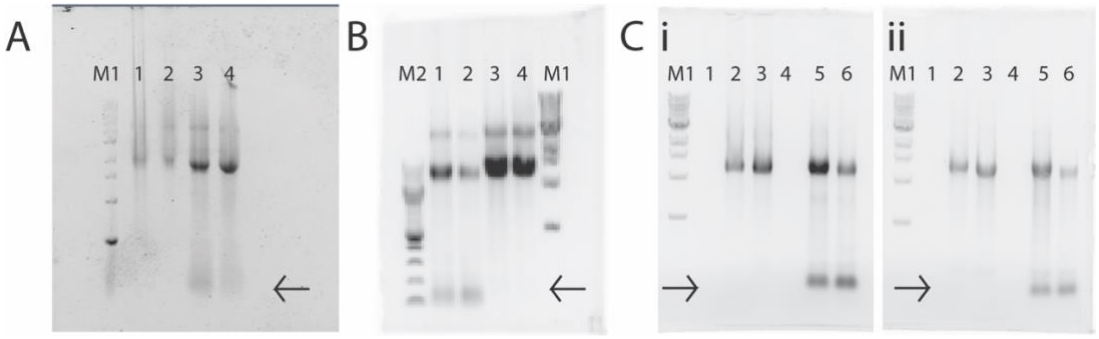



**Figure 3-2. Gel electrophoresis of RNA extraction of *Oncopeltus* ovaries.** RNA was separated on 1% agarose gels; photographs of gels shown. (A-B) Quality of RNA from wild type *Oncopeltus* ovaries. Preparations of total RNA (lanes 1,2) or small RNAs (lanes 3,4) from 8 females (lanes 1,3) or 4 females (lanes 2,4). RNA extraction (A) before and (B) after phenol-chloroform cleanup. (C) Quality of RNA from *Of-ftz-f1* knockdown ovaries. Preparations of total RNA (lanes 2,3) or small RNAs (lanes 5,6) from *gfp* dsRNA treatment (lanes 2,5) or *Of-ftz-f1* dsRNA treatment (lanes 3,6). (Ci) RNA before DNase treatment; (Cii) RNA after DNase treatment. (C) Lane 1 and 4, negative control, with no RNA. M1 is the 1kb DNA ladder marker and M2 is the 100 bp ladder marker. Arrows indicate location of small RNAs.

As there had not been any report, in the literature, of isolation of small RNAs from *Oncopeltus* ovaries, we needed to determine how to prepare the ovaries for RNA isolation. Parameters to be worked out included the amount of ovarian tissue needed for RNA extraction, how to thaw ovary samples from storage at – 80 °C, the final amount of RNA needed, how to optimize the quality of isolated RNA, and more. The RNAzol® RT protocol recommends use of 100 mg tissue per 1 ml of RNAzol. Before injection of *Of-ftz-f1* dsRNA, I dissected up to ~50 mg and ~100 mg of wild type *Oncopeltus* ovaries to determine how many female adults are needed and determine how much RNA was extracted. I found that dissection of 8 adults yielded

~99 mg of ovary tissue and 4 adults yielded ~58 mg of ovary tissue. RNA extraction for *Oncopeltus* ovaries was performed as described in Methods. RNA extraction of 4 adults yielded ~3 ug and 8 adults yielded ~4 ug of RNA with ~1.2 – 1.9 A260/A280 ratios for the different preps, suggesting some impurities in the RNA (Appendix II, Table A). I next performed gel electrophoresis to visualize the RNA, assess the quality of the RNA and determine whether isolation of small RNAs from total RNA was successful. The ribosomal RNA bands were present in some samples (Fig. 3-2A,B) but degraded RNA, indicated by the smears on the gel, was present in all samples, including a smear in the location where small RNAs would be present (Fig. 3-2A, arrow).

We suspected that the fatty tissue surrounding the ovaries was a cause of poor RNA quality. I next performed a phenol-chloroform cleanup during RNA extraction to improve quality of RNA (see Methods). The same conditions for obtaining ~50 mg and ~100 mg of wild type *Oncopeltus* ovarian tissue as above were used with the addition of the phenol-chloroform cleanup. Ovaries were dissected from 7 and 9 day old adults. This time point was chosen because the *Of-ftz-f1* knockdown ovaries were similar to the size of the controls at 7 days (Fig. 3-2B). Absorbance measurements suggested improvements in RNA quality, with readings of ~1.9 – 2 for the A260/A280 ratio (Appendix II, Table B). Gel electrophoresis revealed a band corresponding to small RNAs as well as the ribosomal RNA bands in the samples. There was also a decrease in the smeared appearance of the RNA demonstrating that I isolated intact RNA, including small RNA and total RNA (Fig. 3-2B).

Having established a method to isolate clean small RNA, an experiment was performed to determine if 2°RNAs are produced by using RNA from *Of-ftz-f1* knockdown ovaries and performing a sensitive northern blot technique. *Of-ftz-f1* was knocked down in 45 adult *Oncopeltus* and as a negative control, *gfp* dsRNA was injected into 35 females. The number difference was based on the expected size of the ovaries with those injected with *Of-ftz-f1* being smaller than the wild-type-like ovaries expected for the negative control, *gfp* injections. For each dsRNA treatment, a total of 180 - 210 mg of ovaries were collected from these females. RNA extraction was performed as described above and samples were combined after the phenol-chloroform extraction. These RNA preparations were of good quality, as demonstrated by absorbance measurements made of ~1.9 – 2 for the A260/A280 ratio (Appendix II, Table C). A DNase treatment was performed for each treatment, resulting in cleaner products and RNA concentration decreased to ~ 700 - 1000 ng/µl for the total RNA prep and ~ 2 ug/µl for the small RNA prep. Gel electrophoresis revealed small RNAs separated from the total RNA and the expected bands for the small RNAs indicating successful isolation of intact RNA from ovaries treated with dsRNA (Fig. 3-2 C).

