

Evaluation of RNAi and larvicide enhancements for the biting midge, *Culicoides sonorensis* (Diptera: Ceratopogonidae)

by

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B.S., University of California, Davis, 2013
M.S., California State University, Fresno, 2018

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Abstract

Culicoides biting midges are important livestock pests that transmit viruses that are detrimental to animal welfare and economically impact producers' livelihoods. Current measures to protect livestock are often expensive or ineffective, and traditional chemical insecticides are often harmful to non-target organisms, underlining a need to develop new bio-rational control methods. RNA interference (RNAi) is a promising technique for insect control that uses double-stranded RNA (dsRNA) to suppress physiologically important genes in target insects. This mechanism is not equally effective in all insects, and this research examined RNAi in *Culicoides sonorensis*, a model species for this genus. Cultured insect cells had a robust RNAi response, whereas larval stages only weakly responded to the treatment dsRNA. Several mechanisms contribute to RNAi inefficiency in other insects, including the instability of dsRNA. Accordingly, dsRNA-degrading enzymes (dsRNases) were characterized as potential barriers to RNAi efficiency. Seven putative enzymes were found in *C. sonorensis*, and the transcript expression profile of six of these enzymes shows they are present in many developmental stages, which was confirmed by *ex vivo* dsRNA digest in larval midge gut fluid. Three RNAi enhancers (secondary RNA structures, chitosan nanoparticles, and *E. coli* expressing dsRNA) were evaluated in larval midges, and only chitosan nanoparticles increased RNAi efficacy above baseline treatments. In addition to RNAi, we tested the effectiveness of *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) for *C. sonorensis* larval control. Larvae were tolerant to *Bti* treatments compared to *Aedes aegypti* but increasing the alkalinity of the rearing environment significantly enhanced *Bti* insecticidal activity. These studies identify factors limiting the efficacy of RNAi and *Bti*-based insecticides and provide foundational knowledge for pursuing the development of new larval control methodologies in *C. sonorensis*.

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Dedication

This dissertation is dedicated to my family and friends who kept me going throughout my graduate career. Thank you!

Chapter 1 - Introduction

In preparation as: Osborne, C. J., Cohnstaedt, L. W., and K. S. Silver. Outlook on new strategies for controlling *Culicoides* biting midges. *Pathogens*.

Abstract

Culicoides are small biting midges with the capacity to transmit important livestock pathogens around much of the world, and their impacts on animal welfare are likely to expand. Hemorrhagic diseases resulting from *Culicoides*-vectored viruses, for example, can lead to millions of dollars in economic damages for producers. Chemical insecticides can reduce *Culicoides* abundance but may not suppress population numbers enough to prevent pathogen transmission. These insecticides can also cause negative effects on non-target organisms and ecosystems. RNA interference (RNAi) is a cellular regulatory mechanism which degrades mRNA and suppresses gene expression. Studies have examined the utility of this mechanism for insect pest control, and with it, have described the hurdles towards producing, optimizing, and applying these RNAi-based products. These methods hold promise for being highly specific and environmentally benign when compared to chemical insecticides and are more transient than engineering transgenic insects. Given the lack of available control options for *Culicoides*, RNAi-based products could be an option to treat large areas with minimal environmental impact. Here, we describe the state of current *Culicoides* control methods, successes and hurdles towards using RNAi for pest control, and the necessary research required to bring an RNAi-based control method to fruition for *Culicoides* midges.

Introduction

Livestock animals are susceptible to numerous viral, protozoan, and nematode pathogens that are transmitted by *Culicoides* biting midges (Diptera: Ceratopogonidae) (Mullen and Murphree 2019). Disease in herds such as cattle can impact livestock producers by lowering animal weight gain, decreasing milk production, and increasing animal mortality (Kedmi et al. 2010). These insects occur throughout much of the world and the effects of climate change are likely to exacerbate the risks posed by *Culicoides* (Carpenter et al. 2013, Maclachlan and Mayo 2013, Mayo et al. 2020, Burruss et al. 2021, Jimenez-Cabello et al. 2023). Warmer temperatures are expected to increase seasonal midge activity, shorten overwintering periods, and even enhance viral replication in these insects (Niedbalski and Fitzner 2018, Rozo-Lopez et al. 2022). Effective control strategies are needed to protect susceptible herds from *Culicoides*-transmitted diseases.

There are few products which are labeled to target *Culicoides*. Common chemical insecticides against adult populations include pyrethroids and organophosphates (Venail et al. 2015). These non-specific products are a concern for large-scale application, and even insect growth regulators (IGRs) which are more specific and safer for the environment can affect non-target insect species. Microbially-derived products such as those containing *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) toxins are touted as safer alternatives given their biological nature and specificity against dipterans (Ben-Dov 2014). However, these products are ineffective against midges despite being highly effective in mosquitos (Foxi et al. 2019). Using RNA interference (RNAi) to suppress gene expression in *Culicoides* has only briefly been examined, and its utility as a larval control strategy is worth exploring. In this review, we summarize the growing impacts of *Culicoides* and recent advances in efforts to manage their populations. We

also highlight how RNAi could be used to control *Culicoides* by examining research on mosquitos as a proxy for midges. Finally, we discuss how RNAi-based insecticides in *Culicoides* could be developed and evaluated.

***Culicoides* biting midges**

Culicoides are small, hematophagous flies and the genus is comprised of over 1,300 species (Mullen and Murphree 2019, Borkent and Dominiak 2020). Females taking a blood meal to produce eggs can cause exceptionally irritating bites as they lacerate the skin and use a complex cocktail of salivary proteins to attenuate host responses (Lehiy and Drolet 2014). *Culicoides* feed on many types of hosts (mammals, birds, and reptiles) depending on midge species, locality, and host availability (Mullen and Murphree 2019). Only a few *Culicoides* species are competent to transmit important livestock pathogens, but their behavior and ecology can vary and confound control strategies (Purse et al. 2015).

Blood-fed *Culicoides* develop eggs within a few days at optimal temperatures and oviposit them into moist substrates. The eggs rapidly hatch, and immature midges develop through four larval instars before pupating and emerging as adults. This life cycle may take as little as a couple weeks but can extend many months or even years depending on species, temperature, and resource availability (Mullen and Murphree 2019). Substrate preference for oviposition depends on species. Common substrate types include manure-rich mud with a water-soil interface (e.g., *C. sonorensis*), composted material (e.g., *C. imicola*), and animal dung pats (e.g., *C. brevitarsis*) (Purse et al. 2015). Yet, many larval habitats appear to be undescribed or cryptic (Mullens et al. 2015, Braverman et al. 2018). Larvae are generalist feeders. Their food includes protozoans, nematodes, other insects, bacteria, fungi, algae, and more (Mullen and

Murphree 2019). The hardiness of eggs and immatures have been examined with respect to desiccation and cold tolerance, and these findings lend support to the expectation that these stages overwinter in temperate and subtropical climates (McDermott and Mullens 2014, McDermott et al. 2017).

The seasonality of adult midges is also dependent on species and geography. Common ecological drivers of adult *Culicoides* abundance include warm springs and autumns, higher precipitation, humidity, proximity to aquatic sources, and proximity to hosts such as cattle and sheep (Ayala et al. 2018, Barcelo et al. 2021, Dinh et al. 2021, Lakew et al. 2021). Evaluating midge abundance is accomplished primarily by trapping adult insects using carbon dioxide baited traps, light traps, or combinations of these. Larvae may be collected from suspected habitats (e.g., manure-rich wastewater mud) and sampled directly, or adults may be sampled in emergence traps from these substrates (McDermott and Lysyk 2020). These techniques are necessary to surveil the presence and abundance of *Culicoides*, as well as pathogen prevalence in midge populations to monitor risks to livestock.

Culicoides have been implicated as vectors of numerous pathogens around much of the globe. The most concerning viruses are those affecting livestock, which include bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), vesicular stomatitis virus (VSV), African horse sickness virus (AHSV), Akabane virus (AKV), and Schmallenberg virus (SBV) (Purse et al. 2015, Mullen and Murphree 2019). Oropouche virus (OROV) is one of the few *Culicoides*-transmitted pathogens which can cause disease in humans and is found in South America. Non-viral pathogens include filarial nematodes of humans and livestock, which include species in the genera *Mansonella* and *Onchocerca* (Mullen and Murphree 2019). Other pathogens include lumpy skin disease virus (LSDV), bovine ephemeral fever virus (BEFV), and Simbu serogroup

virus which are concerning for livestock (Behar et al. 2022, Sanz-Bernardo et al. 2022). Clinical presentations in most livestock associated diseases include fever, lesions, edema, and lameness. Disease can lead to mortality in susceptible animals, such as BTV in sheep, EHDV in white-tailed deer, and AHSV in horses (Mullen and Murphree 2019). Interestingly, an outbreak of BTV serotype 8 (BTV-8) in 2006 led to mass livestock disease and mortality in a region of Europe which had not seen this serotype previously, and which underscored the risks of these diseases to spread in naïve livestock populations (Mayo et al. 2017).

Beyond the loss of livestock, further considerations for *Culicoides*-associated diseases include reduced animal weight, lowered milk production, and fetal abortion. These outcomes have significant economic costs for livestock producers. The economic impacts of BTV-8 in France and the Netherlands in 2007 alone was over \$1.4 billion USD, with larger impact estimates at over \$3 billion USD globally (Rushton and Lyons 2015). Livestock producers are also impacted by restrictions on animal and animal product movement and trade. The importance of these restrictions was made apparent after an investigation of an outbreak of BTV-8 in France in 2015 found the virus to be remarkably similar to the 2008 virus, which led researchers to conclude the virus originated from frozen animal products like stored bull semen used for artificial insemination (Pascall et al. 2020). Livestock producers are under immense pressure from *Culicoides*-associated diseases which emphasizes the need for effective population management methods.

Modeling shows strong associations between temperature and *Culicoides* seasonal abundance, as evidenced by recent studies (Barcelo et al. 2021, Gao et al. 2021, Barcelo et al. 2023, Lysyk et al. 2023). Warming climates will likely lead to range expansion for some *Culicoides* species, and even in regions where temperatures become too warm for established

species, others which are better adapted to these conditions may become established (Niedbalski and Fitzner 2018, Mohlmann et al. 2021). These midges will continue to play a major role in animal welfare and a diverse set of control options are necessary to combat these pests.

Current Control Strategies

Most biting midge species are nuisance pests and a few species vector livestock-associated pathogens. As such, most control strategies currently employed for midge control focus on reducing midge abundance in animal agriculture systems. The current strategies can broadly be grouped into cultural control, chemical insecticides, and microbial insecticides (Fig. 1.1). These have been extensively reviewed previously (Carpenter et al. 2008, Pfannenstiel et al. 2015, Harrup et al. 2016, Benelli et al. 2017). Here, we will describe updates to the field and their implications for the state of *Culicoides* control.

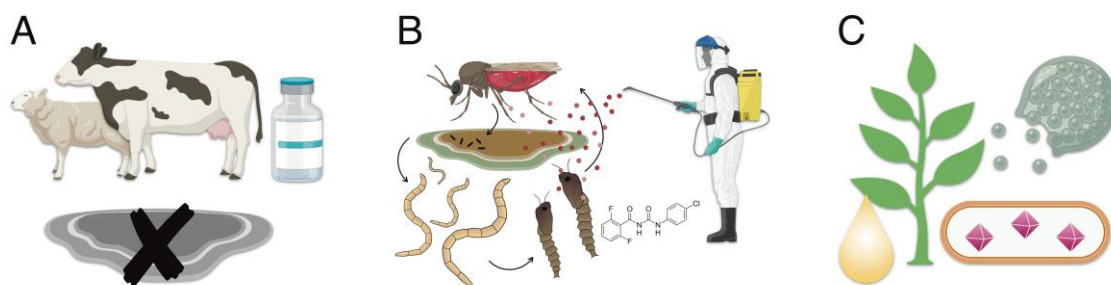


Figure 1.1. Example control options for *Culicoides* biting midges.

A) Cultural control methods include vaccinating livestock against *Culicoides* transmitted viruses and removing larval developmental sites. B) Insecticidal treatments can be applied to control adult midges and immature stages. C) Bio-rational products include plant extracts, microbial toxins, and entomopathogenic fungi.

Cultural Control

Restrictions on animal movement can prevent infection of local *Culicoides* populations with viruses, and vaccinating animals can reduce viral transmission and disease severity. However, both strategies have generally been regarded as ineffective at controlling pathogen spread where employed (McVey and MacLachlan 2015, Mayo et al. 2017). Inactivated vaccines are effective but expensive to produce, while live virus vaccines pose the risk of recombination with wild type viruses (MacLachlan and Mayo 2013, Mayo et al. 2017). This issue is also complicated in regions where multiple viral serotypes are present and vaccinations lack the appropriate coverage. Herd management may play a role in amplifying viral spread. A study in India found buffalo, which are asymptomatic when infected with BTV and were placed as a buffer to susceptible sheep herds, were instead serving as reservoirs for the virus and leading to disease outbreak (Chanda et al. 2019). This finding suggests that effective vaccine campaigns will need to consider local virus reservoirs. Other recent studies have also advised against vaccines even during disease outbreaks given their prohibitive costs and lack of protective immunity on some herd animals (Narladkar 2018, Carvelli et al. 2019).

Advanced warning of outbreaks can be informed by routine serological surveys of sentinel herds or from wildlife. Serological surveys are also complicated by hot zones of civil unrest in BTV endemic regions such as Pakistan and Afghanistan (Sohail et al. 2018). Predictive measures such as disease and vector distribution modeling are increasingly being used by examining past outbreaks to predict future outbreaks (Cappai et al. 2018, Cuellar et al. 2018, Duric et al. 2018, Grewar et al. 2019, Villard et al. 2019). These models are informed by basic *Culicoides* collection efforts globally which link local ecology to species richness and distribution as well as how weather patterns affect midge abundance (Collins et al. 2015, Ayala

et al. 2018, Yang et al. 2018). One study detected *Culicoides* in a region previously thought to have been inhospitable to these midges in high-altitude regions of China (Duan et al. 2019). These data contribute to advances in large, regional modeling efforts that can influence herd movement and vaccination campaigns in a cost-effective manner.

Landscape alteration for *Culicoides* management could potentially reduce midge abundance and subsequently prevalence. In livestock rearing operations, removing manure and other organic waste restricts habitable larval development sites. One study evaluated the impact of removing a wastewater collection site from a California dairy farm and comparing *C. sonorensis* abundance to a control site (Mayo et al. 2014). However, the authors found an opposite effect where more *Culicoides* were collected from the farm lacking a wastewater pond. These findings suggest that *C. sonorensis* are likely using other agricultural associated water sources. Spillover from outside midge populations which develop in often cryptic habitats is another factor to contend with. Efficiently identifying these sites to potentially remove them or treat them may reduce these spillover events (Kameke et al. 2021). Another consideration for landscape alteration is separating livestock animals from wild animals which are hosts for viruses, such as BTV and EHDV in cervids (McGregor et al. 2018, McGregor et al. 2019b, McGregor et al. 2019a, Sloyer et al. 2019). Analysis of midge bloodmeals can better inform producers which wild animals are near managed herds. After a bloodmeal, female midges can be found resting in local vegetation. The microclimate of these environments was examined in one study which found warmer temperatures in these locations likely encouraged virus incubation, a theme commonly discussed with regard to a warming climate (Haider et al. 2018). Studies like these rely on successful midge collection and provide empirical evidence for ecological factors affecting adult midges near livestock herds. In a recent review, McDermott and Mullens show

that standard midge collection by light-attractant suction traps are biasing collections resulting in underestimation of population sizes, sex ratios, infection status, and fed-states (2018). Trapping methods are reviewed here (McDermott and Lysyk 2020), and are an important consideration for monitoring *Culicoides* populations for pathogen surveillance, species distribution, and evaluating control strategies.

Perhaps the most problematic factor contributing to effective *Culicoides* control is the identification of larval habitats. This is substantiated in a recent article where Braverman et al. (2018) review the collection of 58 adult *Culicoides* species in suction traps, but only 15 larval species in substrate collections in the same region of Israel. Their work went on to assess multiple types of substrates, from organic-poor sandy soil to organic-rich manure infused mud, to tree holes and water pools in pockets of vegetation in cropland. The authors found *Culicoides* in all types of substrates with different species preferring different environments. In addition, two articles assessed midge oviposition preference in Florida by using combined field and laboratory experiments (Erram and Burkett-Cadena 2018, Erram et al. 2019). Wong et al. (2018) found that *Culicoides* in Southern California preferred ovipositing onto moist soil within 5 cm of the water line in agricultural wastewater ponds. The authors suggest altering the pond water levels to desiccate freshly deposited eggs from the previous night. A similar tactic was recently described in Australia, where a large lagoon's water level was altered periodically to disrupt egg development (Suara et al. 2018). Measures such as these are relatively simple and may be effective at controlling those species that utilize large water sources as larval habitats. More basic research is needed to identify habitat preference for species with cryptic breeding habitats, but ultimately these data will encourage broader field assessments of potential midge habitat preference and will inform the diversity of habitats needing larval control treatments.

Chemical Insecticides

The most common methods of controlling *Culicoides* have traditionally been through chemical applications in the form of adulticides and repellents on animals and structures, and larvicides in known or suspected habitats suitable for immature development. Deltamethrin, for example, is consistently effective in laboratory settings and most field applications for North American *Culicoides* species (Pfannenstiel et al. 2015). A major issue with adult midge control, however, is effective contact with insecticides on host animals, such as the application of pour-on treatments for cattle. Both insecticides and repellents are quick to wash off in rain and they will naturally degrade under average environmental conditions in the field if they are not reapplied often (Carpenter et al. 2008). Midge biting preferences also complicate these applications as most species of concern prefer the belly, coronary bands on the feet, anus, or other exposed soft tissues of an animal where topical compounds may not naturally diffuse (Elbers et al. 2018). Another application method is spraying the walls of stalls where animals are housed at night. Some species of endophilous midges will enter housing areas to feed on cattle and can contact insecticides when landing on treated surfaces (Carpenter et al. 2008). These insecticides have been shown to be useful in controlling *Culicoides* numbers but do not consistently prevent bites. The use of insecticides also poses a risk for fostering insecticide resistance in *Culicoides* as it has with other pest insects (Benelli et al. 2017). While specific insecticide resistance does not appear to be an imminent threat, laboratory studies have shown variable efficacy of insecticides between *Culicoides* species and within species' populations (Carpenter et al. 2008, Pfannenstiel et al. 2015, Venail et al. 2015). This observation promotes the need for new control measures, including those beyond chemical insecticides.

Topical insecticide application to host animals can create targeted sources of exposure for adult midges. This was examined in a recent study using ivermectin and deltamethrin applied topically to the midline of cattle (Murchie et al. 2019). The authors found no significant differences in the number of blood-fed females collected from treated and control animals, but the midges appeared to have been exposed to lethal levels of insecticide. While a potential route of adult control, these methods would not initially prevent bites from potentially infected females.

Effective population control likely requires employing multiple methods. One recent study used deltamethrin to control adult insects and both *Bti* and diflubenzuron to treat larval habitats (Meloni et al. 2018). These compounds were selected based on their use in mosquito control as few formulated insecticides have been specifically developed for *Culicoides* control. This study found reduced numbers of *Culicoides* on working sheep farms that were treated with all three insecticides compared to untreated farms which served as controls for the experiment. It should be noted that larval treatment areas were selected for their composition of moist habitats with organic matter which were known for the species targeted. This approach may not always be feasible when the breeding habitats for the species of concern is unknown (Mullens et al. 2015).

Given the diversity of *Culicoides* species around the world, much work is ongoing to examine the efficacy of standard insecticidal treatments on endemic populations of concern. Other insect repellent methods have been examined, such as cattle and netting treated with DEET, and these studies show some success in repelling *Culicoides* (Benelli et al. 2017). Compounds such as essential oils derived from eucalyptus and lemon were thought to provide a more natural alternative for repelling adult *Culicoides*, but these were not effective in laboratory

settings. In contrast, an Australian group observed 95% efficacy at repelling adult *Culicoides* in a field trial with an extract of a local tree species (Benelli and Pavela 2018). Benelli and colleagues (2017) sought to find alternative treatments for larval control. Here, the researchers used an inexpensive neem product to treat livestock waste runoff ponds. This treatment successfully discouraged adult oviposition onto the mud substrate when compared to controls. The researchers examined adult emergence as a metric but did not quantify the larval populations around the treated area. The neem cake used was noted to be less expensive than the concentrated active ingredients that are produced, namely azadirachtin, but the adult repellency distance is unknown, so it is unclear how much area needs to be treated and how frequently. No single chemical insecticide or repellent has been identified as an efficient and effective treatment for controlling *Culicoides*. Combination approaches to manage adult and larval stages would be the most effective so long as insecticide resistance and aquatic ecosystem health are monitored closely (Cavallaro et al. 2019).

Treating larval stages of *Culicoides* in semi-aquatic ecosystems is complicated by dilution effects, poor mixing of hydrophobic compounds with water, and environmental degradation. In addition, midges rarely occupy these habitats on their own, and the effects of chemical insecticides on non-target organisms is an important consideration. Studies have found pesticides such as neonicotinoids and fipronil reduced micro- and macro-invertebrate abundance in habitats near agricultural operations (Pisa et al. 2015). Cypermethrin was detected in soil samples more than three months after treatment, and many trophic levels were found to have been negatively affected (Giddings et al. 2013). Semi-natural experiments have evaluated permethrin toxicity in containers containing multiple trophic levels of aquatic animals (Hua and Relyea 2019). Here, the authors show hemipterans and odonates were highly susceptible to the

insecticide, but it should be noted that these systems were stagnant, and the level of insecticide used was higher than the recommended rate. Concerns for off-target environmental effects from chemical insecticides lie not only with the agricultural producers, but with household consumers who may be unaware of insecticide toxicity to non-target species.

Microbial Insecticides

Entomopathogenic fungi and bacterial-derived insecticides are commonly used in mosquito control as bio-rational alternatives to traditional chemical insecticides.

Entomopathogenic fungi appear to kill *Culicoides* and did not have off-target effects on those non-pest insects tested in one recent study (Ansari et al. 2019). Distributing fungal spores into the environment at large scale should be considered carefully but use in livestock housing units may be an effective strategy to infect midges upon contact. While this scenario would not prevent bites, it would control adult populations and kill midges before they can oviposit as mortality was observed in ~24 h.

Perhaps most well-known are those products containing insecticidal toxins from *Bti* which are toxic to dipterans such as mosquitos and black flies (Ben-Dov 2014). Using *Bti* is quite common in large-scale applications and in many types of consumer products for mosquito control. However, microbial *Bti* and commercial formulations of *Bti* appear to have limited efficacy as larvicides against midges. A recent study determined the lethal concentrations of *Bti* in *Culicoides* larvae collected in Sardinia were much higher than those seen in most mosquito species (Foxi et al. 2019). The authors show *Bti* appeared to work best compared to two other candidate bacterial-based insecticides derived from *B. sphaericus* and *B. laterosporus*. Two North American biting midges, one *Culicoides* sp. and one *Leptoconops* sp., were also

unaffected by *Bti* products in one early study (Lacey and Kline 1983). The refractory nature of midges to *Bti* toxicity has been linked to an acidic gut environment which is not permissible for toxin activation (Frouz et al. 2007). Alternative formulations of *Bti* insecticides could alter the larval gut pH, but current *Bti* products appear to be unfavorable for *Culicoides* control. The environmental impacts of biological control agents are expected to be less than those of traditional chemical insecticides, but the risks are not negligible and should be included in any risk assessment.

RNAi for Insect Control

RNA interference (RNAi) is a conserved and highly specific mechanism for suppressing target genes. For use in insect pest control, RNAi causes mortality or other favorable outcomes as a result of suppressing physiologically important proteins and enzymes. RNAi-based insecticides use nucleic acids (e.g., double-stranded RNA, dsRNA) complimentary to the genetic sequence of target genes in the pest to suppress target transcript levels. Thus, the effects can be made highly specific to a target species, and RNA-based treatments would be environmentally labile. However, while promising, there are numerous hurdles to overcome in developing efficient RNAi-based treatment options in insects.

Mechanism and Function

The term RNAi encompasses multiple overlapping pathways wherein RNA molecules are degraded. The major pathways are reviewed by Zhu and Palli (2020) and discussed briefly here. The small interfering RNA (siRNA) pathway begins when dsRNA is bound by accessory proteins, R2D2 or Loquacious, and is enzymatically cleaved by Dicer-2 (Dcr2). These cleavages

result in small dsRNA products of approximately 22 bp and are termed siRNA molecules. Each siRNA molecule is incorporated into a complex of accessory proteins which include the enzyme Argonaute-2 (Ago2). One strand of RNA, termed the passenger strand, is degraded while the remaining strand serves as a guide to bind complementary mRNA molecules. This complex of proteins and RNA is referred to as the RNA-induced silencing complex (RISC). The siRNA pathway can use exogenous or endogenous dsRNA as a starting substrate and has roles in cleaving host mRNA to silence gene expression, cleaving viral RNA products, and silencing transposons (Fig. 1.2).

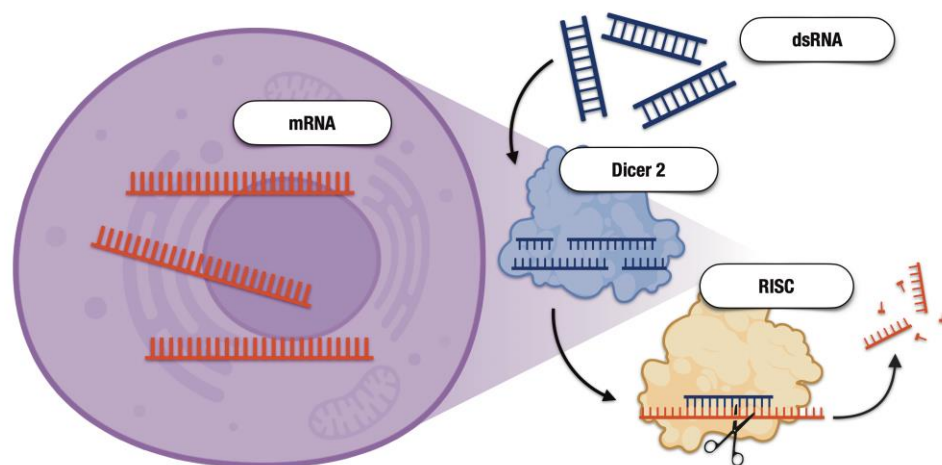


Figure 1.2. Representation of the siRNA pathway inside of the cell.

Double-stranded RNA (dsRNA) is internalized in the cell where it is cleaved into siRNA by Dicer-2. One strand of siRNA serves as a guide for mRNA in the RNAi induced silencing complex (RISC). Argonaute-2 in the RISC cleaves mRNA thus rendering it unusable.

There are two other pathways that overlap in functionality with the siRNA pathway and whose endpoints are the degradation of RNA. The microRNA (miRNA) pathway causes host

gene silencing from host-derived RNA templates. The miRNA pathway resembles the siRNA pathway albeit with a different set of proteins (e.g., Dcr1, Ago1). The final pathway, piwi-interacting RNA (piRNA), has a role in degrading host transposon transcripts. These pathways are conserved in insects, and some proteins have undergone gene duplication in some species (Zhu and Palli 2020). Insecticidal RNAi products are expected to be processed by the siRNA pathway where exogenous dsRNA molecules are taken up by cells, processed as above, leading to gene suppression, though the other two pathways can also play small roles in this function (Gao et al. 2020).

Insecticidal Properties

Any number of physiologically important genes could be targets for RNAi-based suppression. Ideal RNAi targets are genes which are highly expressed with large mRNA pools, sensitive to RNAi, and encode for proteins with short half-lives (Scott et al. 2013). If RNAi is only a transient response in an insect, then long-lived proteins may not be affected by dsRNA treatment. Scott et al. (2013) discussed the identification of vacuolar ATPase (*V-ATPase*) subunit A, a cytochrome P450, and *Snf7* as targets in coleopteran pests. A screen of *Anopheles gambiae* found suppressing actin, a leukocyte receptor complex member, and offtrack genes to be insecticidal in larvae fed on yeast producing short-hairpin RNAs for these targets (Mysore et al. 2017). This study also showed that the treatment resulted in up to 100% mortality in laboratory trials. Chitin synthase genes are also common targets for RNAi in insects. Chitin synthase 1 (*CHS-1*) is involved with cuticular chitin synthesis, whereas chitin synthase 2 (*CHS-2*) synthesizes the chitin of the peritrophic matrix (Zhu et al. 2016). Suppressing these genes in mosquitos can lead to decreased chitin formation and greater susceptibility to insecticides, such

as diflubenzuron (Zhang et al. 2010, Lopez et al. 2019). Other targets in six mosquito species are reviewed in Munawar et al. (2020). Target selection is the first of many important steps towards developing RNAi-based insecticides, and there are many considerations for optimizing their delivery.

Barriers to Implementation

Creating efficient RNAi-based insecticides requires understanding properties of the specific insect pest. For example, coleopterans are usually highly susceptible to dsRNA treatment and target suppression of greater than 90% can be achieved with low doses of dsRNA. In contrast, lepidopterans are quite refractory to treatment (Joga et al. 2016, Cooper et al. 2019). Numerous factors may contribute to these discrepancies, and include the stability of dsRNA molecules, uptake and processing of dsRNA in the insect cells, expression of core RNAi machinery, and viral suppressors of RNAi, among others (Cooper et al. 2019) (Fig. 1.3). Currently, very little is known about how any of these potential barriers may affect RNAi efficiency in *Culicoides* midges, but these are important factors in developing RNAi as a management tool for these livestock pests.

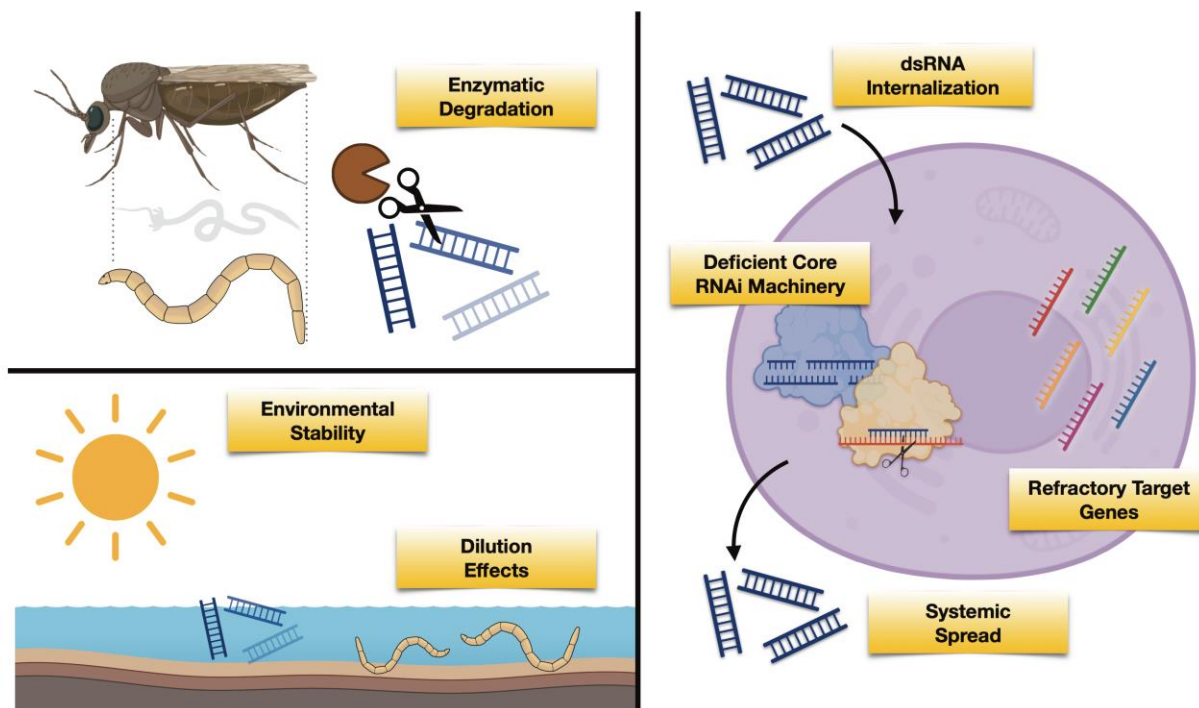


Figure 1.3. Barriers to dsRNA stability and RNAi efficiency in insects.

Enzymatic degradation of dsRNA by enzymes (dsRNases) in hemolymph and gut fluid reduce dsRNA levels. Environmental factors and dilution effects are considerations for real-world treatments. In the insect, barriers to RNAi efficiency include the uptake of dsRNA, processing by core RNAi proteins and enzymes, refractory target genes, and poor systemic spread (adapted from Cooper et al. 2019).

One of these barriers is the expression of enzymes which degrade dsRNA molecules, leading to low RNAi responses in insects. These enzymes are termed dsRNA-degrading enzymes or nucleases (dsRNases), and their abundance, diversity, tissue specific expression, and activity are investigated in many insect orders (Singh et al. 2017, Song et al. 2019, Cooper et al. 2020, Lei et al. 2022). Suppressing these nucleases, by RNAi, to enhance RNAi has been investigated in *Aedes aegypti* (Giesbrecht et al. 2020) and in a tephritid fruit fly (Tayler et al. 2019) with success. Specifically, Giesbrecht et al. (2020) showed midgut specificity for two larval mosquito nucleases which contributed to the degradation of orally delivered dsRNA. Identifying and overcoming these nucleases may be essential for oral delivery of dsRNA-based control agents.

Strategies to Enhance RNAi

Overcoming barriers to dsRNA production, application, environmental stability, and uptake are avenues of ongoing investigation. Specific strategies will likely need to be developed for different *Culicoides* species given their diversity of habitats and behaviors. There are many methods available towards creating more stable RNAi products and producing RNAi products at scale.

Nanoparticles

Free dsRNAs in the insect gut lumen and hemolymph are exposed to enzymes which may degrade these molecules before they can enter cells and initiate an RNAi response. Nanoparticles made of organic or inorganic, natural or synthetic materials can bind dsRNA to reduce enzyme access to these molecules (Christiaens et al. 2020, Silver et al. 2021). Chitosan is a common material for constructing these nanoparticles. Here, electrostatic interactions of the negatively charged dsRNAs and the positively charged amino groups on the chitosan polymer lead to the formation of nanoparticles (Zhang et al. 2015). These nanoparticles can be fed to larval insects in a food source. One study showed two chitin synthase genes in *An. gambiae* were suppressed in larvae fed 5 µg of dsRNA-chitosan nanoparticles in food each day for 4 days (Zhang et al. 2010). The transcripts of both *CHS-1* and *-2* were suppressed by 50-60% in their respective treatments, led to noticeable phenotypes in larvae, and increased larval mortality. Similarly, Das et al. (2015) compared the activities of chitosan nanoparticles to those of carbon quantum dot and silica nanoparticles. The authors showed significant suppression of two genes when dsRNA was bound to chitosan nanoparticles, and one gene with dsRNA bound to carbon quantum dot nanoparticles. Additionally, these nanoparticles were shown to have some insecticidal properties on their own

(i.e., when bound to ds*GFP*), but it's noted that these materials are biodegradable and have low toxicity which could ease worries about their use in the environment (Das et al. 2015).

Developing nanoparticles and carrier compounds with selective charges are hypothesized to create specificity for delivering dsRNA to tissue types in insects (Paquette et al. 2015). These advances are promising routes towards improving dsRNA stability and delivery of insecticidal treatments.

Secondary RNA Structures

Designing dsRNA molecules with sequences of complementary bases separated by a segment of non-complementary bases encourage loop formation. A common example is short hairpin RNA (shRNA) molecules with a single loop at one end of the target sequence formed by the non-complementary bases. These are used extensively in mosquito studies and can be synthesized *in vitro* or expressed in microbial systems (Duman-Scheel 2019, Abbasi et al. 2020, Giesbrecht et al. 2020). These molecules are effective for target gene suppression and have been shown to reduce arboviral replication by RNAi suppression in cells and animal models, which could enhance antiviral activity in insects and reduce viral spread in vector species (Shen et al. 2014, Tng et al. 2022).

As an alternative to shRNAs, Abbasi et al. (2020) constructed RNA molecules containing closed ends at both molecule termini and termed these paperclip RNA (pcRNA). The authors show these pcRNA (23 bp) molecules to be as efficient as long dsRNA (200 bp) molecules, as well as siRNA and shRNA (23 bp) molecules. Importantly, they also show that pcRNA is taken up by cells through a novel mechanism that is different from that of shRNAs and long dsRNAs. This finding is particularly significant because one documented mechanism of resistance to

RNAi is through inhibition of uptake of long dsRNAs. In *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae), beetles exposed to dsRNA targeting *Snf7* became resistant to dsRNA treatment after repeated exposures because reduced dsRNA uptake, not dsRNA degradation, in the gut lumen resulted in lowered RNAi efficacy (Khajuria et al. 2018). The implications of this work will need to be explored in greater detail as RNAi-based insect control products are developed. However, secondary RNA structures may create more resilient dsRNA molecules with alternative uptake in insect cells.

Bacterial Expression

Expressing dsRNA in microorganisms provides an alternative to mass *in vitro* dsRNA production. A common approach uses the L4440 plasmid which contains inverted T7 promoters to create an expression template for a target dsRNA of choice. Once the plasmid is constructed, it can be transformed into bacteria, such as the HT115 (DE3) *Escherichia coli* strain which lacks RNase III, preventing dsRNA product degradation. Synthesis is then induced with isopropyl β -D-1-thiogalactopyranoside (IPTG). Ahn et al. (2019) provide an overview of this process and compare heat and sonication methods for liberating dsRNA from *E. coli* after production. In this study, the authors found that dsRNA yields from a 25 mL culture were estimated at 488 μ g, 238 μ g, and 97 μ g for sonication, heat, and conventional methods, respectively. In a different study, *E. coli* were induced with lactose instead of IPTG and lysed with chlorhexidine to liberate dsRNA, which the authors describe as inexpensive alternatives to those previously described (Lopez et al. 2019). In this latter work, *Ae. aegypti* larvae showed 50% suppression of chitin synthase A when treated with 400 ng of dsRNA in their rearing water, and this was associated with 50% mortality in 4th instar larvae after four days. Direct application of dsRNA-expressing

E. coli into rearing water, or their incorporation into a food source, may also assist with delivery and protection of dsRNA in the insect gut. Taracena et al. (2022) showed this methodology can also be used to feed adult mosquitos through a sugar-*E. coli* meal to achieve significant target knockdown. A recent study also described the production of minicells from *E. coli* producing dsRNA which resulted in protective encapsulations of dsRNA for fungal control (Islam et al. 2021). This technology appears to be adaptable for diverse pests and is worth further investigation for use in *Culicoides* midges.

Researchers are also exploring the utility of engineering symbiotic bacteria to express dsRNA. One study used a symbiont expressing a dsRNA hairpin targeting *Rhodnius prolixus*, a vector of Chagas disease, and the authors found a significant reduction in target mRNA levels and reduced egg production in adult females (Taracena et al. 2015). Another study evaluated symbiote expression in honeybees and their associated gut bacterium, *Snodgrassella alvi*, which produced dsRNA against *Varroa destructor* mites (Leonard et al. 2020). The symbiont successfully colonized the bee guts and significantly reduced feeding mite survival. Symbiotic bacteria may be an ideal delivery system given their close association with target species and certain tissue tropisms in the host, but delivery methods for delivering the engineered symbiotes to their hosts and having these spread from host to host needs further development.

Fungal Expression

Perhaps one of the most promising methods for mass producing dsRNA in a microbial system uses baker's yeast, *Saccharomyces cerevisiae* (Duman-Scheel 2019). This organism is unique for its production scalability and ability to encapsulate dsRNA when dried to enhance stability. When compared to heat-killed bacteria expressing dsRNA, *S. cerevisiae* performed just

as effectively (greater than 75% mortality) in larval mosquito bioassays (Mysore et al. 2017). The same research group later showed high efficacy of yeast-based larvicides expressing shRNA targeting an olfactory receptor neuron (*sema1a*) against *Ae. aegypti*, *Ae. albopictus*, *An. gambiae*, and *Culex quinquefasciatus* with mortality rates greater than 80% (Mysore et al. 2019). This group has gone on to develop multiple yeast-based insecticidal treatments targeting mosquito physiology (Mysore et al. 2020, Mysore et al. 2021a, Mysore et al. 2021b). Yeast provide some increased stability as they can be readily dried and reconstituted in mosquito larval environments (Duman-Scheel 2019).

Viruses

Viruses present a unique delivery option for dsRNA as they can be engineered to deliver dsRNA-expressing nucleic acid constructs and virus-like particles can be used as empty vessels to deliver dsRNA cargo into cells (Kolliopoulou et al. 2017). Gu and colleagues (2011) created recombinant mosquito densoviruses containing shRNA constructs targeting either *Ae. aegypti* or *Ae. albopictus* V-ATPase. Their results show broad viral affinity for multiple mosquito tissues, target suppression in cells and larvae, and an increase in larval mortality after exposure to the virus by soaking. In addition, plant viroids provide a unique scaffold for dsRNA production and have been shown to be insecticidal in *D. virgifera* (Ortola et al. 2021). Virus host specificity, genome type, tissues tropism, infectivity, and persistence are among the considerations that should be investigated as these avenues are developed for RNAi pest control in biting midges.