### 3.3.3 Design of RNA probes

RNA probes were designed to match three regions of the *Of-ftz-f1* gene: a region upstream of the dsRNA sequence, the region matching the dsRNA sequence, and a region downstream of the dsRNA sequence (Fig. 3-3A). Three sense probes were synthesized in order to identify newly synthesized, 2$^{o}$ RNAs. In addition, an upstream antisense probe was synthesized to determine if the dsRNA was effective in

knocking down the endogenous *Of-ftz-f1* transcript. An actin antisense probe was used as a control. To assess quality, all five probes were visualized on an agarose gel. As shown in Figure 3-3Bi, sharp bands corresponding to RNA probes suggest that full length, intact probes were synthesized effectively. Note that the bottom represents the DNA template; when run on a MOPS (3-(N-morpholino) propanesulfonic acid) gel, only the RNA band was observed (Fig. 3-3Bii).

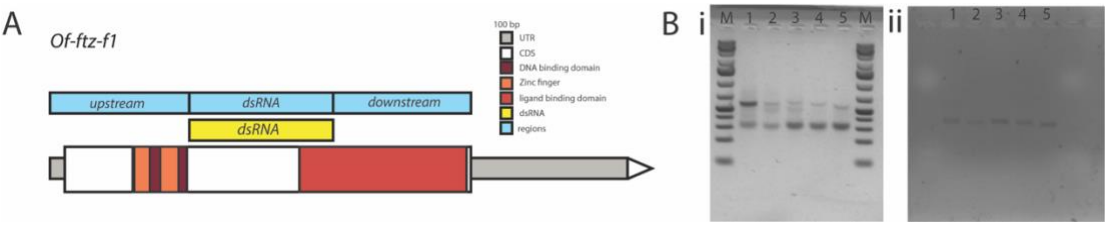



**Figure 3-3. RNA probes used to determine production of 2°RNAs.** (A) Schematic of *Of-ftz-f1* gene with location of dsRNA (yellow) and probe regions (blue) used for Northern Blotting. (B) Gel electrophoresis of RNA probes used for Northern blotting. Probes include the antisense *Of-ftz-f1* "upstream" (lane 1), the sense *Of-ftz-f1* "upstream" (lane 2), the sense *Of-ftz-f1* "dsRNA" (lane 3), the sense *Of-ftz-f1* "downstream" (lane 4), and the control, antisense *Of-actin* "upstream" (lane 5). M, 1kb ladder marker. (B i) 1.5 % agarose gel of the RNA probes. Note that the bottom represents the DNA template. (B ii) 1.5 % MOPS gel showing just the RNA.

### 3.3.4 Using Northern Blots to determine if *Oncopeltus* generates 2° RNAs

Choi et al. (2017) optimized northern blotting techniques to detect sense, anti-sense, and miRNAs from *C. elegans.* They determined that northern blotting has a strong bias at detecting miRNAs and the use of short probes can improve this bias and distinguish miRNAs by a single nucleotide (Choi et al. 2017). Thus, I replicated this technique in *Oncopeltus* to determine whether small RNAs are produced after injection of dsRNA.

The small and total RNA blots from 7 and 9 day old wild type *Oncopeltus* ovaries were probed first with the antisense actin probe (Fig. 3-4Ai), hybridized at 42 °C, stripped, probed with the sense *Of-ftz-f1* "dsRNA" region probe (Fig. 3-4Aii), hybridized at 42 °C, stripped and then probed a third time with the antisense actin probe, hybridized at 37 °C (Fig. 3-4Aiii). There was high background at the top of the blot and two bands at ~1.5 kb and ~1.2 kb were observed for the antisense actin probe. With the *Of-ftz-f1* sense probe "dsRNA" region, one band at ~1.5 kb was observed.

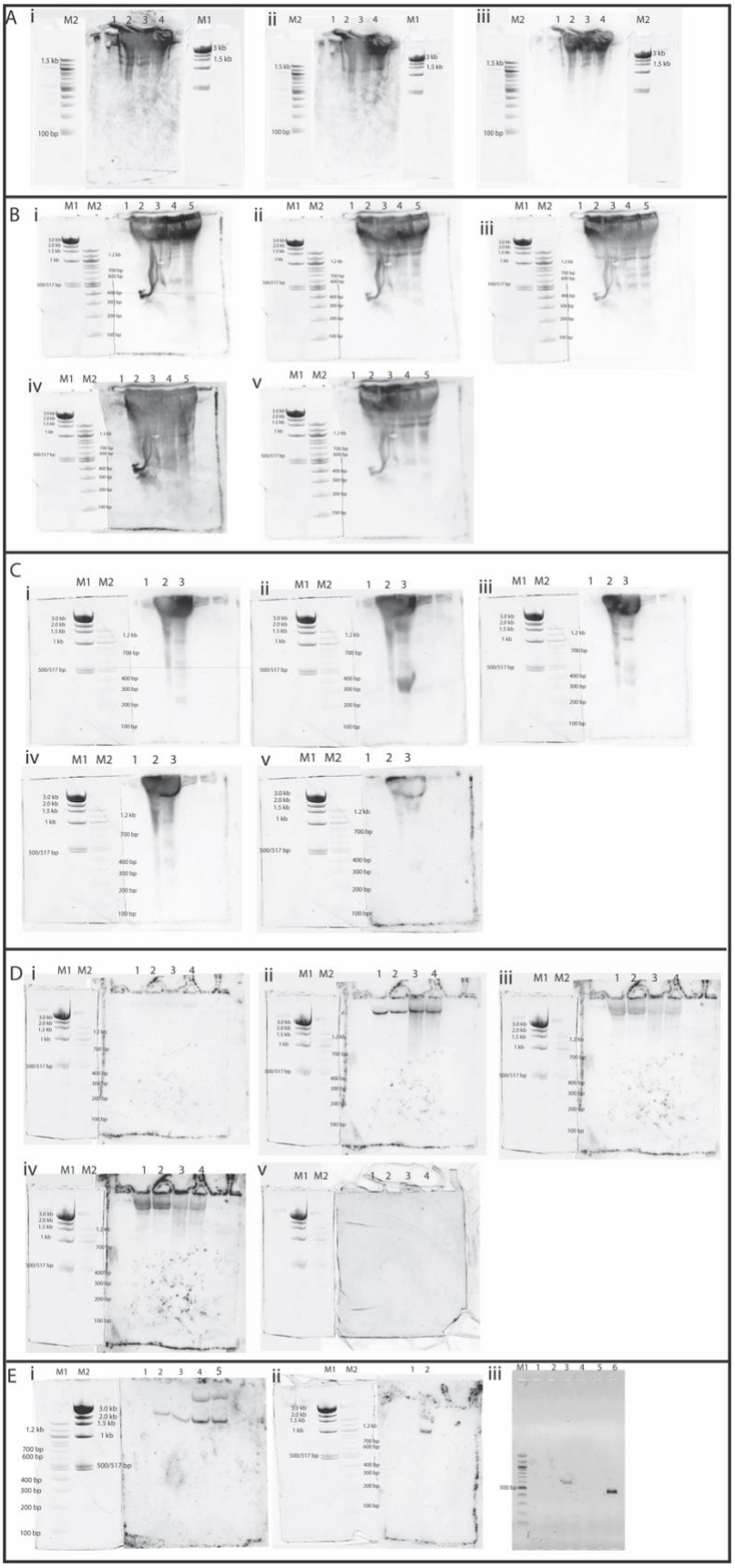

A i
M2 1 2 3 4 M1
1.5 kb
100 bp
3 kb
1.5 kb
ii
M2 1 2 3 4 M1
iii
M2 1 2 3 4 M2
B i
M1 M2 1 2 3 4 5
ii
iii
iv
v
C
i
M1 M2 1 2 3
ii
iii
iv
v
D i
M1 M2 1 2 3 4
ii
iii
iv
v
E i
M1 M2 1 2 3 4 5
3.0 kb
2.0 kb
1.5 kb
1 kb
500/517 bp
ii
M1 M2 1 2
iii
M1 1 2 3 4 5 6
500 bp

**Figure 3-4. Short RNAs were not effectively detected by Northern Blotting.** Photos of northern blots are shown. **(A)** *Oncopeltus* small and total RNA from 7 and 9 day old wild type *Oncopeltus* ovaries. No RNA, negative control (lane 1). Small (lane 2) and total RNA (lane 3, 4) were prepared from wild-type 7 (lane 2,3) and 9 day old (lane 4) ovaries and probed using (i, iii) antisense actin probe at (i) 42 °C and (iii) 37 °C, and (ii) sense *Of-ftz-f1* "dsRNA" region probe at 42 °C. Blotting was performed in the order shown. **(B)** RNA from *gfp* dsRNA and *Of-ftz-f1* dsRNA ovaries probed. No RNA, negative control (lane 1). Total RNA (lane 2-3) and small RNA (lane 4-5) prepared from *gfp* dsRNA (lane 2, 4) or *Of-ftz-f1* dsRNA (lane 3, 5) treated *Of* ovaries were probed using (i) sense *Of-ftz-f1* "dsRNA" region; (ii) sense *Of-ftz-f1* "upstream" region; (iii) sense *Of-ftz-f1* "downstream" region; (iv) antisense *Of-ftz-f1* "upstream" region; and (v) antisense *Of-actin.* **(C)** A *C. elegans* probe was prepared to troubleshoot background issues. *Oncopeltus* (lane 2) or *C. elegans* (lane 3) total RNA prepared and probed in the following order: (i) antisense *Of-actin;* (ii) antisense *C. elegans-actin;* (iii) sense *Of-ftz-f1* "dsRNA" region; (iv) antisense *Of-actin* and (v) no probe used to assess washing. **(D)** Changes to RNA amount and hybridization temperature improved northern blot technique. *C. elegans* (lane 1, 2) or *Oncopeltus* (lane 3,4) total RNA at 0.5 ug and 1 ug, hybridized at 68 °C, and probed in the following order: (i) sense *Of-ftz-f1* "dsRNA" region, (ii) antisense *Of-actin;* and (iii-iv) antisense *C. elegans-actin*. (v) An image of the PAGE gel after the RNA was transferred to the positively charged nylon membrane, demonstrating that all RNA was transferred to the blot. **(E)** An mCherry probe to determine RNA probe specificity. (i) *C. elegans* containing mCherry (lane 2-3) and *C. elegans* wild type (lane 4-5) total RNA hybridized with an mCherry probe. (ii) An mCherry transgenic *C. elegans* RNA probed with mCherry probe. (iii) RT-PCR demonstrating that there are two different types of *C. elegans* RNA, wild type (lane 2, 5) and RNA containing mCherry (lane 3, 6). M1,1kb ladder marker; M2, 100 bp ladder marker.

Next, RNA from the *gfp* dsRNA and *Of-ftz-f1* dsRNA treated ovaries were analyzed (Fig. 3-4B). For these blots, the RNA probes were used in the following order and hybridized at 42 °C: sense *Of-ftz-f1* "dsRNA" region (Fig. 3-4Bi), sense *Of-ftz-f1* "upstream" region (Fig. 3-4Bii), sense *Of-ftz-f1* "downstream" region (Fig. 3-4Biii), antisense *Of-ftz-f1* "upstream" region (Fig. 3-4Biv), and antisense *Of-actin* (Fig. 3-4Biv). This blot resulted in high background at the top of the blot and extending throughout. Different sized bands were observed for the different probes, suggesting some specificity for the different probes. However, for the blots probed with sense *Of-ftz-f1* "upstream" region (Fig. 3-4Bii) and the antisense *Of-actin (*Fig. 3-4 Biv), two bands of the same sizes (~1.5 and 2 kb) were observed for the *gfp* dsRNA and *Of-ftz-f1* dsRNA treatments. In sum, the same sized bands were observed

regardless of dsRNA treatment and the high level of background precluded analysis of specific products for the different probes. Therefore, the blots were inconclusive and impossible to interpret without a reduction in the background.