Algal Expression

Aquatic insects or those with aquatic life stages are likely to encounter algae in natural ecosystems. These organisms have been investigated as dsRNA production systems for mosquito control in several studies. One study engineered *Chlamydomonas reinhardtii* chloroplasts to produce dsRNA targeting *3-HKT* in *An. stephensi* (Kumar et al. 2013). The authors report significant target suppression and increases in larval mortality when larvae were fed transgenic algae. Another study used the same microalgae to express dsRNA against *HR3* in *Ae. aegypti*, and similarly found target suppression and increased larval mortality compared to controls (Fei et al. 2020). Other studies have used both *Chlamydomonas* and *Chlorella*, which includes strains harvested from water sources near the associated research institutions to kill *Ae. aegypti* and *Ae. albopictus* larvae (Fei et al. 2021, Fei et al. 2023). These studies are promising because they have shown successful transformation of both laboratory and natural algal strains. In addition, Fei et al. (2023) found their transformed *Chlorella* sp. to be larvicidal in semi-field trials. While these studies are promising, transforming other algal species and strains which are specific to a pest's natural environment may not be as easy. There are a number of considerations towards the successful engineering of these algae, reviewed here (Mosey et al. 2021).

Outlook for Controlling *Culicoides* using RNAi

The first publication to examine RNAi in *Culicoides* used cultured cells to show BTV (a dsRNA virus) was likely degraded by Dcr2 and resulted in 21 nt virus-derived small interfering RNA (viRNA) molecules (Schnettler et al. 2013). Mills et al. (2015) described work to knock down transcript levels of a target gene in adult *C. sonorensis* by RNAi. The authors showed that the core RNAi machinery is present in *C. sonorensis* (e.g., *Ago2*, *Dcr2*, *R2D2*) and that

intrathoracic injection of dsRNA led to approx. 45% transcript suppression after 5 days. These results show that *C. sonorensis* are sensitive to dsRNA, at least by injection, and that RNAi pathways are functional.

Translating these findings into treatment options for controlling *Culicoides* requires more foundational work, however. Reviewed in Silver et al. (2021) and here, approaches include naked dsRNA treatments, complexing dsRNA to nanoparticles, using viral vectors, and expressing dsRNA in microbial systems. These treatments could be applied to potential or known *Culicoides* larval habitats to control larval midges. *Culicoides* are generalist feeders and therefore are expected to consume bacteria, fungi, and algae. Hydrogel baits for larval mosquitos have been developed to hold phagostimulants and insecticides (Piazzoni et al. 2022). However, these are formulated to float on the surface of water. Larval *Culicoides* inhabit just the first few centimeters of substrate to facilitate respiration. Ideally treatments would be spread into these systems and incorporated into the shallow substrates of ponds, muddy depressions, compost, and manure. Alternatively, sugar baits or similar attractants containing dsRNA could be used to target adults (Pfannenstiel et al. 2015).

Desired outcomes for RNAi-based control methods include mortality and altering pathogen transmission. In larval *Culicoides* these could target physiologically important genes which have been explored in mosquito literature. For example, suppressing chitin synthases could affect cuticle formation during molting between larval instars and during pupation, and reducing peritrophic matrix formation in the larval gut. Combinatorial treatments may prove useful in increasing efficacy. Bacteria and other disruptors of peritrophic matrix integrity could degrade *Culicoides* gut integrity, and chemical chitin synthase inhibitors such as diflubenzuron can alter molting efficiency (Zhang et al. 2010, Erlandson et al. 2019). One interesting target is

the sex selection pathway. In mosquitos, studies have targeted genes resulting in altered male:female ratios and which show promise for reducing female populations (i.e., the blood feeding stage) (Whyard et al. 2015, Mysore et al. 2022). Other targets may influence pathogen fitness in the host, insect fecundity, and insect host seeking ability.

Perhaps the most important aspect of RNAi-based insect control is its high specificity and low off-target effects when compared to traditional insecticides. The regulatory framework for reviewing dsRNA products in the United States and in the European Union are reviewed by Dietz-Pfeilstetter et al. (2021). These authors, and others, encourage toxicity studies on non-target organism to ensure specificity (Romeis and Widmer 2020). One study also examined oral toxicity in a mammalian system, which found no negative outcomes (Petrick et al. 2016). These considerations are not only important for the scientific community, but for public perception as well. One study engaged community members in a potential treatment site where yeast-based dsRNA insecticides were planned to be used (Stewart et al. 2020). The authors found that the community was well versed in mosquito biology and open to new insecticides if they were safe. Next-generation approaches to control *Culicoides* have been proposed to include sterile insect technique, *Wolbachia*-based sterility, and transgenic alterations (Shults et al. 2021). Many of these techniques have been untested in large systems and could draw public criticism. RNA interference approaches may be more tolerated by the community as they do not affect insects' genomes and have transient properties.

Conclusions

Culicoides are an important pest of livestock which impact animal welfare and livestock producers' livelihoods. A changing climate is likely to expand the ranges for these insect vectors

which brings with them the risk of pathogen transmission to susceptible herds. The global movement of animals and animal products is an ongoing concern for introducing pathogens to vector competent *Culicoides* species in new environments. Effective control by cultural methods and chemical and bio-rational insecticides has been challenging for these insects. RNAi-based insecticides are an exciting avenue for advancement in the field but are not without hurdles towards widespread implementation. Effective control will require finding ideal genes for suppression in target midge species, optimizing delivery methods, designing application methods which are efficient and stable in the environment, monitoring the effects on target and non-target species, and working with regulatory bodies and the public for approvals and acceptance (Fig. 1.4). Many foundational studies on RNAi in *Culicoides* remain to be examined, but this field has great potential as such products could drastically reduce the need for broad-spectrum insecticides that have detrimental effects on non-target organisms and the environment. Given the current lack of effective control strategies, *Culicoides* are an important target for advanced insecticide development.

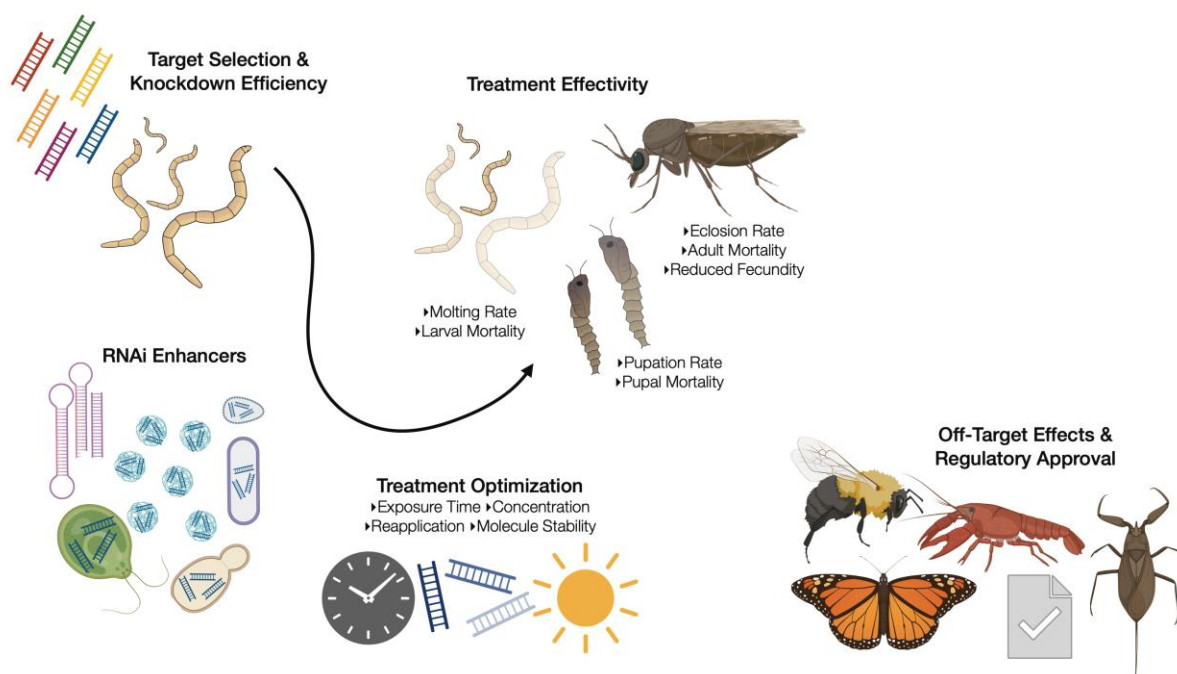


Figure 1.4. Example development plan for an RNAi-based insecticide.

Genes targeted for suppression by RNAi should be screened. RNAi enhancers include RNA structures (e.g., shRNA, pcRNA), nanoparticle carriers, and heterologous expression in microbial systems. Multiple factors should be considered towards developing RNAi insecticides, which include optimizing treatments and endpoints to evaluate efficacy. The specificity of RNAi products should be examined in multiple arthropods which could be exposed to these products. Regulatory approval is needed for field applications.

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Chapter 2 - Evaluation of potential reference genes in the biting midge *Culicoides sonorensis* for real-time quantitative PCR analyses

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Abstract

Studies examining differentially expressed genes and gene silencing by RNA interference (RNAi) require a set of stably expressed reference genes for accurate normalization. The biting midge *Culicoides sonorensis* is an important vector of livestock pathogens and is often used as a model species for biting midge research. Here, we examine the stable expression of six candidate reference genes in *C. sonorensis*: *actin*, *β -tubulin*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ribosomal protein subunit (*RPS*) 18, vacuolar ATPase subunit A (*VhaA*), and elongation factor 1-beta (*EF1b*). Gene expression was assessed under seven conditions, including cells treated with double-stranded RNA (dsRNA), 3rd and 4th instar larvae treated with dsRNA, six developmental stages, four adult female body parts or tissue groups, and females injected with bluetongue virus or vesicular stomatitis virus. Stable gene expression was assessed using RefFinder, NormFinder, geNorm, and BestKeeper. The ranked results for each analysis tool under each condition and a comprehensive ranking for each condition are presented. The data show that optimal reference genes vary between conditions and that just two reference genes were necessary for each condition. These findings provide reference genes for use under these

conditions in future studies using real-time quantitative PCR to evaluate gene expression in *C. sonorensis*.

Introduction

Real-time quantitative polymerase chain reaction (RT-qPCR) is an invaluable tool used to examine gene expression, but proper use requires optimization of conditions and identification of appropriate reference genes (Taylor et al. 2019). Reference genes are those that demonstrate stable expression across variable conditions and are used to calibrate expression relative to a target gene of interest. This type of calibration intends to account for differences in RNA levels among samples and to eliminate non-biological variations due to quality and quantity of template used, the yield of the extraction process, and differences in enzymatic reactions (Edmunds et al. 2014, Lu et al. 2018). However, the expression of reference gene transcript levels may vary across species, tissues, developmental stages, and in response to biotic and abiotic factors. These variations can subsequently affect measured changes in gene expression and mask real biological significance (Shakeel et al. 2018). Therefore, evaluating and selecting appropriate reference genes for individual study organisms under specific experimental conditions is essential to employ RT-qPCR (Lu et al. 2018, Shakeel et al. 2018).

Culicoides biting midges are economically important livestock pests that have been implicated as vectors of numerous pathogenic viruses and some pathogenic nematodes (Purse et al. 2015, Mullen and Murphree 2019). In the United States, *C. sonorensis* Wirth & Jones (Diptera: Ceratopogonidae) can transmit bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), and vesicular stomatitis virus (VSV), which are concerns primarily for livestock producers (McVey et al. 2015, McGregor et al. 2022). *Culicoides sonorensis* is one of the few

species to be established in colony, and, as such, has been a model species for laboratory-based studies (Nayduch et al. 2014c). Physiological and behavioral changes due to arbovirus infection have been documented previously in *C. sonorensis* (McDermott and Mullens 2018). Nayduch et al. (2019) found that midges infected with EHDV had 2,401 differently expressed unigenes compared to uninfected controls. Many genes associated with tissue structure, sensory processes, vision, and behavior were significantly downregulated in EHDV-infected midges, while genes coding for innate immune responses and olfaction were upregulated. Transcriptome studies like this one are often paired with RT-qPCR to confirm the up- and down-regulation of genes of interest and rely on having a set of stably expressed reference genes for normalization.

Using reference genes in relative RT-qPCR is necessary to fulfill the minimum information for publication of quantitative real-time PCR experiments (MIQE) (Bustin et al. 2009). Common reference gene candidates in insects include those involved in cytoskeleton structure formation, protein synthesis, and metabolism (Lu et al. 2018). In *C. sonorensis*, several ribosomal proteins (*Rp*) were identified in a transcriptome study of adult females but were found to be unsuitable as reference genes in later evaluation due to their variable expression across feeding conditions (Nayduch et al. 2014b). This same study found elongation factor 1-beta (*EF1b*) to be a suitable option based on its stable expression across several conditions including unfed or sugar or blood-fed midges, as determined by comparative transcriptomics. *EF1b* was subsequently used as a reference gene in studies of humoral immune responses (Nayduch et al. 2014b), EHDV infection (Mills et al. 2017b, Nayduch et al. 2019), and RNA interference (RNAi) studies (Mills et al. 2015, Mills et al. 2017a). Other reference genes used in *C. sonorensis* studies include heat shock protein 60 (*HSP60*), cytochrome (*CytB5*), *RpL13*, *RpL21*, *RpS8*, and vacuolar type ATPase (*V-ATPase*) subunits (Vermaak et al. 2017, Morales-Hojas et

al. 2018). Only one study appears to have used the widely accepted analysis tools NormFinder, geNorm, and BestKeeper to determine suitability of reference genes for normalization of RT-qPCR data in *C. sonorensis*, and this study focused on the vector competence of susceptible and refractory individuals (Morales-Hojas et al. 2018). Since *C. sonorensis* is a model organism for studies involving biting midge behavior, vector competence, population control, and more, additional work is needed to identify stably expressed reference genes suitable for normalization across a broader range of experimental conditions.

The purpose of the present study is to examine six candidate *C. sonorensis* genes for their suitability as reference genes in RT-qPCR-based relative gene expression analyses under laboratory conditions. We determined the relative expression of two structural genes (*actin* and *β-tubulin*), a metabolic enzyme (*GAPDH*), a ribosomal protein subunit (*RPS18*), the previously described *EF1b*, and subunit A of *Vha*. The expression of these genes was examined in cells and larvae treated with double-stranded RNA (dsRNA); in larval, pupal, and adult *C. sonorensis*; in four adult female body parts or tissue groups (head, midgut, remaining gut tract, and the remaining body); and in female midges injected with BTV or VSV. These data identify the most suitable reference genes for examining relative transcript levels using RT-qPCR, will enhance the utility of *C. sonorensis* as a model system, and will aid in the efforts of other researchers to study the midge physiology and the virus-vector interactions that occur in this veterinary pest.

Materials and Methods

Insect Cells and Life Stages

Culicoides sonorensis cells (W8A) are of embryonic origin and were maintained in Schneider's Insect Media (Sigma-Aldrich, St. Louis, MO, USA) (24.5 g/L) supplemented with 0.4 g/L sodium bicarbonate, 0.0585 g/L L-glutamine, 0.006 g/L reduced glutathione, 0.03 g/L L-asparagine, 0.6 g/L calcium chloride, 18 µL of 10 mg/L bovine insulin and 15% fetal bovine serum at 28 °C (McHolland and Mecham 2003). The AK line of *C. sonorensis* was used for all developmental stages, body part and tissue group collection, and virus injection studies (Nayduch et al. 2014c). Insects were kept at 25 °C and 60-80% relative humidity with a 14:10 light:dark cycle during the duration of the experiments unless otherwise noted. Both cultured cells and insects were sourced from the USDA-ARS facility in Manhattan, Kansas where they are actively maintained.

DsRNA Synthesis

Pooled cDNA (described below) was used to synthesize dsRNA. Briefly, primers containing a T7 promoter sequence were used to amplify DNA template from *C. sonorensis* cDNA (ds*IAP1* and ds*VhaA*) or from a plasmid containing *eGFP* (Table A.1). Reaction mixtures were as follows: 25 µl of 2X PCR MasterMix with Dye (abm), 2.5 µl each of forward and reverse primer (10 µM stock), 2 µl of cDNA template, and 18 µl of nuclease-free water. Thermal cycling conditions were 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, and a final extension step of 72 °C for 5 min. The PCR product was run on and extracted from a 1.5% agarose gel and purified with a gel

extraction kit (QIAGEN, Hilden, Germany). Double-stranded RNA was synthesized from 1 µg of cDNA template using a HiScribe T7 High Yield RNA synthesis kit (NEB, Ipswich, MA) according to manufacturer's recommendations. The dsRNA product was purified by sodium acetate-ethanol precipitation overnight at -20 °C, pelleted by centrifugation at 17,000 x g for 10 min, washed twice with 70 % ethanol, and reconstituted in nuclease-free water. Then, dsRNA was quantified by spectroscopy (Implen, Westlake Village, CA) and evaluated for specificity by gel electrophoresis.

Cells Treated with dsRNA

Cultured *C. sonorensis* cells were seeded into 12-well culture plates in 1 mL of modified Schneider's media and grown to ~75% confluence. The first experiment examined cell responses to non-target dsRNA by treating wells with 1 µg (i.e., 1 µg/mL), 100 ng, or 10 ng of *dseGFP*, or nuclease-free water as a control. Each treatment and control were replicated in triplicate. Cells were harvested 48 h post-treatment (hpt) in 400 µl of TRIzol reagent (Invitrogen, Waltham, MA) and processed for RNA extraction as below. By this time point cells had reached ~90% confluency. A second experiment examined cell response to an endogenous target by using *dsVhaA*. Wells were treated with 10 µg, 1 µg, or 100 ng of *dsVhaA*, or 1 µg of *dseGFP* as a control. Cells were harvested at 48 hpt as above.

Larvae Treated with dsRNA

Instar 3 (L₃) or 4 (L₄) larvae were aliquoted into wells of a 12-well cell culture plate (n=20 each) and the rearing water was aspirated and replaced with 1 mL nuclease-free water. The experimental layout consisted of 3 biological replicates (pools of 20 larvae) for each instar

and each condition (control or treatment). Each well was treated with 10 µg (i.e., 10 µg/mL) of dsRNA targeting either *IAP1* or *eGFP*. Larvae were maintained in a growth chamber for 72 h before being harvested in 400 µl TRIzol reagent. All larvae survived the treatments, and the total pool was used for each biological replicate. Larvae were homogenized with a tissue pestle, centrifuged at 16,000 x g for 5 min to pellet the bulk insect tissue, and then the liquid portion was moved to a new, clean tube for extraction of total RNA.

Developmental Stage and Body Part or Tissue Group Collection

L₃ and L₄ larvae, sex-sorted male and female pupae (Shults et al. 2016), and adult male and female midges (sugar-fed) were collected directly into 400 µl TRIzol reagent. Three pools of each stage were collected and consisted of 20 individuals per pool (n=20). Pooled insects were homogenized with two 2.4 mm stainless steel beads in a Bead Mill Homogenizer (Omni International, Kennesaw GA). Homogenized pools were centrifuged as above to pellet bulk tissue and the liquid portion was moved to a new tube for total RNA extraction. Adult female midges (n=50) were individually dissected in drops of nuclease-water to yield four body parts or tissue groups: 1) heads with salivary glands attached, 2) midguts separated from 3) remaining gut and associated organs (foregut, hindgut, and Malpighian tubules), and 4) the remaining body of the midge. Three pools of each tissue were collected in 400 µl of TRIzol, homogenized with a pestle, and processed as above.

Virus Injected Midges

Adult *C. sonorensis* midges were obtained from the AK colony maintained by USDA-ARS in Manhattan, KS. Mated female midges (3-4 days post-eclosion) were anesthetized with

CO₂ and injected with 60 nl of either VSV-NJ (2.52×10^8 PFU/ml) grown in porcine epithelial cells (AG08113; Coriell Institute, Camden, NJ) or BTV-17 (6.63×10^5 PFU/ml) grown in baby hamster kidney cells (BHK, American Type Culture Collection, Manassas, VA). Injections were delivered intrathoracically into the soft cuticle between the wing base and second pleural sclerite using a hand-held Nanoject II (Drummond Scientific Company, Broomall, PA). After treatment, midges were offered 10% sucrose *ad libitum* and were maintained in environmental chambers at 25 ± 1 °C and $75 \pm 5\%$ relative humidity with a 13:11 light:dark cycle. Six days post-injection (dpi), three pools of each treatment were collected and consisted of 20 individuals per pool (n=20). Midge pools were homogenized with two stainless steel beads in 400 µl TRIzol as above.

RNA Extraction and cDNA Synthesis

Total RNA was isolated using TRIzol according to the manufacturer's instructions (Fisher Scientific, Pittsburgh, PA). Total RNA was reconstituted in 50 µl of nuclease-free water. Final RNA concentrations were measured by spectroscopy, and cDNA was synthesized using 1 µg of RNA with an OneScript® Plus cDNA Synthesis Kit (abm, Richmond, BC, Canada). Residual genomic DNA was removed by incubation with DNaseI (Thermo Fisher Scientific, Waltham, MA) for 30 min at 37 °C followed by enzyme inactivation with EDTA at 65 °C for 10 min. The resulting cDNA was diluted 1:3 with nuclease-free water to create the template for RT-qPCR.

Genes Selection and RT-qPCR Primers

Candidate reference genes were selected based on published literature on mosquitoes, other insects, and other studies using *C. sonorensis* (Nayduch et al. 2014a, Dzaki et al. 2017, Lu

et al. 2018). These candidate genes were from multiple functional classes, including structural proteins, enzymes involved in metabolism, subunits of the ribosome and vacuolar ATPase, and a protein elongation factor. Transcript sequences were obtained from a transcriptome shotgun assembly (Accession: PRJNA238338) which is hosted on the National Center for Biotechnology Information (NCBI) database, and primers were designed using the Primer-BLAST web tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Optimal primers were designed to have 18-22 nucleotide bases, an amplicon size of 80-200 bp, 60 °C melt temperature, 50-60% GC content, and specificity to *C. sonorensis*. The RT-qPCR primers, amplicon size, and transcript accessions are reported in Table 2.1.

Real-time Quantitative PCR

Primers were validated using pooled cDNA from multiple midge developmental stages. The cDNA was initially diluted 1:20 with nuclease-free water and then used to create a 1:10 dilution series for a standard curve. Melt curve analyses were performed to ensure primer specificity. All quantifications were performed on a QuantStudio 7 Pro (Thermo Fisher Scientific). Reaction mixtures were as follows: 10 µl of BlasTaqTM 2X RT-qPCR MasterMix (abm) with 1 µl of ROX reference dye per 1.25 mL MasterMix, 0.5 µl of each primer (10 µM stocks), 1 µl of cDNA template, and 8 µl of molecular grade water. Thermal cycling conditions consisted of a 3 min hold at 95 °C, 40 cycles of denaturing at 95 °C for 15 sec and annealing and extension at 60 °C for 1 min. All sample reactions were performed in duplicate (technical replicates).

Data Analysis

Technical replicate Ct values were averaged for analysis. No-template controls were all negative or had Ct values above 36 which were considered negligible. Raw Ct values were used for RefFinder (Xie et al. 2012), BestKeeper (Pfaffl et al. 2004), and geNorm (Vandesompele et al. 2002). Data were transformed using the equation $RQ = E^{-(Cq_{\min} - Cq_{\text{sample}})}$ for NormFinder (Andersen et al. 2004), where relative quantity (RQ) is determined using the primer efficiency (E) and the difference between the minimum Ct value in the gene group and each Ct sample. RefFinder generates outputs for ideal reference genes using a comparative delta Ct method, and outputs for each of the other analyses (BestKeeper, geNorm, and NormFinder). Both geNorm and NormFinder analyses were performed in R Studio (version 2022.07.2+576). The most stable genes from BestKeeper were ranked first by the lowest standard deviation and then by the lowest coefficient of variance. Candidate reference genes with a standard deviation greater than one are not recommended for use (Pfaffl et al. 2004). Stability rankings from each analysis were assigned to each gene under each condition tested, and the geometric mean of these values was used to create a cumulative ranking. This is the same method used for RefFinder. The gene stability ranks determined in this study were compared to the ranks generated by RefFinder to evaluate their consensus.

Results

Primer Efficiencies and Ct Values

All candidate reference gene RT-qPCR primers had efficiency values between 94.7% and 101.4% (Table 2.1). Melt curves showed distinct unique peaks for every target analyzed (Fig. A.1). The spread of Ct values in each experiment and for each target are shown in Figure 2.1. All

Ct values were below 30, except for *RPS18* in the heads of female midges which were 31.6, 32.9, and 35.2 for the three biological replicates (Appendix A). These values were included in our analysis. The lowest determined Ct value was 12.9 for *GAPDH*, which belonged to the study of cultured cells treated with varying concentrations of *dseGFP*.

Table 2.1. Gene target accessions, primers, amplicon size, and primer characteristics of candidate reference genes for *Culicoides sonorensis*.

Abbreviations: bp, base pair; %E, primer efficiency; Amp.; amplification factor; *β-tubulin*, beta-tubulin; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *RPS18*, ribosomal protein S18; *VhaA*, vacuolar-type ATPase subunit A; *EF1b*, elongation factor 1-beta (Nayduch et al 2014a).

Target	Accession	Primer Sequence (5'→3')	Product Size (bp)	Slope	%E	Amp.	R ²
<i>Actin</i>	GAWM01012737.1	F: CGATCTGTTGATGCCCCGACT	148	3.29	101.39	2.01	0.9982
		R: ATTCGGGCGTGGAAGCTAAC					
<i>β-tubulin</i>	GAWM01003055.1	F: CAATCTGGTGCAGGAAACAAC	172	3.46	94.66	1.95	0.9996
		R: GAAGGGTTCCCATGCCTGAA					
<i>GAPDH</i>	GAWM01011279.1	F: ACTTGACATGCCGATTGGGT	167	3.34	99.22	1.99	0.9998
		R: GGCCTTGGCGTCAAAGATTG					
<i>RPS18</i>	GAWM01018992.1	F: GGCTTAAAACAGAGAAAGGTCTAT	167	3.44	95.28	1.95	0.9989
		R: AATTTGCACAGAATGCAGACTT					
<i>EF1b</i>	GAWM01007628.1	F: ATCCGTGAAGAACGTCTCAAA	95	3.37	97.87	1.98	0.9998
		R: CATGGCTTAACTTCGAGGATG					
<i>VhaA</i>	GAWM01018658.1	F: GTATGTTGCAAGTGTGGCCTG	86	3.43	95.79	1.96	0.9994
		R: ACGCTGACCAGTTAACAATGGA					

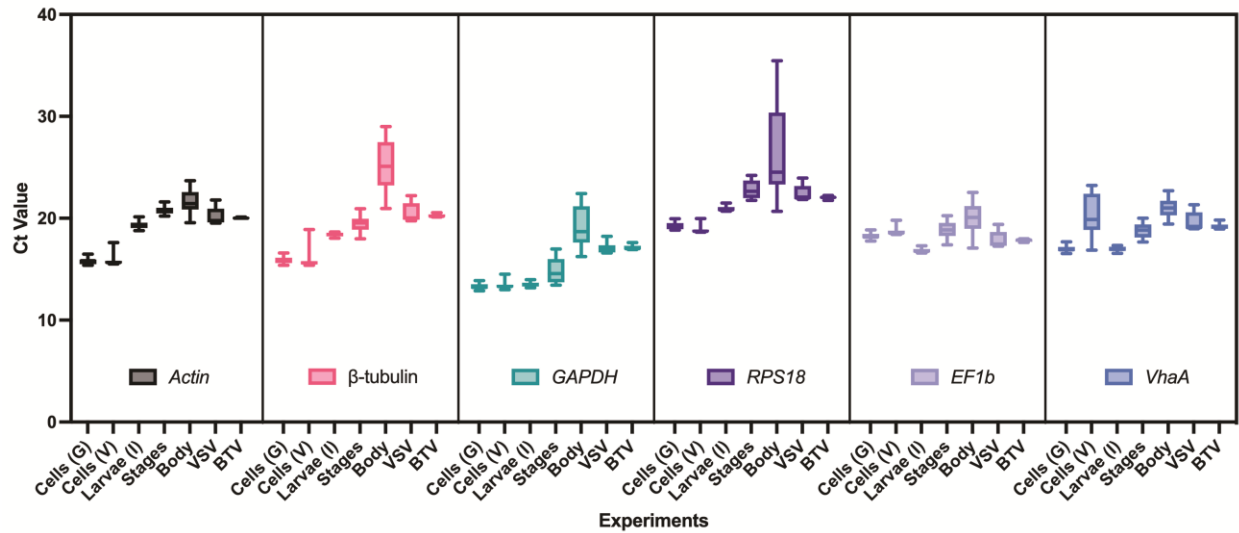


Figure 2.1. Distribution of Ct values for six *Culicoides sonorensis* candidate reference genes for each of seven experiments.

Box and whisker plots showing the 25th to 75th percentiles (box), minimum and maximum Ct values (whiskers), and median Ct value (line). The experiments were cells treated with water or varying concentrations of dseGFP [Cells (G)], cells treated with dseGFP or varying concentrations of dsVhaA [Cells (V)], larvae treated with dseGFP or dsIAP1 [Larvae (I)], midge developmental stages (Stages), midge body parts or tissue groups (Body), and female midges injected with vesicular stomatitis virus (VSV) or bluetongue virus (BTV).

DsRNA Treatments

Comprehensive rankings for the three RNAi experiments are reported in Table 2.2. We examined whether there would be a difference in the stable gene ranking between cells treated with dsRNA against an exogenous RNAi target (*dseGFP*) and an endogenous target (*dsVhaA*). The *VhaA* target was selected for these experiments due to considerable cell death (and thus low cellular material) that occurs after *dsIAP1* treatment. As expected, *VhaA* was the least stable of the genes tested in this experiment as it was the target for RNAi-based transcript suppression. Overall, the two most stable genes for water- or *dseGFP*-treated cells were *GAPDH* and *β-tubulin*, and the two for *dseGFP*- or *dsVhaA*-treated cells were *EF1b* and *GAPDH* (Table 2.2). The most stable gene in the larval RNAi experiment was *VhaA*. Our findings suggest *β-tubulin*

and *EF1b* were the next most stable genes in that order. These gene ranks were inverted in the RefFinder comprehensive ranking (Table A.2).

Table 2.2. Data output from RefFinder, NormFinder, geNorm, and BestKeeper showing key values used to determine stability, and the rankings (from 1 highest stability to 6 lowest stability) of each candidate reference gene evaluated in three RNAi experiments in *Culicoides sonorensis* cultured cells and larvae.

*These experiments contained samples treated with ds*VhaA*. The top two candidate genes share the same Avg. M values in the geNorm output. Underlined values are above the 1.0 threshold for recommended use. Abbreviations: M, stability measure; SD, standard deviation; CP, crossing point (i.e., Ct value), Geo Mean, Geometric mean; *β-tubulin*, beta-tubulin; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *RPS18*, ribosomal protein S18; *VhaA*, vacuolar-type ATPase subunit A; *EF1b*, elongation factor 1-beta.

	RefFinder		NormFinder		geNorm		BestKeeper					Overall	
Gene	Delta Ct	Rank	Stability Value	Rank	Avg. M	Rank	SD [± CP]	r	p-value	power [x-fold]	Rank	Geo Mean	Rank
Cells treated with water or ds <i>eGFP</i>													
<i>Actin</i>	0.106	6	0.08	6	0.087	6	0.24	0.970	0.001	2.01	4	5.42	6
<i>β-tubulin</i>	0.075	1	0.04	1	0.070	4	0.27	0.999	0.001	2.09	6	2.21	2
<i>GAPDH</i>	0.084	2	0.05	3	0.047	1	0.22	0.990	0.001	1.83	1	1.57	1
<i>RPS18</i>	0.087	5	0.05	2	0.078	5	0.27	0.988	0.001	2.05	5	3.98	5
<i>EF1b</i>	0.086	4	0.06	5	0.047	2	0.23	0.982	0.001	1.89	2	2.99	3
<i>VhaA</i>	0.085	3	0.05	4	0.065	3	0.23	0.986	0.001	1.96	3	3.22	4
Cells treated with ds <i>eGFP</i> or ds <i>VhaA</i>													
<i>Actin</i>	0.682	3	0.37	2	0.181	4	0.32	0.800	0.002	1.87	4	3.13	4
<i>β-tubulin</i>	0.947	5	0.61	5	0.334	5	0.51	0.819	0.001	2.83	5	5.00	5
<i>GAPDH</i>	0.677	2	0.41	4	0.117	2	0.23	0.787	0.002	1.50	1	2.00	2

<i>RPS18</i>	0.687	4	0.38	3	0.134	3	0.24	0.687	0.014	1.41	2	2.91	3
<i>EF1b</i>	0.655	1	0.31	1	0.117	1	0.24	0.857	0.001	1.56	3	1.32	1
<i>VhaA*</i>	2.31	6	1.66	6	0.993	6	<u>1.67</u>	0.461	0.132	3.73	6	6.00	6
<i>L₃ and L₄ larvae treated with dseGFP or dsIAP1</i>													
<i>Actin</i>	0.254	5	0.13	2	0.233	6	0.27	0.900	0.001	3.02	6	5.48	6
<i>β-tubulin</i>	0.217	3	0.09	3	0.172	2	0.15	0.777	0.003	1.62	1	2.06	2
<i>GAPDH</i>	0.266	6	0.13	6	0.215	5	0.19	0.679	0.015	1.71	4	5.18	5
<i>RPS18</i>	0.242	4	0.11	4	0.190	4	0.21	0.785	0.002	1.99	5	4.23	4
<i>EF1b</i>	0.201	2	0.07	2	0.180	3	0.17	0.862	0.001	1.87	2	2.21	3
<i>VhaA</i>	0.193	1	0.07	1	0.172	1	0.19	0.897	0.001	2.00	3	1.32	1

Life Stages and Body Parts and Tissue Groups

Comprehensive rankings for the life history and body parts or tissue groups studies are reported in Table 2.3. The consensus ranking from this study and RefFinder found *actin* and *VhaA* to be the most stable candidates when comparing *C. sonorensis* developmental stages (Table A.2). *GAPDH* was the least stable, and the BestKeeper reported standard deviation (SD) value was above the acceptable threshold (Table 2.3). Our body part and tissue group study found four of the six candidate genes were unacceptable when using the BestKeeper SD cutoff. Only *actin* and *VhaA* were deemed acceptable according to the BestKeeper SD cutoff, but the overall ranking based on all analyses for *VhaA* was fourth. In contrast, even though the BestKeeper SD was greater than one for *EF1b*, *EF1b* and *actin* were ranked as first and second most stable in the overall rankings, respectively. The tissue study also found *RPS18* to be the worst candidate, and the Ct values for this gene were the most variable of those examined (Fig. 2.1).

Table 2.3. Data output from RefFinder, NormFinder, geNorm, and BestKeeper showing key values used to determine stability, and the rankings of each candidate reference gene evaluated in six developmental stages and four female body parts or tissue groups of *Culicoides sonorensis*.

The top two candidate genes share the same Avg. M values in the geNorm output. Underlined values are above the 1.0 threshold for recommended use. Abbreviations: M, stability measure; SD, standard deviation; CP, crossing point (i.e., Ct value), Geo Mean, Geometric mean; *β-tubulin*, beta-tubulin; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *RPS18*, ribosomal protein S18; *VhaA*, vacuolar-type ATPase subunit A; *EF1b*, elongation factor 1-beta.

	RefFinder		NormFinder		geNorm		BestKeeper					Overall	
Gene	Delta Ct	Rank	Stability Value	Rank	Avg. M	Rank	SD [± CP]	r	p-value	power [x-fold]	Rank	Geo Mean	Rank
Developmental Stages													
<i>Actin</i>	0.765	1	0.12	1	0.487	3	0.31	0.887	0.001	1.96	1	1.32	1
<i>β-tubulin</i>	1.071	5	0.71	5	0.652	4	0.67	0.441	0.068	2.05	4	4.47	5
<i>GAPDH</i>	1.681	6	1.41	6	1.061	6	<u>1.06</u>	-0.018	0.945	0.96	6	6.00	6
<i>RPS18</i>	0.99	3	0.66	4	0.331	2	0.77	0.457	0.056	2.11	5	3.31	3
<i>EF1B</i>	1.001	4	0.62	3	0.750	5	0.63	0.836	0.001	3.76	3	3.66	4
<i>VhaA</i>	0.855	2	0.5	2	0.331	1	0.59	0.624	0.006	2.29	2	1.68	2
Female Body Parts and Tissue Groups													
<i>Actin</i>	1.487	3	0.9	3	0.525	1	0.86	0.960	0.001	1.48	2	2.06	2
<i>β-tubulin</i>	1.813	5	0.98	4	1.118	5	<u>1.86</u>	0.932	0.001	2.27	5	4.73	5
<i>GAPDH</i>	1.421	2	0.37	1	0.801	4	<u>1.66</u>	0.984	0.001	2.01	4	2.38	3

<i>RPS18</i>	3.345	6	2.21	6	1.861	6	<u>3.63</u>	0.963	0.001	4.73	6	6.00	6
<i>EF1B</i>	1.396	1	0.76	2	0.525	2	<u>1.15</u>	0.957	0.001	1.66	3	1.86	1
<i>VhaA</i>	1.702	4	1.3	5	0.623	3	0.70	0.880	0.001	1.33	1	2.78	4

Virus Infections

Comprehensive rankings for the two virus injection studies are reported in Table 2.4. The top three candidate reference genes for both virus experiments tested were *EF1b*, *actin*, and *RPS18*. Our analyses found *EF1b* to be the most stable in both experiments. *RPS18* was the second most stable for VSV-infected midges, and *actin* was the second most stable for BTV-infected midges. Injecting virus into midges was chosen over the more natural route of feeding virus on virus-spiked blood. A previous study found significant variability in virus infection rates when *C. sonorensis* fed on virus-spiked blood in an artificial system (Rozo-Lopez et al. 2021). Virus injection is often preferred to ensure consistent, high levels of infection in *C. sonorensis*; however, it induces higher levels of trauma. We determined the infection rates were more important to the study's outcome and opted for this method.

Table 2.4. Data output from RefFinder, NormFinder, geNorm, and BestKeeper showing key values used to determine stability, and the rankings of each candidate reference gene evaluated in *Culicoides sonorensis* females injected with vesicular stomatitis virus (VSV) and media controls, and bluetongue virus (BTV) and media controls.

The top two candidate genes share the same Avg. M values in the geNorm output. Abbreviations: M, stability measure; SD, standard deviation; CP, crossing point (i.e., Ct value), Geo Mean, Geometric mean; *β-tubulin*, beta-tubulin; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *RPS18*, ribosomal protein S18; *VhaA*, vacuolar-type ATPase subunit A; *EF1b*, elongation factor 1-beta.

	RefFinder		NormFinder		geNorm		BestKeeper					Overall	
Gene	Delta Ct	Rank	Stability Value	Rank	Avg. M	Rank	SD [± CP]	r	p-value	power [x-fold]	Rank	Geo Mean	Rank
VSV-Injected Females													
<i>Actin</i>	0.204	3	0.13	3	0.085	1	0.69	0.992	0.001	2.07	4	2.45	3
<i>β-tubulin</i>	0.234	5	0.16	5	0.143	5	0.80	0.995	0.001	2.19	6	5.23	6
<i>GAPDH</i>	0.458	6	0.36	6	0.248	6	0.37	0.927	0.008	1.56	1	3.83	4
<i>RPS18</i>	0.188	2	0.06	2	0.085	2	0.66	0.997	0.001	1.95	2	2.00	2
<i>EF1b</i>	0.185	1	0.03	1	0.106	3	0.68	0.999	0.001	2.00	3	1.73	1
<i>VhaA</i>	0.221	4	0.16	4	0.133	4	0.79	0.995	0.001	2.18	5	4.23	5
BTV-Injected Females													
<i>Actin</i>	0.184	2	0.02	2	0.110	2	0.05	0.733	0.098	1.27	1	1.68	2
<i>β-tubulin</i>	0.206	4	0.07	4	0.113	3	0.12	0.591	0.218	1.63	4	3.72	4
<i>GAPDH</i>	0.277	5	0.12	5	0.177	5	0.19	0.641	0.170	2.48	5	5.00	5

<i>RPS18</i>	0.199	3	0.06	3	0.136	4	0.10	0.725	0.102	1.83	2	2.91	3
<i>EF1b</i>	0.167	1	0.02	1	0.110	1	0.12	0.954	0.003	2.10	3	1.32	1
<i>VhaA</i>	0.325	6	0.15	6	0.226	6	0.21	0.596	0.213	2.73	6	6.00	6

Optimal Number of Reference Genes

The geNorm package reports pairwise variation scores (V scores) to inform the number of genes to use for optimal normalization. Scores above the suggested 0.15 cutoff suggest that another gene needs to be added in experimental analysis (Vandesompele et al. 2002). None of the V scores in this study reached the 0.15 cutoff. The highest V score among all the experiments was 0.02785, which is well below the cutoff value (Table A.3). These results suggest that just two reference genes are sufficient to yield accurate results for all the conditions examined here.

Discussion

This study provides a comprehensive examination of candidate reference genes for analysis of gene expression in *C. sonorensis*. We included five different functional classes of genes and examined their stability in RNAi studies, midge developmental stages, female midge body parts and tissue groups, and with and without virus infections. We excluded teneral, sugar-fed, and blood-fed females from this study as these conditions were examined in a previous transcriptome study (Nayduch et al. 2014a). It is widely accepted that at least two reference genes are necessary for adequate normalization when performing relative quantification of transcript levels (Bustin et al. 2009). Our results suggest that including only two reference genes is suitable for the conditions we tested based on pairwise V scores. Future studies can use the most stably expressed genes described here when normalizing gene expression across similar treatments or evaluate new reference genes using the analysis tools described here.

Our study evaluated two genes that have previously been studied as candidate reference genes. First, *EF1b* was used as a reference gene for viral infection studies, and our findings support this as the most stable gene for these analyses, albeit with a similar but not identical virus

(Mills et al. 2017b, Nayduch et al. 2019). Another study used *EF1b* as a reference gene for tissue-specific expression of antimicrobial peptide genes (Nayduch et al. 2014b). Second, a V-ATPase subunit was examined for stability among four other candidate reference genes (Morales-Hojas et al. 2018). The authors found V-ATPase unsuitable for inclusion in their study as it ranked poorly against the others when examined with BestKeeper, geNorm, and NormFinder. Of note, that study appears to have used the gene coding subunit C, whereas our study used the gene for the A subunit. *VhaA* was in the top two most stable reference genes in two of our experiments (developmental stages and larvae treated with *dsIAP1*).