To determine whether the background issues were caused by the *Oncopeltus* tissue preparations or the blots themselves, *C. elegans* RNA was prepared. This RNA was run side-by-side on a gel with the *Oncopeltus* ovary RNA (Fig. 3-4C). Blots were probed with antisense *Of-actin* (Fig. 3-4Ci), a new *C. elegans-actin* antisense probe (Fig. 3-4Cii), sense *Of-ftz-f1* "dsRNA" region (Fig. 3-4Ciii), antisense *Of-actin* (Fig. 3-4Civ) and, as a negative control, no probe (Fig. 3-4Cv). This blot still showed the high background at the top of the blot as well as where the RNA was transferred to. The same bands in both the *Oncopeltus* and *C. elegans* RNA were also observed, possibly due to the similarity of sequence found between actin homologs. The negative control also demonstrated that some bands were still seen after stripping was performed. Because the *C. elegans actin* probe was different from the previously designed probes, the RNA probe design appears to have been successful, assuming cross-reactivity between the actin probes from the two species.

All previous blots were hybridized at 42 °C. Given the high background in all of these northern blotting experiments, I decided to test whether increasing the hybridization temperature would reduce the background (Fig. 3-4D). *Oncopeltus* and *C. elegans* total RNA was probed with antisense *Of-actin* probe previously used (Fig. 3-4Dii) and the *C. elegans* antisense actin probe (Fig. 3-4Diii-iv) and the hybridization temperature was increased to 68 °C. In addition, two amounts of RNA were tested, 0.5 ug and 1 ug of RNA (Fig. 3-4D). This treatment drastically reduced

the background seen in previous experiments. The same band was observed for both species for both the *Oncopeltus* and *C. elegans actin* probes, but the strength of the band differed between species. For the *Oncopeltus*-specific probe, the band was stronger for the *Oncopeltus* RNA (Fig. 3-4Dii) and for the *C. elegans*-specific probe, the band was stronger for the *C. elegans* RNA (Fig. 3-4Diii,iv). This suggested that the probes are, at least to some extent, species-specific and demonstrated that I can successfully perform a northern blot without background.

To further troubleshoot the northern blotting protocol, RNA was prepared from wild type *C. elegans* and *C. elegans* containing an mCherry transgene. RNA was extracted from wild type *C. elegans* RNA and *C. elegans* carrying an mCherry transgene. This RNA was probed with a newly synthesized mCherry probe (Fig. 3-4Ei). This blot showed differences between the wild type (Fig. 3-4Ei, lane 2-3) and the mCherry *C. elegans* transgene RNA (Fig. 3-4Ei, lane 4-5) with a band > 3kb in the wild type total RNA. However, there was also a band of > 1.5 kb seen for both the wild type RNA and for the mCherry transgene, although no bands were expected for the wild type RNA. This was repeated with just the mCherry transgene RNA probed with the mCherry probe (Fig. 3-4Eii). At least two major bands were detected on this blot. Therefore, the samples in the first blot (Fig. 3-4Ei) may have been mixed up but this would not explain the band at the 2kb size seen in both the wild type and the mCherry transgene RNA. To confirm if there was contamination of RNA samples, an RT-PCR was performed, demonstrating that the samples were not mixed up - the DNA from the wild type *C. elegans* did not contain the mCherry transgene (Fig. 3-4Eiii). In conclusion, the quality of the northern blotting was improved but not to the

extent that allowed us to determine whether 2º RNAs were synthesized in *Oncopeltus*.

### 3.4 Discussion

It is not known whether 2º small RNAs are biosynthesized in insects. If such RNAs are synthesized in response to dsRNA treatment, this would increase the risk associated with exposure to dsRNA via the environment. We therefore wanted to use *Oncopeltus* to test whether this species generates 2º small RNAs, especially since it is a species that is highly sensitive to small amounts of dsRNA.

We determined that *Of-ftz-f1* is expressed throughout oogenesis and knockdown resulted in blocks in oogenesis, demonstrating that it plays a role in ovary development. We used this to test whether 2º small RNAs are produced as part of the RNAi response in *Oncopeltus*. I developed a procedure for RNA extraction from *Oncopeltus* ovaries and I was successful at separating small RNA from large RNAs (Fig. 3-2). I next attempted to adapt a sensitive northern blotting technique developed for *C. elegans* (Choi et al. 2017) to *Oncopeltus* ovaries. Although this northern blotting for detection of 2º small RNAs remains inconclusive, troubleshooting that I carried out improved this protocol. For example, it was previously thought that 10 ug of RNA was needed to see RNA on a northern blot but I determined that a minimum of 500 ng is sufficient to see a band for use with long RNA probes. Background was reduced and RNA probe specificity improved by raising the hybridization to temperature 68 °C. At this temperature, there were no small bands seen when *Oncopeltus* total RNA was probed with *Of-actin* compared to blots hybridized at lower temperatures (37 °C and 42°C). Further experiments could improve this

technique for use with *Oncopeltus* ovary RNA. Alternatively, the RNA isolation protocol I established could be used to identify new small RNAs with RNA sequencing.

# Chapter 4: Conclusions and future directions

Here I examined the effectiveness of RNAi in the milkweed bug, *Oncopeltus fasciatus*. I examined the susceptibility of *Oncopeltus* to pRNAi using developmental regulatory genes *eve*, *Scr*, *ftz-f1*. Knockdown of these genes resulted in specific morphological defects that ranged in severity allowing us to assess subtle dsRNA-induced effects. The duration of dsRNA response increased as dsRNA amounts increased, and the effectiveness of dsRNA-induced effects decreased when the length of the dsRNAs was shortened to a 150 bp and 75 bp range. As little as 1 ng of dsRNA injected into female milkweed bugs resulted in defects in offspring. Further, *Oncopeltus* is susceptible to dsRNA via feeding. Surprisingly, adult females fed eggs from mothers treated with dsRNA were able to transmit the RNAi effect to their offspring. The trophic experiment was performed a total of three times, with slight variations in the protocols, including a blinded experiment to rule out investigator bias or technical errors. Additional improvements such as feeding eggs from freshly injected females or even increasing the length of collection could result in a higher percentage. Because this experiment was done as a worst-case scenario that was within a species, based on cannibalization of *Oncopeltus*, in an agricultural setting this would not necessarily pose a risk because the RNAi effect is targeting the pest of interest. However, this experiment should be replicated in species that would mimic an agricultural setting, using two different species, such as a natural predator (e.g., lady beetle) and its prey (e.g., aphid). Use of developmental genes, suggested here, would allow for the detection of subtle dsRNA-induced effects.