Female midge body parts and tissue groups were the most inconsistent samples tested for stable gene expression. The distribution of Ct values from these samples was broad for *β-tubulin*, *GAPDH*, *RPS18*, and *EF1b* (Fig. 2.1). Unsurprisingly, these four genes were flagged as unacceptable in the BestKeeper software (Table 2.3). The two remaining genes, *VhaA* and *actin*, were below the suggested cutoff threshold and are, therefore, the only two candidate genes acceptable for female midge tissues studies using the BestKeeper criteria. These insects are incredibly small, just 2 mm long, and dissecting them for individual tissues can be challenging. While every effort was made to preserve the integrity of the samples, it is possible that contaminants from other tissues were included in pooled tissue samples. Evaluating additional reference genes and examining more tissue types is warranted, given our findings.

We found that some of the spreadsheet-based tools could not be used on some computers (e.g., NormFinder), and that one hosted on a website had discontinued support (e.g., GeNorm). Both of these tools have been adapted for use in R and we successfully ran scripts provided by developers (geNorm, <https://search.r-project.org/CRAN/refmans/ctrlGene/html/00Index.html>; NormFinder, <https://www.moma.dk/software/normfinder>). The NormFinder package for R also

offers flexibility in using raw Ct or linearly transformed values. Access and ease of use of these tools are critical for producing accurate gene expression stability data.

The RefFinder website provides a simple user interface for examining stable expression among candidate reference genes using only raw Ct values. RefFinder generates a report for the other analysis tools (NormFinder, BestKeeper, geNorm) and gives a stability rank for each gene submitted. Additionally, the website incorporates a comparative delta Ct analysis, yielding a fourth stability ranking. The geometric mean of each rank is used to create a comprehensive stability rank for the submitted genes. However, the tool's input field does not use transformed data (e.g., as in NormFinder), nor does it have an input for primer efficiencies (e.g., as in BestKeeper). We examined how the RefFinder ranks compared to those generated in our analyses and reported the findings in Table A.2. Our dsRNA-treated cell experiments, developmental stage analysis, body part and tissue group analysis, and BTV-injected females experiment had identical comprehensive rankings. The remaining two experiments, dsRNA-treated larvae and VSV-injected females, had two inverted and one inverted gene ranks, respectively. The gene rank disagreement in VSV-injected females did not alter which genes are recommended for normalization (i.e., *RPS18* and *EF1b*). Taken together, these findings suggest that the RefFinder website tool alone may be adequate for preliminary stable reference gene stability analyses.

Around the world, *Culicoides* are a significant threat to livestock production and, in some instances, human health. Ongoing and future research will rely on the use of reference genes in gene expression studies to examine vector competency, behavior, RNAi-based gene suppression, and other studies of midge biology and ecology. We have found only a handful of studies that have evaluated reference genes for differential gene analysis in *C. sonorensis*. This species will

continue to play a prominent role as a model for numerous studies given that it is one of the few midge taxa that can be colony reared. Each of the six candidate genes examined in this study appear at least once as the most stable gene among all of our experimental conditions. This finding indicates that there is no singular reference gene, or set of genes, which could be considered universal for all *C. sonorensis* experiments. In fact, no universal reference genes demonstrating stable expression across species, developmental stages, tissues, and experimental conditions have been identified in any species to date, reinforcing the need to validate reference gene expression stability before every RT-qPCR experiment (Lu et al. 2018, Shakeel et al. 2018).

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Chapter 3 - Examining the efficiency of RNAi in cultured cells and larvae of the biting midge *Culicoides sonorensis* (Diptera: Ceratopogonidae)

Abstract

Culicoides sonorensis are small biting midges which are vectors of livestock pathogens in the United States. The pathogens transmitted by midges can lead to poor herd animal health with significant economic impacts for livestock producers. RNA interference (RNAi) is a mechanism where target transcripts are suppressed after induction with double-stranded RNA (dsRNA). This technique is an important tool for unraveling insect genetics, determining physiological processes, and even in pest control applications. RNAi responses can be highly variable in different insects and remain poorly characterized in *C. sonorensis*. The objectives of this study were to examine the responses of cultured *C. sonorensis* cells and late-stage larvae to dsRNA treatment, as well as the expression of core RNAi machinery in cultured cells and insect developmental stages. Larvae responded poorly to dsRNA ($\leq 20\%$ suppression) when compared to cells (approx. 90% suppression) even at 10 times the dsRNA concentration. Transcript levels of four RNAi-associated proteins were determined by RT-qPCR in *C. sonorensis* W8A cells and six developmental stages with varying expression profiles for each gene and stage examined. A dsRNA-binding protein of interest, Staufén homolog, was expressed significantly lower in fourth instar larvae when compared to other life stages, but the implications for this disparity remain to be investigated. These results show differential RNAi efficiency between *C. sonorensis* cells and

larvae and will inform future studies which seek to use RNAi for genetic studies and insecticide development.

Introduction

Culicoides sonorensis Wirth & Jones (Diptera: Ceratopogonidae) are very small biting midges which are primarily pests of livestock animals and important vectors of livestock pathogens (Mullen and Murphree 2019). Examples of *C. sonorensis* transmitted viruses include bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), and vesicular stomatitis virus (VSV), which are concerns primarily for cattle, sheep, deer, and horses (McVey et al. 2015, Rozo-Lopez et al. 2018, McGregor et al. 2022). This species is one of the few to have been successfully established in colony and it will continue to be an important model for *Culicoides* spp. research in other important vector species around the world (Nayduch et al. 2014b, Purse et al. 2015). These studies include determining factors influencing vector competency, sensory mechanisms and their effects on behavior, and methods for controlling pest populations.

One important tool for understanding gene and cellular function is RNA interference (RNAi). This conserved mechanism in insects refers to the processing of small RNA molecules which leads to the degradation of larger RNA molecules, reviewed in (Zhu and Palli 2020). The short/small interfering RNA (siRNA) pathway processes exogenous dsRNA molecules such as those from viral infection or designed molecules introduced by researchers. Briefly, a core set of proteins and enzymes process exogenous dsRNA molecules in the cytosol of the cell beginning with the enzyme Dicer-2 (Dcr2) and the dsRNA-binding protein, R2D2. The dsRNA is cleaved into 19-21 bp siRNA products, which are loaded into a complex of proteins termed the RISC (RNA-induced silencing complex). Within the RISC, a single guide strand of the siRNA is

retained. When bound to complimentary mRNA, the enzyme Argonaute-2 (Ago2) destroys the mRNA molecule. Barriers to effective RNAi responses in insects include the low expression of core RNAi machinery (i.e., Dcr2, R2D2, Ago2), among other potential mechanisms such as dsRNA uptake, enzymatic degradation of dsRNA, and systemic spread of the RNAi signal (Cooper et al. 2019). Previous work has shown that both *C. sonorensis* cells (Schnettler et al. 2013) and adult female midges (Mills et al. 2015) produce RNAi responses upon treatment with dsRNA, and transcripts of the core components of the RNAi machinery are expressed in adult female midges (Nayduch et al. 2014a, Nayduch et al. 2019). However, RNAi mechanisms have yet to be well characterized in *C. sonorensis*.

Traditional strategies for controlling *Culicoides* include reducing larval habitats, and chemical treatments of livestock, livestock housing units, and larval habitats (Pfannenstiel et al. 2015). While modestly effective in reducing midge populations, these efforts may not suppress populations enough to reduce pathogen spread amongst herds (Carpenter et al. 2008). Traditional chemical insecticides can also have detrimental effects on non-target organisms. Environmentally safer control techniques, such as essential oils and bacterial toxins (e.g., *Bacillus thuringiensis*, *Bt*), have been examined with mixed results (Benelli et al. 2017, Meloni et al. 2018, Foxi et al. 2019). RNAi-based insecticides for *Culicoides* control have not yet been examined, but their use in mosquitos is a potentially promising and active area of investigation (Munawar et al. 2020). These products are expected to be both highly specific and environmentally benign when compared with traditional methods. While a promising advance for insect control, much of the foundational work on RNAi in *C. sonorensis* needs to be examined.

This study examines RNAi responses in *C. sonorensis*, with a focus on cultured cells and late larval instars. The efficiency of RNAi responses was examined in cultured cells and 3rd and 4th instar larvae using two targets (vacuolar-type ATPase subunit A, *VhaA*; inhibitor of apoptosis protein 1, *IAP1*). We examined the expression of core RNAi proteins and enzymes in cells and six developmental midge stages (and 3rd and 4th instar larvae, male and female pupae, and male and female adults). We also briefly examined the expression and role of a dsRNA-binding protein, Staufén (Stau). The results suggest that larvae are less susceptible to target suppression by RNAi when compared to cultured cells, and that the high expression of *Ago2* in cells could be a factor. While its function was not well characterized here, transcript levels of a Staufén homolog were found to be dramatically reduced in 4th instar larvae. Our findings suggest that mechanisms other than limited expression of core RNAi machinery are responsible for the low efficiency of RNAi responses in *C. sonorensis* larvae and more work is necessary to characterize other potential mechanisms limiting RNAi responses.

Materials and Methods

Cultured Insect Cells and Developmental Stages

Cultured *C. sonorensis* cells (W8A line), larvae, pupae, and adults (AK line, actively maintained in colony) were sourced from the USDA-ARS laboratories in Manhattan, Kansas (Center for Grain and Animal Health Research). Cells were grown at 28 °C in Schneider's Insect Media (Sigma-Aldrich, St. Louis, MO, USA) modified as previously described (Chapter 2). When reared for experiments, insects were maintained at 25 °C, ~70% relative humidity, and with a 14:10 light:dark cycle in an incubator.

DsRNA Synthesis

Three targets and one control for RNAi-based knockdown were selected (Table B.1). Endogenous target dsRNA was synthesized using template cDNA pooled from multiple *C. sonorensis* developmental stages, and control dsRNA (*eGFP*) was synthesized from a plasmid template. Methods for dsRNA synthesis and purification are described in (Chapter 2).

Cells treated with dsRNA

Cultured W8A cells were seeded on 6-well or 12-well cell culture plates and grown in 1 mL media to ~75% confluence before being treated with dsRNA. In dose response studies, cells were treated with variable concentrations of *dsIAP1* (1 ng/mL – 1 µg/mL), variable concentrations of *dsVhaA* (100 ng/mL – 10 µg/mL) or control *dseGFP* (1 µg/mL). Each treatment or control was replicated in triplicate. Cells treated with *dsIAP1* were imaged 24 h post treatment (hpt) to observe cytopathic effects (CPE). At 48 hpt, cell media was removed by vacuum aspiration and 400 µl of TRIzol reagent was added to the wells for 5 min to lyse cells. The lysed cells in TRIzol were transferred to 1.5 mL tubes for total RNA isolation following the manufacturer's recommendations.

Larvae Treated with dsRNA

Pools of larvae (20 individuals) were treated with dsRNA in wells of cell culture plates. Here, 3 biological replicates each of third (L₃) and fourth (L₄) instar larvae were treated with 10 µg/mL *dsIAP1*, 10 µg/mL *dsVhaA*, or 10 µg/mL *dseGFP* in 1 mL of nuclease-free water. Anecdotally, larvae were observed to be long-lived without food and that cannibalism was not a major concern for losing individual larvae. Larvae were harvested 72 hpt by placing the total

pool of larvae into a single 1.5 mL tube and homogenizing the larvae with a tissue pestle in 400 µl of TRIzol. Bulk insect tissue was separated from the liquid portion by centrifugation at 16,000 x g for 5 min, and the liquid portion was moved to a new, clean 1.5 mL for extraction of total RNA.

Developmental Stage Collection

Pools of 20 individual L₃, L₄, male pupae, female pupae, adult males, or adult females were harvested directly into 400 µl of TRIzol. These collections were made in triplicate for each stage and processed as previously described (Chapter 2).

RNA Extraction and cDNA Synthesis

Total RNA was isolated from samples and eluted in 50 µl of nuclease-free water. 1 µg of RNA was used to synthesize cDNA for all samples. The methods have been previously described (Chapter 2).

Phylogenetic Analysis

The hypothetical amino acid sequence for Staufen was determined using the Expasy Translate tool (<https://web.expasy.org/translate/>, Swiss Institute of Bioinformatics). The top 20 related sequences to the Staufen translated amino acid sequence were obtained from BLAST-x (National Center for Biotechnology Information, NCBI, <https://blast.ncbi.nlm.nih.gov>). These 20 sequences and the *C. sonorensis* Staufen sequence were aligned in MEGA X (Stecher et al. 2020) using MUSCLE. The best model prediction was determined using the “Find Best

DNA/Protein Model” tool. A maximum likelihood tree was constructed using the LG+G model, as it was the highest rated model.

Real-time Quantitative PCR

Primers for RT-qPCR were designed for *Dcr2*, *Ago2*, two *R2D2* genes, and a Staufen homolog in this study (Table B.2). Primers for *IAP1* were previously described in Mills et al. (2015) and for *VhaA* in Chapter 2 and are reported in Table B.2. Six *C. sonorensis* reference genes have previously been described, and five were used in this study (Chapter 2). *Actin*, *EF1b*, and *GAPDH* were used as reference genes for studies where cells were treated with dsRNA; β -*tubulin* and *EF1b* were reference genes for studies where larvae were treated with dsRNA; and *actin* and *VhaA* were used in gene of interest expression studies in cells and insect developmental stages. Two technical replicates were performed for each sample, and all quantifications were performed as previously described (Chapter 2).

Data Analysis

Technical replicate Ct values were averaged for analysis. No-template controls had no amplification or had Ct values above 35 which were considered negligible. Gene expression was analyzed one of two ways, which are noted in the legends of each figure. The delta-Ct (Δ Ct) method was used to show relative gene expression normalized to the geometric mean of two reference genes for cell and developmental stage expression profiles. The delta-delta-Ct ($\Delta\Delta$ Ct) method was used to calibrate normalized gene expression to either a control group or insect stage, again using two reference genes to determine normalized expression. Normalized relative expression or fold change were used for statistical analysis. Data were analyzed in Prism 9

(Version 9.5.1, GraphPad Software, LLC, Boston, MA). Datasets which met the criteria for normality by the Shapiro-Wilk test and equal variance by the Brown-Forsythe test were evaluated with a one-way ANOVA and Tukey's multiple comparisons *post hoc* test. Datasets which did not meet these requirements were evaluated by a Kruskal-Wallis one-way ANOVA with a Dunn's multiple comparisons *post hoc* test. Unpaired, two-tailed t tests were used for experiments which compared two groups, and which met the criteria for normality and equal variance as above. Statistical significance levels were set to 0.05.

Results

RNAi in Cultured *C. sonorensis* Cells

Cultured *C. sonorensis* W8A cells treated with varying concentrations of *dsIAP1* responded to higher dsRNA concentrations with increased target suppression (Fig. 3.1A). Appreciable CPE were observed in cells treated with 1 µg/mL *dsIAP1* compared to control cells (Fig. 3.1B and C). Treatment with *dsIAP1* (1µg and 100 ng) suppressed target transcript levels by ~50%, but this response was not statistically significant despite the noticeable CPE ($F = 3.063$; $df = 4, 10$; $P=0.0688$). The CPE, however, reduced the number of cells in culture resulting in more limited RNA yields which may have affected our results.

Because of the CPE caused by *dsIAP1*, we also tested a second target that did not cause CPE. Here, *dsVhaA* treated cells had target suppression of approximately 74% for the 100 ng/mL treatment, and greater than 90% for the 1 µg/mL and 10 µg/mL treatments when compared to the control (Fig. 3.1D). Only the 10 µg/mL treatment was statistically significant ($P = 0.0134$) when examined with a Dunn's multiple comparison test. These results show cultured *C. sonorensis*

cells respond to dsRNA treatment in as little as 24 h (CPE in *dsIAP1* treated cells) and with concentrations as low as 100 ng/ μ L yielding 74% target suppression after 48 h (*dsVhaA* in cells).

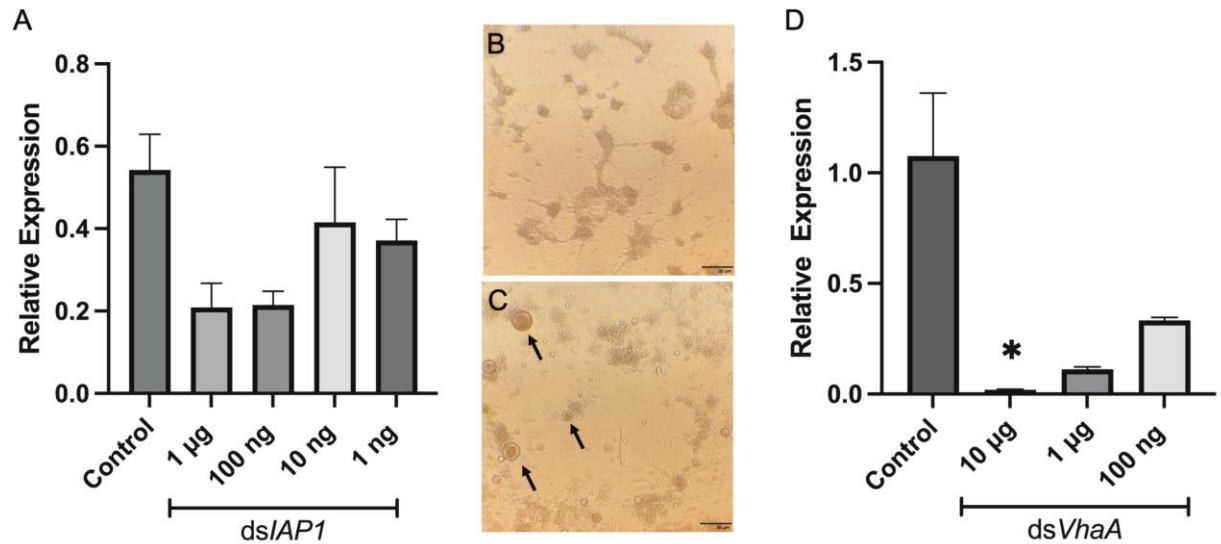


Figure 3.1. RNA interference (RNAi) responses to dsRNA in cultured W8A *C. sonorensis* cells.

A) Relative (fold change) expression of *IAP1* after treatment with multiple concentrations of *dsIAP1* or control *dseGFP* (1 μ g) in 1 mL cell culture media (i.e., 1 μ g/mL) for 48 h. Means with SEM are shown. Phenotype of W8A cells 24 h after treatment with B) 1 μ g/mL of *dseGFP* or C) 1 μ g/mL *dsIAP1*. Arrows show examples of cytopathic effects (CPE). D) Cells treated with variable concentrations of *dsVhaA* or control *dseGFP* (1 μ g) in 1 mL cell culture media (i.e., 1 μ g/mL) for 48 h. * $P=0.0134$ by Dunn's multiple comparison test.

RNAi in *C. sonorensis* Larvae

Instar 3 and 4 larvae were treated with 10 μ g of dsRNA targeting either *IAP1* or *VhaA* and a control (*dseGFP*). A slight, yet non-significant suppression of the *IAP1* target was appreciable in both L₃ ($t = 0.4195$; $df = 4$; $P = 0.6964$) and L₄ ($t = 1.049$ $df = 4$; $P = 0.3534$) larvae (Fig. 3.2A). By comparison, there was significant suppression of *VhaA* transcript levels in L₄ larvae ($t = 5.977$; $df = 4$; $P = 0.0039$) but only to about 83% of the control (Fig. 3.2B). The response in L₃ larvae was non-significant ($t = 0.9052$; $df = 4$; $P = 0.4166$).

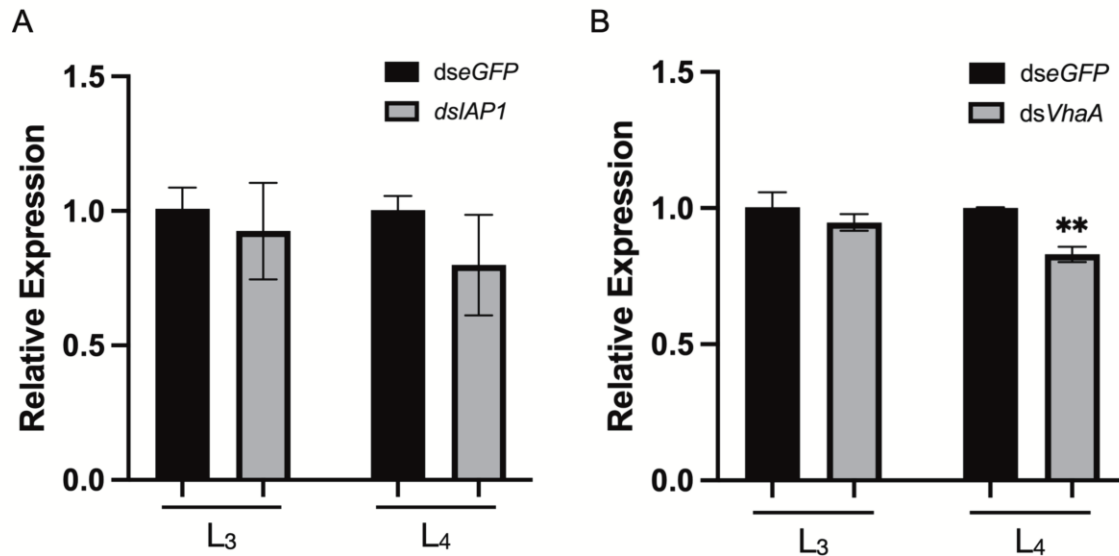


Figure 3.2. Relative (fold) expression of target genes after attempted RNAi knockdown in *C. sonorensis* third (L3) and fourth (L4) instar larvae.

Means with SEM are presented. Pools of 20 larvae were treated with 10 μ g of control (*dseGFP*) or treatment (B. *dsIAP1*, C. *dsVhaA*) dsRNA in 1 mL of nuclease-free water for 72 h. Only *dsVhaA* was significantly decreased in L4 larvae (** $P=0.0039$ by t test).

Core RNAi Machinery in Cultured Midge Cells and Life Stages

The transcript levels of *Dcr2*, *Ago2*, and two *R2D2* targets were examined in untreated W8A cells. Here, *Dcr2* had the lowest transcript levels, *Ago2* was the highest, and both *R2D2* targets had comparable transcript levels (Fig. 3.3A). We also measure the transcript levels of these targets in L3, L4, male and female pupae, and adult male and female midges. *Dcr2* exhibited the lowest transcript levels in the larval stages and highest in the adult stages (Figure 3.3B-G). In contrast, transcript levels of *Ago2* were highest in the larvae and lowest in the pupal and adult stages (Figure 3.3B-G). Both *R2D2* targets had similar transcript profiles in all life stages and were only significantly different from each other in female midges ($P = 0.0458$). Overall, *Ago2* was expressed highest in cells.

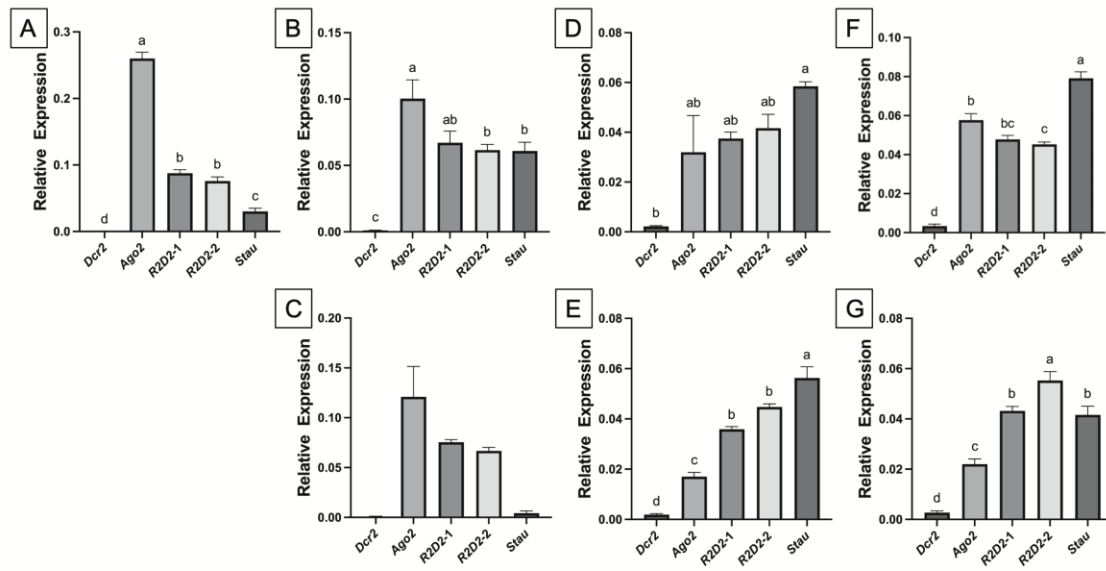


Figure 3.3. Relative expression of core RNAi genes and associated proteins in W8A cells and insect developmental stages.

Expression was determined by the ΔC_t method to two reference genes. Shown are means with SEM and lowercase letters indicate levels of statistical significance. Panels are A) cultured W8A cells, B) L₃ larvae, C) L₄ larvae, D) male pupae, E) female pupae, F) adult males, and G) adult females. Statistics are reported in Table B.3.

Staufen homolog in *C. sonorensis*

A dsRNA-binding Staufen homolog was found in a previously published *C. sonorensis* transcriptome (Nayduch et al. 2014a). The top 20 closest sequences to the predicted amino acid sequence were determined using BLAST-x (<https://blast.ncbi.nlm.nih.gov/>). These related sequences included Staufen homolog 2 (*Stau-2*) in termites, true bugs, and beetles, as well as unnamed or hypothetical proteins from other groups including cockroaches, flies, mayflies, crickets, and stick insects. The closest related sequence was from *Polypedilum vanderplanki* (GenBank: KAG5679232.1), a chironomid midge (Fig. B.1). In cells, *Stau* transcript levels were significantly lower than those of *Ago2* and both *R2D2* targets, but significantly higher than *Dcr2* (Fig. 3.3A). In the developmental stages, the *Stau* transcript levels were drastically lower in L₄

larvae compared to all other life stages (Fig. 3.3B-G). The expression ratios are *Stau* and the four core RNAi genes examined here are supported in Table B.4. We examined if dsRNA would trigger a noticeable change in expression of *Stau* in cultured cells (Fig. B.2), but there was no significant difference after 48 h of exposure ($F = 0.38131$; $df = 3, 8$; $P = 0.7694$).

To evaluate the role this protein may have in RNAi efficiency we examined if its suppression would decrease the sensitivity of cells to treatment with dsRNA. Treatment of W8A cells with 1 $\mu\text{g/mL}$ of ds*Stau* for 48 h resulted in significant suppression ($P < 0.001$) of *Stau* transcript levels (Fig. 3.4A). Subsequently, cells were treated with a priming dose of 1 $\mu\text{g/mL}$ dsRNA (*dseGFP* or *dsStau*) and a treatment dose of 1 $\mu\text{g/mL}$ target dsRNA (*dseGFP* or *dsIAP1*) 24 h later and the effect on target transcript levels were assessed. The suppression of the target (*IAP1*) was approx. 25% when pretreated with *dseGFP*, but that response was reduced to 17% when primed with ds*Stau* (Fig. 3.4B). While these values were significantly different from the control, ($P = 0.0329$ and $P = 0.0057$ for pretreatment with *dseGFP* or ds*Stau*, respectively), they did not significantly differ from one another ($P = 0.3094$).

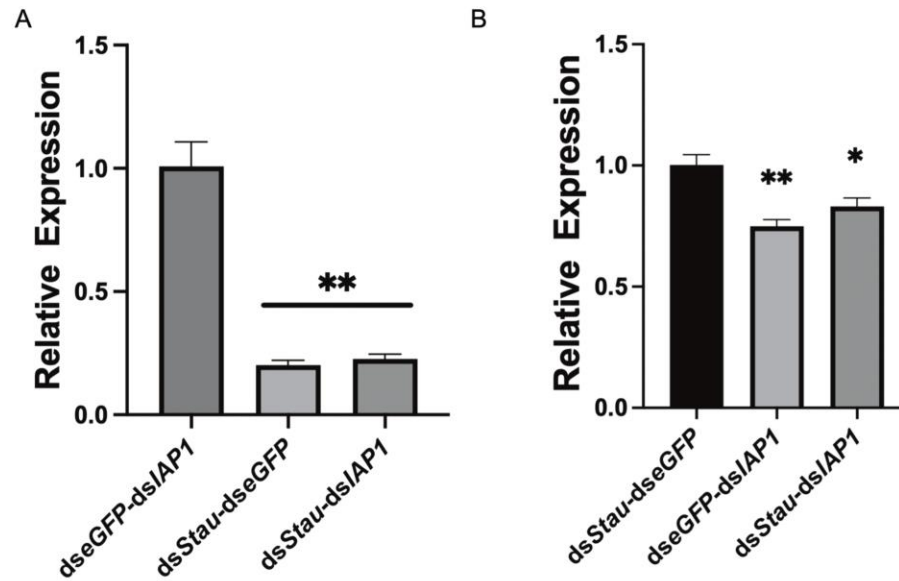


Figure 3.4. Relative (fold) expression of target genes after attempted RNAi knockdown in *C. sonorensis* W8A cells.

Cells were treated with a 1 $\mu\text{g}/\text{mL}$ priming dose of dsRNA, a 1 $\mu\text{g}/\text{mL}$ treatment dose 24 h later, and were harvested 24 h after the treatment dose. Notation is “Priming-Treatment.” Means with SEM are presented. A) Significant knockdown of *Stau* (** $P < 0.001$). B) *IAP1* knockdown efficiency is reduced slightly in cells with knocked-down *Stau* (* $P = 0.0329$) compared to those without *Stau* knockdown (** $P = 0.0057$).

Discussion

This study examines the RNAi response in cultured cells and late-larval stages of *C. sonorensis* using traditional RNAi-based gene knockdown. Treatment of W8A cells with three different dsRNA constructs (*IAP1*, *VhaA*, and *Stau*) caused greater than 70% suppression using dsRNA concentrations of 1 $\mu\text{g}/\text{mL}$ for ds*Stau* and 100 ng/mL for ds*VhaA*. However, larvae soaked with 10 $\mu\text{g}/\text{mL}$ of dsRNA for 72 h showed only modest target suppression ($\leq 20\%$). In contrast to our soaking experiments, Mills et al. (2015) observed ~45% target suppression when adult females were injected with 150 ng ds*IAP1* intrathoracically, a concentration that was 10- to 100-fold lower than our soaking experiments. Whereas these data suggest that RNAi pathways

are active in *C. sonorensis* and can process applied dsRNAs to produce suppression of target transcripts, it is likely that physiological differences between larvae and adults in response to treatment with dsRNA as well as the different delivery methods are likely responsible for differences in RNAi efficiency between life stages. These discrepancies have been observed in *Drosophila* larvae versus adults (Miller et al. 2008), and between injection versus ingestion in weevils (Prentice et al. 2017), for example, with many more studies extensively reviewed in Cooper et al. (2019). In the former study, most *Drosophila* tissues were refractory to RNAi after injection of larvae which was attributed to the lack of dsRNA uptake by most cell types. In the latter study, oral ingestion of dsRNA was less effective than by injection in the same organism which was attributed to enzymatic degradation of the treatment in the insects' guts. The mechanisms for the observed disparities in *C. sonorensis* RNAi efficiency *in vitro* and with different delivery methods *in vivo* still require further investigation.

Previous reviews of RNAi in insects have postulated several mechanisms that can limit RNAi efficiency, including dsRNA degradation by nucleases, poor uptake of dsRNA into cells, low core RNAi machinery expression, and lack of systemic RNAi (Cooper et al 2019). Here we examined whether differential expression of the core RNAi machinery may be responsible for the variation in RNAi efficiency observed between *in vitro* and *in vivo* experiments. This, however, does not appear to be the cause as the core proteins and enzymes of the siRNA pathway are consistently expressed in cultured cells and midge developmental stages. While *Dcr2* was found to be expressed lowest in larvae when compared to pupae and adults, *Dcr2* was also expressed the lowest in cultured cells. Cultured cells appear to produce robust RNAi responses indicating that the observed "low" transcript levels for *Dcr2* are not likely to be the primary cause of reduced efficiency of RNAi responses in *Culicoides* larvae. However, *Ago2*

was highest in cells compared to all insect development stages. This finding could be a reason for the observed higher RNAi efficiency in cultured cells. We anticipate that other mechanisms such as nuclease digestion of dsRNA treatments may reduce RNAi efficiency in larval stages of these insects as well (Cooper et al. 2019).

The core RNAi proteins and enzymes in the siRNA pathway may have multiple genes in different insects. Cooper et al. (2019) summarizes the findings from numerous studies which show gene duplication in Diptera, including two *Ago2* genes in *Musca domestica*, three *Ago2* genes in *Glossina morsitans*, and two *Ago2* genes in *Culex pipiens*. The remainder of species cited in this review have one gene each of *Dcr2*, *Ago2*, and *R2D2*. In a transcriptome (Accession: PRJNA238338) from adult female *C. sonorensis*, three *Ago2*, one *Dcr2*, and two *R2D2* accessions were identified (Nayduch et al. 2014a, Mills et al. 2015). We designed primers to each of the *R2D2* transcripts, but the resulting melt curves of the products had the same dissociation temperature. There were, however, slight differences in the expression profiles of each of these targets which suggests we are detecting each independently. These data suggest that *C. sonorensis* may have two *R2D2* genes which was not noted in any other dipteran species (Cooper et al. 2019). The implications for this likely gene duplication in RNAi efficiency remains to be investigated. Future experiments could knockdown each target to confirm each transcript is expressed independently, and changes in RNAi efficiency could be examined in each condition to determine the influence two *R2D2* proteins have on dsRNA processing.

A Staufen homolog in *C. sonorensis* was investigated for its potential role in affecting RNAi efficiency. The *C. sonorensis* Staufen is predicted to have five dsRNA binding domains (dsRBDs) as determined by ProSite (<https://prosite.expasy.org>), which is consistent with the provided functional description for this protein. There was only one sequence for Staufen in a

previously published *C. sonorensis* transcriptome (Nayduch et al. 2014a). Related sequences in other insects had functions such as maternal effect protein Staufen (*Gryllus bimaculatus*) and dsRNA binding protein Staufen homolog 2 (*Diabrotica virgifera virgifera*, *Zootermopsis nevadensis*, *Leptinotarsa decemlineata*, and *Halyomorpha halys*). These accessions are from large scale projects (e.g., transcriptomes) and do not appear to have associated functional experiments.

In our experiments, suppression of *Stau* in *C. sonorensis* cells by RNAi had little effect on RNAi efficacy in our experiments, making it unclear what its role might be in RNAi responses or midge physiology. In previous studies, maternal effects Staufen *Drosophila* played a role in localizing specific mRNAs during cell division (Li et al. 1997). In *C. elegans*, a Staufen 1 (Stau-1) protein was shown to bind dsRNA with high affinity and that no single dsRBD was required to do so (LeGendre et al. 2013). The authors in this study also suggested this Staufen protein responds to dsRNA but does not play a direct role in RNAi. In contrast, LeGendre et al. (2013) showed that suppressing *Stau-1* in *C. elegans* enhanced RNAi efficiency likely by reducing sequestration of dsRNA products and allowing more dsRNA to be processed by the RNAi machinery. In coleopterans, StaufenC (*StauC*) has been shown to be necessary to process dsRNA for RNAi (Yoon et al. 2018). Here, the authors demonstrate that suppressing *StauC* in cultured cells of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) by RNAi reduced the quantity of siRNA products from a treatment at 24 h, and completely suppressed siRNA product production at 48 h. In addition, knocking down *StauC* in cells was protective against apoptosis after subsequent *dsIAP1* treatments. The authors show, however, that StaufenC is not the same as other Staufen homologs in insects and is only detected in coleopterans. The role *Stau* plays in *C. sonorensis* RNAi efficiency, if any, remains to be seen.

Factors limiting RNAi have been reviewed extensively and may contribute to lowered RNAi efficiency in multiple ways (Cooper et al. 2019). These mechanisms of inefficiency remain to be investigated in *C. sonorensis*, including dsRNA uptake, the presence and activity of dsRNA-degrading nucleases, and systemic spread of RNAi signals in the insect. Here, we show that each component of the siRNA pathway is expressed in cultured cells and developmental stages of *C. sonorensis*, and that RNAi can suppress gene expression more efficiently in cells when compared to late-larval stages. These data add to the growing knowledge around RNAi in insects as a reverse genetics tool and possible pest control application. Additionally, our findings help bolster the research around RNAi in *C. sonorensis*, and related *Culicoides* spp., which will inform future studies on these important livestock pests.

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Chapter 4 - Characterization of putative double-stranded RNA degrading enzymes in the biting midge *Culicoides sonorensis*

Abstract

RNA interference (RNAi)-based approaches show promise for highly specific, environmentally benign control of pest insects. The biting midge, *Culicoides sonorensis* is an important vector of livestock pathogens in the United States, but the potential for RNAi-based control of this species remains understudied. Research in other insect species suggests that there are hurdles limiting RNAi efficiency, including the presence of double-stranded RNA-degrading enzymes (dsRNases) that destroy the double-stranded RNA (dsRNA) molecules used to induce RNAi. We examined the *C. sonorensis* transcriptome to identify putative dsRNases and examined their expression in third and fourth instar larvae, male and female pupae, and adult male and female midges. Using tissue fluid collected from larval gut tracts, we quantitatively and qualitatively show *ex vivo* digestion of dsRNA by RT-qPCR and gel electrophoresis, respectively. We show that two dsRNases can be suppressed by RNAi in cultured *C. sonorensis* cells, but a similar treatment in larval midges yielded inconclusive results. We attempted to suppress dsRNases in cultured cells to protect subsequent dsRNA treatments from degradation, but the cell media appeared to contribute to dsRNA degradation and interfered with this experiment. Further work to evaluate the expression, tissue tropism, and function of these putative dsRNases will inform future RNAi-based studies in *C. sonorensis* focused on developing effective RNAi-based insecticidal treatments for these important arthropod vectors.

Introduction

Broad-spectrum chemical insecticides can harm non-target organisms and are coming under greater scrutiny by an environmentally conscious public. RNA interference (RNAi) is a conserved post-transcriptional regulatory mechanism with potential applications for insecticidal products (Christiaens et al. 2020). To induce RNAi responses, double-stranded RNA (dsRNA) is internalized by pest organisms through feeding, soaking, or topical application where a series of processing steps ultimately triggers the degradation of target gene mRNA. DsRNAs are designed based on the nucleotide sequences of target genes and, as a result, are expected to be both highly specific and environmentally benign when compared to traditional chemical insecticides.

Numerous gene targets have been examined in insects with the expectation that their suppression by RNAi will lead to increased mortality or reduced fitness (Das and Sherif 2020, Munawar et al. 2020, Nitnavare et al. 2021). The diversity of physiologically important targets (e.g., chitin synthase enzymes, structural proteins, membrane transporters, insecticide detoxification enzymes, etc.) makes RNAi-based insecticides an exciting area of study, but unfortunately there are numerous barriers that can limit RNAi efficiency in some insects (Cooper et al. 2019, Zhu and Palli 2020).

Multiple barriers have been evaluated as contributing to reduced dsRNA sensitivity, including reduced dsRNA cellular internalization, low dsRNA stability from environmental factors and enzymes, low expression of core RNAi-associated enzymes, and lack of systemic RNAi spread (Cooper et al. 2019). The contribution of dsRNA-degrading enzymes (dsRNases) to degrading these molecules before they're processed by the RNAi-associated proteins and enzymes are of particular interest due to their widespread prevalence in insects. These enzymes have been characterized in multiple insect taxa and have been shown to readily degrade dsRNA

substrates (Singh et al. 2017, Song et al. 2019, Cooper et al. 2020a), though their characterization in dipterans is not well studied and is worthy of further investigation (Tayler et al. 2019, Giesbrecht et al. 2020). Multiple putative dsRNases are typically identified in each insect, but their expression differs between tissues and life stages, impacting their contribution to dsRNA degradation. For example, using RNAi to knock down dsRNases which were highly expressed in the guts of *Aedes aegypti* larvae appeared to enhance the stability of dsRNA (Giesbrecht et al. 2020). This study also identified 10 putative dsRNases with variable expression in different developmental stages and insect tissues. The roles these enzymes play in RNAi efficiency are an important area of investigation towards developing RNAi-based treatments for pests.

Culicoides are small flies in the biting midge family Ceratopogonidae, and are members of the suborder Nematocera, which includes non-biting midges, mosquitos, sand flies, and black flies (Mullen and Murphree 2019). These midges are vectors of numerous viral and nematode pathogens primarily of livestock (Purse et al. 2015). In the United States, *Culicoides sonorensis* Wirth & Jones is one of the most well studied species and is a vector of serious viral livestock pathogens such as bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), and vesicular stomatitis virus (VSV) (McGregor et al. 2022). Many methods have been used or proposed for *C. sonorensis* population control, including insecticidal sprays, on-animal insecticides and deterrents, and larval habitat treatments (Pfannenstiel et al. 2015, Benelli et al. 2017). RNAi-based insecticides could prove useful in supplementing current methods or supplanting more harmful products, but these have not been well studied in *C. sonorensis*. We have previously shown that dsRNA can elicit target gene suppression in cultured *C. sonorensis* cells but is not efficient in larval stages (Chapter 3), suggesting that there are mechanisms that

limit RNAi efficiency. Accordingly, we hypothesize that *C. sonorensis* dsRNases may play a role in degrading dsRNA during oral delivery as has been observed in other insect species.

In this study, we use previously described *dsRNase* transcript sequences in mosquitos and other insects to identify potential *dsRNases* in *C. sonorensis* transcriptome and genome sequence data. We describe seven of these putative enzymes and further show the expression profiles of six of these in *C. sonorensis* developmental stages and female body parts and tissue groups. Additionally, we used RNAi to suppress three of these dsRNases and examined dsRNA stability. Our results show that dsRNases likely contribute to instability of dsRNA in *C. sonorensis*.

Materials and Methods

Bioinformatics, Physiological Inferences, and Phylogenetic Analysis

Previously described *dsRNases* of mosquitos were used to query *C. sonorensis* transcriptome sequences (Accession: PRJNA238338) using BLAST-n (National Center for Biotechnology Information, NCBI) (Cooper et al. 2020b, Giesbrecht et al. 2020). The resulting transcripts were then examined using BLAST-x to check for the presence of the DNA/RNA non-specific endonuclease (Endonuclease_NS) domain. Those transcripts containing this domain were then examined against a published *C. sonorensis* genome (Accession: PRJEB7005) using BLAST-n. Here, transcripts that appear to overlap on the same genome scaffold were combined for downstream analysis. These steps resulted in seven putative dsRNases which are identified in this study using the characters A-F. Transcripts were processed using a translation tool (Expasy, SIB, Swiss Institute of Bioinformatics, <https://web.expasy.org/translate/>) to identify likely amino acid sequences (Appendix C) for each putative dsRNase. Amino acid sequences were used to confirm domains using InterProScan (EMBL-EBI, European Bioinformatics Institute,

<https://www.ebi.ac.uk/interpro/search/sequence/>), and the predicted characteristics (e.g., cellular localization) were examined using DeepLoc (Version 2.0, DTU Health Tech, <https://services.healthtech.dtu.dk/service.php?DeepLoc-2.0>).

Amino acid sequences for dsRNases from related nematoceran taxa (mosquitos specifically), other insects, and other arthropods were collected from previously published literature and from highly related sequences in BLAST-x. Sequences were aligned by MUSCLE using default parameters and multiple maximum likelihood (ML) trees were constructed in MEGA (Stecher et al. 2020).

Insect Developmental Stage and Body Part and Tissue Groups

Culicoides sonorensis cultured cells (W8A) and developmental stages were sourced from the USDA-ARS laboratories in Manhattan, Kansas where each are actively maintained. Cultured cells and insects were maintained in our lab using previously described conditions (Chapter 2). Third (L₃) and fourth instar (L₄) larvae, male and female pupae, and adult males and females were collected into 400 µl of TRIzol reagent (Invitrogen, Waltham, MA) to create three pools of each developmental stage (20 individuals per pool). Fifty adult female *C. sonorensis* were dissected in drops of nuclease-free water to yield four pools of body parts or tissue groups containing 1) heads 2) midguts 3) reminder of gut tract, and 4) remaining body. This was repeated twice more and resulted in three pools for each body part or tissue group. All pools were transferred into 400 µl of TRIzol. Developmental stage, body part, and tissue group pools were homogenized and processed for total RNA isolation as previously described (Chapter 2).

DsRNA Synthesis and RNAi-based Treatments

Primers containing a T7 promoter sequence were used to amplify DNA template from *C. sonorensis* cDNA derived from combined life stages or from the Ac5-STABLE1-neo plasmid containing the *eGFP* gene (Table C.1). Treatment and control dsRNA were synthesized as previously described (Chapter 2). Cultured cells were grown to ~75% confluence in 12-well cell culture plates in 1 mL of modified Schneider's media (Chapter 2) and treated with 1-2 µg of dsRNA (i.e., 1-2 µg/mL). These treatments were either 2 µg *dseGFP* (control), 2 µg dsRNA targeting *CsdsRNase-A* or -B, or a combination of *CsdsRNase-A* and -B (1 µg/ea). Cells were harvested 72 h post treatment in 400 µl TRIzol and processed for total RNA isolation.

Third instar larvae were placed into wells of a 6-well cell culture plate (20 larvae per well) and each well was filled with 1 mL of nuclease-free water. Larvae were treated with 15 µg of dsRNA (i.e., 15 µg/mL) consisting of either 5 µg/ea dsRNA targeting *CsdsRNase-A*, -B, and -G, or 15 µg of *dseGFP* (control). Larvae were harvested 4 days post treatment into 400 µl of TRIzol, homogenized with a tissue pestle, and processed for total RNA isolation.

***Ex-vivo* dsRNA Stability**

Fifty (50) L₄ larvae were dissected in nuclease-free water by snipping the heads off their bodies using a small pair of dissecting scissors and gently pulling their extruding gut from their body cavity. This was repeated twice more to yield three total pools. The pools of gut tissue were centrifuged at 17,000 x g for 10 min and the supernatant was removed to yield a fluid enriched for larval gut contents. The total protein concentration from an aliquot of each pool was determined using a Pierce™ BCA Protein Assay Kit (Fisher Scientific, Pittsburgh, PA). 500 ng of *dseGFP* was incubated with 3 µg of larval gut fluid and nuclease-free water in a total volume

of 15 μ l for 60 min at 28 °C. Controls consisted of a nuclease-free water only, 3 μ g of heat-inactivated (95 °C for 10 min) larval gut fluid, and 3 μ g of larval gut fluid and EDTA to a final concentration of 20 mM. 2 μ l of each treatment or control was used to synthesize cDNA for RT-qPCR and the remaining product was mixed with 3 μ l loading dye (6x TriTrack, Thermo Scientific) and loaded onto a 1.5 % agarose gel. After electrophoresis, the resulting gel was visualized on an iBright Imaging System (Thermo Fisher Scientific). For quantitative analysis, a serial dilution was made of seven *dseGFP* concentrations (200 ng/ μ l to 0.2 pg/ μ l) and 2 μ l of each dilution step were processed for cDNA synthesis and downstream RT-qPCR to create a standard curve using a method described previously (Cooper et al. 2020a).

Cultured W8A cells were grown in 1 mL media in a 12-well cell culture plates. When cells reached ~75% confluence the media was aspirated and replaced with 1mL fresh media. Cells were treated with dsRNA as previously described to knockdown *CsdsRNase-A* and -B or *dsCHS-1* (control). After 24 h, the media from half of the wells was collected and replaced with 1 mL fresh media. After an additional 24 h the media from all the wells was harvested. The final samples were 24 h (initial), 24 h (media change), and 48 h conditioned media from cells treated with dsRNA to suppress two dsRNases or a control which is not expressed in cells. Total protein concentrations were determined as above. Volumes of conditioned cell media equivalent to 20 μ g of protein were used for a dsRNA degradation study using 300 ng of *dseGFP*. The treatment schema was similar to that of the larval gut fluid study except with 25 μ l reaction volumes, incubation for 90 min, and the EDTA control had a final concentration of 50 mM. Digest products were examined by gel electrophoresis and RT-qPCR as above.

RT-qPCR

Three biological replicates were used in each study, and two technical replicates for each biological replicate. Gene expression and knock-down studies were assayed by RT-qPCR, and two reference genes were examined for each sample. These reference genes were *actin* and *VhaA* for dsRNase expression in cells and insect developmental stages, *actin* and *EF1b* for body part and tissue group dsRNase expression, *GAPDH* and *EF1b* for cell culture-based RNAi studies, and β -*tubulin* and *VhaA* for dsRNA treated larvae (Chapter 2). The two technical replicates were averaged for analysis. Either the delta-Ct (Δ Ct) or delta-delta-Ct ($\Delta\Delta$ Ct) methods were used to examine relative gene expression showing fold change between a control and sample group (Livak and Schmittgen 2001).

Detecting residual dsRNA levels after *ex-vivo* digest was ascertained by creating a linear regression from the log quantity of *dseGFP* (in ng) and resulting Ct values for each sample by RT-qPCR. The slope and y-intercept from the model were used to create an equation where the residual dsRNA could be determined from the Ct value in each sample from the digest experiments using the slope and y-intercepts of the regression line (Cooper et al. 2020a).

Statistical Analyses

All statistical tests were performed in PRISM 9 (Version 9.5.1, GraphPad Software, LLC, Boston, MA). Each dataset was subjected to tests to determine if criteria for normality and equal variance were met. If data were normal and variances were equal, a one-way ANOVA was used with a Tukey's post-hoc multiple comparison test (MCT). If data were not normal, a Kruskal-Wallis tests was used with a Dunn's MCT. For comparisons of two groups, a two-tail t test was

used if assumptions for normality and equal variance were met, or a Mann-Whitney test when data were not normal. The test used for each analysis is noted in Table C.2.

Results

Putative dsRNases in *C. sonorensis*

Seven putative dsRNases were identified from the available *C. sonorensis* genomic and transcriptomic data. The predicted proteins all had a high likelihood of being secreted extracellularly based on the presence of signal sequences (Table 4.1). The predicted proteins also each contained an “Endonuclease_NS” domain and were of similar amino acid length (Fig. 4.1A). An effort was made to avoid duplicate discoveries by mapping each transcript to the scaffold in a published *C. sonorensis* genome and combining sequences when they appeared to overlap (Table 4.1). However, our work did not attempt to distinguish between isoforms or alleles of the same gene. Only *CsdsRNase-B* lacked a stop codon, and the sequence appears to end within the “Endonuclease_NS” domain, though the substrate binding sites, Mg²⁺ binding site, and active site are all contained in the sequence. When aligned, all seven *C. sonorensis* dsRNases had a conserved histidine (H) in the active site which has been shown to be required for activity in *Serratia marcescens* (Friedhoff et al. 1994), and each had the same gene structures such as substrate binding sites, active sites, and Mg²⁺ binding sites (Fig. 4.1).

Table 4.1. Predicted protein characteristics of seven putative dsRNases in *Culicoides sonorensis*.

Abbreviations: *Cs*, *Culicoides sonorensis*; dsRNase, double-stranded RNA-degrading enzyme; Endo_NS, DNA/RNA non-specific endonuclease; AA, amino acid; EC, extracellular. Multiple accessions indicate two or more transcripts were used to construct a final predicted sequence. “Endonuclease_NS” domain observed in NCBI BLAST-p^A. Predicted AA sequence and start and stop codons (Yes/No) determined by ExPasy^B. Likelihood of EC secretion determined by DeepLoc2.0^C. Signal peptides determined by SignalP 5.0^D. Solubility determined by DeepLoc1.0^E. **CsdsRNase-F* is constructed from two sequences which had a 20 bp overlap.

Target	Accession Number	“Endo_NS” Domain ^A	AA Length ^B	Start/Stop Codon Present ^B	Likelihood of EC Secretion ^C	Signal Peptide Present ^D	Solubility Likelihood ^E
<i>CsdsRNase-A</i>	GAWM01000334.1 GAWM01000335.1	Yes	425	Y/Y	92.2%	Yes	97.0%
<i>CsdsRNase-B</i>	GAWM01006805.1	Yes	250	Y/N	90.2%	Yes	99.2%
<i>CsdsRNase-C</i>	GAWM01002773.1	Yes	376	Y/Y	87.2%	Yes	83.2%
<i>CsdsRNase-D</i>	GAWM01017632.1 GAWM01013642.1 GAWM01013640.1	Yes	364	Y/Y	90.4%	Yes	100%
<i>CsdsRNase-E</i>	GAWM01005324.1	Yes	422	Y/Y	94.3%	Yes	99.6%
<i>CsdsRNase-F*</i>	GAWM01007140.1 GAWM01007142.1	Yes	394	Y/Y	83.3%	Yes	99.9%
<i>CsdsRNase-G</i>	GAWM01011845.1	Yes	459	Y/Y	84.4%	Yes	99.8%

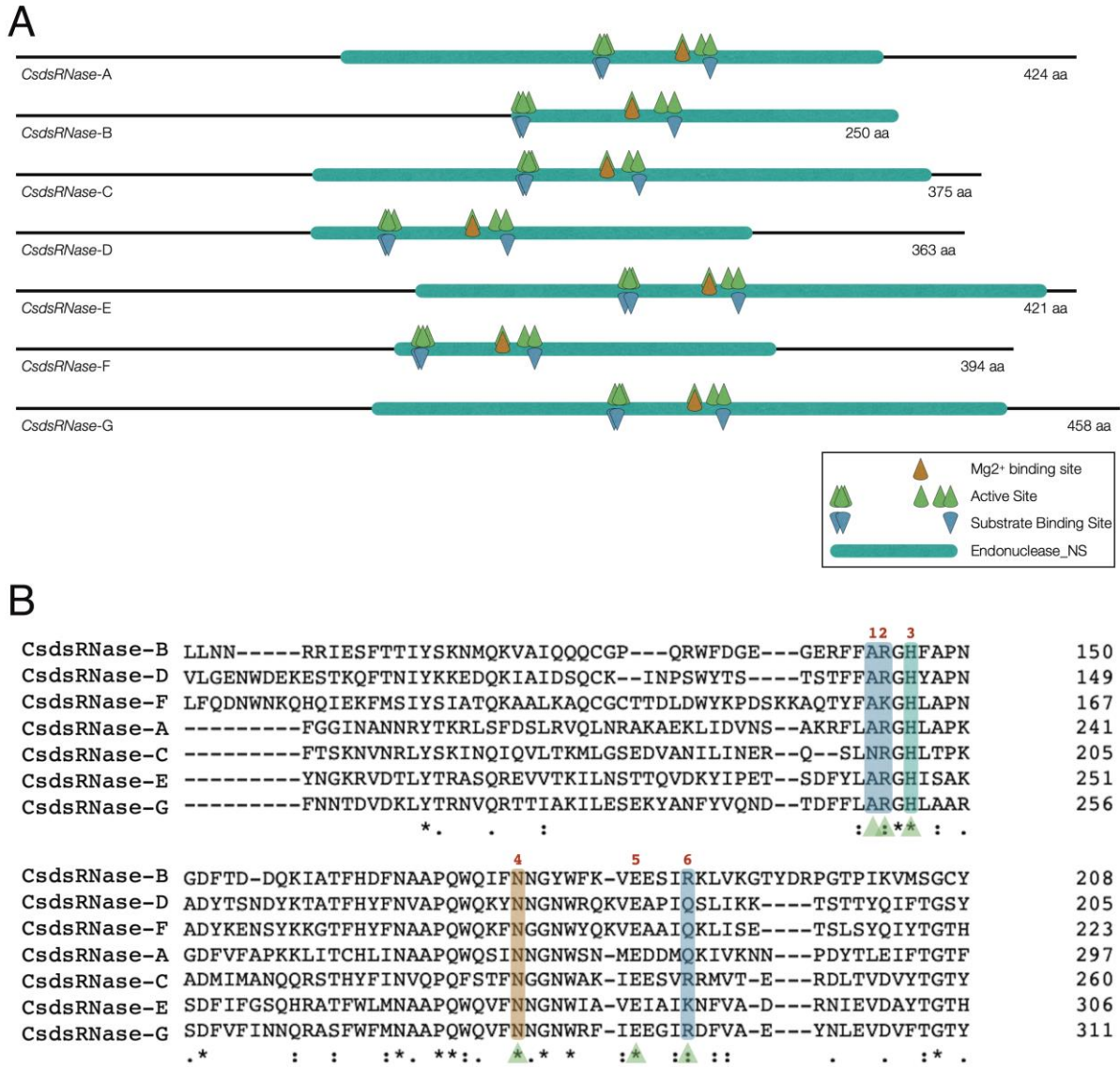


Figure 4.1. Predicted amino acid sequences of seven (A-F) putative dsRNases in *Culicoides sonorensis* were determined.

A. Schematic showing DNA/RNA non-specific endonuclease (Endonuclease_NS), active sites, substrate binding sites, Mg^{2+} binding site, and amino acid (aa) length as predicted by NCBI BLAST-p. B. CLUSTAL-Omega alignment of the seven sequences which are trimmed to show the sites of interest and annotated to the residue level using the NCBI conserved domain viewer. Substrate binding sites are highlighted blue and are labeled 1-2 and 6. The predicted proton acceptor residue is highlighted in teal and is labeled 3. The Mg^{2+} binding site is highlighted in brown and labeled 4. The active sites are denoted with a green triangle and are labeled 1-6. CLUSTAL-Omega annotations show fully conserved residues (*), highly similar amino acids (:), and weakly similar amino acids (.).

The majority of similar sequences found using a BLAST search were from Diptera (Fig. 4.2). *CsdsRNase* -A, -B, -D, and -F clustered at the base of the tree, which was rooted with the previously characterized bacterial nuclease from *S. marcescens*. *CsdsRNase*-C appeared to cluster with the higher flies, while *CsdsRNase*-E and -G clustered together with the lower flies. Most higher fly sequences appear to cluster together. When compared with a similar investigation of *Ae. aegypti* dsRNases (Giesbrecht et al. 2020), *CsdsRNase*-E and -G, and *CsdsRNase*-B, -D, and -F cluster together (Fig C.1A). Separately, we examined our sequences against dipteran nucleases from a previous study (Cooper et al. 2020b) and found *CsdsRNase*-E and -G clustered with mosquito sequences, but the remaining 5 sequences were separate (Fig. C.1B). In each of our three trees, *CsdsRNase*-E and -G cluster closely together, and *CsdsRNase*-B, -D, and -F cluster together.



Figure 4.2. Maximum likelihood tree constructed from the top 20 related protein sequences to each of the seven *C. sonorensis* nucleases described in this study (bolded).

Duplicate sequences were removed prior to analysis. A related *Serratia marcescens* nuclease is included as an outgroup. GenBank accessions are listed with each taxon. Colors delineate the outgroup (light grey), non-Diptera taxa (dark grey), non-mosquito basal Diptera taxa (blue), *Anopheline* mosquitos (yellow), *Aedine* mosquitos (orange), and higher derived Diptera taxa (pink). Sequences were aligned in MEGA X using MUSCLE and the tree was constructed using the LG+G+I model with partial deletion, and all other parameters set to default.

Expression of dsRNases

Only transcripts for *CsdsRNase-A*, -B, and -G were detected in cultured W8A cells by RT-qPCR. *CsdsRNase-A* had the highest relative expression in cultured cells, followed by *CsdsRNase-B* and *CsdsRNase-G* (Fig. C.2). Each nuclease had a unique expression profile in all the developmental stages examined (Fig. 4.3). Two enzymes (*CsdsRNase-E* and -G) had the highest relative transcript levels in larvae, and *CsdsRNase-B* showed the highest transcript levels in the L₄ stage, albeit with considerable variation. *CsdsRNase-D* had the lowest expression in larvae. *CsdsRNase-A* and -C were the most consistent in their expression across all developmental stages. These expression profiles are shown in Figure 4.3, and their statistical tests are described in Table C.2. The expression of each nuclease in body parts and tissue groups from dissected female midges was variable (Fig. C.3). Here, expression of *CsdsRNase-G* showed the highest transcript levels followed by -A and -B, with -C, -D, and -E having lower transcript levels.

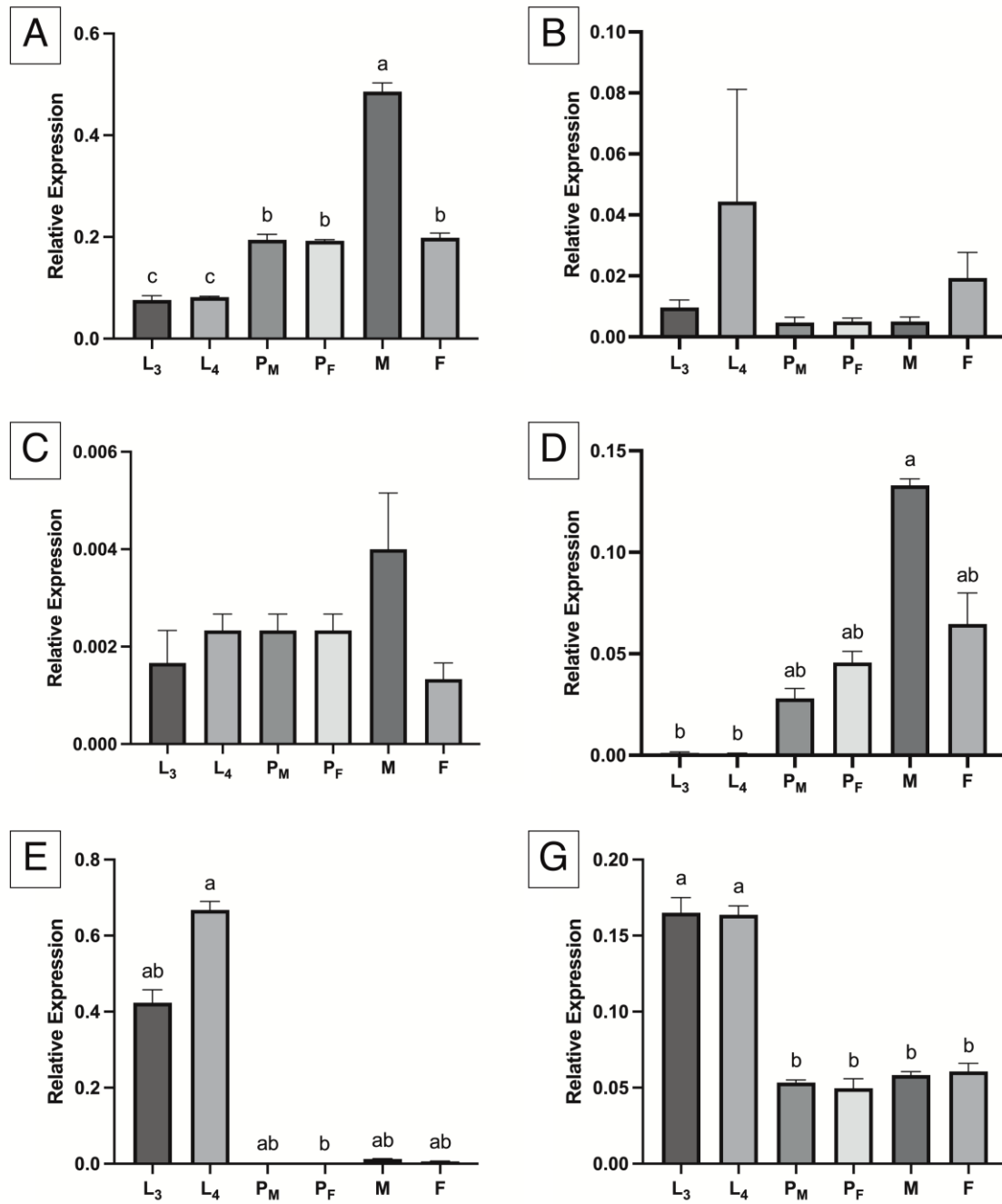


Figure 4.3. Expression of *CdsRNases* in six developmental *C. sonorensis* life stages.

Each nuclease is identified by the letter in the box (i.e., *CdsRNase-A*). The developmental stages are instar 3 (L₃) and 4 (L₄) larvae, male (P_M) and female (P_F) pupae, and adult males (M) and females (F). Relative expression (ΔCt) is to two reference genes. Bars are SEM. Letters over each bar indicate statistical significance. Statistics are reported in Table C.2.

Larval Gut Fluid Digest of dsRNA

When dsRNA was digested in the presence of active gut fluid, a marked decrease in the target band was observed on the agarose gel (Fig. 4.4A). The water control and heat inactivated control appear similar, which suggests that enzymes are the primary driver of dsRNA degradation in this fluid. The EDTA treated gut fluid was expected to reduce dsRNA degradation, but surprisingly appeared to have no effect on dsRNA stability. This observation was corroborated with multiple additional attempts (not shown). We are unable to provide a definitive reason for this other than the presence of other unidentified enzymes which do not require divalent cations may be degrading the dsRNA.

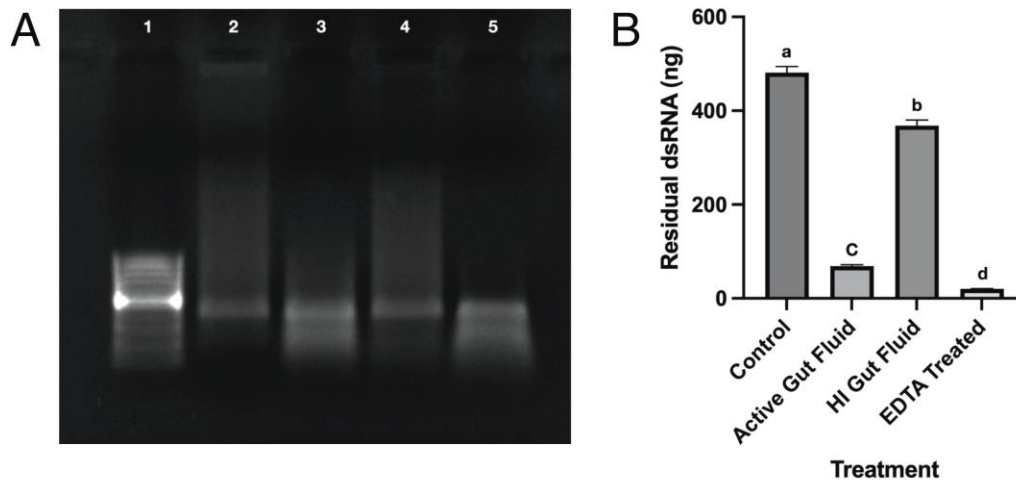


Figure 4.4. Residual dsRNA after degradation of 500 ng *dseGFP* in 3 µg of gut fluid from L4 *C. sonorensis* for 60 min at 28 °C. A) 1.5% agarose gel containing digested dsRNA products.

Lanes are 100 bp ladder with brightest band at 500 bp [1], water only control [2], gut fluid [3], heat-inactivated (95 °C, 10 min) gut fluid [4], and gut fluid with a final EDTA concentration of 20 mM [5]. B) Residual dsRNA values determined by RT-qPCR from a standard curve of *dseGFP* concentrations. Letters over bars indicate statistical significance levels. Bars are averages and lines are SEM. ANOVA $F(3, 8) = 620.7$, $P < 0.0001$.

The findings in the gel were corroborated by the quantitative values determined from the RT-qPCR standard curve (Fig. 4.4B). Here, there were significant differences ($P < 0.0001$) in the yield of dsRNA from each treatment. There was a significant difference ($P < 0.0001$) between the water control and heat-inactivated gut fluid control, although the loss in detectable yield was only ~23%. When comparing the detectable dsRNA yield between the heat-inactivated control and active gut fluid, there was ~81% loss ($P < 0.0001$). And when the heat-inactivated control was compared with the EDTA treatment, there was ~95% loss in yield ($P < 0.0001$). The difference between the active gut fluid and EDTA treatment was also significant ($P = 0.0216$).

Suppression of Nuclease Transcripts in W8 Cells

Transcripts of two nucleases (*CsdsRNase-A* and -B) were successfully suppressed by RNAi (Fig. 4.5). Cells only responded to dsRNA matching the intended target as shown by treating cells individually with dsRNA against *CsdsRNase-A* or -B and measuring transcript levels. Additionally, both targets could be suppressed together with a combination of each dsRNA construct. Transcript levels for *CsdsRNase-A* or *CsdsRNase-B* were reduced by more than 85% or 75%, respectively. There were only slight, non-significant differences in transcript levels between the single and combinatorial treatments (2% for *CsdsRNase-A* and 5% for *CsdsRNase-B*) despite the single treatments having double the dsRNA concentration (i.e., 2 $\mu\text{g/mL}$ target dsRNA for single and 1 $\mu\text{g/mL}$ target dsRNA for combination treatment). Interestingly, there was a slight increase in expression of *CsdsRNase-A* (~15%) and *CsdsRNase-B* (~9%) in cells when treated with their non-target counterpart, though neither were statistically significant.

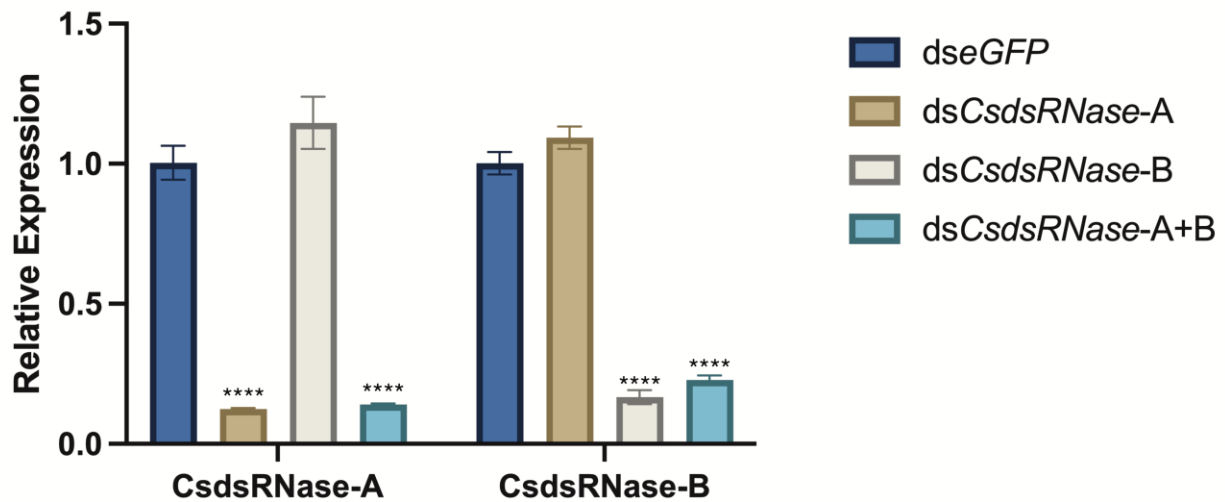


Figure 4.5. Relative expression (fold change) of *C. sonorensis* nucleases in W8A cultured cells after treatment with 2 $\mu\text{g/mL}$ *dseGFP* (control), 2 $\mu\text{g/mL}$ *dsCsdsRNase-A* or -B, or a combination of each *dsCsdsRNase* (1 $\mu\text{g/mL}$ each).

Cells were harvested after 72 h. Relative expression is $\Delta\Delta\text{Ct}$ method normalized to two reference genes. Bars are mean with SEM. Tests are ANOVAs and **** $P < 0.0001$.

We then assessed the protective value in suppressing these nucleases for non-target dsRNA in W8 cells. Here, *CsdsRNase-A* and -B were both targeted by RNAi as before, and then *dseGFP* was incubated in conditioned media derived from treated cells and appropriate controls. As expected, the water only control had the highest band brightness on the agarose gel (Fig. C.4A). However, it was difficult to perceive differences in other bands. The RT-qPCR experiment showed similar findings (Fig. C4B). Here, heat-inactivated conditioned cell media had the highest residual dsRNA value and media only (non-conditioned) had ~53% lower dsRNA yield. There were no differences in the dsRNA stability between any of the treatments, but one treatment in the 48 h changed media experiment had a single outlier which gave some indication of lower yield.

Nuclease Suppression in Larvae

Three dsRNA constructs (*CsdsRNase* -A, -B, and -G) were combined and used to treat L₃ larvae. This stage was found to be more susceptible to *CsdsRNase*-G transcript suppression when compared to L₄ larvae (Chapter 5). The relative expression of each of these nucleases and the change in transcript levels between treatment and control are shown in Figure C.5. Here, we used the Δ Ct method to highlight the expression of each target in L₃. *CsdsRNase*-G was the most highly expressed, followed by *CsdsRNase*-A and then *CsdsRNase*-B. Each target appears to have some level of transcript suppression compared to their respective control; however, there is very high variability in each sample. no significant differences in transcript level were detected in any pair (Table C.2).

Discussion

Instability of dsRNA attributed to dsRNases in insects has been observed in a number of different orders, including Lepidoptera (Arimatsu et al. 2007, Guan et al. 2018, Cooper et al. 2020b, Cooper et al. 2020a), Orthoptera (Wynant et al. 2014, Song et al. 2017, Song et al. 2019), Coleoptera (Spit et al. 2017, Peng et al. 2020), Hymenoptera (Lei et al. 2022), and Diptera (Tayler et al. 2019, Giesbrecht et al. 2020, Yoon et al. 2021). The expected number of putative dsRNases appears variable in insects. For example, Cooper et al. (2020b) identified three to four enzymes in *Ostrinia nubilalis*, and other reports show one to three enzymes in other species. In contrast, Giesbrecht et al. (2020) found 10 putative dsRNases in *Aedes aegypti*. We identified seven putative dsRNases in *C. sonorensis*. Each putative dsRNase had a unique predicted protein sequence that mapped to different areas on the *C. sonorensis* genome. Our findings are consistent with other studies which show identical or functionally similar amino acids in each of the

important functional domains of dsRNases (Song et al. 2019, Cooper et al. 2020b, Giesbrecht et al. 2020, Lei et al. 2022). Additionally, each of the six putative dsRNases that we examined displayed unique expression profiles in midge developmental stages and tissues or body parts examined.

Two studies in dipteran taxa found the suppression of dsRNases appear to enhance dsRNA stability in RNAi experiments (Tayler et al. 2019, Giesbrecht et al. 2020). These studies examined co-feeding dsRNA to knockdown gut-specific dsRNases which effectively enhanced RNAi efficiency. Our findings suggest *CsdsRNase-E* and *-G*, and to some extent *CsdsRNase-B*, have the highest transcript levels in larval midges, though *CsdsRNase-A* and *-C* are still present. Additionally, dsRNA was degraded in the fluid collected from larval guts, suggesting that the dsRNases present were active. However, unlike previous reports that increased dsRNA stability by suppressing dsRNase expression, we were unable to suppress transcript levels of three dsRNases in larvae by RNAi using orally delivered dsRNA. Suppressing two *CsdsRNases* in W8 cells was successful but a follow-up study to assess the stability of dsRNA in conditioned media was complicated by instability from cell media factors. Our results suggest that our suppression of dsRNases *in vivo* and *in vitro* were insufficient to accurately measure nuclease activity. Future experiments should focus on activity of individual dsRNases by expressing them independently in a homologous or heterologous expression systems.

Interestingly, we observed a slight increase in *dsRNase* expression in cultured cells that were treated with dsRNA to suppress a separate *dsRNase*. This increase was observed in both treatments and was above the baseline of control group (Fig. 4.5). These data suggest that different *dsRNases* could serve a compensatory role following the loss of expression of a particular *dsRNase*, or that treatment with dsRNA enhances expression of one or more *dsRNases*.

Accordingly, effectively suppressing multiple dsRNases at the same time may be necessary to enhance dsRNA stability and improve RNAi efficiency in these insects.

One interesting finding was the persistence of dsRNase activity in our experiments where samples had been treated with EDTA. Heat inactivation of larval gut fluid or conditioned cell media prevented dsRNA degradation in our experiments, but EDTA, which has been used to reduce dsRNA degradation in similar experiments in other insects (Garbutt et al. 2013, Lei et al. 2022), was apparently ineffective. Each of the seven putative *C. sonorensis* dsRNases that we identified include a Mg^{2+} binding site, so we expected a similar outcome with these enzymes following exposure to EDTA concentrations used in previous studies (Garbutt et al. 2013, Lei et al. 2022). The fact that degradation activity is lost by heat inactivation suggests that enzymes are responsible for the digestion we observe in physiologically active fluids. However, the inability of EDTA to suppress the degradative activity in physiological fluids indicates that the degradative activity observed is independent of the availability of Mg^{2+} , suggesting either that the putative dsRNases that we have identified do not require Mg^{2+} or that other enzymes are involved in the degradation of dsRNA. Future work should examine the activities of the putative dsRNases present in *C. sonorensis* on different substrates (ssRNA, dsRNA, and DNA) and in different conditions (e.g., pH) to evaluate their roles in dsRNA degradation.

Dipterans are regarded as having lower RNAi efficiency compared to more susceptible insect orders, but they are amenable to gene knockdown particularly in injection studies (Swevers et al. 2013). In this same review, the authors discuss the role dsRNA plays in triggering RNAi-associated core protein expression and the shared responses cells have when sensing exogenous dsRNA of designed or viral origin. *Culicoides* spp. are vectors of numerous dsRNA viruses, including BTV, EHDV, African Horse Sickness, and Equine encephalosis (Mullen and

Murphree 2019). The presence of these viruses in cells should initiate an RNAi response, and even some positive sense, single stranded RNA viruses may make dsRNA intermediates (Weber et al. 2006). While it would follow that an inefficient RNAi response would make a host more permissible to viral infection (i.e., a good vector), there are other factors that must be considered. Many arboviruses (e.g., bluetongue) are asymptomatic in the insect vector which minimize selection pressures to suppress viral spread, though other viruses (e.g., West Nile virus) encode viral suppressors of RNAi to actively combat the RNAi mechanism. Further analysis here may give clues to the role, if any, that the dsRNases described in this study play in insect immunity.

An additional dsRNase role in insects is the digestion of nucleic acids for bioavailability (Arimatsu et al. 2007). We hypothesized that dsRNase expression in the gut associated tissues of female *C. sonorensis* would be higher as this would be the site of both initial viral introduction and metabolism after a bloodmeal. We previously discuss here the issues with cleanly dissecting these insects as they are very small and cross contamination between body parts and tissue groups is difficult to avoid. The dissected females were also only offered a maintenance sucrose solution from our colony source. Changes in the expression of dsRNases and RNAi associated proteins may occur after blood feeding, though this was not commented on in a transcriptome study which investigated differential gene expression in teneral, sugar-fed, and blood-fed female *C. sonorensis* (Nayduch et al. 2014). Larvae are expected to encounter large quantities of organic material and associated microbial organisms in both their natural and laboratory rearing environments. We expected to see higher dsRNase expression in these stages to process this material. Five dsRNases were expressed in larval stages, and only *CsdsRNase-D* was expressed at lower levels in larvae when compared to the other developmental stages (Fig. 4.3). The functional role of these enzymes in *C. sonorensis* remains an open question.

Culicoides are important livestock pests which will continue burdening producers and posing risks for animal herds. RNAi is an important tool for determining gene function and potentially developing insecticidal compounds, both of which have utility in *Culicoides* research. Our findings show that at least six putative dsRNases are expressed in *C. sonorensis*, and that dsRNA is enzymatically degraded in larval gut fluid. These reinforce the idea that many hurdles exist towards developing effective RNAi strategies in insects. Our results serve as a firm foundation for exploring many investigative avenues which could better characterize the tissue-level expression, substrate specificity, and activity of the dsRNases we describe here. Understanding the function of these enzymes should inform methods to overcome them. These include combination treatments to knockdown nucleases and target genes, protecting dsRNA using nanoparticle carriers, and expressing dsRNA in microbial systems (Giesbrecht et al. 2020, Silver et al. 2021). While many hurdles towards an effective RNAi-based insecticide remain, the prospects of a highly specific, environmentally benign insecticide should be a priority for investigation.

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Chapter 5 - Evaluating RNAi targets, treatments, and enhancers in the biting midge *Culicoides sonorensis*

Abstract

RNA interference (RNAi) can be used to suppress physiologically important target genes in insects, and its utility for insecticidal products is an area of active development. Previous studies suggest that RNAi responses are not highly efficient in the biting midge, *Culicoides sonorensis* (Diptera: Ceratopogonidae). This may be caused by the expression of multiple double-stranded RNA (dsRNA) degrading nucleases in *C. sonorensis* life stages, but other mechanisms, including poor dsRNA uptake or target gene selection may also have a role. Here, we examine the expression of six target genes (chitin synthases 1 and 2, a chitinase, vacuolar type ATPase subunit A, inhibitor of apoptosis protein 1, and a putative dsRNA-degrading nuclease) in six developmental stages and evaluate their sensitivity to suppression by RNAi in 3rd and 4th instar larvae. In addition, three RNAi-enhancing methods were tested for their ability to increase target transcript suppression in larvae, including using short hairpin (sh-) and paperclip RNA (pcRNA), dsRNA bound to chitosan nanoparticles, and dsRNA expressed in *E. coli*. Target gene expression was variable among life stages. Target suppression was highest when treating L₄ larvae with dsRNA for chitin synthase 1. RNAi suppression profiles in 3rd instar larvae were more variable and without a clear indication that any target evaluated was susceptible to RNAi suppression. Of the shRNA, pcRNA, combination of dsRNA with nanoparticles, or *E. coli* expressed dsRNA enhancements used, only the dsRNA/chitosan nanoparticles enhanced suppression of target transcript levels in larvae. Our findings suggest more optimization of larval

treatments are needed to improve RNAi efficiency in larval *C. sonorensis* to realize its potential as a novel control strategy for this important agricultural pest.

Introduction

The biting midge *Culicoides sonorensis* Wirth & Jones (Diptera: Ceratopogonidae) is an economically important vector of livestock pathogens in much of the United States (McGregor et al. 2022). Traditional chemical insecticides are harmful to non-target species and have largely shown to be ineffective at controlling transmission of pathogens by *Culicoides* on livestock operations (Carpenter et al. 2008). RNA interference (RNAi) is a conserved mechanism in insects that can be manipulated to produce highly specific insecticide products (Christiaens et al. 2020, Zhu and Palli 2020). These methods have been explored in many insects such as agriculture pests in cropping systems, toxic baits for urban pests, and control for medically important vector species (Scott et al. 2013). Developing insecticidal products for *C. sonorensis* could supplement conventional methods by targeting physiologically important genes (e.g., chitin synthases) to induce mortality or to act in synergy with other insecticides (e.g., suppressing detoxification enzymes). The foundational work to characterize RNAi in *C. sonorensis* is only recently being explored.