I also determined that *Of-ftz-f1* is expressed throughout oogenesis. Knockdown resulted phenotypic defects demonstrating that it plays a role throughout ovary development. Our northern blotting technique to determine whether small RNAs are generated in *Oncopeltus* was inconclusive but I successfully prepared small RNAs and separated them from large RNAs. I was able to modify the RNA extraction protocol generated by the Jose lab to extract RNA from *Oncopeltus* ovaries. This RNA protocol can now be adjusted to extract RNA for RNA sequencing.

We compared the effectiveness of *V-ATPase* to developmental genes *eve* and Scr and found that developmental genes are better for testing RNAi effectiveness. Even though we saw that the housekeeping gene *V-ATPase* is not effective to test effectiveness of RNAi using death as an assay, we observed that knockdown of *Of-Vha68-1* also blocked egg laying. Therefore, besides sequencing RNA from *Of-ftz-f1* and *gfp* dsRNA treated females, *Of-Vha68-1* dsRNA treated females could also be used.

Investigating the potential for unexpected effects of RNAi on non-target organisms is important to avoid unintended consequences from use of RNAi insecticides. Our results show that low doses of dsRNA can induce effects in targeted species and it can be passed across generations based through ingestion of dsRNA. This suggests that risk to non-target insects in field settings may occur due to the environmental risk of ingesting low doses of dsRNA. Our observation of multigenerational dsRNA effects suggests that insects may also be capable of amplifying the effects of dsRNA. Lastly trophic exposure to dsRNA may be a threat caused by RNAi based insecticides.

# Appendix I. List of Primers

**Table A. Primers used for gene isolation.**

| Gene Name | Primer Sequence |
|---|---|
| *Of-eve* | Forward: 5'TCGTGTTTGGATCTGGCAGC<br>Reverse: 5'CTAGCGAGTTTTGAACATAAAACTCCTGG |
| *Of-Scr* | Forward: 5'GGACATAGTGATGTGTCCGC<br>Reverse:<br>5'AACACAAATGAGATAATTAATACACTTATAGTGTC |
| *Of-Vha68-1*<br>5'RACE | 5'AGCTCCCAGGTGGAGCAAGGTAGGTGAC |
| *Of-Vha68-1*<br>3'RACE | 5'TCCCGATGGGCAGAGGCCCTCAGAGAGA |
| *Of-Vha68-1*<br>3'RACE | 5'CGTTTCTGCCCATTCTACAAGACTGTCGGA |
| *Of-Vha68-1* | Forward: 5'CCCGTGGAGTAAACATAA<br>Reverse: 5' TATGAAATAAGCTTAAAAATTAAAAGATAT |
| *Of-ftz-f1* | Forward: 5'GGGCTTTATGGTGTTGAGAAGG<br>Reverse: 5' CAATCAAGTGTAAGACTGGAATGTAAACTA |

**Table B. Primers used for dsRNA templates.** ** Represent Ultramer DNA oligos (IDT)

| dsRNA Name | Primer Sequence |
|---|---|
| *Of-ds-eve* | Forward: 5'taatacgactcactatagggagaGGATGAAGGACAAACGGCAG Reverse: 5'taatacgactcactatagggagaCGAGTGACAAATCCTCCATGTAGG |
| *Of-ds-Scr* 600 bp | Forward: 5'taatacgactcactatagggagaAGTTCGTCAATTCGCTGGC Reverse: 5'taatacgactcactatagggagaGTCCGAGGTGAACTCTCTTCAT |
| *Of-ds-Scr* 300 bp A | Forward: 5'taatacgactcactatagggagaAGTTCGTCAATTCGCTGGC Reverse: 5'taatacgactcactatagggagaCGCCGCTCAGGTTTGTC |
| *Of-ds-Scr* 300 bp B | Forward: 5'taatacgactcactatagggagaGGTCCTCGTGCAAGTTCG Reverse: 5'taatacgactcactatagggagaGTCCGAGGTGAACTCTCTTCAT |
| *Of-ds-Scr* 150 bp A | Forward: 5'taatacgactcactatagggagaGGTCCTCGTGCAAGTTCG Reverse: 5'taatacgactcactatagggagaCTGGAGCCGACGGAGCAC |
| *Of-ds-Scr* 150 bp B | Forward: 5'taatacgactcactatagggagaCTCCCGCTTCCCAGACAT Reverse: 5'taatacgactcactatagggagaGTCCGAGGTGAACTCTCTTCAT |
| *Of-ds-Scr* 75 bp A | Forward: 5’taatacgactcactatagggagaCTCCCGCTTCCCAGACAT Reverse: 5'taatacgactcactatagggagaCCGCTGCACCTTGGGTGG |
| *Of-ds-Scr* 75 bp B | Forward: 5'taatacgactcactatagggagaCCAAGAGCCCCGGACAGC Reverse: 5'taatacgactcactatagggagaGTCCGAGGTGAACTCTCTTCAT |
| *Of-ds-Scr* 38 bp A** | Forward: 5'taatacgactcactatagggagaCCAAGAGCCCCGGACAGCAGGCTTCCAGTAATCCTCCTtctccctatagtgagtcgtatta Reverse: 5'taatacgactcactatagggagaAGGAGGATTACTGGAAGCCTGCTGTCCGGGGCTCTTGGtctccctatagtgagtcgtatta |

| | |
|---|---|
| *Of-ds-Scr*<br>38 bp B** | Forward:<br>5'taatacgactcactatagggagaTCAGATATACCCCTGGATGAAGA<br>GAGTTCACCTCGGACtctccctatagtgagtcgtatta<br>Reverse:<br>5'taatacgactcactatagggagaGTCCGAGGTGAACTCTCTTCATCC<br>AGGGGTATATCTGAtctccctatagtgagtcgtatta |
| *Of-ds-Vha68-1* | Forward: 5'<br>taatacgactcactatagggagaGCACAAGATGATCCTTCCTCCT<br>Reverse: 5'<br>taatacgactcactatagggagaCCTGAGATCTCTCTGAGGGC |
| *Of-ds-ftz-f1* | Forward:<br>5' taatacgactcactatagggagaGACAGGATGCGAGGCGG<br>Reverse:<br>5'<br>taatacgactcactatagggagaTTTACACATAAGTTCAAATAAGTC |