The first studies to investigate RNAi in *C. sonorensis* focused on antiviral responses in cultured cells (Schnettler et al. 2013) and suppressing targets in the adult female midge (Mills et al. 2015). In the later study, the authors found modest gene target suppression when female midges were injected intrathoracically with 150 ng of target dsRNA. Our recent work observed that RNAi was effective at suppressing target gene expression to comparable levels with a similar treatment dose in cultured cells, but that larval midge stages had a poor response even at

ten times the concentration (Chapter 3). Numerous factors can affect the efficiency of RNAi in insects, including dsRNA uptake, enzymatic degradation of dsRNA, insufficient expression of core RNAi machinery, and poor gene target selection (Cooper et al. 2019). We recently explored the role of dsRNA-degrading enzymes (dsRNases) in *C. sonorensis* (Chapter 4) where we found seven likely dsRNases and characterized the expression of six of these in cultured cells and six developmental stages. Three dsRNases had the highest expression in larval stages and another two were consistently expressed in all stages. We also found dsRNA was degraded in extracts from larval gut tracts. Taken together, these findings suggest that dsRNases likely degrade dsRNA treatment in *C. sonorensis* and contribute to lower RNAi efficiency, but other mechanisms that limit RNAi efficiency in *C. sonorensis* also need evaluation.

Multiple methods have been explored or proposed to enhance RNAi in insects. Some examples include creating RNA structures with closed loop ends to prevent exonuclease degradation, binding dsRNA to nanoparticle carriers to prevent enzymatic degradation, and expressing dsRNA in microbial systems (Silver et al. 2021). In *Aedes aegypti*, both short hairpin RNA (shRNA) with a single closed loop end and paperclip RNA (pcRNA) were able to suppress target genes at comparable levels to traditional dsRNA (Abbasi et al. 2020). Chitosan nanoparticles carry a slight positive charge which attracts the negatively charged backbone of dsRNA and forms nanoparticle constructs when mixed (Zhang et al. 2015). These have been shown to increase stability and uptake of dsRNA in *Anopheles gambiae* (Zhang et al. 2010). *Escherichia coli* (*E. coli*) is also a common system for expressing dsRNA where target dsRNA sequence is inserted into the L4440 plasmid between two, inverted T7 promoters. This engineered plasmid can be transformed into the HT115 strain of *E. coli* which lacks *RNaseIII* to prevent degradation of expressed products (Ahn et al. 2019). Other expression systems include

yeast (Duman-Scheel 2019), plants (Das and Sherif 2020), and algae (Fei et al. 2020). These methods provide numerous routes for enhancing RNAi in refractory insect species towards creating better insecticidal products.

In the present study, we investigated six potential targets for RNAi-based suppression by characterizing their expression profiles in cultured cells and six developmental stages. We then screened these six dsRNA products in third and fourth instar larvae to examine the knockdown efficiency of each. We also examined the ability of shRNA and pcRNA structures and *E. coli*-expressed dsRNA and chitosan/dsRNA nanoparticles to evaluate their ability to enhance RNAi efficiency in cultured *C. sonorensis* cells or in third and fourth instar larvae, respectively. Though chitosan/dsRNA nanoparticles improved RNAi efficiency in larval *C. sonorensis*, outcomes with the other enhancers were less effective. These findings highlight the need for additional research into RNAi-based control methods for this livestock pest.

Materials and Methods

Insect Cells and Life Stages

Culicoides sonorensis from the AK line and cultured W8A cells (embryonic origin) are maintained at the USDA-ARS laboratories in Manhattan, KS. Methods for maintaining each have been previously described (Chapter 2). Cells and six developmental midge stages were harvested for transcript level expression studies by total RNA extraction, cDNA synthesis, and RT-qPCR as previously described (Chapter 2).

Target Genes and Gene Expression

A published *C. sonorensis* transcriptome (Accession: PRJNA238338) was used to identify nucleic acid sequences for targets of interest for RNAi-based suppression (Nayduch et al. 2014). Three of the genes of interest have previously been described: *IAP1* (Mills et al. 2015), *VhaA* (Chapter 2), and *CsdsRNase-G* (Chapter 4). Chitin synthase (*CHS*) genes from *Ae. aegypti* were referenced in Lopez et al. (2019) and were used to query the *C. sonorensis* transcriptome using BLAST (NCBI, National Center for Biotechnology Information). Two *C. sonorensis* transcripts of interest were found, and when queried using BLAST-p, both showed conserved domains for *CHS*. Other mosquito transcripts for *CHS-1* and -2 were used to query the *C. sonorensis* transcriptome and showed GAWM01003557.1 is likely *CHS-1*, and GAWM01015448.1 is likely *CHS-2*. Unigenes with predicted domains from the previously described transcriptome were queried for the term “chitinase.” Numerous targets were found, and one was chosen for this study given its complete coding sequence and presence of a chitin binding domain GAWM01000884.1. The *C. sonorensis* sequence and chitinase sequences from other insect taxa were used to construct a phylogeny in MEGA X (Stecher et al. 2020).

DsRNA, shRNA, and pcRNA Synthesis

Primers with T7 promoter sequences were used to synthesize dsRNA in this study. For naked dsRNA, total *C. sonorensis* RNA was used to create cDNA which was then used as a template for PCR using primers with T7 promoters, described in (Chapter 2). The two secondary RNA structures, shRNA and pcRNA, were synthesized using a single primer as template. 1 µg of template (PCR product or primer) was used to synthesize RNA overnight using a HiScribe T7 High Yield RNA synthesis kit (NEB, Ipswich, MA) according to manufacturer's

recommendations. To form the secondary RNA structures, products from each reaction were heated to 95 °C and slowly cooled in a thermocycler at -1 °C/2 min to a final temperature of 25 °C (Abbasi et al. 2020). The final product was purified and cleaned as previously described (Chapter 2). Primers can be found in Table D.2.

Chitosan Nanoparticle Synthesis

Chitosan nanoparticles containing dsRNA were formed using procedures described in Zhang et al. (2015). Briefly, deacetylated ($\geq 75\%$) chitosan (Sigma-Aldrich, Milwaukee, WI) was dissolved in 0.1 M sodium acetate buffer to create a solution of 0.02% (w/v) chitosan. Separately, 32 μg of dsRNA was suspended in 50 μl of water, and 50 μl of 100mM sodium sulfate was added to the dsRNA. One hundred (100) μl of the chitosan solution was added to the dsRNA solution (100 μl), the tube was incubated at 55 °C for 1 min, and then the mixture was vortexed immediately at RT for 30 sec. Chitosan/dsRNA was pelleted by centrifugation at 13,000 x g for 10 min at RT. The supernatant was decanted, and the remaining pellet was dried for downstream use. An aliquot of the supernatant was used to determine the unbound RNA concentration by spectroscopy (Implen, Westlake Village, CA). Loading efficiency was determined using the equation $(32 \mu\text{g} - X \mu\text{g in supernatant}) / (32 \mu\text{g}) \times 100 = \% \text{ loading efficiency}$. A small amount of powdered larval food (Hunt 1994) was mixed with the dried chitosan pellet, and 30 μl of 2% melted agarose (cooled to ~ 50 °C to prevent dsRNA strand dissociation) was mixed with the food to create a gel pellet. The gel pellet was cut into six equal pieces and fed to larval *C. sonorensis*.

Vector Construction and dsRNA Expression in *E. coli*

Target and control nucleotide sequences were screened for restriction enzymes (REs) which would not cleave within the dsRNA sequence, and which were present in the L4440 plasmid [L4440 was a gift from Andrew Fire (Addgene plasmid # 1654 ; <http://n2t.net/addgene:1654> ; RRID:Addgene_1654)]. The REs selected were high fidelity (HF) *BmtI*-HF and *MluI*-HF (NEB) and PCR primers containing digest sites were designed for each plasmid insert (Table D.3). Template DNA was amplified using these primers with high fidelity PlatinumTM SuperFiTM II (Invitrogen, Thermo Fisher Scientific, Waltham, MA) master mix according to the manufacturer's supplied protocol. Product was purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). 1 µg of plasmid DNA or cloning template DNA (i.e., PCR product) was digested in 50 µl volumes containing 5 µl of rCutSmart buffer (NEB), 1 µl of *BmtI*-HF, 1 µl of *MluI*-HF, and nuclease-free water to the final volume. The reaction proceeded for 10 min at 37 °C . Digest products were run on a 1% agarose gel and products were extracted and purified with a QIAquick Gel Extraction Kit (Qiagen). Cloning inserts were ligated into the plasmid using the Quick LigationTM Kit (NEB) with 40 ng of linearized pL4440, 17 ng of insert, 1 µl of ligase, 10 µl of ligase buffer, and nuclease free water up to 20 µl total volume. Reactions proceeded for 5 min at RT. This was done for each of six plasmid constructs.

To make competent cells, an overnight culture of HT115 *E. coli* was started in LB broth and grown at 37 °C. Twenty-five mL of LB were spiked with the overnight culture the next morning. When cells reached an optical density (OD) of 0.4-0.9 the sample was pulled and centrifuged at 3000 rpm for 10 min at 4 °C . The media was decanted, and the pellet was resuspended in 12.5 mL of cold, sterile 50 mM CaCl₂. After 30 min on ice, the tube was centrifuged again as above, the liquid portion decanted, and the pellet resuspended in 2.5 mL 50

mM CaCl₂. 50 µl of cells were mixed with 5 µl of ligated product from above for 30 min. Tubes were transferred to a 37 °C water bath for exactly 1 min, after which they were placed back onto ice for 2 min. 1 mL of pre-warmed (37 °C) S.O.C. media (Thermo Fisher) was added to each tube and cells were incubated at 37 °C for 1 hr on a plate shaker (225 rpm). 150 µl of transformed cells were spread onto pre-warmed LB plates containing ampicillin (amp⁺). Colonies observed over the next two days were streaked onto new amp⁺LB plates for isolation. Successful transformation was confirmed by colony PCR using SK-KS primers (Table D.3), and the purified PCR product was sent for Sanger sequencing (Azenta Life Sciences, Burlington, MA).

To express dsRNA, transformed *E. coli* from single colonies were inoculated into amp⁺LB broths to create overnight cultures. The next morning, 100 µl of overnight culture was inoculated into flasks containing 25 mL pre-warmed 2xYT media (Sigma-Aldrich) containing ampicillin. Flasks were placed into a shaker incubator at 37 °C and 190 rpm. Mid-log phase was observed after ~4 h by spectroscopy at which point flasks were treated with either IPTG (isopropyl-β-D-thiogalactopyranoside, Fisher Scientific) to a final concentration of 1 mM, or lactose (Fisher Scientific) to a final concentration of 10 g/L. Bacterial cells were harvested after 5 hours by centrifuging the media at 3000 x g for 15 min. Pelleted cells were resuspended in nuclease-free water and used for treatment or dsRNA was harvested using the protocol described in Ahn et al. (2019) using heat to liberate the dsRNA.

Cell Treatments

W8A cultured cells were seeded into wells of a 24-well cell culture plate in 1 mL of media and grown to ~75% confluence. Cells were treated with 200 ng/mL *dseGFP* (control), 200

ng/mL dsRNA (*IAP1* or *VhaA*), or an equivalent concentration of sh*IAP1* or pc*VhaA*. The shRNA and pcRNA equivalents were determined by finding the molar equivalent of each dsRNA construct and multiplying by an anticipated number of Dicer products (i.e., ~nineteen 21 nt products in a 409 bp dsRNA molecule). The determined equivalents were 177 ng/mL sh*IAP1* or 442 ng/mL pc*VhaA*. Cells were harvested 48 h later and processed for total RNA extraction in 400 µl of TRIzol (Invitrogen) as described previously (Chapter 2). In a separate experiment, cells were treated with 100 ng/mL dseGFP (control), 100 ng/mL of *in vitro* (*iv*) synthesized ds*IAP1*, or 100 ng/mL of *E. coli* (*Ec*) produced ds*IAP1*. Cells were harvested as previously described 48 h after treatment. Each treatment or control had three biological replications.

Larval Treatments

Third (L₃) and fourth (L₄) instar *C. sonorensis* larvae were obtained directly from a colony source. Larvae were pooled (~20 individuals per pool) and placed into 12-well cell culture plates in 1 mL of nuclease-free water. Each experiment consisted of three treated pools for biological replication. In a range finding study, L₃ larvae were treated with 10 µg/mL dseGFP (control), 10 µg/mL ds*CHS-2*, or 1 µg/mL ds*CHS-2*. Larvae were collected after 72 h and homogenized in 400 µl TRIzol with a tissue pestle as previously described (Chapter 2). The 10 µg/mL concentration was used for further studies. For larval screens, L₃ and L₄ larvae were treated with 10 µg/mL dsRNA for *eGFP*, *IAP1*, *VhaA*, *CHS-1*, *CHS-2*, *CHT*, and *CsdsRNase-G*. For additional transcript suppression studies, L₃ larvae were treated with either 10 µg/mL dseGFP or ds*CHS-2*. A second experiment treated L₃ larvae with 100 µl of *E. coli* expressing dseGFP or ds*CHS-2*. A third experiment treated both L₃ and L₄ larvae with chitosan/dseGFP or chitosan/ds*CHS-1* (~3-4 µg dsRNA per chitosan pellet).

In larval mortality experiments, pools of 10 larvae (L₃ or L₄) were treated with either 1 µg/mL, 3 µg/mL, or 5 µg/mL each of dsRNA for *CHS-1*, *CHS-2*, and *CHT*. Control larvae were treated with 15 µg/mL *dseGFP* to equal the maximum amount of the treatment population. Larvae were examined each of seven days and mortality was counted in each well. Dead larvae were removed each day.

RT-qPCR

All gene expression studies were examined by RT-qPCR as previously described (Chapter 2). Two RT-qPCR technical replicates for each biological replicate were completed for each sample. Technical replicates were averaged for analysis. Two *C. sonorensis* reference genes were used for each experiment (Chapter 2). These were *actin* and *VhaA* for comparative gene expression profiles in developmental stages (*EF1b* was used as a reference for *VhaA* expression), *EF1b* and *β-tubulin* for gene expression studies in larvae screened by RNAi knockdown (where *VhaA* was a target), *EF1b* and *GAPDH* for cells treated with dsRNA/shRNA/pcRNA, and *EF1b* and *VhaA* for larvae treated with dsRNA.

Statistical Analysis

Statistical tests were performed in PRISM 9 (Version 9.5.1, GraphPad Software, LLC, Boston, MA). Data were subjected to a Shapiro-Wilk test to test for normality, and a Brown-Forsythe test for equal variance. If met, pairwise comparisons were examined with a two-tail t test, and group comparisons with a one-way ANOVA and a Tukey's post-hoc multiple comparison test (MCT). Non-normal data were examined with a Mann-Whitney test (for pairwise comparison) or a Kruskal-Wallis test and a Dunn's MCT (for group comparisons).

Statistical significance levels were set to 0.05. For developmental stage expression profiles, the delta-Ct (Δ Ct) method was used to show relative gene expression normalized to the geometric mean of two reference genes. The delta-delta-Ct ($\Delta\Delta$ Ct) method was used to calibrate normalized gene expression to either a control group or insect stage. The method used is noted in each figure legend. The test used and outcome for each analysis is described in Table D.5.

Results

Target Expression Profiles

The *IAP1* and *VhaA* targets were expected to have the most consistent expression profiles across all *C. sonorensis* developmental stages given their importance in cell maintenance, and *VhaA* was described as an ideal RT-qPCR reference gene in *C. sonorensis* (Chapter 2). Both targets had similar expression levels across all the developmental stages (Fig. 5.1A-B), albeit with statistically significant differences between some stages. In insects, *CHS-1* is involved with exoskeletal formation and *CHS-2* synthesizes the peritrophic matrix in larval stages (Hegedus et al. 2009, Zhu et al. 2016). Chitin synthase-1 was expressed in all *C. sonorensis* stages and was highest in pupae and males (Fig. 5.1C). By comparison, *CHS-2* was highest in larval stages and lowest in pupae (Fig 5.1D). Adult stages had intermediate *CHS-2* expression. These data reinforce our gene assignments since we'd expect larval stages to be actively synthesizing peritrophic matrix (PM) while feeding, and pupae to be actively synthesizing cuticle. The *CHT* target we chose appeared to have highest expression in pupae and males, was lowest in L₄ and females, and L₃ larvae had intermediate and variable expression (Fig. 5.1E). This chitinase had a predicted “endochitinase” domain, and when compared with other predicted chitinase sequences in *C. sonorensis* and related taxa, this domain was most abundant (Fig. D.1). The predicted

protein also had a chitin binding domain. The function of this enzyme in *C. sonorensis* is unknown but does appear to have an expression profile resembling *CHS-1*. We also investigated a single nuclease (*CsdsRNase-G*) which was described previously (Chapter 4). This dsRNase was expressed highest in larval stages and all other stages similar expression levels (Fig. 5.1F). We found that the only meaningful target expression in cultured cells were *IAP1* and *VhaA*, which both have important cellular functions. All the remaining targets described here had little or no expression in cells (data not shown).

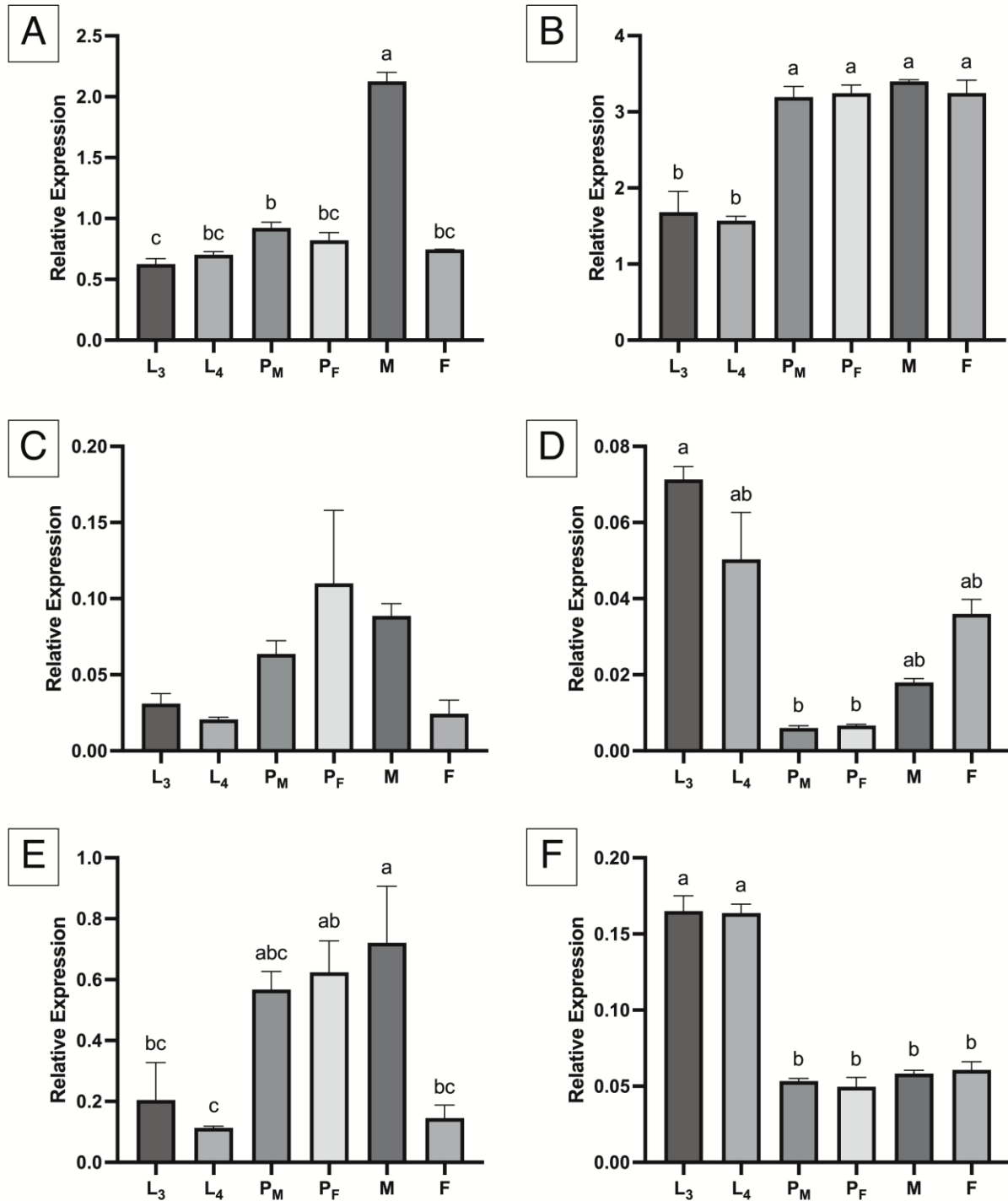


Figure 5.1. Expression of candidate RNAi target genes in six developmental *C. sonorensis* life stages.

Gene targets are (A) *IAP1*, (B) *VhaA*, (C) *CHS-1*, (D) *CHS-2*, (E) *CHT*, and (F) *CsdsRNase-G*. The developmental stages are instar 3 (L₃) and 4 (L₄) larvae, male (P_M) and female (P_F) pupae, and adult males (M) and females (F). Relative expression (ΔCt) is to two reference genes. Bars

are SEM. Letters over each bar indicate statistical significance. Statistics are reported in Table D.5.

RNAi Target Screen in Larvae

We examined which concentration of dsRNA had the optimal target knockdown efficiency in *C. sonorensis* larvae by doing a small-scale range finding study. Here, 10 µg/mL showed the highest knockdown efficiency (~55% suppression) when compared to 1 µg/mL (~30% suppression), and both treatments were statistically significant from the control (10 µg/mL: $P = 0.0006$; 1 µg/mL: $P = 0.0122$) (Fig. 5.2). Next, L₃ larvae were treated with 10 µg/mL of dsRNA to either a target or control. Each pairwise comparison was subjected to statistical analysis, and all results were plotted on the same figure (Fig. 5.3). There were no statistically significant differences in any pairwise comparison in L₃ larvae. Both *IAP1* and *VhaA* appear unchanged, while *CHT* and *CsdsRNase-G* had modest suppression. Both *CHS* genes had higher transcript levels in treated groups, but both (especially *CHS-1*) had large variations in their expression values. In the L₄ group, *VhaA* transcript levels were significantly suppressed, but *CsdsRNase-G* transcript levels were significantly higher than their relative controls (Fig. 5.4), though these differences were small. There was some suppression in *IAP1* and *CHS-2*, and very high (yet non-significant) suppression in *CHS-1*. The expression of *CHT* was very high in the treatment compared to the control, but with high variability.

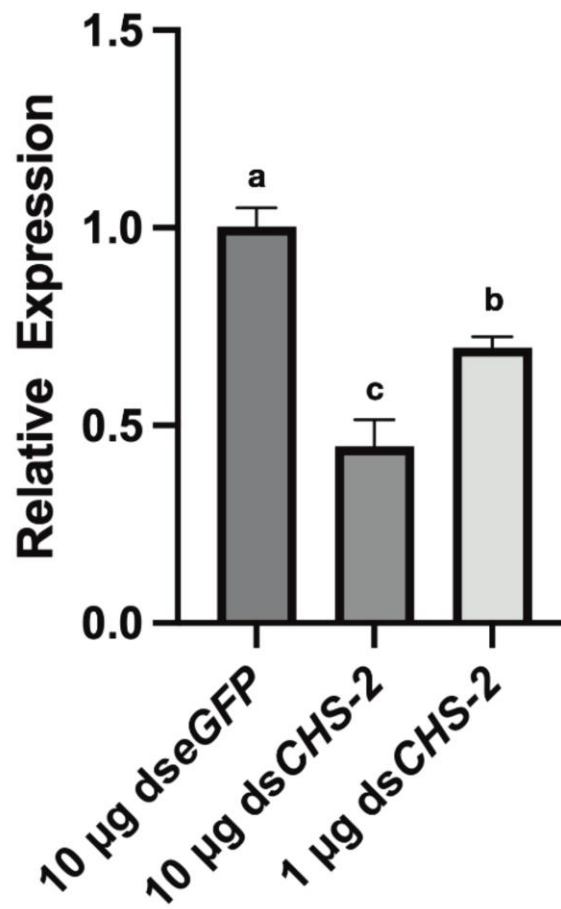


Figure 5.2. Expression (fold) change of *CHS-2* in *L3* after treatment with two concentration of target dsRNA.

Relative expression ($\Delta\Delta C_t$) is to two reference genes, and all values are normalized to the control. Bars are SEM. Letters over each bar indicate statistical significance. Statistics are reported in Table D.5.

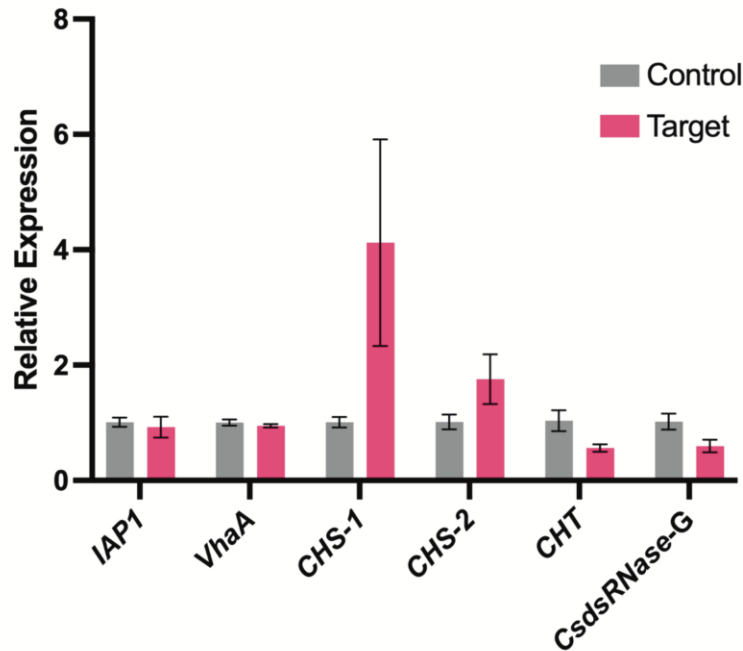


Figure 5.3. Expression (fold) change of numerous gene targets in L₃ after treatment with 10 µg/mL of either control (*dseGFP*) or target dsRNA.

Relative expression ($\Delta\Delta C_t$) is to two reference genes, and all values are normalized to the control. Bars are SEM. Statistics are reported in Table D.5.

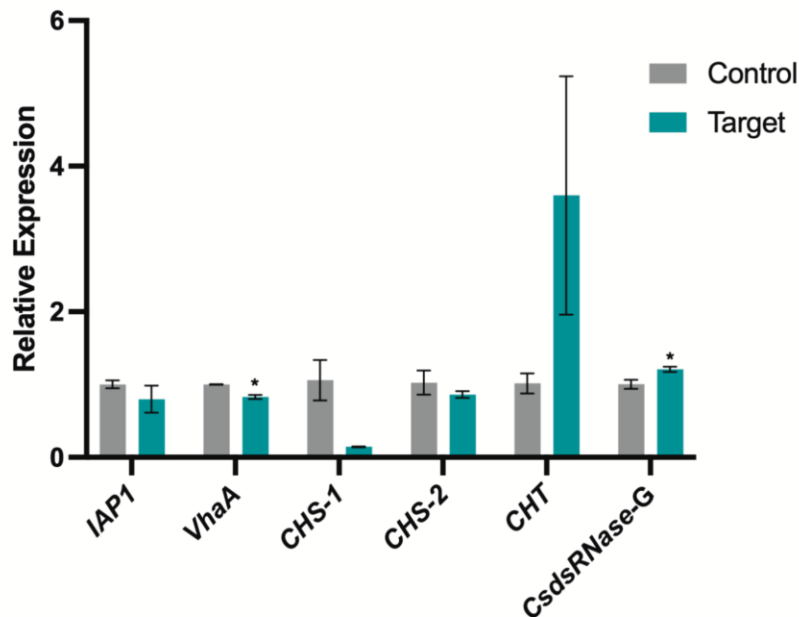


Figure 5.4. Expression (fold) change of numerous gene targets in L₄ after treatment with 10 µg/mL of either control (*dseGFP*) or target dsRNA.

Relative expression ($\Delta\Delta C_t$) is to two reference genes, and all values are normalized to the control. Bars are SEM. *P<0.05. Statistics are reported in Table D.5.

RNAi Enhancers – Secondary RNA Structures

We designed primers to synthesize shRNA and pcRNA for four targets (Fig. D.2A). These sequences are predicted to produce constructs with the desired folding structure when analyzed using RNAfold WebServer (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>). RNA was synthesized from 1 µg of primer template but interestingly we were unable to visualize any of these constructs on agarose gels stained with SafeView™ Classic nucleic acid stain (abm, Richmond, BC, Canada). We increased the agarose concentration to 4% and ran the gel for a short time to better control for possible product runoff and were still unable to visualize product. We detected over 500x more RNA in the reactions after synthesis and purification and were therefore convinced that synthesis was successful (Table D.4). We then used two of these constructs in cells to examine their effects on target suppression. There was a ~35% decrease in *IAP1* expression using dsRNA and a ~27% decrease when using a molar equivalent amount of sh*IAP1* (Fig. D.2B). Only the dsRNA suppression was statistically significant ($P = 0.0321$). We used a similar experiment to assess the activity of pc*VhaA* (Fig. D.2C). Here, we observed ~70% target suppression with the dsRNA treatment and ~39% suppression with pcRNA. Again, only the dsRNA treatment was significantly different ($P = 0.0118$). The respective dsRNA outperformed shRNA and pcRNA in RNAi efficiency and thus we opted not to continue investigating these constructs.

RNAi Enhancers – *E. coli* Expression

We successfully transformed *E. coli* (*Ec*) to express six dsRNA constructs (*eGFP*, *IAP1*, *VhaA*, *CHT*, *CHS-1*, *CHS-2*). Both lactose and IPTG induction produced large quantities of dsRNA product which could be detected by spectroscopy (Table D.4). However, the amount of

target dsRNA versus off-target dsRNA varied widely. We show that bands of target dsRNA derived from *Ec* expression are likely less than 25% of total dsRNA (Fig. 5.3A). We also used an RT-qPCR standard curve approach and observed values of as little as 0.01% of target dsRNA versus total dsRNA (data not shown). When W8A cells were treated with *in vitro* (*iv*) synthesized *dsIAP1* and *dsIAP1* purified from *Ec*, we observed similar suppression of the target (Fig. 5.3B).

The variable expression of target dsRNA makes standardization difficult. We examined the knockdown of a target (*CHS-2*) in L₃ larvae using standard dsRNA and compared this to treatment with *Ec* expressing either *dseGFP* or *dsCHS-2*. The dsRNA from *Ec* was not purified and instead bacterial pellets had been heat killed and resuspended in 1 mL of nuclease-free water. We used 100 µl of resuspended *Ec* per treatment well (1 mL). After 72 h there was a slight suppression of target in naked dsRNA treated larvae (Fig. 5.5A) but a very large increase in target expression in *Ec* treated larvae (Fig. 5.5B). Neither treatment was significant (Table D.5).

RNAi Enhancers – Chitosan Nanoparticles

Chitosan nanoparticles were successfully synthesized using dsRNA for *eGFP* and *CHS-1* (Table D.4). Food pellets containing these nanoparticles were difficult to evenly cut and we observed that larvae were not able to consume the entire pellets which suggests only small exposure to these dsRNA/nanoparticles. Nevertheless, target transcript levels were suppressed by ~45% or greater in L₃ and L₄ larvae (Fig. 5.5C), though these effects were not statistically significant (Table D.5).

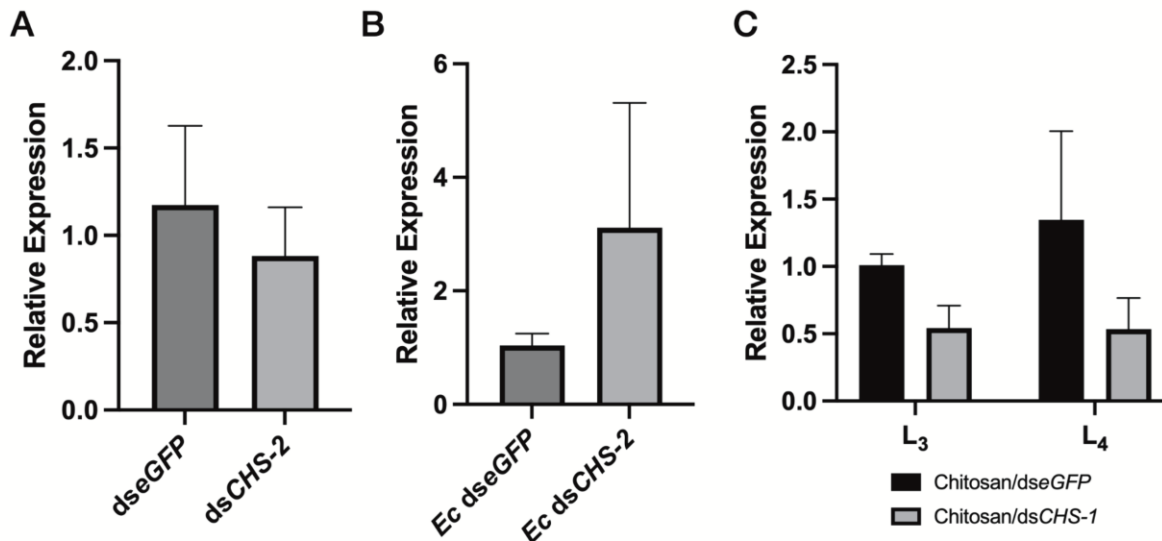


Figure 5.5. Relative expression (fold) of target genes after three types of dsRNA treatments.

A) Knockdown of *CHS-2* using 10 µg/mL of naked dsRNA in L₃ larvae for 72 h. **B)** Attempted knockdown of *CHS-2* using 100 µl of *E. coli* (*Ec*) expressing dsRNA in L₃ larvae for 72 h. **C)** Attempted knockdown of *CHS-1* using ~3-4 µg/mL of dsRNA bound to chitosan nanoparticles in L₃ and L₄ larvae for 72 h. Bars are SEM. Statistics are reported in Table D.5.

Larval Mortality

As a complement to our gene knockdown studies, we examined whether treatments with three dsRNA constructs were larvicidal when delivered together. Here, we found no significant effects of dsRNA concentration on larval mortality. There was significantly more ($P=0.0474$) mortality in the L₄ larvae compared to L₃ larvae (Fig. 5.6); however, this includes some effect of increased mortality in the L₄ control group. The highest mean mortality was observed in the 5 µg ds*CHS-1/CHS-2/CHT* treatment (15 µg/mL total) at 28%. The highest individual well mortality value was 33%, also in the 5 µg ds*CHS-1/CHS-2/CHT* group in L₄ larvae. Almost all conditions had wells with 0% larval mortality at the conclusion of the study with the exceptions of the 3 µg ds*CHS-1/CHS-2/CHT* in the L₃ group and 5 µg ds*CHS-1/CHS-2/CHT* in the L₄ group. Interestingly, the condition with the highest L₄ group mean mortality was the lowest mean

mortality condition in the L₃ group (5 µg ds*CHS-1/CHS-2/CHT*). Overall, the high variability in mortality in L₃ and L₄ larvae made interpreting the impact of these treatments difficult to determine.

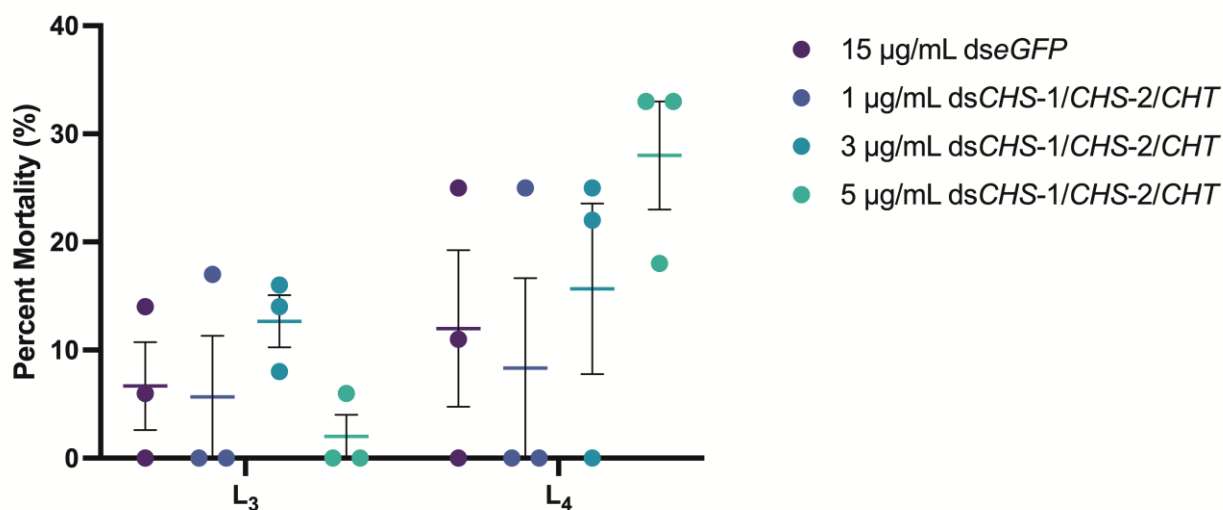


Figure 5.6. Seven-day mortality of L₃ and L₄ larvae treated with dsRNA targeting *CHS-1*, *CHS-2*, and *CHT* at three concentrations, or *dseGFP* (15 µg/mL) control.

Individual points are percent mortality in each replicate well, bars are mean mortality, and error bars are SEM. There was a significance difference in mortality in the L₄ larvae (F 1, 19 = 4.493; P=0.0474). Full statistics are reported in Table D.5.

Discussion

In this study we set out to examine RNAi targets in *C. sonorensis*, their effects in larval stages, three methods of enhancing RNAi, and if a combination of dsRNA constructs had larvicidal properties. We successfully characterized the expression of six RNAi targets in six midge developmental stages. This is an important step in target selection as ideal targets for insect control should be highly expressed, sensitive to RNAi, and have proteins with short half-lives (Scott et al. 2013). The targets screened were meant to encompass diverse mechanisms with the potential to increase larval mortality, including molting, gut integrity, and cell metabolism.

The chitin synthase genes appear to be ideal targets given their importance in molting and adult development (*CHS-1*) and larval peritrophic matrix development (*CHS-2*). We observed *CHS-2* to be highly expressed in the larval stages which is consistent with high PM turnover during development in actively feeding larvae (Fig. 5.1D). We anticipate that failure to produce PM would leave larvae susceptible to gut damage which could increase larval mortality. The expression of *CHS-1* increased between the larval and pupal stages and the suppression of these enzymes in the pupae would be expected to be detrimental to cuticle development. There are numerous chitinase enzymes in insects (Zhu et al. 2016), and the putative chitinase gene we chose appears to be related to other insect enzymes with endochitinase activity (Fig. D.1). Transcript levels were highest in pupae and males but were also present in the remaining life stages. This putative chitinase may have activity in processing chitin during molting, but we did not characterize its function further. The remaining targets (*IAP1*, *VhaA*, and *CsdsRNase-G*) had more consistent expression in each stage, albeit with statistically significant differences between some stages (Fig. 5.1). Constitutive expression indicates the physiological importance of these genes and could make them candidates for RNAi-based suppression to reduce midge fitness. Other studies have done large-scale screens of targets in mosquitos to find optimal targets (Mysore et al. 2017) which would be an ideal future approach in *C. sonorensis* for identifying gene targets that are most susceptible to RNAi in this species.

The results from our knockdown screens in both L₃ and L₄ were highly variable. An excellent example of this is our efforts to suppress transcript levels of *CHS-2* in L₃. Our initial range finding study (Fig. 5.2) suggested that this would be a good target for RNAi, but subsequent tests suggested that *CHS-2* transcript levels were upregulated with dsRNA treatment at the same concentration (Fig. 5.3). In addition, there were no clear patterns in suppression of

transcript levels between L₃ and L₄ treated larvae either. We are confident that larvae are exposed to dsRNA through soaking experiments because previous observations showed clear evidence of internalization of food dyes from rearing water and of food contents in larval guts when fed agarose embedded with untreated food. Experiments to show uptake of fluorescently labeled dsRNA by gut epithelial cells were not performed in this work, so it is currently unknown whether the dsRNA that the larvae are treated with is degraded prior to uptake or if it can penetrate the gut epithelia and then fails to have an effect inside the larvae. Future experiments may focus on the uptake of dsRNA by cells lining the alimentary tract and the fate of dsRNA in the gut. One study suggests Diptera lack the SID-1 protein necessary for systemic RNAi spread, and which could contribute to some of the poor RNAi responses observed here (Dowling et al. 2016). While *Ae. aegypti* appear to be susceptible to target suppression by dsRNA application in the cell and larval stages (Abbasi et al. 2020), pupae (Arshad et al. 2021), and adults (Coy et al. 2012) stages, we've shown previously that there is a large discrepancy in RNAi efficacy between cultured *C. sonorensis* cells and larvae (Chapter 3). There are numerous treatment variables which remain to be explored, including reapplication of dsRNA throughout the duration of the experiment, types of food offered to *C. sonorensis* which may alter target gene expression (i.e., encourage growth and molting), and the persistence of dsRNA in larval rearing water. Our results are a firm foundation for these future studies that evaluate the mechanisms that limit RNAi efficiency in *C. sonorensis* larvae, and given the high variability observed here, it's necessary to explore enhancers of RNAi for use in *Culicoides*.

Efforts have been made to use secondary RNA structures, chitosan nanoparticles, and *E. coli* expressing dsRNA to enhance RNAi in other target insects. Abbasi et al. (2020) treated *Ae. aegypti* cultured cells and larvae with long dsRNA or equivalent amounts of siRNA, shRNA, or

pcRNA and saw target transcript suppression was significant in all treatments. However, their long dsRNA treatments performed the best in all treatments, and pcRNA treatments were comparable to the observed transcript suppression in cultured cells. We treated cultured W8A cells with molar equivalents of shRNA or pcRNA to observe activity comparable to their dsRNA counterparts. Though we were unable to visualize the short, secondary structures of the RNAs, computer modeling and the observation of activity suggest they were functional constructs. However, their lack of enhanced activity makes them less attractive targets for development of RNAi-based insecticides for *C. sonorensis*.