**Table C. Primers used for RT-PCR.**

| **dsRNA Name** | **Primer Sequence** |
|---|---|
| *Of-ftz-f1* | Forward: 5'CTCCAGATCAAGCAGGAGATC<br>Reverse: 5'CAGAACCAGCATATCCGACC |
| *Of-actin* | Forward: 5'GCACCAAGGGCTGTCTTC<br>Reverse: 5'GCAGGGCATAACCTTCGTAG |

**Table D. Primers used for RNA probe synthesis template.**

| RNA probe Name | Primer Sequence |
|---|---|
| antisense *Of-ftz-f1, "upstream"* | Forward:<br>5'GGGCTTTATGGTGTTGAGAAGG<br>Reverse:<br>5' taatacgactcactatagggagaCGCTCGAACAGCTTCGAG |
| sense *Of-ftz-f1, "upstream"* | Forward:<br>5'taatacgactcactatagggagaGGGCTTTATGGTGTTGAGAAGG<br>Reverse:<br>5' CGCTCGAACAGCTTCGAG |
| sense *Of-ftz-f1, "dsRNA"* | Forward:<br>5' taatacgactcactatagggagaGACAGGATGCGAGGCGG<br>Reverse:<br>5' TTTACACATAAGTTCAAATAAGTC |
| sense *Of-ftz-f1, "downstream"* | Forward:<br>5'taatacgactcactatagggagaGTGTTGGACCAAAATCTTTTCT<br>Reverse:<br>5' CTACTTTCTTTTTGCATGGAGC |
| antisense *Of-actin* | Forward:<br>5'CAAGGCATCAGGGAGTGATG<br>Reverse:<br>5' taatacgactcactatagggagaCAAAGTCAAGGGCAACATAGCA |
| antisense *C. elegans actin* probe | Forward:<br>5'CCGCTCTTGTGGTGGACA<br>Reverse:<br>5' taatacgactcactatagggagaGTGGTGAAGGAGTAACCGC |
| antisense mCherry probe | Forward:<br>5' CATACGAGGGAACCCAGACC<br>Reverse:<br>5' taatacgactcactatagggagaTTGTAGGTGGTCTTGACCTCAG |

# Appendix II. Quality of RNA from *Oncopeltus* Ovaries

**Table A: RNA quality of Wild Type *Oncopeltus* ovaries.**

| **sample** | **Name** | **Concentration [ng/ul]** | | **A260/A280** | |
|---|---|---|---|---|---|
| | | Pre-DNase Treatment | Post-DNase Treatment | Pre-DNase Treatment | Post-DNase Treatment |
| 1 | total RNA #1 | 3249 | 4224 | 1.204 | 1.201 |
| 2 | total RNA #2 | 4670 | 4930 | 1.876 | 1.941 |
| 3 | miRNA #1 | 3936 | 3513 | 1.946 | 1.987 |
| 4 | miRNA #2 | 4265 | 3554 | 1.934 | 1.996 |

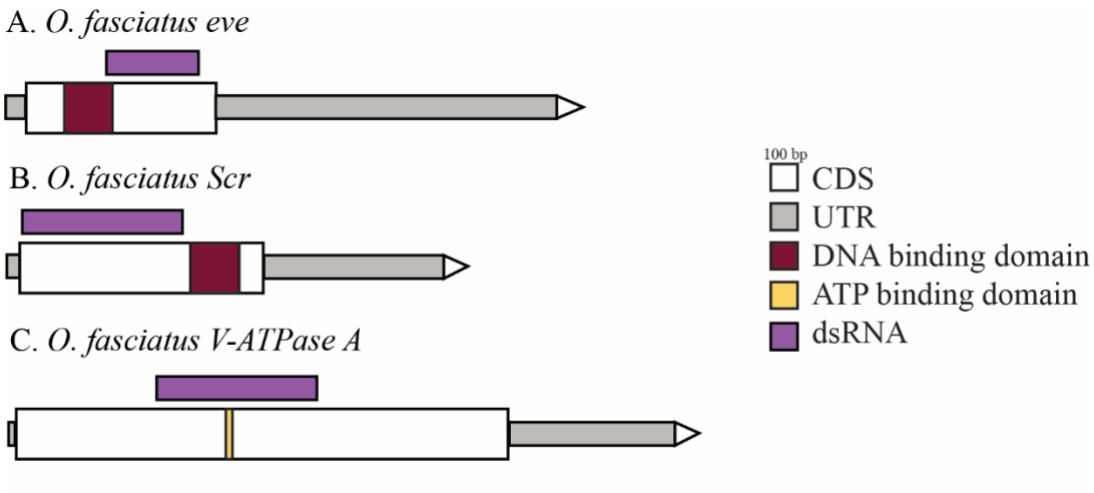
**Table B: Quality of RNA from wild type *Oncopeltus* ovaries after phenol chloroform cleanup.**

| **sample** | **Name** | **Concentration (ng/ul)** | | **A260/A280** | |
|---|---|---|---|---|---|
| | | Pre-DNase Treatment | Post-DNase Treatment | Pre-DNase Treatment | Post-DNase Treatment |
| 1 | 7 day old, miRNA | 8108 | 7088 | 2.021 | 2.022 |
| 2 | 9 day old, miRNA | 3452 | 2800 | 1.993 | 1.978 |
| 3 | 7 day old, total | 12420 | 14430 | 2.014 | 2.02 |
| 4 | 9 day old, total | 8728 | 7328 | 2.017 | 2.02 |

**Table C: *Of* RNA ovary quality from *gfp* dsRNA and *Of-ftz-f1* injected adults.**

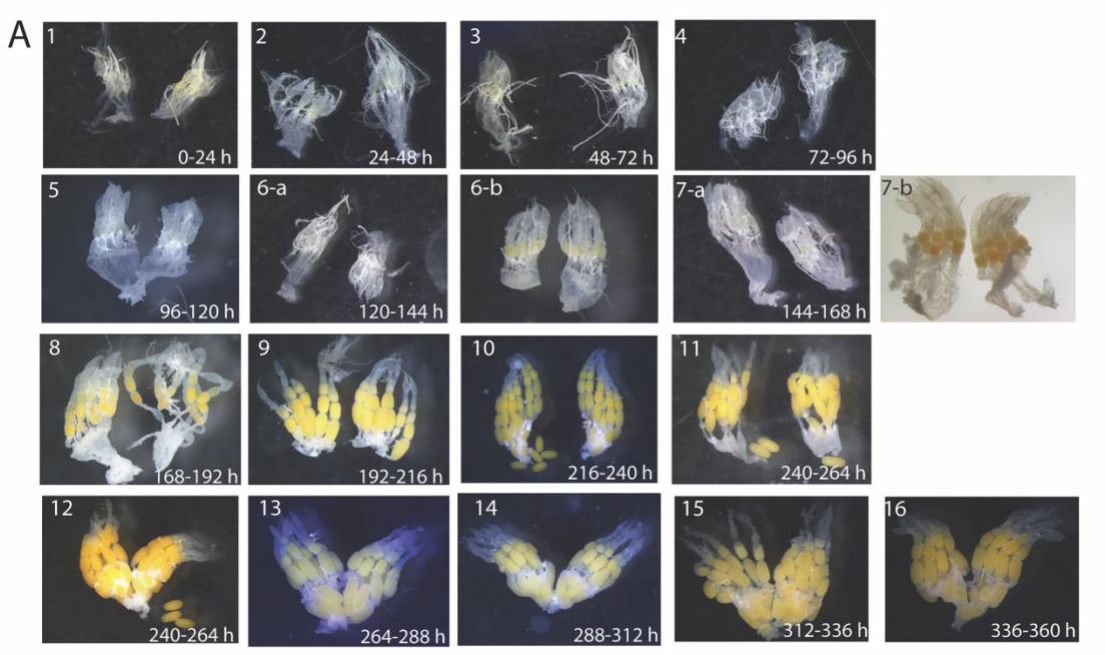
| sample | Name | 1:10 ng/ul | Final [ng/ul] | A260/A280 | A260/A230 |
|---|---|---|---|---|---|
| Pre DNase treatment | | | | | |
| 1 | neg. control total | 3.7 | 37 | 1.661 | 0.344 |
| 2 | gfp total | 270.4 | 2704 | 1.899 | 2.246 |
| 3 | ff1 total | 376.4 | 3764 | 1.882 | 2.438 |
| 4 | neg. control miRNA | 1.4 | 14 | 2.615 | 0.159 |
| 5 | gfp miRNA | 636.4 | 6364 | 1.959 | 2.035 |
| 6 | ff1 miRNA | 453.6 | 4536 | 1.925 | 1.868 |
| Post DNase treatment | | | | | |
| 1 | neg. control total | 0.2 | 2 | 1 | 0.074 |
| 2 | gfp total | 209.2 | 2092 | 1.989 | 2.117 |
| 3 | ff1 total | 270.8 | 2708 | 1.997 | 2.242 |
| 4 | neg. control miRNA | 1.9 | 19 | 1.88 | 0.251 |
| 5 | gfp miRNA | 460.4 | 4604 | 2.002 | 1.944 |
| 6 | ff1 miRNA | 290 | 2900 | 1.965 | 1.773 |

# Appendix III. Maps of genes



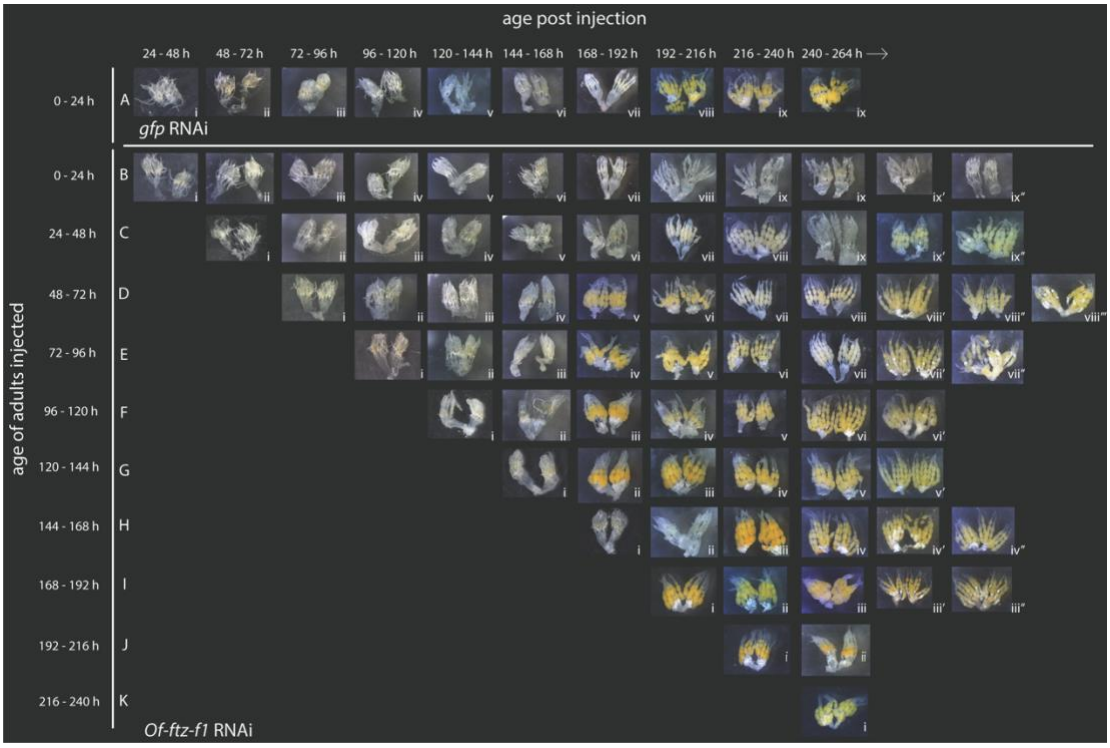

Developmental regulatory genes (A) *even-skipped (eve), (B) Sex combs reduced (Scr)* and (C) *V-ATPase subunit A* (Vha68-1) (D) *fushi-tarazu factor 1 (ftz-f1)* were isolated from *Oncopeltus.* White rectangles indicate coding regions. Gray rectangles indicate UTRs. Colored bars on the transcript indicate the location of signature domains. Purple bar above the transcript indicates the location of the dsRNA.