Chitosan nanoparticles complexed with dsRNA were successfully used in *An. gambiae* larval feeding experiments to suppress transcripts of two chitin synthase genes (Zhang et al. 2010). Here, we observed appreciable, but non-significant, suppression of targets compared to controls (Fig. 5.5). One concern with these experiments was that the pellets of agarose containing chitosan/dsRNA and food were difficult to evenly divide and larvae were unable to eat large amounts of these pellets during the experiments' duration likely as a result of their small size. Larval mosquitos—which this technique was first developed for—are much larger than *C. sonorensis* and can eat more material. We cannot anticipate the actual concentration of chitosan/dsRNA that larval midges consumed. However, these results are promising especially given that treatments concentrations were much smaller (~3-4 µg/well) than naked dsRNA (10 µg/well) in similar experiments. The chitosan nanoparticles are anticipated to protect dsRNA from degradation in the gut and perhaps increase cellular uptake of dsRNA (Zhang et al. 2010). These nanoparticles are potentially a good candidate for future investigation for RNAi-based insecticides for *C. sonorensis*.

Expressing dsRNA, and shRNA, in microbial (e.g., bacteria, algae, fungi) systems has become widespread as these provide an avenue to produce large quantities of RNAi treatment constructs relatively inexpensively (Ahn et al. 2019, Duman-Scheel 2019, Lopez et al. 2019). Six dsRNA constructs were successfully cloned into plasmids and *E. coli* were successfully transformed with these plasmids. Ahn et al. (2019) compared conventional, heat, and sonication methods to liberate dsRNA from *E. coli* cells. The authors found that heat and sonication yielded higher dsRNA quantities, and we used heat to liberate dsRNA followed by isolation steps described by the authors. Synthesizing dsRNA in vitro produced very consistent values of around 270 µg (SD ± 14.0) per two reactions, or approx. 135 µg per single reaction (Table D.4). By comparison, *E. coli* expressed dsRNA yields ranged from 48 µg to 352 µg for 25 mL cultures. Lactose induction also produced higher dsRNA yields over the short term compared to IPTG induced samples. This may have been caused by an increase in bacterial growth associated with the addition of lactose, but since lactose can be metabolized, we would expect the increased production to decrease over time compared to IPTG. There are many optimization steps that can be explored using this system.

A major issue with the *E. coli* system was accurately determining the quantity of target dsRNA expressed amongst the host nucleic acid. The protocol described by Ahn et al. (2019) uses DNaseI and RNaseA to degrade DNA and single stranded RNA, ideally leaving only intact dsRNA. We found that only 25% or less of the product produced was target specific when comparing *E. coli* and in vitro synthesized dsRNA using gel electrophoresis (Fig. D.3), and yields may be as little as less than 1% when we used standardized RT-qPCR approach (data not presented here) according to other protocols developed for accurately quantifying dsRNA (Cooper et al. 2020). The time and resources necessary to accurately quantify bacterial products

conflicted with the anticipated low cost and ease of use for producing large quantities of dsRNA in a short period of time. We show here that *E. coli* derived dsRNA performs equally as well as in vitro synthesized dsRNA (Fig. D.3). Treating larvae with *E. coli*, however, presented a challenge. Similar to feeding chitosan nanoparticles, we could not accurately quantify the feeding rate or behavior of larvae during treatment. Two studies in Diptera which successfully reduced target transcripts by feeding *E. coli* expressing dsRNA ensured insects were exposed to treatments by applying concentrated bacteria to food sources (Li et al. 2011, Taracena et al. 2022). We opted for an approach which deposited heat-killed *E. coli* suspended in water into the treatment wells. Ultimately, this treatment had no effect in suppressing the gene target in larvae (Fig. 5.5B), but optimization for delivering these treatments could yield better transcript target suppression.

The results presented here suggest RNAi responses in *C. sonorensis* are highly variable. Use of chitosan nanoparticles was the only RNAi enhancement method which had positive effects on target gene suppression. Using combinations of three dsRNA constructs did not significantly impact larval mortality either. There are many other methods being explored to enhance RNAi by stabilizing the molecules in the environment and insect gut, to enhance uptake of molecules, and to mass produce dsRNA (Silver et al. 2021). The semi-aquatic habitats of many *Culicoides* species are often rich in organic matter. Using microbial expression systems which are adapted to these environments (e.g., microalgae and bacteria) could be useful in developing site-specific treatments. Chitosan nanoparticles, which are constructed from a natural material, are expected to be biodegradable and likely safe for environmental use. These could be explored for field applications. Ultimately, our findings show larval *C. sonorensis* do not appear to be amenable to RNAi-based gene suppression in most instances but there are many avenues

left to explore towards bringing this technology to fruition in this important livestock pest. Future directions include investigating the uptake mechanisms, transport, and systemic responses to dsRNA, how suppressing detoxification genes could enhance insecticides, and altering the physiology or structure of the larval gut to make this stage more susceptible to insecticides.

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Chapter 6 - Variable gut pH as a potential mechanism of tolerance to *Bti* in the biting midge *Culicoides sonorensis*

Abstract

Bio-rational insecticides derived from toxins of *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) are safer alternatives for non-target organisms when compared to traditional chemical insecticides for controlling dipteran pests such as black flies and mosquitoes. The biting midge *Culicoides sonorensis* (Diptera: Ceratopogonidae) is an important pest of livestock in much of the United States and larval midges utilize semi-aquatic habitats which are permissive for *Bti* product application. Reports suggest that *Bti* and *Bti* products are ineffective in biting midges despite their close taxonomic relation to black flies and mosquitoes. Here, we investigate the toxicity of the *Bti*-based insecticide VectoBac® WDG and its active ingredient (*Bti* strain AM65-52) in larval *C. sonorensis*. A suspected mechanism of *Bti* tolerance is an acidic larval gut, and we used a pH indicator dye to examine larval *C. sonorensis* gut pH after exposure to *Bti*. The LC₉₀ of VectoBac® WDG (386 mg/L) was determined to be almost 10,000 times more than that of some mosquito species, and no concentration of active ingredient tested achieved 50% larval mortality. The larval gut was found to be more acidic after exposure to *Bti* which inhibits *Bti* toxin activity. By comparison, 100% mortality was achieved in larval *Aedes aegypti* at the product's label rate for this species and mosquito larvae had alkaline guts regardless of treatment. Altering the larval rearing water to alkaline conditions enhanced *Bti* efficacy when using the active ingredient. We conclude that *Bti* is not practical for larval *C. sonorensis* control at the same rates as mosquitos but show that alterations or additives to the environment could make the products more effective.

Introduction

Chemical insecticides can be detrimental to non-target organisms and can pollute water systems, making pests with aquatic live stages (i.e., mosquitos, black flies, etc.) difficult to target without broader impacts. Bio-rational insecticides are those which are typically safer for non-target organisms and the environment and include essential oils, entomopathogenic fungi, and bacterial entomotoxins. A notable example are the toxins derived from the bacterium *Bacillus thuringiensis* (*Bt*), of which the subspecies *israelensis* (*Bti*) expresses toxins which kill dipteran pests (Ben-Dov 2014). Products containing *Bti* are effectively used for mosquito and black fly control throughout much of the world. Despite its widespread use in the field, resistance to *Bti* products have not been reported (Ben-Dov 2014). This insecticide is an important tool for controlling major vectors of human pathogens.

Bacillus thuringiensis is a gram negative, spore forming bacterium which produces crystalline protoxins during sporulation (Palma et al. 2014, Silva-Filha et al. 2021). When ingested by insects, protoxins are solubilized under alkaline conditions, cleaved by host enzymes to form active toxins, and these active toxins bind to membrane bound receptors where they form pores in the gut membrane (Bravo et al. 2007, Bravo et al. 2011). The major toxins expressed in *Bti* are Cry4Aa, Cry4Ba and Cry11Aa and Cyt1Aa, and are suggested to work synergistically and help reduce resistance development to any single toxin (Ben-Dov 2014). These toxins are effective specifically against larvae in the families Culicidae, Simuliidae, and Chironomidae (Berry et al. 2002). However, studies have shown that larval chironomid midges (i.e., non-biting midges) have guts with variable pH, including acidic conditions which prevent protoxin solubilization (Frouz et al. 2007). A study of two biting midges, a *Leptoconops* sp. and a *Culicoides* sp. (Family: Ceratopogonidae), found no larvicidal activity of *Bti* strain H-14 in either

species (Lacey and Kline 1983). Toxicity to *Bti* in European *Culicoides* spp. was examined in field collected larvae in northern Sardinia (Foxi et al. 2019). The authors found the lethal concentration to kill 50% percent of larvae (LC₅₀) to be 14.8 parts per million (ppm) active ingredient (a.i.) and 26.3 ppm a.i. for the LC₉₀. By comparison, another study found the LC₉₅ in multiple mosquito species to be 0.019 ppm for *Culex quinquefasciatus*, 0.031 ppm for *Aedes aegypti*, and less than 0.040 ppm for four other mosquito species (Russell et al. 2003). These data reinforce the lack of *Bti* efficacy in biting midges and the high sensitivity of mosquitoes.

Culicoides are important vectors of pathogens around much of the world where they primarily impact livestock herds (Purse et al. 2015, Mullen and Murphree 2019), and *C. sonorensis* is an important vector of livestock pathogens in much of the United States (McGregor et al. 2022). Chemical insecticides and other control methods are moderately effective at reducing pest populations but do not always reduce abundance to levels that prevent pathogen spread (Pfannenstiel et al. 2015). New and optimized methods to control *Culicoides* populations are still desired, including those that are safer for non-target organisms and the environment. In this study we examine the toxicity of a.i. of *Bti* as well as a commercial formulation (VectoBac® WDG) in *C. sonorensis* larvae. We also investigated the gut pH of larvae after treatment with *Bti* and evaluate if changes in the pH of the larval rearing water enhance the efficacy of *Bti*.

Materials and Methods

Insects

Colony reared *C. sonorensis* (AK line) larvae were sourced directly from the USDA-ARS laboratory in Manhattan, KS. Eggs from *Ae. aegypti* (Rockefeller strain) were provided by researchers at Tulane University and were reared by a collaborator at Kansas State University.

For our experiments, larvae were maintained in nuclease-free water in an incubator set to 25 °C with a 14:10 light:dark cycle. In some experiments, larval *C. sonorensis* were given a small amount of a powdered diet sprinkled into their water and larval *Ae. aegypti* were given fish flakes.

***Bti* Products**

The commercial product VectoBac® WDG (Valent BioSciences, Libertyville, IL) contains *Bti* strain AM65-52 at 37.4% active ingredient (a.i.) and has an advertised potency of 3,000 international toxin units (ITU) per mg for *Ae. aegypti*. We also sourced primary powder (i.e., technical grade active ingredient) which had a listed potency of 7,000 ITU/mg. This powder contains fermentation solids, spores, and insecticidal toxins. Both products were stored at 4 °C.

Bti* Toxicity in *C. sonorensis

Instar 3 larvae (L₃) were obtained directly from the insectary source and were aliquoted into wells of 24-well cell culture plates. Each well contained approx. 15 larvae. Rearing water was aspirated from each well. A 1:10 dilution series of technical grade *Bti* (4.83 mg/mL – 0.0483 µg/mL) was made in seven steps for an initial range finding study. Larvae were treated with 1 mL of the dilution step or control (water only) and each dilution step or control was used four times (four biological replicates). Larval mortality was assessed daily for seven days, and dead larvae were removed from each well. This experiment was replicated for VectoBac® WDG where the highest initial concentration was 11 mg/ml. A second, 1:2 dilution series (1.0 mg/ml – 0.0625 mg/ml) was made for VectoBac® WDG in five steps and was used to treat larvae as

above. Log PROBIT analysis was conducted in SAS[®] Studio (SAS Institute Inc., v3.8) to determine LC₅₀ and LC₉₀ values, associated 95% confidence intervals, and model statistics.

PH Indicator Dye

Universal pH Indicator Solution (ThermoFisher Scientific, Waltham, MA), containing approx. 42% isopropanol, 3.5% phenol, and 2% methanol, was aliquoted into 1.5 mL microcentrifuge tubes in 1 mL volumes. Tubes were left open in a fume hood to allow volatiles to evaporate until the volume was ~0.5 mL (50% of original). This concentrated dye was expected to primarily be the aqueous component of the dye and to be less toxic to larvae. 1 mL of concentrated dye was added to 450 mL of water in a beaker on a stir plate. A pH probe (accumet[®] AB150, Fisher Scientific) was inserted into the water, and the pH of the water was adjusted with 0.1 N HCl or 0.1 N NaOH through a range of pH values (4.52 – 9.34). Pictures of the beaker's water were captured with an iPhone 12 Pro (Apple, Cupertino, CA) for 16 pH values (Fig. E.1). A reference chart for pH and dye color was constructed from actual values captured during this experiment (Fig. 6.1).

Larval Gut pH

Fourth instar (L₄) *C. sonorensis* larvae (~20/pool) were aliquoted into wells of a 6-well cell culture plate. Each well (A-F) received a control or treatment as follows: A-C) 1 mL water only, D) 1 mL water with larval food (Hunt 1994), E) 1 mL of 177 mg/L technical grade *Bti*, and F) 1 mL of 177 mg/L VectoBac[®] WDG. Plates with larvae were left overnight in an incubator as previously described. After ~12 h larvae were aspirated and moved into 1.5 mL microcentrifuge tubes. Water was removed and replaced with 200 µl of store-bought green food coloring (Group

B) or 200 µl of concentrated pH indicator dye (Groups C-F). Larvae were soaked for 1 h, transferred to a clean 6-well cell culture plate with their respective groups, and washed with deionized water. Representative samples from each larval group were placed onto a glass slide with a cover slip, examined with a dissecting scope, and imaged with an iPhone 12 Pro.

Mosquito comparisons

Fourth instar *Ae. aegypti* were used for this study. First, five larvae were placed into wells of 12-well cell culture plates. Wells were treated with 1 mL of water (control), technical grade *Bti* (8 mg/L), or VectoBac® WDG (8 mg/L). Three biological replicates for each condition were performed and mortality was assessed after 24 h. Second, *Ae. aegypti* larvae were placed into wells of a 6-well cell culture plate (10 larvae each) and treated with a scheme as above for *C. sonorensis*, with the exception that both *Bti* products were used at a concentration of 0.8 mg/L. Living larvae were soaked in food color or dye and imaged as above.

Enhancing *Bti* with pH Changes

Approximately 20 L₃ *C. sonorensis* larvae were placed into each well of three 24-well plates. Plate 1 was treated with a 1:2 dilution series of VectoBac® WDG (62.5 mg/L – 1000 mg/L) in five steps. Four replicate wells were treated for each concentration, and four wells were water only controls. Each well was then treated with 40 µl of 0.1 N NaOH and 5 µl of concentrated pH indicator dye. Plate 2 used the same layout as Plate 1 but with technical grade *Bti* at 10x the concentration of VectoBac® WDG (625 mg/L – 10,000 mg/L). A third plate used the same layout as Plate 2 but NaOH was substituted with 100 µl of heat killed *E. coli* suspended in water. The *E. coli* were strain HT115 from a study previously described (Chapter 5). Briefly,

E. coli were grown in 25 mL flasks for 9 h, pelleted by centrifugation, and resuspended in 2 mL water. The pellet weight was 1 g which gave an approximate bacterial concentration of 500 mg/mL. The *E. coli* mixture was heat killed at 70 °C for 10 min and mortality was confirmed by streaking this mixture onto a LB agar plate and allowing it to grow overnight. Daily larval mortality was assessed for seven days for each plate.

Results

Lethal concentrations of *Bti* in *C. sonorensis*

The LC₅₀ for VectoBac® WDG in *C. sonorensis* was 177 mg/L (95% CI: 151-205 mg/L) and 386 mg/L (95% CI: 319-503 mg/L) for the LC₉₀ after seven days of exposure (Table 6.1). By comparison, no pool of larvae treated with technical grade *Bti* had mortality above 50% at any concentration tested after seven days. The highest mortality recorded was 45% in a larval pool treated with 4,830 mg/L. These results meant we were unable to determine an LC₅₀ for technical grade *Bti* under the concentration range tested. To ensure that both *Bti* products tested here were active, *Ae. aegypti* larvae were treated with the VectoBac® WDG label rate of 8 mg/L. All mosquito larvae tested were dead after 24 h and zero larvae were dead in control wells.

Table 6.1. The concentration range tested and calculated lethal concentration (LC) values for VectoBac® WDG tested against L3 instar *C. sonorensis* larvae.

Log PROBIT test outputs are reported as well. Abbreviations: n, sample size (total insects used including from control conditions); SE, standard error; LC, lethal concentration; CI, confidence interval; X^2 , Pearson Chi-Square. A non-significant p-value suggests no statistically significant difference between the data and the model.

Range Tested	Number of Concentrations	n	Slope ($\pm$ SE)	LC ₅₀ (95% CI)	LC ₉₀ (95% CI)	X ²	p-value	R ²
0.625-1.0 mg/L	5	360	3.78 ($\pm$ 0.43)	177 mg/L (151-205 mg/L)	386 mg/L (319-503 mg/L)	25.18	0.1199	0.80

***C. sonorensis* larval gut pH**

Larvae were treated in four conditions and imaged after exposure to water (control), food coloring (ingestion control), or pH indicator dye (Fig. 6.2). Green food coloring can be seen through much of the alimentary tract (Fig. 6.2B) except in the foregut, and there is a delineation between the foregut and midgut indicating the presence of a sphincter. The concentrated pH dye was active throughout a range of pH values and was used as a reference for larval gut pH (Fig. 6.1). Larvae exposed only to water had blue guts which were approx. pH 8 (Fig. 6.2C). Larvae which were fed or exposed to VectoBac® WDG or technical grade *Bti* had yellow guts which were approx. pH 6 (Fig. 6.2D-F). By comparison, all *Ae. aegypti* larvae exposed to the same conditions as *C. sonorensis* larvae had blue guts of approx. pH 8 (Fig. 6.3A). Mosquito larvae were more difficult to see through than midge larvae. Only 1 larva from each group are shown in Figure 6.2 and these larvae were representative of most larvae in the group. However, there was variability in the gut coloration in *C. sonorensis* larvae treated with *Bti* products. Figure 6.3B shows multiple *C. sonorensis* larvae which were exposed to VectoBac® WDG overnight and soaked in pH dye as described previously. These larvae have areas of yellow (acidic), green (neutral), and blue (alkaline) within the same individuals' guts.



Figure 6.1. Universal indicator dye colors corresponding to pH values.

Colors are derived from actual observations during a validation experiment.

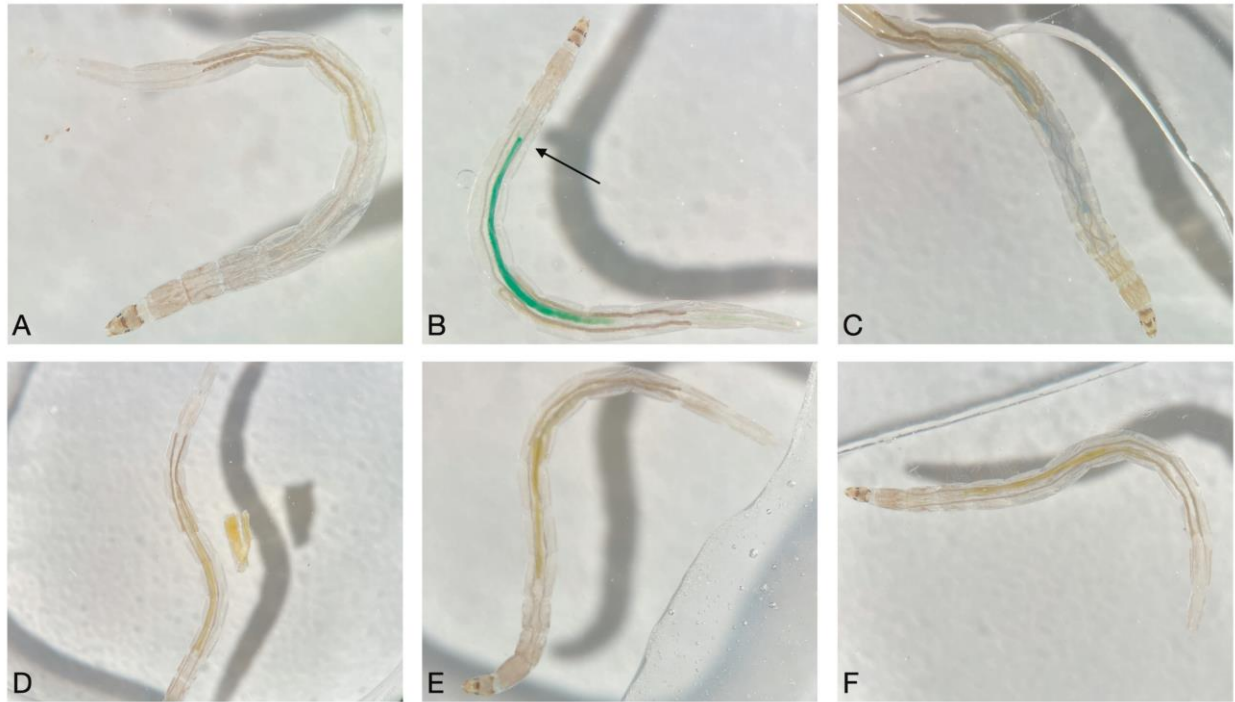


Figure 6.2. L4 *C. sonorensis* larvae were soaked overnight in A-C) water, D) water with food, E) water with technical grade *Bti*, and F) water with VectoBac® WDG.

After 12 h, larvae were soaked in water (A), green food coloring (B), or pH dye (C-F) for 1 hr, washed, and imaged live. Larvae are ~4 mm in length. Arrow shows the junction of the foregut and midgut.

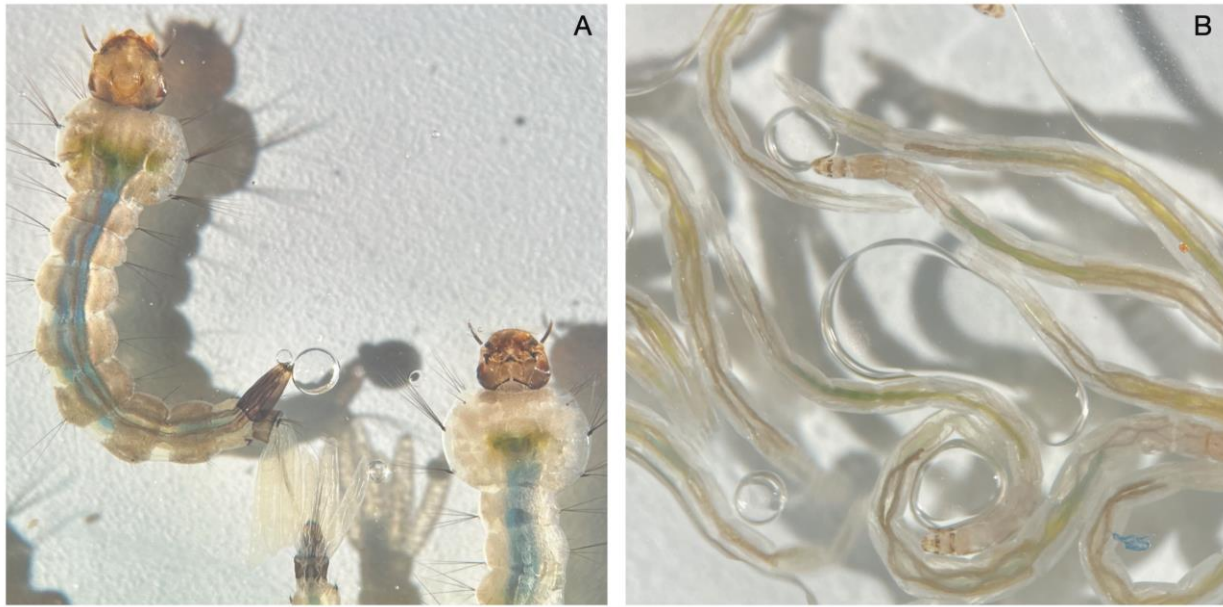


Figure 6.3. Larvae of *Ae. aegypti* (A) and *C. sonorensis* (B) after 12 hr exposure to *Bti* followed by a 1 hr soak in pH indicator dye.

Alkaline pH and *Bti* efficacy in *C. sonorensis*

The observation that *C. sonorensis* larval guts tended to be acidic suggested that this feature may be a potential reason for the observed low efficiency of *Bti* treatment. Accordingly, we attempted to determine LC₅₀ and LC₉₀ values for VectoBac® WDG and technical grade *Bti* when rearing conditions were made alkaline. The results of each of three experiments were subjected to log PROBIT analysis and the results of all three analyses had significant p-values (Table E.1). These results suggest the data differ significantly from the model and we opted to report daily mean survival for the results instead (Fig. 6.4). Alkaline conditions from NaOH alone (controls) did not greatly affect larvae during the study. The highest mean control mortality was 10% on day 7 in plate 2. For VectoBac® WDG + NaOH treated larvae, only the 500 mg/L treated group achieved 50% mortality after 7 days (Fig. 6.4A). By comparison, the LC₅₀ for

VectoBac[®] WDG was 177 mg/L after 7 days. This suggests no enhancement to VectoBac[®] WDG with NaOH.

Four tested concentrations achieved greater than 50% mortality by day 7 in the technical grade *Bti* + NaOH plate (Fig. 6.4B). Only two treatments did not achieve 50% mortality, which were the control and the intermediate technical grade *Bti* concentration (2,500 mg/L). These results show some enhancement to the efficacy of technical grade *Bti*. In our original study we were unable to achieve 50% mortality in any group after 7 days with the highest concentration of 4,830 mg/L.

Preliminary experiments performed in our lab found *Bti* products were enhanced in *C. sonorensis* when larval water was treated with heat-killed *E. coli* suspended in water (which created an alkaline environment). Treating wells with *E. coli* killed larval *C. sonorensis* in the absence of *Bti* (Fig. 6.4C). These control wells experienced immediate larval die-off after 24 h with a mean mortality of 15%. Mortality stabilized for most of the remainder of the study period and only increased at day 6 and again at day 7. The final mean mortality for the control was 19%. Every technical grade *Bti* concentration achieved greater than 50% mean mortality by day 3, and two concentrations achieved $\geq 90\%$ mean mortality (10,000 mg/L at day 4 and 5,000 mg/L at day 7). These data show that heat-killed *E. coli* can enhance *Bti* in killing *C. sonorensis*. A blue color (alkaline condition pH 8) was observed in all wells throughout the experiment (representative example in Fig. E.2). At the conclusion of the experiment 40 μ l of 0.1 N HCl was added to wells to check the integrity of the dye, and well colors changed towards neutral/acidic immediately suggesting the dye was still pH sensitive.

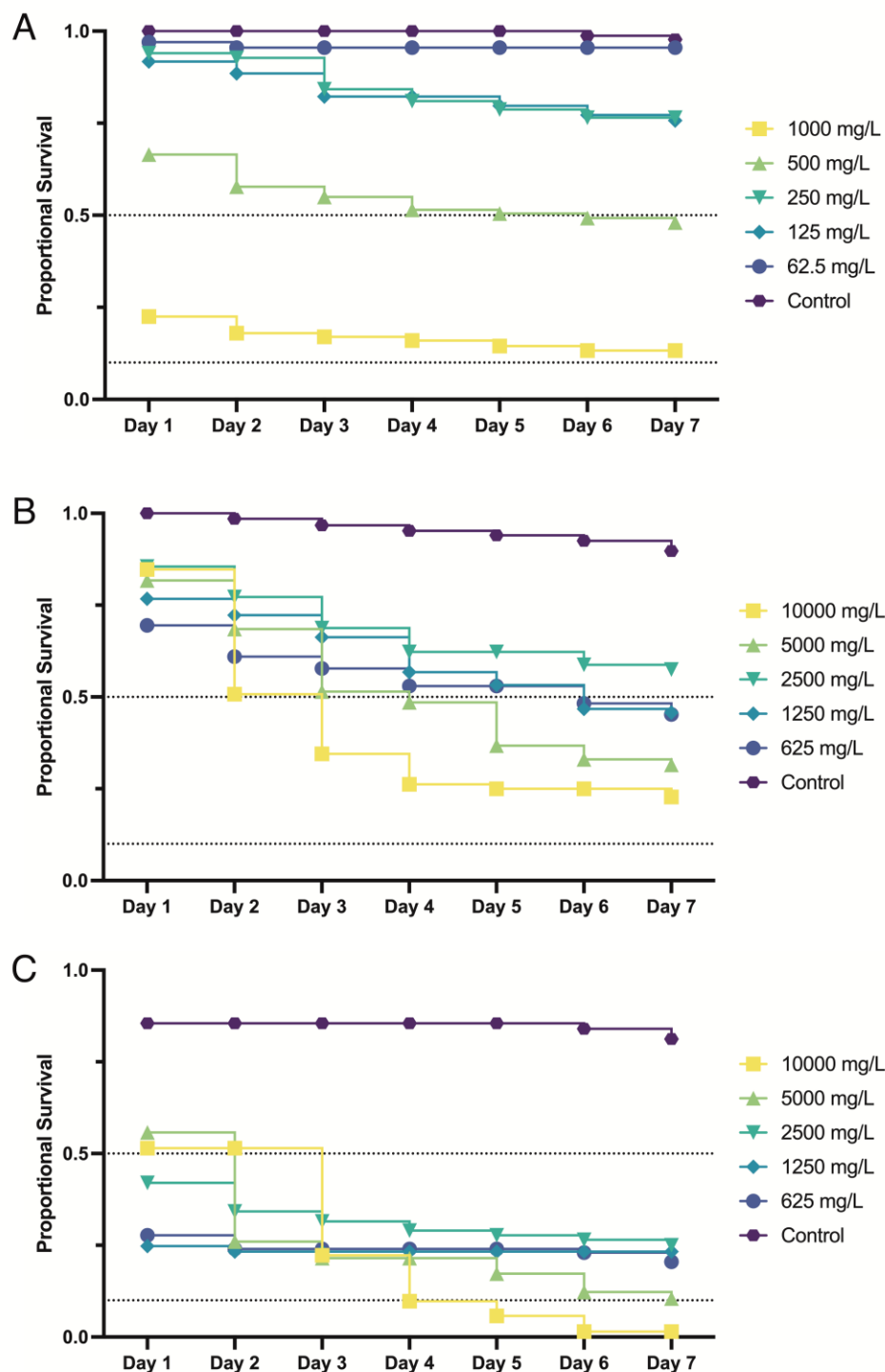


Figure 6.4. Daily mean proportional survival of *C. sonorensis* larvae treated with *Bti* products and alkaline enhancers (four biological replicates for each).

Dotted lines indicate 50% (approx. LC_{50}) survival and 10% survival (approx. LC_{90}). **A)** Larvae treated VectoBac® WDG + 40 µl NaOH. **B)** Technical Grade *Bti* + 40 µl NaOH. **C)** Technical Grade *Bti* + 100 µl heat-killed *E. coli* in water. Additional data are in Table E.2.

Discussion

In this study we determined the LC_{50} and LC_{90} of VectoBac[®] WDG against *C. sonorensis*. We also showed that technical grade *Bti* was ineffective at killing *C. sonorensis* larvae even at extremely high concentrations (<50% mortality at 4,830 mg/L). Experiments found *Ae. aegypti* larvae can be killed with both *Bti* products in 24 h using the label rate of 8 mg/L. One published LC_{95} value in *Ae. aegypti* was 0.031 ppm (approx. equivalent to 0.031 mg/L), which is over 10,000 times less material than was necessary to achieve the LC_{90} value in *C. sonorensis* (Russell et al. 2003). Black flies are effectively controlled by using *Bti* products in running water (e.g., streams), and one study replicated this in a microcosm to assess *Bti* efficacy (Iburg et al. 2010). Here, greater than 75% larval black fly mortality was observed after treatments with less than 0.5 mg/L of active ingredient. These findings suggest some physiological difference is likely making *Culicoides* tolerant to *Bti*.

We examined if larval gut pH could play a role as a tolerance mechanism and found that unfed *C. sonorensis* larvae had alkaline guts, but larvae had acidic guts after being fed a larval diet and after exposure to both *Bti* products tested here. Neither the larval food nor the *Bti* products were acidic on their own. It appears that the larval gut pH is highly variable and becomes more acidic when the larvae are actively feeding, but that *Ae. aegypti* gut pH is unaltered by exposure to food or *Bti* products. We then examined if creating an alkaline rearing environment would enhance the efficacy of *Bti* products. It appears that the addition of both NaOH and heat-killed *E. coli* created alkaline conditions which enhanced technical grade *Bti* but not VectoBac[®] WDG. When compared to our original findings, we were able to achieve 50% larval mortality using technical grade *Bti* concentrations as low as 625 mg/L. These values are still much higher than the concentrations necessary to kill mosquito larvae.

Larval gut pH was not homogenous in our studies. Individuals shown in Figure 6.2 were representative of the others in the same treatment group, but Figure 6.3B shows more heterogeneity of the larval gut. In this figure larvae can be seen with three distinct colors (pH levels) within the same gut tract and variations in regional coloration between individuals (i.e., proximal to distal). We are confident that larvae take up their rearing water as 100% of individuals can be observed with food coloring in their guts after ~2 h of soaking. It's not clear, however, if larvae actively consume *Bti* products or cease eating when exposed to certain concentrations of the products. This is evidenced by variations in daily mean mortality values that did not appear to be concentration dependent. For example, 50% mean mortality was observed by day 1 with technical grade *Bti* concentrations of 625-2,500 mg/L but not with concentration 5,000-10,000 mg/L (Fig. 6.4C). This could also be a factor in our investigation of alkaline enhancers. It's unclear if simply raising the pH (more alkaline) enhances *Bti* toxin solubility prior to ingestion or if there's another mechanism at play (e.g., anoxic conditions). Nevertheless, both NaOH and *E. coli* additions improved the efficacy of technical grade *Bti*. These findings could translate to future research on insecticide additives to increase gut pH or identify new avenues of suppressing gut acidification.

Numerous *Bti* products are available for commercial use. Most appear to use the AM65-52 strain but with different concentrations of active ingredient and proprietary additives. Valent BioSciences, the manufacturer of VectoBac® WDG, produces a product for chironomid midges (Bactimos®) which sinks to the bottom of water systems to interact with larvae in substrate. This product is designed to be more potent for midges as the accompanying material cites 15-75 times less sensitivity of chironomids to *Bti* when compared to mosquitos (<https://www.valentbiosciences.com/publichealth/products/bactimos/>). This product could be

explored for *C. sonorensis*, though we show that this biting midge may be thousands of times less sensitive to *Bti* than mosquitos. Foxi et al. (2019) examined VectoBac[®] 12 AS (1.2% a.i.; 1,200 ITU/mg) in mid-stage *Culicoides* larvae as they're expected to be actively feeding compared to L₄ larvae, a method we replicated for our LC studies. The LC₉₀ was ~26.3 ppm of a.i. which is approximately 2,192 mg/L of VectoBac[®] 12 AS. Our LC₉₀ for VectoBac[®] WDG (37.4% a.i.) was 386 mg/L, or ~144.4 ppm a.i. This is roughly 5.5 times more a.i. than for the Sardinian *Culicoides* sp. used in the previous study. The authors also show a ~40% loss in effectivity when larvae were reared with mud substrate compared to water. It's expected *Bti* in the environment would interact with organic matter and be broken down by sunlight, reducing its efficacy in field conditions (Lacey 2007, Su 2017, Foxi et al. 2019). In one practical field trial, VectoBac[®] DT (3.4% a.i.; 3,400 ITU/mg) was used in combination with Flubex[®] (a.i. diflubenzuron) to treat larval breeding sites and Decaflow[®] (a.i. deltamethrin) for adult treatment on livestock farms in northern Sardinia (Meloni et al. 2018). The authors saw significant adult midge reductions on each site studied, showing this method could be useful for *Culicoides* control. This is important as there are no registered control options for larval biting midges, and our findings may inform development of enhancers for *Bti* against biting midges.

Methods for *Culicoides* control have been reviewed previously but there are few or no highly effective strategies or products available (Carpenter et al. 2008, Pfannenstiel et al. 2015). In part, the ecology of these midges is complicated by cryptic breeding sites which make field treatments of larval stages difficult (Mullens et al. 2015). Exchanging traditional chemical insecticides (e.g., organophosphates, pyrethroids) for more targeted insect growth regulators should be safer for the environment, but these products still have off-target effects on non-target insects. Biological insecticides such as *Bti* have a narrower range of taxa but their efficacy in

Culicoides is limited, as evidenced by this study. Our observations suggest new avenues for enhancing the efficacy of existing insecticide chemistries for use on *Culicoides* midges, but ultimately more investigation is necessary to develop new control options for livestock producers.

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Chapter 7 - Conclusions and Future Directions

Global food systems are threatened by insects which can deplete crops, destroy stored products, and affect livestock health. Some biting midges in the genus *Culicoides* are competent vectors of animal viruses around much of the globe, and outbreaks of viral pathogens threaten the livelihoods of livestock producers (Purse et al. 2015, Rushton and Lyons 2015, Mullen and Murphree 2019). Traditional strategies to control midge populations include habitat modification, larvicidal treatments, and chemical insecticide applications (Carpenter et al. 2008). These methods are not always effective and thus new control options are sought for these important pests. RNA interference (RNAi) is a potential tool for developing highly specific insecticides that do not have the same impact on non-target species and the environment as other methods (Zhu and Palli 2020). The research described in this dissertation explored the foundations of RNAi efficiency, barriers to double-stranded RNA (dsRNA) stability, and methods to enhance RNAi delivery in the model biting midge species, *C. sonorensis*. Specifically, methods to kill larval *C. sonorensis* were prioritized to investigate possible novel larvicidal products against this developmental stage.

RNAi suppresses targeted gene expression by reducing transcript abundance. Real-time qPCR (RT-qPCR) was instrumental in determining the effect of RNAi treatments. In Chapter 2, reference genes necessary for accurate RT-qPCR analysis were described for seven broad experimental applications. The results were critical for all further gene expression analyses conducted in Chapters 3-5, and broadly the findings will be applicable to researchers around the world. Research described in Chapter 3 built on earlier work which found RNAi mechanisms to be active in *C. sonorensis* (Schnettler et al. 2013, Mills et al. 2015). These results showed that RNAi effectively knocked down target genes in cultured *C. sonorensis* cells (W8), but there was

a limited response in larval midges. Gene expression profiles showed that core RNAi-associated proteins and enzymes were present in all major developmental stages and were not the likely reason for the disparate responses. The dsRNA binding protein, Staufén, had dramatically lower expression in fourth instar larvae compared to all other developmental stages. Whereas this differential expression could have implications for RNAi efficiency, suppression of Staufén transcripts in W8 cells had no effect on RNAi efficiency, but more mechanistic experiments may clarify its role. These data suggest another mechanism is reducing RNAi efficiency in larvae.

Chapter 4 describes experiments to identify and characterize putative dsRNA-degrading enzymes (dsRNases) which may reduce the stability of dsRNA products used for RNAi studies (Cooper et al. 2019). Seven putative enzymes were found using a published transcriptome and genome for *C. sonorensis* (Nayduch et al. 2014, Morales-Hojas et al. 2018). The expression profiles for six dsRNases were characterized in six midge developmental stages, cultured cells, and four female tissue groups or body parts. The expression patterns were varied and mostly unique. Functional experiments found dsRNA was degraded in fluid derived from larval guts, supporting the idea that dsRNases are important inhibitors of effective RNAi responses when feeding larvae. Suppressing two dsRNases was successful in cultured cells which is a potential method for enhancing dsRNA stability, but dsRNase transcript suppression was inconclusive in larval stages. Overcoming these enzymes has been identified as an important step towards enhancing RNAi in other insects, and our data suggests that this is the case in *Culicoides* as well.

Protecting dsRNA from degradation was explored using specialized RNA structures, a nanoparticle carrier, and heterologous expression in *E. coli* in Chapter 5, methods that have previously been used to enhance RNAi in insects (Silver et al. 2021). Short-hairpin RNA (shRNA) and paperclip RNA (pcRNA) were easy to synthesize *in vitro* but did not enhance

RNAi efficiency above their traditional dsRNA counterparts. Chitosan nanoparticles bound to dsRNA have been successfully used in mosquito treatments and suppressed some gene targets in third and fourth instar *C. sonorensis*. Notably, suppression in larvae was achieved at lower concentrations than with naked dsRNA treatments. Expressing dsRNA in *E. coli* was explored as a method to increase dsRNA production and to protect dsRNA while in the alimentary tract of *C. sonorensis*. Six dsRNA constructs were successfully cloned and expressed in *E. coli*, but standardizing treatments were difficult and gene suppression in larvae was not observed. No results clearly show RNAi to be effective for *C. sonorensis* control, but the increase in efficiency shown by complexation of dsRNA with nanoparticles suggest that future research could employ these methods.

Other novel larval control methods were explored in Chapter 6. Here, the toxicity of *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) was determined using PROBIT analysis to find lethal concentrations for a commercial formulation and its active ingredient. The results showed 10,000 times more material was required to kill *C. sonorensis* when compared with many mosquito species. The *C. sonorensis* gut environment was shown to be more acidic than a mosquito's gut, which is a likely mechanism that explains the reduced sensitivity of *C. sonorensis* larvae to *Bti*. Increasing the pH (more alkaline) of the larval rearing system enhanced the toxicity of *Bti*. These findings could inform future product development which is specific for treating these refractory insects.

This research contributes to the growing body of knowledge regarding varied RNAi efficiency among insect taxa and highlights potential mechanisms for these disparities. In addition, this work creates foundations for future studies which will use RNAi in *Culicoides* as a molecular tool for genetic studies, control methods, and more.

These include describing effective dsRNA concentrations to achieve desired gene suppression and which strategies effectively enhanced RNAi. Future research should focus on optimizing RNAi-based treatment strategies. These include 1) describing which gene candidates are both insecticidal and most sensitive to RNAi suppression, 2) reapplication of dsRNA treatments for continuous exposure to enhance gene suppression, 3) employing additional enhancement strategies not explored here (e.g., other nanoparticles, fungal expression, etc.), 4) enhancing dsRNA stability in field conditions, 5) evaluating off-target effects on other *Culicoides* species and in non-target organisms, and 6) investigating RNAi mechanisms in other important *Culicoides* species. There is still an immense amount of research needed to characterize RNAi in *C. sonorensis*. The research presented here provides new understanding of RNAi mechanisms in dipterans and makes inroads towards developing new control strategies for this biting midge and making animals and humans safer from this pest.

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Appendix A - Supplemental Information for Chapter 2

Supplementary Data A.1. All determined Ct values for six candidate reference genes in *Culicoides sonorensis*.

Reference genes are *Actin*, beta-tubulin (*β-tubulin*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ribosomal protein S18 (*RPS18*), vacuolar-type ATPase subunit A (*VhaA*), and elongation factor 1-beta (*EF1b*). The experiments were cells treated with water or varying concentrations of *dseGFP* (Cells (G)), cells treated with *dseGFP* or varying concentrations of *dsVhaA* (Cells (V)), larvae treated with *dseGFP* or *dsIAP1* (Larvae (I)), midge developmental stages (Stages), midge body parts or tissue groups (Body), and female midges injected with vesicular stomatitis virus (VSV) or bluetongue virus (BTV).

<i>Actin</i>							<i>β-tubulin</i>						
Cells (G)	Cells (V)	Larvae (I)	Stages	Body	VSV	BTV	Cells (G)	Cells (V)	Larvae (I)	Stages	Body	VSV	BTV
15.78	15.99	19.62	20.72	21.63	19.78	20.01	15.79	15.93	18.61	19.72	24.82	19.75	20.19
16.48	17.62	19.62	20.76	21.02	20.67	19.97	16.59	18.90	18.66	18.99	23.77	21.22	20.13
15.87	15.69	18.95	21.00	21.70	21.78	20.03	15.74	15.49	18.21	19.78	25.25	22.22	20.21
16.29	15.76	18.79	21.12	19.56	19.77	20.10	16.26	15.69	18.04	19.73	20.97	20.05	20.54
15.95	15.73	20.14	20.89	20.54	19.63	20.12	16.15	15.63	18.50	19.60	21.37	19.97	20.32
15.91	15.52	19.40	20.96	21.83	19.52	19.98	16.05	15.38	18.45	19.36	23.03	19.89	20.13
15.88	15.63	19.25	20.52	20.82			15.91	15.43	18.38	18.58	25.51		
15.76	15.57	19.03	20.93	21.25			15.78	15.60	18.21	19.18	25.01		
15.47	15.58	19.34	20.84	21.06			15.38	15.44	18.28	18.91	25.19		
15.47	15.82	19.41	20.38	23.68			15.58	15.84	18.46	17.99	29.00		
15.59	15.60	19.06	20.51	22.83			15.69	15.48	18.64	18.79	28.27		
15.39	15.64	19.40	20.21	22.85			15.44	15.65	18.35	18.05	28.13		
			21.60							20.95			
			21.18							20.68			
			21.28							20.80			
			20.36							19.83			
			20.22							19.49			
			20.55							20.29			
<i>GAPDH</i>							<i>RPS18</i>						
Cells (G)	Cells (V)	Larvae (I)	Stages	Body	VSV	BTV	Cells (G)	Cells (V)	Larvae (I)	Stages	Body	VSV	BTV
13.16	13.45	13.77	13.73	19.77	16.59	17.03	19.18	19.21	21.44	23.66	26.06	21.91	22.06
13.88	14.51	13.59	14.01	19.04	17.08	16.94	19.96	19.97	21.50	23.64	25.08	22.89	21.75
13.20	13.30	13.21	14.25	20.00	18.22	17.01	19.20	18.88	20.77	23.81	26.54	23.95	22.02

13.59	13.52	13.18	13.56	16.24	17.06	17.19	19.65	18.82	20.84	24.21	20.69	22.01	22.07
13.57	13.26	13.46	13.44	17.30	16.68	17.62	19.56	18.69	21.14	23.98	21.77	21.99	22.05
13.43	13.25	13.49	13.55	18.34	17.01	16.92	19.43	18.63	20.96	23.83	23.32	21.84	22.24
13.32	13.22	13.31	16.11	17.59			19.38	18.73	20.80	21.99	23.95		
13.19	13.06	13.30	16.99	17.65			19.21	18.73	20.83	22.44	23.33		
12.88	12.99	13.97	15.97	17.71			18.89	18.72	20.70	22.36	23.43		
13.07	13.49	13.68	16.53	22.43			18.91	18.85	21.03	21.84	31.65		
13.24	13.33	13.44	16.62	21.58			19.01	18.63	20.72	22.19	32.87		
13.00	13.38	13.64	15.89	21.80			18.84	18.71	20.79	21.76	35.46		
			14.97							23.52			
			13.83							22.85			
			13.64							22.97			
			15.36							21.86			
			14.85							21.93			
			14.30							22.20			
EF1b							VhaA						
Cells (G)	Cells (V)	Larvae (I)	Stages	Body	VSV	BTv	Cells (G)	Cells (V)	Larvae (I)	Stages	Body	VSV	BTv
18.16	18.88	17.29	18.24	20.50	17.25	17.70	16.92	17.18	17.20	19.12	21.66	19.04	18.97
18.85	19.81	17.12	18.28	20.05	18.41	17.63	17.69	19.57	17.21	18.93	21.04	20.34	18.95
18.10	18.50	16.68	18.45	20.69	19.39	17.82	16.85	16.87	16.78	20.00	21.73	21.32	19.17
18.48	18.82	16.64	18.94	17.08	17.54	17.99	17.39	23.01	16.55	19.65	19.70	19.11	19.16
18.50	18.65	17.02	18.81	18.92	17.31	17.94	17.24	23.23	17.34	19.61	19.43	18.97	19.23
18.42	18.57	16.90	18.78	20.11	17.38	17.92	17.01	23.22	16.99	19.64	20.99	19.05	19.82
18.23	18.48	16.70	19.03	19.12			16.99	20.18	16.75	18.35	20.15		
18.12	18.41	16.67	19.45	18.77			16.86	20.41	16.87	18.75	20.96		
17.77	18.46	16.83	19.80	19.30			16.53	20.56	16.86	18.62	20.68		
18.05	18.63	16.60	18.63	22.54			16.74	19.20	17.00	18.14	22.71		
18.16	18.44	16.64	18.97	21.36			16.91	18.81	16.92	18.16	21.36		
17.84	18.51	16.84	18.91	21.79			16.69	18.97	17.26	18.00	21.80		
			20.26							19.32			
			19.85							18.92			
			20.16							19.04			
			17.39							17.99			
			17.72							17.67			

			17.82							18.00			
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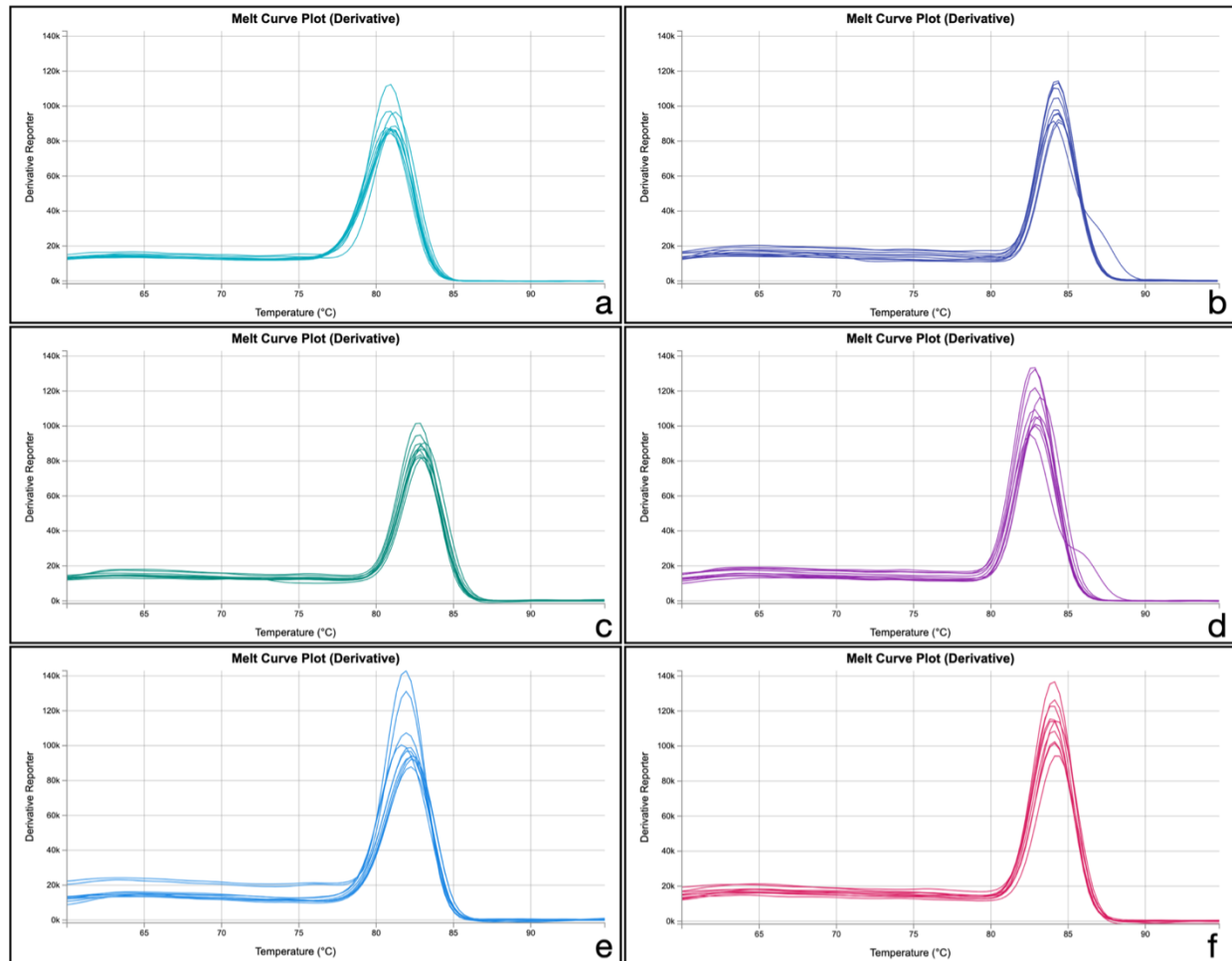


Figure A.1. Melt curve analyses of six *Culicoides sonorensis* candidate reference genes.

Analysis was performed on a QuantStudio 7 Pro (Thermo Fisher Scientific). Outputs are from the Design & Analysis Software (Release Version: 2.6.0, Thermo Fisher Scientific). Figure shows the melt curves for *EF1b* (a), *β-tubulin* (b), *VhaA* (c), *actin* (d), *RPS18* (e), and *GAPDH* (f).

Table A.1. Primers for dsRNA synthesis for *Culicoides sonorensis* experiments.

Abbreviations: bp, base pair; ds, double-stranded; *IAP1*, inhibitor of apoptosis 1; *VhaA*, vacuolar-type ATPase subunit A; *eGFP*, enhanced green fluorescent protein; [T7], T7 Promoter Sequence (TAATACGACTCACTATAGGG). Primer references are superscripted. Ac5-STABLE1-neo was a gift from Rosa Barrio & James Sutherland (Addgene plasmid # 32425 ; <http://n2t.net/addgene:32425> ; RRID:Addgene_32425).

Target	Accession/Source	Primer Sequence (5'→3')	Product Size (bp)
ds <i>IAP1</i> ¹	GAWM01009039.1	F: [T7]-AGTTGAAGAACAACCTTGAGATGG	344
		R: [T7]-GCCAATCTTCATACGACACC	
ds <i>VhaA</i>	GAWM01018658.1	F: [T7]-CAACTGCAATACCAGGAGCG	409
		R: [T7]-CGTGACCTAAATATGCGGG	
ds <i>eGFP</i> ²	Ac5-STABLE1-neo (Plasmid #32425)	F: [T7]-TGACCACCCTGACCTAC	304
		R: [T7]-TTGATGCCGTTCTTCTGC	

Table A.2. Rankings from 1 (best) to 6 (worst) of six candidate *C. sonorensis* reference genes under seven experimental conditions as determined by four analysis tools (RefFinder, BestKeeper, NormFinder, and geNorm).

Comprehensive rankings are reported from RefFinder and this study's analyses. ^ARefFinder (RF) reports rankings for each of the other analysis tools (BestKeeper, NormFinder, geNorm) and includes the delta Ct ranking when creating a comprehensive rank. Bolded text call attention to comprehensive ranks

Experiment	Algorithm	Ranking					
		1	2	3	4	5	6
Cells (dseGFP)	RF ^A : Delta Ct	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>EF1b</i>	<i>RPS18</i>	<i>Actin</i>
	RF: BestKeeper	<i>GAPDH</i>	<i>EF1b</i>	<i>VhaA</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>RPS18</i>
	RF: Normfinder	<i>β-tubulin</i>	<i>VhaA</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>
	RF: geNorm	<i>GAPDH</i>	<i>EF1b</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>
	RF: Comprehensive	<i>GAPDH</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>RPS18</i>	<i>Actin</i>
	BestKeeper	<i>GAPDH</i>	<i>EF1b</i>	<i>VhaA</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>
	NormFinder	<i>β-tubulin</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>EF1b</i>	<i>Actin</i>
	geNorm	<i>GAPDH</i>	<i>EF1b</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>
	Comprehensive	<i>GAPDH</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>RPS18</i>	<i>Actin</i>
Cells (dsVhaA)	RF: Delta Ct	<i>EF1b</i>	<i>GAPDH</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>VhaA</i>
	RF: BestKeeper	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
	RF: Normfinder	<i>EF1b</i>	<i>GAPDH</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>VhaA</i>
	RF: geNorm	<i>GAPDH</i>	<i>EF1b</i>	<i>RPS18</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
	RF: Comprehensive	<i>EF1b</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>

	BestKeeper	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
	NormFinder	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>VhaA</i>
	geNorm	<i>EF1b</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
	Comprehensive	<i>EF1b</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>
Larvae (<i>dsIAP1</i>)	RF: Delta Ct	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>
	RF: BestKeeper	<i>β-tubulin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>Actin</i>
	RF: Normfinder	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>
	RF: geNorm	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>
	RF: Comprehensive	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>
	BestKeeper	<i>β-tubulin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>GAPDH</i>	<i>RPS18</i>	<i>Actin</i>
	NormFinder	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>
	geNorm	<i>VhaA</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>Actin</i>
	Comprehensive	<i>VhaA</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>Actin</i>
Life Stages	RF: Delta Ct	<i>Actin</i>	<i>VhaA</i>	<i>RPS18</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	RF: BestKeeper	<i>Actin</i>	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>GAPDH</i>
	RF: Normfinder	<i>Actin</i>	<i>VhaA</i>	<i>EF1b</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	RF: geNorm	<i>RPS18</i>	<i>VhaA</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>GAPDH</i>
	RF: Comprehensive	<i>Actin</i>	<i>VhaA</i>	<i>RPS18</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	BestKeeper	<i>Actin</i>	<i>VhaA</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>GAPDH</i>
	NormFinder	<i>Actin</i>	<i>VhaA</i>	<i>EF1b</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>

	geNorm	<i>VhaA</i>	<i>RPS18</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>EF1b</i>	<i>GAPDH</i>
	Comprehensive	<i>Actin</i>	<i>VhaA</i>	<i>RPS18</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>GAPDH</i>
Female Tissues	RF: Delta Ct	<i>EF1b</i>	<i>GAPDH</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>RPS18</i>
	RF: BestKeeper	<i>VhaA</i>	<i>Actin</i>	<i>EF1b</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>RPS18</i>
	RF: Normfinder	<i>GAPDH</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>Actin</i>	<i>VhaA</i>	<i>RPS18</i>
	RF: geNorm	<i>Actin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>RPS18</i>
	RF: Comprehensive	<i>EF1b</i>	<i>Actin</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>RPS18</i>
	BestKeeper	<i>VhaA</i>	<i>Actin</i>	<i>EF1b</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>RPS18</i>
	NormFinder	<i>GAPDH</i>	<i>EF1b</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>VhaA</i>	<i>RPS18</i>
	geNorm	<i>Actin</i>	<i>EF1b</i>	<i>VhaA</i>	<i>GAPDH</i>	<i>β-tubulin</i>	<i>RPS18</i>
	Comprehensive	<i>EF1b</i>	<i>Actin</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>RPS18</i>
VSV Injected	RF: Delta Ct	<i>EF1b</i>	<i>RPS18</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	RF: BestKeeper	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>
	RF: Normfinder	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	RF: geNorm	<i>Actin</i>	<i>RPS18</i>	<i>EF1b</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	RF: Comprehensive	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>β-tubulin</i>
	BestKeeper	<i>GAPDH</i>	<i>RPS18</i>	<i>EF1b</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>
	NormFinder	<i>EF1b</i>	<i>RPS18</i>	<i>Actin</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	geNorm	<i>Actin</i>	<i>RPS18</i>	<i>EF1b</i>	<i>VhaA</i>	<i>β-tubulin</i>	<i>GAPDH</i>
	Comprehensive	<i>EF1b</i>	<i>RPS18</i>	<i>Actin</i>	<i>GAPDH</i>	<i>VhaA</i>	<i>β-tubulin</i>

BTV Injected	RF: Delta Ct	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	RF: BestKeeper	<i>Actin</i>	<i>RPS18</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	RF: Normfinder	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	RF: geNorm	<i>Actin</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>VhaA</i>
	RF: Comprehensive	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	BestKeeper	<i>Actin</i>	<i>RPS18</i>	<i>EF1b</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	NormFinder	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>
	geNorm	<i>EF1b</i>	<i>Actin</i>	<i>β-tubulin</i>	<i>RPS18</i>	<i>GAPDH</i>	<i>VhaA</i>
	Comprehensive	<i>EF1b</i>	<i>Actin</i>	<i>RPS18</i>	<i>β-tubulin</i>	<i>GAPDH</i>	<i>VhaA</i>

Table A.3. Pairwise V score values for each of the experiments in this study as determined by the geNorm analysis.

A value of 0.15 or greater suggests a significant contribution of adding another reference gene. The experiments were cells treated with water or varying concentrations of *dseGFP* (Cells (G)), cells treated with *dseGFP* or varying concentrations of *dsVhaA* (Cells (V)), larvae treated with *dseGFP* or *dsIAP1* (Larvae (I)), midge developmental stages (Stages), midge body parts or tissue groups (Body), and female midges injected with vesicular stomatitis virus (VSV) or bluetongue virus (BTV).

	Cells (G)	Cells (V)	Larvae (I)	Stages	Body	VSV	BTV
V2/V3	0.00213	0.00562	0.00482	0.01246	0.01660	0.00222	0.00273
V3/V4	0.00180	0.00487	0.00329	0.01410	0.01861	0.00304	0.00249
V4/V5	0.00132	0.00932	0.00328	0.01223	0.01615	0.00291	0.00376
V5/V6	0.00147	0.02785	0.00366	0.02411	0.02514	0.00489	0.00391

References

1. **Mills, M. K., D. Nayduch, and K. Michel. 2015.** Inducing RNA interference in the arbovirus vector, *Culicoides sonorensis*. *Insect. Mol. Biol.* 24: 105-114.
2. **Yao, J., Rotenberg, D., Afsharifar, A., Barandoc-Alviar, K., and A. E. Whitfield. 2013.** Development of RNAi methods for *Peregrinus maidis*, the corn planthopper. *PLoS One*. 8: e70243.

Appendix B - Supplemental Information for Chapter 3

Table B.1. Target accessions, RT-qPCR primers, dsRNA synthesis primers, and amplicon size for *C. sonorensis* RNAi associated genes examined in this study.

Abbreviations: bp, base pair; *IAP1*, inhibitor of apoptosis protein 1; *VhaA*, vacuolar-type ATPase subunit A; *eGFP*, enhanced green fluorescent protein; dsRNA, double-stranded RNA. [T7] Promoter Sequence: TAATACGACTCACTATAGGG. Citations are superscripted. Accessions are from NCBI and plasmids are from AddGene (addgene.org).

Target	Role	Accession/Source	Primer Sequence (5'→3')	Product Size (bp)
<i>IAP1</i> ¹	RT-qPCR	GAWM01009039.1	TTGTGGACATATTATTGCCTGTG	85
			CATGACTTTCGTGAATGGTTGT	
<i>VhaA</i> ²	RT-qPCR	GAWM01018658.1	GTATGTTGCAAGTGTGGCCTG	86
			ACGCTGACCAGTTAACAATGGA	
ds <i>IAP1</i> ¹	dsRNA	GAWM01009039.1	F: [T7]-AGTTGAAGAACACTTGAGATGG	344
			R: [T7]-GCCAATCTTCATACGACACC	
ds <i>VhaA</i> ²	dsRNA	GAWM01018658.1	F: [T7]-CAACTGCAATACCAGGAGCG	409
			R: [T7]-CGTGCACCTAAATATGCGGG	
ds <i>Stau</i>	dsRNA	GAWM01016436.1	F: [T7]-GGCAGAAAAAGGTCCACCTCA	367
			R: [T7]-TTCTCGCTTCCTAGGTGCTC	
dse <i>GFP</i> ³	dsRNA	Ac5-STABLE1-neo (Plasmid #32425)	F: [T7]-TGACCACCCTGACCTAC	304
			R: [T7]-TTGATGCCGTTCTTCTGC	

Table B.2. Target accessions, RT-qPCR primers, and amplicon size for *C. sonorensis* RNAi associated genes examined in this study.

Abbreviations: bp, base pair; *Ago2*, Argonaute-2; *Dcr2*, Dicer-2.

Target	Accession	Primer Sequence (5' -> 3')	Product Size (bp)
<i>Ago2</i>	GAWM01012835.1	F: TCAATCATGAAGGAATTGCAACGA	166
		R: ATGAGTCGCCATTACACCAGG	
<i>Dcr2</i>	GAWM01016560.1	F: TGGATCGACCTCCTGCATTG	150
		R: TTTTACCAGCACCAGTCGGA	
<i>R2D2-1</i>	GAWM01017705.1	F: TGCAAAACACAGTGCAGCTC	172
		R: GGGACAATGAGACGGTCCTG	
<i>R2D2-2</i>	GAWM01013568.1	F: TTCGAAGTCGTGTCTCAGCA	197
		R: AAGAGGCCAATTTTCGCTCCA	
<i>Staufen</i>	GAWM01016436.1	F: TCCTCATCCACCACCAAAATTGA	105
		R: CCACGTTTCATTGCAAGAGCA	

Table B.3. Statistics for Figure 3.2. Abbreviations: D.o.F., degrees of freedom (between group, within group); MCT, multiple comparisons test.

Panel	Test Type	D.o.F.	Test Value	P-value	Post-hoc Test
A	One-way ANOVA	4, 10	283.1	< 0.0001	Tukey's MCT
B	One-way ANOVA	4, 10	18.76	0.0001	Tukey's MCT
C	Kruskal-Wallis	4, 10	11.50	0.0013	Dunn's MCT
D	Kruskal-Wallis	4, 10	9.30	0.0229	Dunn's MCT
E	One-way ANOVA	4, 10	90.17	< 0.0001	Tukey's MCT
F	One-way ANOVA	4, 10	129.8	< 0.0001	Tukey's MCT
G	One-way ANOVA	4, 10	65.59	< 0.0001	Tukey's MCT

Table B.4. Expression ratios for four RNAi genes and a Staufén homolog in cultured *C. sonorensis* cells and six insect developmental stages.

Shown are the averages of three replicates of proportional expression values relative to *Ago2*. Data are derived from Figure 3.2. Abbreviations: *Ago2*, Argonaute-2; *Dcr2*, Dicer-2; *Stau*, Staufén.

	<i>Dcr2</i>	<i>Ago2</i>	<i>R2D2-1</i>	<i>R2D2-2</i>	<i>Stau</i>
Cells	0.00	1.00	0.34	0.29	0.12
L ₃ Larvae	0.01	1.00	0.67	0.61	0.60
L ₄ Larvae	0.01	1.00	0.62	0.55	0.03
Male Pupae	0.07	1.00	1.16	1.29	1.82
Female Pupae	0.11	1.00	2.10	2.61	3.29
Males	0.06	1.00	0.83	0.79	1.37
Females	0.12	1.00	1.96	2.50	1.88



Figure B.1. Maximum likelihood tree constructed with the putative *C. sonorensis* Staufen protein and 20 related amino acid (AA) sequences from other taxa.

Taxa orders are Blattodea (*C. secundus*, *Z. nevadensis*), Coleoptera (*P. striolata*, *P. chrysocephala*, *D. virgifera*, *L. decemlineata*, *R. ferrugineus*, *C. assimilis*), Diptera (*C. sonorensis*, *P. vanderplanki*), Ephemeroptera (*E. danica*), Hemiptera (*H. halys*), Orthoptera (*G. bimaculatus*), and Phasmatodea (*T. shepardi*). All unreported protein names are hypothetical or unnamed, and *C. sonorensis* is bolded.

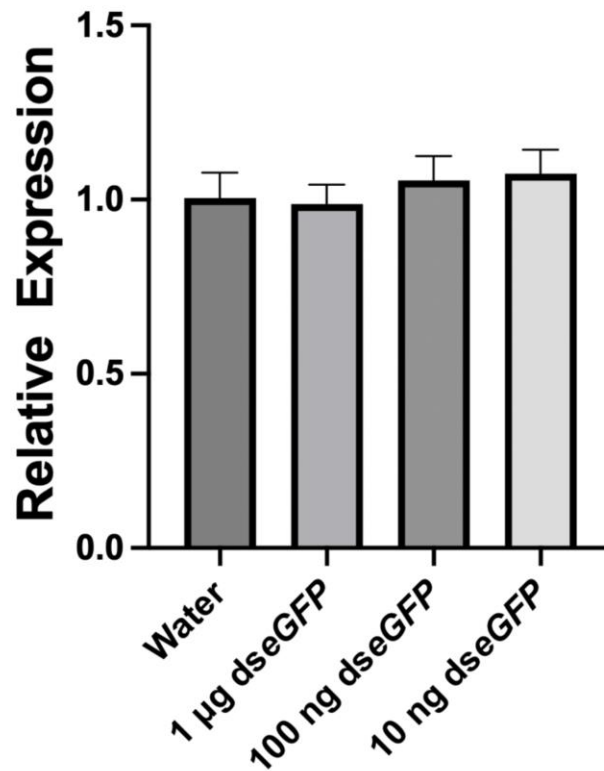


Figure B.2. Relative expression (fold) of *Stau* in cultured W8A cells after treatment with varying concentrations of *dseGFP*.

All doses are in 1 mL of media (i.e., 1 µg/mL) and cells were harvested after 48 h. Shown are means with SEM.

References

1. **Mills, M. K., D. Nayduch, and K. Michel. 2015.** Inducing RNA interference in the arbovirus vector, *Culicoides sonorensis*. *Insect. Mol. Biol.* 24: 105-114.
2. Chapter 2
3. **Yao, J., Rotenberg, D., Afsharifar, A., Barandoc-Alviar, K., and A. E. Whitfield. 2013.** Development of RNAi methods for *Peregrinus maidis*, the corn planthopper. *PLoS One*. 8: e70243.

Appendix C - Supplemental Information for Chapter 4

Table C.1. Primers used in this study for RT-qPCR and dsRNA synthesis. Primer citations are superscripted. All reference gene primers are in (Chapter 2).

Abbreviations: *Cs*, *Culicoides sonorensis*; dsRNase, double-stranded RNA degrading enzyme; bp, base pair; *CHS-1*, chitin synthase-1; *eGFP*, enhanced green fluorescent protein; dsRNA, double-stranded RNA; [T7], T7 Promoter Sequence (TAATACGACTCACTATAGGG). Accessions are from NCBI and plasmids are from AddGene (addgene.org).

Target	Role	Accession/Source	Primer Sequence (5'→3')	Product Size (bp)
<i>CsdsRNase-A</i>	RT-qPCR	GAWM01000334.1	F: GCACGTGGACATTTAGCACC	96
			R: TGA CTGCCATTGAGGAGCTG	
<i>CsdsRNase-B</i>	RT-qPCR	GAWM01006805.1	F: GCGAATTGCACCCCATTACC	142
			R: ACTCTGGCGTATGTAATGCCT	
<i>CsdsRNase-C</i>	RT-qPCR	GAWM01002773.1	F: ACTCAACGATATTCGTCGTGATTA	106
			R: TGCATAATAATACCCGTCAGGAA	
<i>CsdsRNase-D</i>	RT-qPCR	GAWM01017632.1	F: CTGGCATGGTTCAGTCAAAGT	188
			R: TCCAACGATTGTGTAATGGAAT	
<i>CsdsRNase-E</i>	RT-qPCR	GAWM01005324.1	F: CCCAAAAGATGATGGTTGCCA	115
			R: GATTCCGCGTTCATCATCCG	
<i>CsdsRNase-G</i>	RT-qPCR	GAWM01011845.1	F: GCTCGTGGACATTTAGCTGC	89
			R: CATTGTGGTGCTGCGTTCAT	
<i>CsdsRNase-A</i>	dsRNA	GAWM01000334.1	F: [T7]-CCGAAAGATGGAAGTGGGGT	323
			R: [T7]-TCGTGACGGACAAATATCGGG	
<i>CsdsRNase-B</i>	dsRNA	GAWM01006805.1	F: [T7]-CAATTCAGCAACAATGTGGACC	300
			R: [T7]-CCAAAGTTTCCTGAGTTGCTGC	
<i>CsdsRNase-G</i>	dsRNA	GAWM01011845.1	F: [T7]-CAAGGGTACGAGCAAGTCCA	358
			R: [T7]-CCAATGTTGATGATCGTTGCGT	
<i>CHS-1</i>	dsRNA	GAWM01003557.1	F: [T7]-CAAGATCTGATCAAGCTCGGC	339
			R: [T7]-CTACAAAGGCACCCACCAAC	
<i>eGFP¹</i>	RT-qPCR	Ac5-STABLE1-neo (Plasmid #32425)	F: TGACCACCCTGACCTAC	304
			R: TTGATGCCGTTCTTCTGC	
<i>dseGFP¹</i>	dsRNA	Ac5-STABLE1-neo (Plasmid #32425)	F: [T7]-TGACCACCCTGACCTAC	304
			R: [T7]-TTGATGCCGTTCTTCTGC	

Table C.2. Results for statistical analyses performed throughout this study. Abbreviations: D.o.F., degrees of freedom (between group, within group); C, Appendix C; MCT, multiple comparisons test.

Figure	Test Type	D.o.F.	Test Value	P-value	Post-hoc Test
C2	None				
3A	One-way ANOVA	5, 12	232.3	<0.0001	Tukey's MCT
3B	Kruskal-Wallis	5, 12	9.560	0.0887	Dunn's MCT
3C	Kruskal-Wallis	5, 12	7.311	0.1985	Tukey's MCT
3D	Kruskal-Wallis	5, 12	15.60	0.0081	Dunn's MCT
3E	Kruskal-Wallis	5, 12	16.52	0.0055	Dunn's MCT
3G	One-way ANOVA	5, 12	90.15	<0.0001	Tukey's MCT
C3	None				
4B	One-way ANOVA	3, 8	620.70	<0.0001	Tukey's MCT
C4B	Kruskal-Wallis	8, 18	11.72		
5A	One-way ANOVA	3, 8	98.82	<0.0001	Tukey's MCT
5B	One-way ANOVA	3, 8	235.30	<0.0001	Tukey's MCT
C5-A	Mann-Whitney			0.4000	
C5-B	t test	4	0.98	0.3825	
C5-G	Mann-Whitney			0.7000	

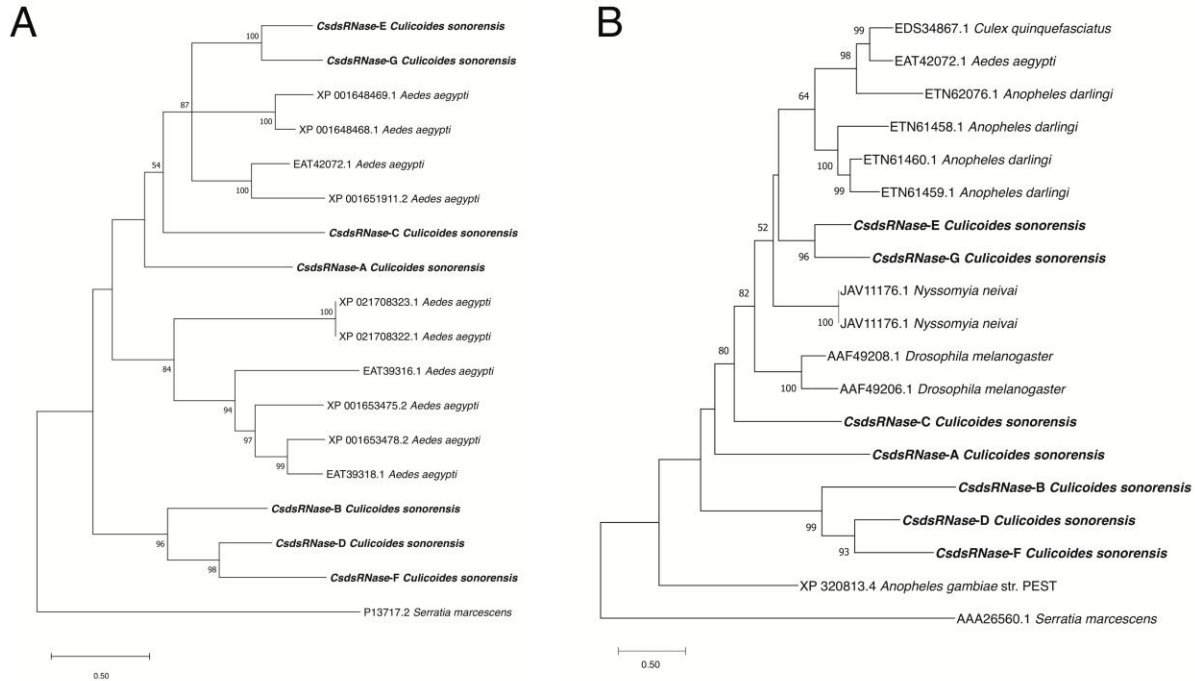


Figure C.1. Maximum likelihood trees constructed from protein sequences of dipteran dsRNases and seven *C. sonorensis* nucleases described in this study (bolded).

The confidence values of each branch are based on 100 bootstrap values, and values below 50 are unreported. The scale of each branch corresponds to the number of substitutions per site. Accessions are listed with each taxon. A. The amino acid sequences of *Aedes aegypti* dsRNases and a *Serratia marcescens* nuclease outgroup from Giesbrecht et al. 2020. B. The dipteran sequences collected and used in Cooper et al. 2020.

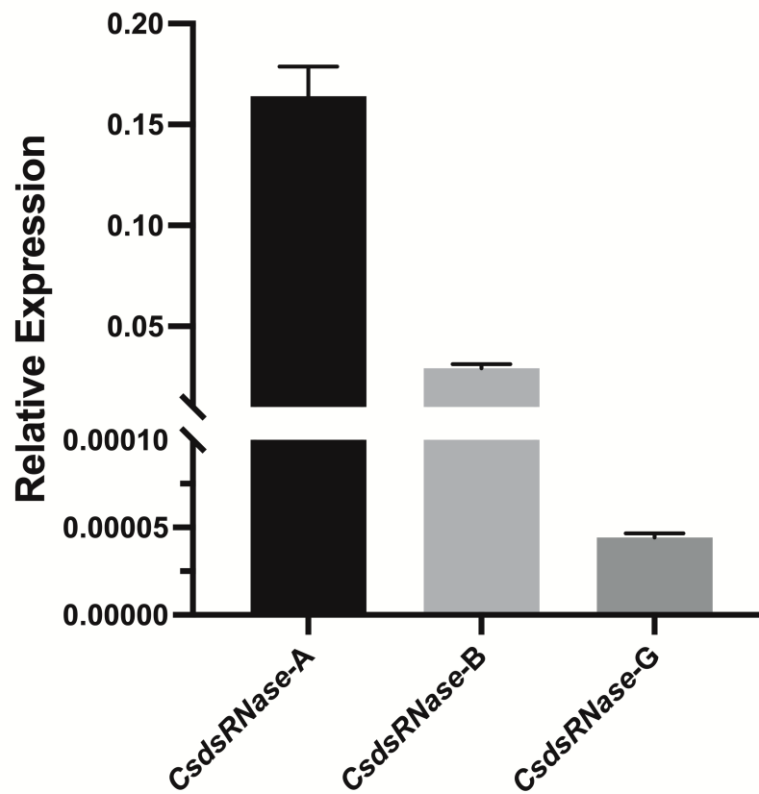


Figure C.2. Expression of *CdsRNases* in cultured W8A cells.

Relative expression (ΔC_t) is normalized to the expression of two reference genes. Bars are SEM. Statistics were not performed.

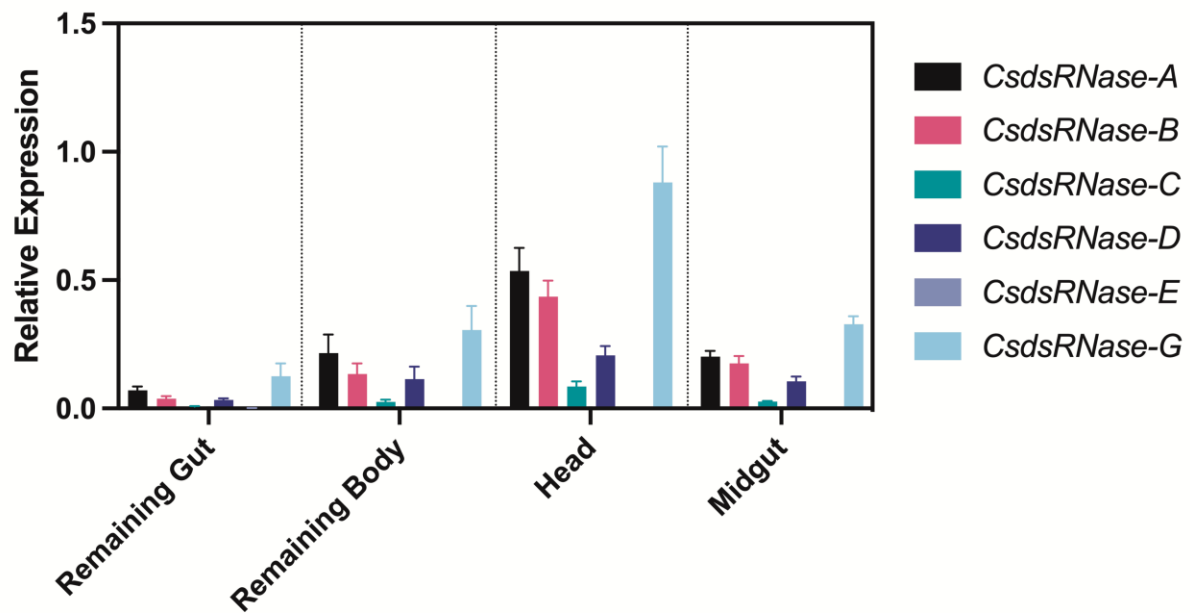


Figure C.3. Expression of *CsdRNases* in four *C. sonorensis* body parts or tissue groups.

Relative expression (ΔCt) is normalized to the expression of two reference genes. Bars are SEM. Data did not meet the test assumptions for a two-way ANOVA.

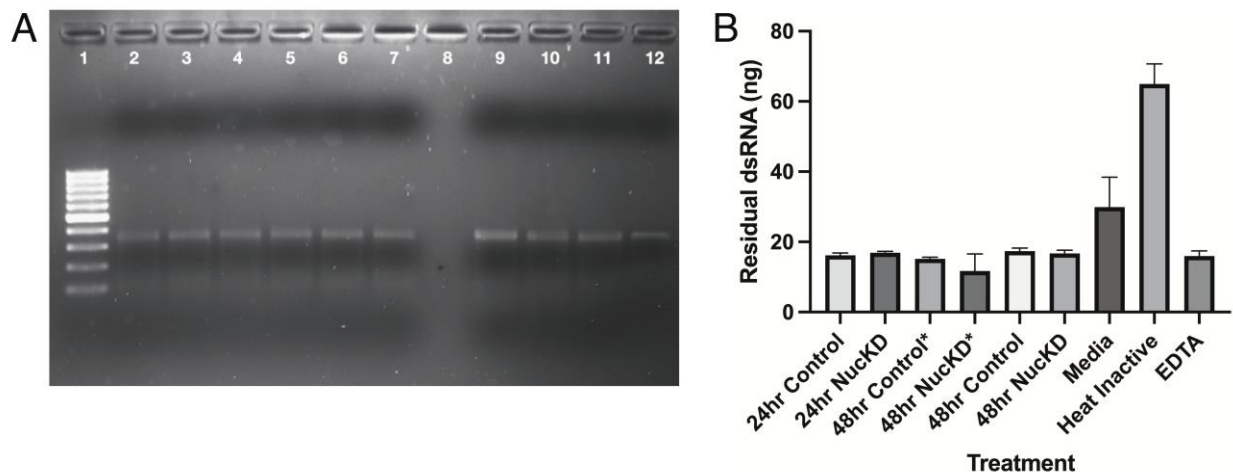


Figure C.4. Residual dsRNA products after degradation of 300 ng *dseGFP* in 20 µg of conditioned cell culture media for 90 min at 28 °C .