# Appendix IV. Ovary development of wildtype *Oncopeltus*




(A) Time course of ovarian development. Ovaries were dissected from *Oncopeltus* female adults every 24 h for 16 days: 1) 0 – 24 h; 2) 24 – 48 h; 3) 48-72 h; 4) 72 – 96 h ; 5) 96 – 120 h; 6) 120 – 144 h (6-a, 6-b show variation of ovaries seen within this time point); 7) 144 – 168 h (7a, 7b show variation of ovaries within this time point); 8) 168 – 192 h; 9) 192 – 216 h; 10) 216 – 240 h; 11) 240 – 264 h; 12) 240 – 264 h; 13) 264 – 288 h; 14) 288 – 312 h; 15) 312 – 336 h; 16) 336 – 360 h. Photographs taken with Olympus SZX16 microscope are shown.

# Appendix V. Effects of *Of-ftz-f1* knockdown across ovary development




Injection of 10ug of (A) *gfp* dsRNA to 0-24h, 1 day old female *Oncopeltus* adults and of *(*B-K) *Of-ftz-f1* dsRNA to different aged female *Oncopeltus* adults ranging from (B) 0-24h, (C) 24-48h, (D) 48-72h, (E) 72-96h, (F) 96-120h, (G) 120-144h, (H) 144-168h, (I) 168-192h, (J) 192-216h, and (K) 216-240h. (i-ix) represents the age of the ovary post injection. (‘) represents extra 240-264 h ovaries. Photographs taken with Olympus SZX16 microscope are shown.

# Appendix VI. Gene Sequences

**>Of-eve**

5’

TCGTGTTTGGATCTGGCAGCAATGGACTTCGCCTATGCCACCATGGACTCG
AGGATGTCACAGCAAGCCTCGTCACTGCTCAAGTCTAATTCCGTGCCACC
TTTAGTTGTCGATATCATGCCGTCGGCCTATCAGAAGTCAATGGCGCAGG
ACAACTTGGATTCCCCTAAGAAGGACGAAATTAAACATGACGGCAACAAT
AGTGCTAACGAAGTGAATATAAGGCGATACAGGACGGCCTTCACGAGAG
AACAGCTCACTAGACTAGAAAAGGAGTTTTTCAAAGAAAACTACGTTTCA
CGACCAAGGCGTTGCGAGCTGGCAGCTCAACTGGGTCTTCCAGAATCAAC
TATAAAGGTGTGGTTCCAGAACCGAAGGATGAAGGACAAACGGCAGAGG
ATGGCCATGGCCTGGCCTTACGCGGTCTACACAGACCCGACGCTGGCCGC
CTCCCTCCTGAGCTCCCTGCACTACCCCTATCCTCCCTACTACCCTCGCTA
CTACCTCCCGCCGCCACCGGTGGTCCCTCCCGCCTGCGATGGCAGCACCT
CCTGCAGGTGCGGGATCGTCAACTGCGTGGCCGCCTTCCCTGGTCACCGC
ACCCCTCCGCTCCACCACCCTCCGACTCACCTCGGTCTGCAGATCCCCTCG
TCTCCCGAGTCGCCGGAGCCCGCTCAGAAGAAGCGCCTCTTTCAGCCCTA
CATGGAGGATTTGTCACTCGAGAAGGCATAGCCAGAGCTTATCGATCTAT
CTGTAAATAGTCCTCGTGGATCTCTCGTTACCCAGAGTCATTCTCCTGCTT
AGTGGGAATCTTCACTATTCTCTTTCAAGACGCAAGGAATTTTTCCGACCG
CCATATTGGAAACCGTTCCCCAATAAAAGTTGAATGGACTTAAGTAACTC
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CCATCCGAACTTGTCTTTAACAATAACATTGATATAACAAAAAATAAACA
TATCTTCAGTAAAGTTTAGAGAGCTCCAAATCCAATGCGGTAATAATCAC
TGTATTATGTTTATGTAATTTTATTATCACATAAAATATGTTTTTTTTTGTG
AACCAGTTGTTGAATAAAAAAAATCATTTTTAGACGGGGCTTTTTAATAA
ATAAGAGCAGCTGTGCTGAAAAGAGAGAATGAAGAAGAGTTTAAGAAGT
ATAAATAAATATTGTTTTGTTAACTTTTCATTGTATGTTACAATTTTAAGA
GTTTAAAATATTGATTATTTTTTGCTCGGGAAGGAGGGGATAACAAGTAA
AATAAGTATTAAACTTACTAAGGTTTTTCAAACCGTAAGACAGAATCTTT
ATTTATTTATTGTTCATAACCTCTTCTTCTTCTTTTTCTTAACCCGTTTATAT
AAATCTTAAGGCAATTTCATTTTTGTAGCATTTTAATTTTATATTTTCATAT
CATTGTTGTATTTAATTAAATACTTAAAGCAATTGCCTATGTAAATGATTT
TTACCAGGAGTTTTATGTTCAAAACTCGCTAG

**>Of-Scr**

5’

GGACATAGTGATGTGTCCGCTCTGATTACTCCAAGTCCAGTTCGCGTCATG
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**>Of-actin**

5’

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**>C. elegans-actin**
5’
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**>mCherry**
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