Samples (3 biological replicates) are controls treated with 2 µg *dsCHS-1* and treatments were 1 µg/ea of *dsCdsRNase-A* and *-B* (NucKD, Nuclease Knockdown). A) 1.5% agarose gel containing digested dsRNA products. Lanes are: 100 bp ladder with brightest band at 500 bp and products between 300-400 bp [1], 24 hr conditioned media with control [2] and attempted dsRNA knockdown treatment [3], 24 hr conditioned media after a media change* at 48 h post treatment (hpt) for control [4] and treatment [5], 48 hr conditioned media for control [6] and treatment [7], empty lane [8], water only control [9], media only control [10], heat-inactivated conditioned media (95 °C for 10 min) [11], and conditioned media with a final EDTA concentration of 50 mM [12]. B) Residual dsRNA values determined by RT-qPCR from a standard curve of *dseGFP* concentrations. Water control average was 300 ng (not shown). Bars are averages with SEM.

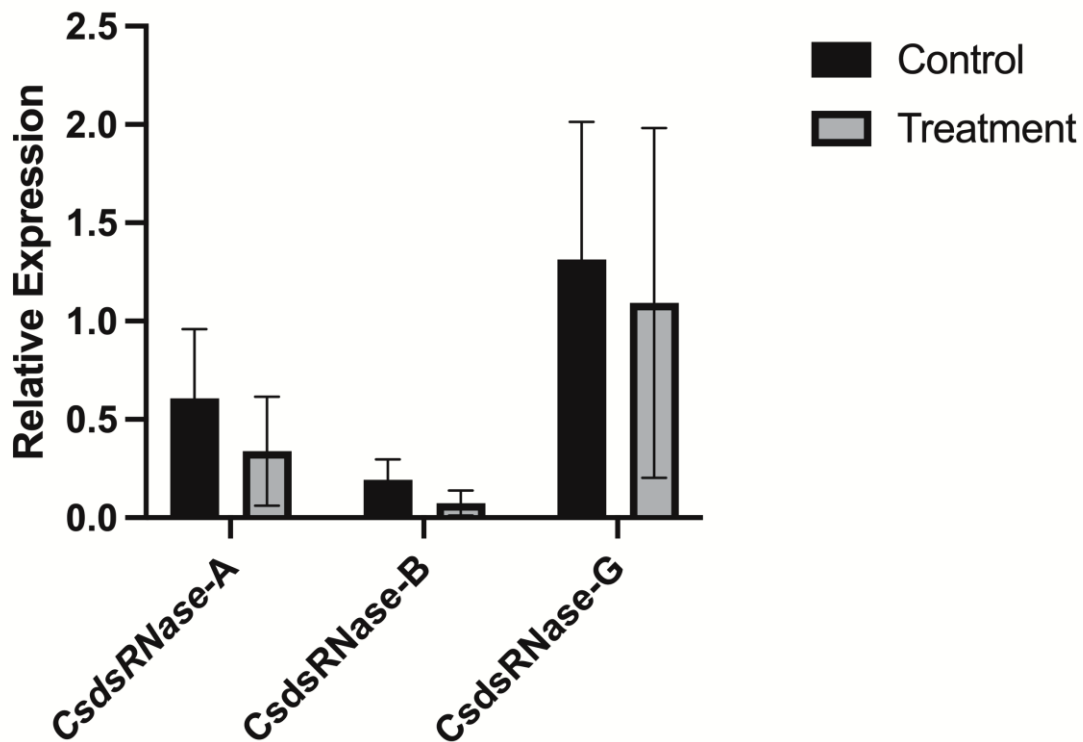


Figure C.5. Relative expression of *C. sonorensis* nucleases in L₃ larvae after treatment with 15 µg/mL *dseGFP* (control) or 5 µg/mL *dsCdsRNase A*, *B*, and *G*.

Larvae were harvested after 4 days. Relative expression is ΔC_t method normalized to two reference genes. Bars are mean with SEM.

Supplemental Information C.1. Predicted amino acid sequences for putative *dsRNases* in *Culicoides sonorensis* determined in this study.

>*CdsRNase-A* predicted amino acid sequence

MERRLRSFNAAMWLTYVVVILELFRLLSCALPSQKGCQIKVTDKFKPRQPLFLSSDLRHIL
 LPKDGSGVINVTHGQEIGVCCPEFCEMMECLSGTSYQTPDGTMDLLEDFTCTSNFEPHI
 LHTTTECSNKGTLRLVGFDLFIAGAKKFRRLYDVCYDMEASNPIFVRHEFSKTNLKQQP
 NVDCPDNWKYRGYFGGINANNRYTKRLSFDSLRVQLNRAKAEKLIDVNSAKRFLARGH
 LAPKGDFVFAPKKLITCHLINAAPQWQSINNGNWSNMEDDMQKIVKNNPDYTLIFTGT

FGIFTIDNVPLYLIPGNPDKVPIPKAFYKIIHILQTDEYFVIIGVNNPYFKRETIENPANGYM
ICNDICGQKYSQYYKCDDKYKMDVSKKAEKLYVEAGYMYMCDLDDFLYGLRQHYNV
QLNLNLSRK

> *CsdsRNase-B* predicted amino acid sequence

MKIIIFPRKLVVLMLYQIIFGQVDAATPVANCTPLPDGNLTCAISFNSEIIYNSKISLQTV
NQNDEFLFQALHTPEYLSSVPGSVVTPNPDWTNNGLKRLNNRRIESFTTIYSKNMQKV
AIQQQCGPQRWFDGEGERFFARGHFAPNGDFTDDQKIATFHDFNAAPQWQIFNNGYWF
KVEESIRKLVKGTYDRPGTPIKVMSCYDQLTCSNAATQETLVYLNNTQRTRGYVHFLI
PIPKVIFYKVVK

> *CsdsRNase-C* predicted amino acid sequence

MTHHLDGSKRDYLEPVNSTKGLIYLNSSQAIELHCSHGFKNYVNVNSLKANCLDRKTFV
INGEKRALNEIACNRNTFHTTKTVGKCKNDGHLIEIGYEIPSLTRDVNNNFDKSFHKLITI
CRNNLTDTTFWSHNILSKANSNMNQNGVPRPSFLMGRDHFTSKNVNRLYSKINQIQVLTK
MLGSEDVANILINERQSLNRGHLTPKADMIMANQQRSTHYFINVQPQFSTFNGGNWAKI
EESVRRMVTERDLTVDVYTGTYGIVSYKDMNGQKKDFYLYDDNGNGLIPVPKIFYKI
VIDRKSLNGIALIGVNNPYVTLNDIRRDYIFCTDISYKIKWVHNIQERIPDGYYYACDVNE
LTKVVDTLPKLNVTGLLI

> *CsdsRNase-D* predicted amino acid sequence

MIFKILLFLLIVYGTKIHGKPEAEDCFESPNNWNCNMRANGNEVLWTNIKFVKNQKEIT
MQTLFTTQVLSSTSLEFSPGRGRPDEPWSTAGLVSVLGENWDEKESTKQFTNIYKKEDQ

KIAIDSQCKINPSWYTSTSTFFARGHYAPNADYTSNDYKTATFHYNVAPQWQKYNNG
NWRQKVEAPIQSLIKKTSTTYQIFTGSYGQLKCPNNQNVKLFLTDSSPQPNFSVKIPIPIY
FRIIAPSPLRDQDTPIFIATSNHPTWTKPEIEQYLRTNFCDPRTDLCDKIPQKKNDLAWFSQ
SDFRCDKGTGTTFIEKGYTYACHANADILNKFGFSLFEGTPKRRKFGSLYSITQSLDRTRI
G

> *CsdsRNase-E* predicted amino acid sequence

MLRTIQSLSVVLLISVTNARVALKLNENEILSEINRLSDPKDDGCQIDINKDLNEPQPLVI
VPGTDKFLLPDDERGIIFLEKGAQIELYCSKSFHGMTNVQFRNITCENGNFVENGQLFQY
RDFNCTEYPFHVAVRTGKKCFNDASVLDVGFEVGSRFLKVYEICFDEVLETTHYVTHEF
TPPYKTHQAGFPRPGWLNTGYNGKRVDTLYTRASQREVVTKILNSTTQVDKYIPETSD
FYLARGHISAKSDFIFGSQHRATFWLMNAAPQWQVFNNGNWIAVEIAIKNFVADRNIEV
DAYTGTHGVTTLWDEQNKPEIYLAFDENNNGLIPVPKIYYKILVERKTKFGIVLIGVNN
PYLDLKEIESDYVLCKDVSDQINWVSWDKKNITLGYSYACDVNEFMAVIKHIPDLTVKG
LLV

> *CsdsRNase-F* predicted amino acid sequence.

MNFNLNNIRQLILLFLLIATSELNGRRLVLAGPEATKCVWPAKWWACRIESLNKEIFA
AKVKLEESPGHITIKSRLSTQIMNKETLFSPGKGRSSDSWSIGGLKELFQDNWNKQHQIE
KFMSIYSIATQKAALKAQCGCTTDLWDWYKPDSKKAQTYFAKGHLAPNADYKENSYKK
GTFHYFNAAPQWQKFNGGNWYQKVEAAIQKLISSETLSYQIYTGTHGHLACPNDPTKH
VFIMSDQQNSGQVQVKIPIPKIYFKVIAPYRLGEGQKPIFIATSNHPSWTKGEIENYLKDR

FCDPAFDLCAAIPMRTPGFEWFSKSGFLCDKGTGRRTTFIEKGYTYACHANAKILDAVKV
TIAHTVESPNKRRKVEEVSSLCQVFDGLRLNCPYRSRG

> CsdRNase-G predicted amino acid sequence

MTRFIIVFTFISAFIITNAFSIKFGNLWKETQENVVSRVRASPMATGCEIDIKEEVKEAEPL
ILIPDKNEFLPSDASGRILLKPSDTIELACTEGFKDNSSINFRKVTCVDGTFMENNMQV
DFKTLKCVKDSIHVANRISDKKCFNNATIINIGFDIQKRFFKVYEICFDEVLERTHYVTHE
FTPGYKWFQNPRPDVKFISTGFFNNTDVKLYTRNVQRTTIAKILESEKYANFYVQNDT
DFFLARGHLAARSDFVFINNQRASFWFMNAAPQWQVFNNGNWRFIEEGIRDFVAEYNL
EVDVFTGTYGTTTLLDENNKEHQIYLRFDENNNGLIPVPKLYYKILVDRKSQKGIVLIGV
NNPYLYWLDLLKGYVLCPDVSHKVKWLSNFEWQKNNLTGYSYACDVNEFRKVVKH
HPDLNVTGLLSKGETPVKSVLLVFSMLLYVPQIESTKISI

References

1. Yao, J., Rotenberg, D., Afsharifar, A., Barandoc-Alviar, K., and A. E. Whitfield.
2013. Development of RNAi methods for *Peregrinus maidis*, the corn planthopper. PLoS
One. 8: e70243.

Appendix D - Supplemental Information for Chapter 5

Table D.1. Primers used in this study for RT-qPCR.

Abbreviations: bp, base pair; *IAP1*, inhibitor of apoptosis protein 1; *CHS*-, chitin synthase-; *CHT*, chitinase; *Cs*, *Culicoides sonorensis*; dsRNase, double-stranded RNA degrading enzyme; *eGFP*, enhanced green fluorescent protein. Accessions are from NCBI and plasmids are from AddGene (addgene.org). Primer citations are superscripted.

Target	Accession/Source	Primer Sequence (5'->3')	Product (bp)
<i>IAP1</i> ¹	GAWM01009039.1	F: TTGTGGACATATTATTGCCTGTG	85
		R: CATGACTTTCGTGAATGGTTGT	
<i>VhaA</i>	GAWM01018658.1	F: GTATGTTGCAAGTGTGGCCTG	86
		R: ACGCTGACCAGTTAACAATGGA	
<i>CHS-1</i>	GAWM01003557.1	F: GGAGCAGCTTGTGGTCGTAT	96
		R: TGCCTTTTGAAGCCAATGACC	
<i>CHS-2</i>	GAWM01015448.1	F: TGCATCCTCAAGAAATCGGC	123
		R: GCGTACCCCATGAAACATCA	
<i>CHT</i>	GAWM01000884.1	F: GCGTCCAAC TACAAGAGCACC	96
		R: CTTCTTCTCGTTGTGCTTTGCC	
<i>CsdsRNase-G</i> ²	GAWM01011845.1	F: GCTCGTGGACATTTAGCTGC	89
		R: CATTGTGGTGCTGCGTTCAT	
<i>eGFP</i> ³	Ac5-STABLE1-neo (Plasmid #32425)	F: TGACCACCCTGACCTAC	304
		R: TTGATGCCGTTCTTCTGC	

Table D.2. Primers for dsRNA, shRNA, and pcRNA synthesis.

*Region with host sequence specificity Abbreviations: VhaA, vacuolar ATPase subunit A; dsRNA, double-stranded RNA; shRNA, short hairpin RNA; pcRNA, paperclip RNA; [T7], T7 Promoter Sequence (TAATACGACTCACTATAGGG). Additional abbreviations and citations as above.

Target	Type	Accession/Source	Primer Sequence (5'→3')	Product (bp)
<i>IAP1</i> ¹	dsRNA	GAWM01009039.1	F: [T7]-AGTTGAAGAACAACCTTGAGATGG	344
			R: [T7]-GCCAATCTTCATACGACACC	
<i>VhaA</i>	dsRNA	GAWM01018658.1	F: [T7]-CAACTGCAATACCAGGAGCG	409
			R: [T7]-CGTGCACCTAAATATGCGGG	
<i>CHS-1</i>	dsRNA	GAWM01003557.1	F: [T7]-CAAGATCTGATCAAGCTCGGC	339
			R: [T7]-CTACAAAGGCACCCACCAAC	
<i>CHS-2</i>	dsRNA	GAWM01015448.1	F: [T7]-TGACCCACATCTTGGGGCT	380
			R: [T7]-TCCATCGCCTACGCTGGTT	
<i>CHT</i>	dsRNA	GAWM01000884.1	F: [T7]-GAAATGGGATAACGGTGGCG	334
			R: [T7]-TGCTCTTGTAGTTGGACGCA	
<i>CsdsRNase-G</i> ²	dsRNA	GAWM01011845.1	F: [T7]-CAAGGGTACGAGCAAGTCCA	358
			R: [T7]-CCAATGTTGATGATCGTTGCGT	
<i>eGFP</i> ³	dsRNA	Ac5-STABLE1-neo (Plasmid #32425)	F: [T7]-TGACCACCCTGACCTAC	304
			R: [T7]-TTGATGCCGTTCTTCTGC	
<i>IAP1</i>	shRNA	GAWM01009039.1	[T7]- CTAGTTCTAGTAGTGGAAGTAGTAAAAAAAAAACTACTTCCACTACTA GAACTAG	23*
<i>VhaA</i>	pcRNA	GAWM01018658.1	[T7]- CAACTGCAATACCAGGAGCGTTTAAAAAAAAAAAAACGCTCCTGGTATT GCAGTTGAGAATAAAAAAAAAAAATTCT	23*

<i>CHS-1</i>	pcRNA	GAWM01003557.1	[T7]- CAAGATCTGATCAAGCTCGGCATAAAAAAAAAAATGCCGAGCTTGATCA GATCTTGAGAATAAAAAAAAAAAATTCT	23*
<i>CHS-2</i>	pcRNA	GAWM01015448.1	[T7]- GCTTGTGGTCGCATTCATCCAATAAAAAAAAAAATTGGATGAATGCGAC CACAAGCAGAATAAAAAAAAAAAATTCT	23*

Table D.3. Primers for cloning or colony PCR and DNA sequencing. Abbreviations: MCS, multiple cloning site.

Additional abbreviations as above. Buffer region for digest site: GTTGTT. Restriction enzyme digest sequences are bolded. Forward primer with *MluI* cut site (|): A|CGCGT. Reverse primer with *BmtI* cut site (|): GCTAG|C.

Target	Role	Accession/Source	Primer Sequence (5'→3')
<i>IAP1</i>	Cloning	GAWM01009039.1	F: GTTGTT ACGCGT AGTTGAAGAACACTTGAGATGG
			R: GTTGTT GCTAG CGCCAATCTTCATACGACACC
<i>VhaA</i>	Cloning	GAWM01018658.1	F: GTTGTT ACGCGT CAACTGCAATACCAGGAGCG
			R: GTTGTT GCTAG CCGTGCACCTAAATATGCGGG
<i>CHS-1</i>	Cloning	GAWM01003557.1	F: GTTGTT ACGCGT CAAGATCTGATCAAGCTCGGC
			R: GTTGTT GCTAG CCTACAAAGGCACCCACCAAC
<i>CHS-2</i>	Cloning	GAWM01015448.1	F: GTTGTT ACGCGT TGACCCACATCTTGGGGCT
			R: GTTGTT GCTAG CTCCATCGCCTACGCTGGTT
<i>CHT</i>	Cloning	GAWM01000884.1	F: GTTGTT ACGCGT GAAATGGGATAACGGTGGCG
			R: GTTGTT GCTAG CTGCTCTTGTAGTTGGACGCA
<i>eGFP</i>	Cloning	Ac5-STABLE1-neo (Plasmid #32425)	F: GTTGTT ACGCGT TGACCACCCTGACCTAC
			R: GTTGTT GCTAG CTTGATGCCGTTCTTCTGC
MCS of L4440 Plasmid	Colony PCR	L4440 (Plasmid #1654)	pBluescriptKS: TCGAGGTCGACGGTATC
			pBluescriptSK: TCTAGAACTAGTGGATC

Table D.4. Example yields of dsRNA, shRNA, pcRNA, and chitosan nanoparticles from *in vitro* and *E. coli* production.

Abbreviations: IPTG, isopropyl- β -D-thiogalactopyranoside; n, sample size; min, minimum; max, maximum; SD, standard deviation. Additional abbreviations as above. *In vitro* samples are the product of two reactions.

Type	n	Min	Max	Mean	Median	SD
<i>In vitro</i> dsRNA Synthesis						
dsRNA	9	251.0	293.0	271.4	264.0	± 14.0
shRNA	1	592.0	592.0	592.0	592.0	
pcRNA	3	501.0	560.0	537.0	550.0	± 31.6
IPTG vs. Lactose Induction						
IPTG	3	79.0	174.0	99.8	79.0	± 66.3
Lactose	3	151.0	231.0	193.7	199.0	± 40.3
Lactose Induced <i>E. coli</i>						
Multiple	8	48.0	352.5	145.2	103.0	± 106.0
Chitosan Nanoparticle Preparation						
dsRNA		Unbound dsRNA	Bound dsRNA	Loading Efficiency	dsRNA in each of 6 pellets	
<i>eGFP</i>		9.6	22.4	70%	3.73	
<i>IAP1</i>		3.4	28.6	89%	4.77	
<i>CHT</i>		8.2	23.8	74%	3.97	
<i>CHS-1</i>		3.8	28.2	88%	4.70	

Table D.5. Statistics performed throughout this manuscript.

Abbreviations: D, Appendix D; D.o.F., degrees of freedom; MCT, multiple comparisons test.

Figure	Test Type	D.o.F.	Test Value	Test P-value	Post-hoc Test	Notes
1A	One-way ANOVA	5, 12	132.8	<0.0001	Tukey's MCT	
1B	One-way ANOVA	5, 12	31.72	<0.0001	Tukey's MCT	
1C	Kruskal-Wallis	5, 12	12.63	0.0272	Dunn's MCT	
1D	Kruskal-Wallis	5, 12	15.89	0.0072	Dunn's MCT	
1E	One-way ANOVA	5, 12	6.737	0.0033	Tukey's MCT	
1F	One-way ANOVA	5, 12	90.15	<0.0001	Tukey's MCT	
2	One-way ANOVA	2, 6	30.58	0.0007	Tukey's MCT	
3: <i>IAP1</i>	t test	4	0.4195	0.6964		
3: <i>VhaA</i>	t test	4	0.9052	0.4166		
3: <i>CHS-1</i>	Welch's t test	2.010	1.737	0.2239		Corrected DoF
3: <i>CHS-2</i>	t test	4	1.646	0.1752		
3: <i>CHT</i>	t test	4	2.491	0.0674		
3: <i>CsdsRNase-G</i>	t test	4	2.385	0.0756		
4: <i>IAP1</i>	t test	4	1.049	0.3534		
4: <i>VhaA</i>	Welch's t test	2.051	5.977	0.0253		Corrected DoF
4: <i>CHS-1</i>	Welch's t test	2.003	3.287	0.0813		Corrected DoF
4: <i>CHS-2</i>	t test	4	0.9551	0.3936		
4: <i>CHT</i>	Welch's t test	2.029	1.572	0.2549		Corrected DoF
4: <i>CsdsRNase-G</i>	t test	4	2.847	0.0466		
D2B	One-way ANOVA	2, 6	6.585	0.0307	Tukey's MCT	
D2C	One-way ANOVA	2, 6	9.375	0.0142	Tukey's MCT	

D3D	One-way ANOVA	2, 6	12.03	0.0080	Tukey's MCT	
5A	t test	4	0.5504	0.6113		
5B	Welch's t test	2.036	0.9396	0.4451		Corrected DoF
5C: L ₃	t test	4	2.516	0.0656		
5C: L ₄	t test	4	1.163	0.3093		
6	Two-way ANOVA	1, 19	4.493	0.0474		Life Stage
		3, 19	0.7745	0.5226	Tukey's MCT	Treatment

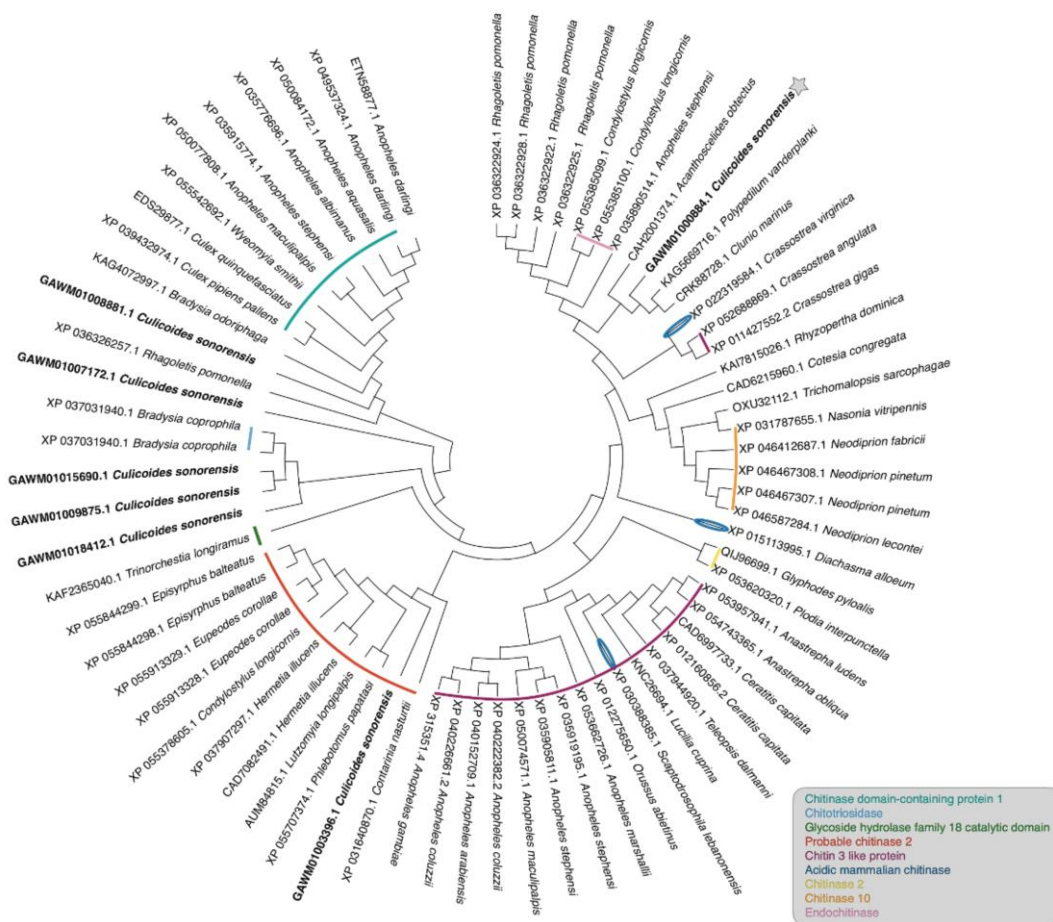


Figure D.1. Cladogram showing seven predicted *C. sonorensis* proteins (bolded) with chitinase domains and their nearest related sequences.

Proteins domains are colored in the key and correspond to taxa. Each taxon is accompanied by their accession. Tree was constructed in MEGA using an LG+G model with partial deletion. The starred accession is the proteins used in this study.

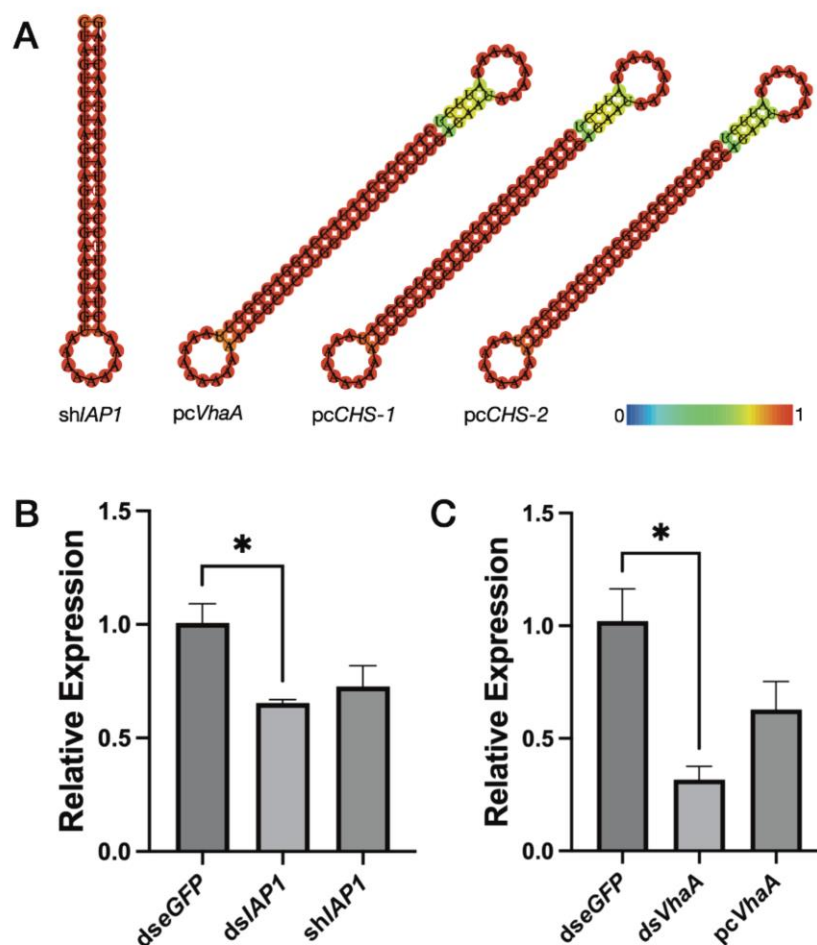


Figure D.2. Secondary RNA structures designed for RNAi in *C. sonorensis*.

A) Predicted RNA structure for four designed structures using the RNAfold WebServer. Colors show probability of base pairing from 0 (blue) to 100% (red). B) Relative expression (fold) of *IAP1* in W8A cells after treatment with 200 ng/mL dsRNA or molar equivalent of sh*IAP1* (**P* = 0.0321). C) Relative expression (fold) of *VhaA* in W8A cells after treatment with 200 ng/mL dsRNA or molar equivalent of pc*VhaA* (**P* = 0.0118). Bars are SEM. Statistics are reported in Table D.5.

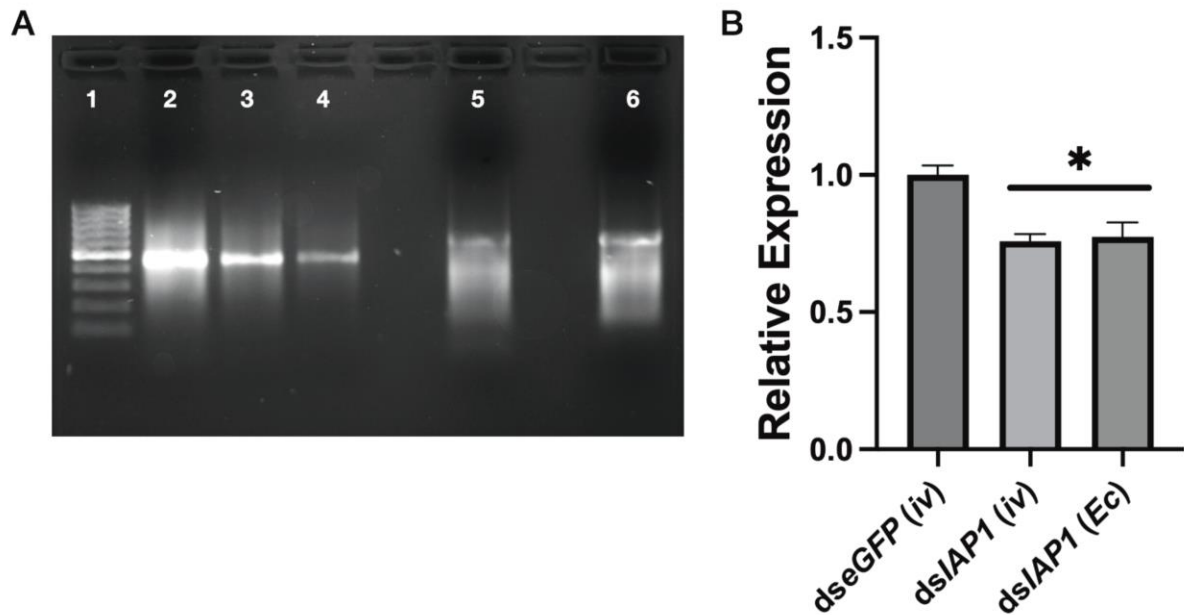


Figure D.3. Yield and activity of *E. coli* (*Ec*) derived dsRNA products.

A) 1.5% agarose gel with 100 bp ladder [Lane 1], 1 µg of *in vitro* (*iv*) synthesized dsRNA [2], 500 ng *iv*-dsRNA [3], 250 ng *iv*-dsRNA [4], 1 µg *Ec*-dsRNA from bacteria induced by lactose [5], and 1 µg *Ec*-dsRNA from bacteria induced by IPTG [6]. The brightest band in the ladder is 500 bp. Note smearing in *Ec*-derived dsRNA and the larger product (arrows) is caused by extra sequence in the plasmid flanking the insertion site. B) Relative expression (fold) of *IAP1* in W8A cells after treatment with 100 ng/mL dsRNA derived from either *iv* synthesis or *Ec* (**P* < 0.05). Bars are SEM. Statistics are reported in Table D.5.

References

1. Mills, M. K., D. Nayduch, and K. Michel. 2015. Inducing RNA interference in the arbovirus vector, *Culicoides sonorensis*. *Insect. Mol. Biol.* 24: 105-114.
2. Chapter 4
3. Yao, J., Rotenberg, D., Afsharifar, A., Barandoc-Alviar, K., and A. E. Whitfield. 2013. Development of RNAi methods for *Peregrinus maidis*, the corn planthopper. *PLoS One*. 8: e70243.

Appendix E - Supplemental Information for Chapter 6

Supplemental Information E.1. SAS Studio code used for log PROBIT analysis.

```
data Name;  
input conc total dead; mort=dead/total; lconc=log10 (conc);  
datalines;  
;  
run;  
proc probit log10 c=0;  
var conc total dead;  
proc reg data=Name; model mort=lconc;  
run;
```

Table E.1. Log PROBIT outputs and statistics for alkaline enhancers of *Bti* products in *C. sonorensis*.

Abbreviations: [c], concentrations; : n, sample size (total insects used including from control conditions), SE, standard error; LC, lethal concentration; CI, confidence interval; X^2 , Pearson Chi-Square. Some values were undetermined by the model (N/A). A significant p-value suggests statistically significant difference between the data and the model.

Plate	Range Tested	Number of [c]	n	Slope ($\pm$ SE)	LC ₅₀ (95% CI)	LC ₉₀ (95% CI)	X^2	p-value	R ²
1) VectoBac® WDG + NaOH	62.5–1000 mg/L	5	480	2.15 ($\pm$ 0.38)	402 mg/L (296 - 575 mg/L)	1,582 mg/L (970 - 4,218 mg/L)	58.60	<.0001	0.71
2) Technical Grade <i>Bti</i> + NaOH	625– 10,000 mg/L	5	480	0.58 ($\pm$ 0.22)	1,599 mg/L (152 - 3,646 mg/L)	>200,000 mg/L	29.28	0.0451	0.28
3) Technical Grade <i>Bti</i> + <i>E. coli</i>	625– 10,000 mg/L	5	480	0.51 ($\pm$ 0.39)	N/A	N/A	63.40	<.0001	0.13

Table E.2. Summary statistics for average daily larval *C. sonorensis* survival after exposure to *Bti* products with alkaline enhancers.

Mean and standard deviation (SD) of four biological replicates is reported for each day at each concentration or control for three experiments (Plates). The treatments are described fully in the main text, and briefly are 1) VectoBac® WDG + NaOH, 2) Technical Grade *Bti* + NaOH, and 3) Technical Grade *Bti* + *E. coli*.

Plate 1	Control		1000 mg/L		500 mg/L		250 mg/L		125 mg/L	
Day	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	1.00	0.00	0.23	0.13	0.67	0.32	0.94	0.02	0.92	0.04
2	1.00	0.00	0.18	0.10	0.58	0.33	0.93	0.04	0.89	0.06
3	1.00	0.00	0.17	0.09	0.55	0.33	0.84	0.05	0.82	0.08
4	1.00	0.00	0.16	0.08	0.52	0.32	0.81	0.06	0.82	0.08
5	1.00	0.00	0.14	0.08	0.51	0.33	0.79	0.10	0.80	0.09
6	0.99	0.02	0.13	0.07	0.49	0.35	0.76	0.15	0.77	0.09
7	0.98	0.03	0.13	0.07	0.48	0.33	0.76	0.15	0.76	0.11
Plate 2	Control		10000 mg/L		5000 mg/L		2500 mg/L		1250 mg/L	
Day	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	1.00	0.00	0.85	0.08	0.82	0.05	0.86	0.07	0.69	0.10
2	0.99	0.03	0.51	0.17	0.68	0.11	0.77	0.03	0.61	0.10
3	0.97	0.04	0.35	0.05	0.51	0.17	0.69	0.08	0.58	0.05
4	0.95	0.03	0.26	0.14	0.48	0.18	0.62	0.07	0.53	0.08
5	0.94	0.05	0.25	0.13	0.37	0.11	0.62	0.07	0.53	0.08
6	0.92	0.07	0.25	0.13	0.33	0.09	0.59	0.09	0.48	0.11
7	0.90	0.12	0.23	0.10	0.31	0.08	0.57	0.11	0.45	0.13
Plate 3	Control		10000 mg/L		5000 mg/L		2500 mg/L		1250 mg/L	
Day	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	0.85	0.07	0.51	0.16	0.56	0.07	0.42	0.17	0.24	0.16
2	0.85	0.07	0.51	0.16	0.26	0.06	0.34	0.20	0.23	0.16
3	0.85	0.07	0.22	0.05	0.22	0.12	0.31	0.19	0.23	0.16

4	0.85	0.07	0.10	0.03	0.22	0.12	0.29	0.19	0.23	0.16
5	0.85	0.07	0.06	0.05	0.17	0.13	0.28	0.18	0.23	0.16
6	0.84	0.05	0.01	0.03	0.12	0.10	0.27	0.19	0.23	0.16
7	0.81	0.04	0.01	0.03	0.10	0.07	0.25	0.17	0.23	0.16

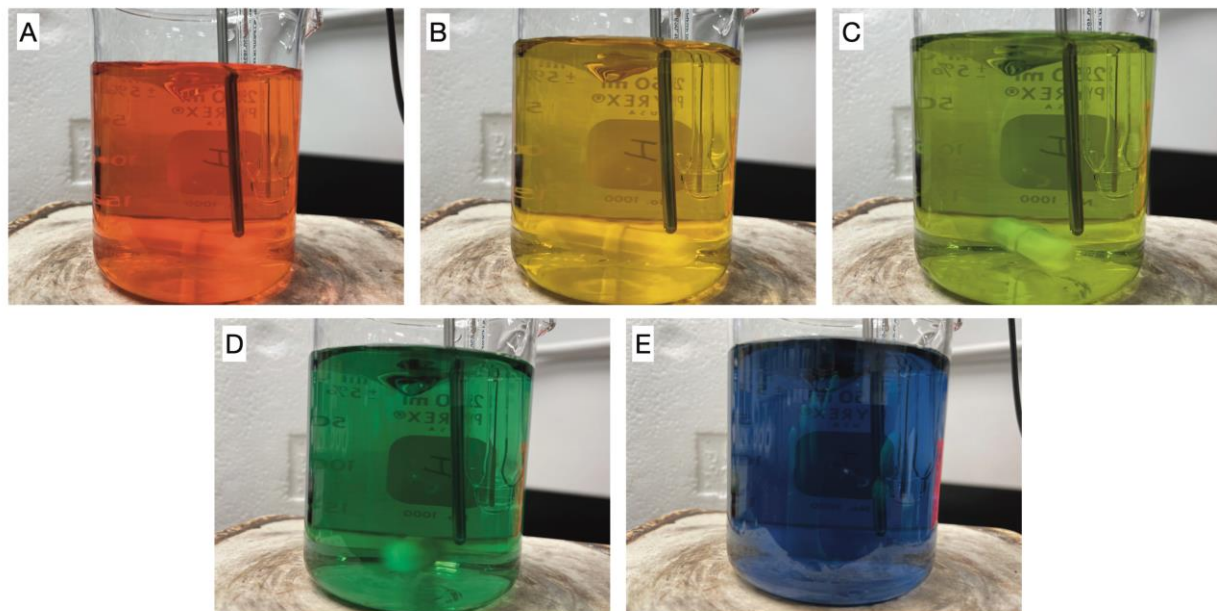


Figure E.1. Concentrated universal pH indicator in water at registered values of A) 5.13, B) 6.24, C) 6.83, D) 7.50, and E) 9.34.

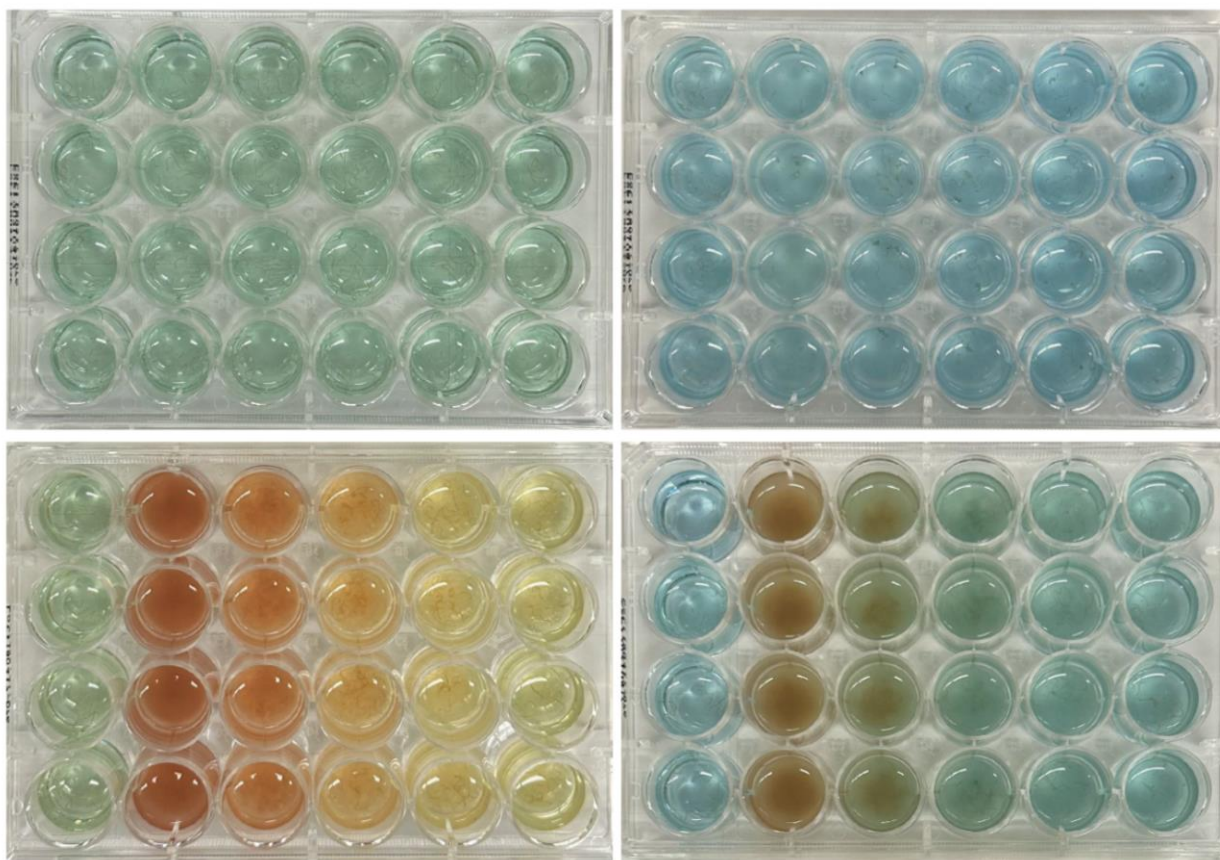


Figure E.2. Plates with *C. sonorensis* larvae treated with VectoBac® WDG (top) and technical grade *Bti* (bottom) before NaOH (left) and after the addition of 40 µl NaOH per well at day 7 (right).

Columns are control or treatment concentrations with the leftmost column being the control and concentrations from high to low occurring left to right. *E. coli* treated wells were similar to the bottom row (not show here).