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In Vivo Gene Knockdown Techniques and the Establishment of Cryopreservation Methods in *Culex* spp. Mosquitoes

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by

Roxana Sandoval

Thesis Advisor: Dr. Claudia Rückert

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We recommend that the thesis
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Roxana Sandoval

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Master of Science

Dr. Claudia Rückert
Advisor

Dr. John Cushman
Committee Member

Dr. Monika Gulia-Nuss
Committee Member

Dr. Claus Tittiger
Committee Member

Dr. Andrew B. Nuss
Graduate School Representative

David W. Zeh, Ph.D., Dean
Graduate School

May, 2022

Abstract

Culex (Cx.) pipiens, known as the common house mosquito (subspecies *Cx. pipiens quinquefasciatus and Cx. pipiens pipiens*) affects humans and animals through its ability to transmit numerous viruses and parasites such as West Nile Virus (WNV), filarial nematodes, and *Plasmodium relictum*, the causative agent of avian malaria. RNA interference (RNAi) is an antiviral pathway that is well studied in most arthropods such as *Aedes aegypti* mosquitoes. In *Culex.* spp., aspects of the RNAi mechanism are not completely understood, including the role of a potentially antiviral protein, P-element-Induced Wimpy Testis-2 (PIWI2). One of the objectives of this thesis was to knock down *PIWI2* gene expression *in vivo* to investigate its role during virus infection. This was implemented by introducing double-stranded RNA (dsRNA) into mosquitoes to stimulate RNAi-mediated gene silencing of *PIWI2*. In this study, dsRNA was introduced into adult *Cx. quinquefasciatus* mosquitoes and pupae via intrathoracic injection or soaking in dsRNA, respectively. However, a significant knockdown of *PIWI2* was not achieved using either dsRNA delivery method. Additional experiments testing dsRNA knockdown efficiency in *Cx. quinquefasciatus* Hsu cells indicated that the dsRNA used for our studies may not have been effective. The next approach would be to conduct further *in vivo* tests utilizing a different dsRNA production technique and increased quantities of dsRNA. In this study we additionally aimed to contribute to mosquito related research methods. Continuous maintenance of *Culex* eggs is required for such *in vivo* studies but is labor-intensive and costly. Thus, the second objective of this thesis was to establish a method for the cryopreservation of *Cx. pipiens* embryos.

Cryopreservation would permit extended storage of *Culex* mosquito lines without the burden of continual colony rearing. The methodology and effects of dechorionation, permeabilization, and handling on embryo viability were tested. Lab grade sodium hypochlorite and heptane successfully permeabilized 6-9 hr old *Culex* embryos, but this treatment was lethal. Additional experimentation is needed to determine the optimal conditions for the permeabilization of *Culex* embryos.

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Chapter 1. Introduction

1. Introduction

In most regions of the globe, mosquitoes are vectors for the transmission of a wide range of viruses and pathogens that may cause disease in humans, birds, horses, and other animals (CDC, 2022). *Culex* mosquitoes are important vectors of arthropod-borne viruses (arboviruses). They are profoundly understudied with respect to other mosquito vectors (Ruckert et al., 2019), although they are heavily involved in the transmission of viruses such as WNV, an arbovirus that affects most parts of the world, including the United States (CDC, 2022).

Many studies have focused on reducing mosquito vector competence, the ability of a vector to transmit viruses or parasites, to minimize the health implications on infected populations (Beerntsen et al., 2000). In mosquitoes, gene knockdowns using RNAi are commonly performed to understand more about the proteins involved in viral transmission or other mechanisms (Airs & Bartholomay, 2017; Attardo et al., 2003; Chen et al., 2019). RNAi studies in arthropods are performed by introducing *in vitro* transcribed dsRNA into cells/organisms, to feed into the RNAi pathway, resulting in a knockdown of target genes. RNAi also occurs naturally when dsRNA viral intermediates are generated in the cell during virus replication, resulting in the degradation of viral RNA (Blair, 2011).

Analysis of key protein functions in the RNAi pathway may help elucidate further antiviral mechanisms in *Culex* spp. mosquitoes. *P-element-induced wimpy testis-2* (*PIWI2)* is a gene in *Culex* mosquitoes that encodes for a RNAi pathway protein and has

an orthologue gene that has been described to encode for an antiviral protein in *Aedes (Ae.) aegypti* (PIWI4) (Varjak et al., 2017). Members of the Rückert Lab have shown that the *Culex* PIWI2 protein is also antiviral in cell culture, but this has not been investigated *in vivo*. To investigate PIWI2 *in vivo*, dsRNA pupae soak and intrathoracic micro injection were used in this study and have been used in *Ae. aegypti* to suppress target gene expression via RNAi (Arshad et al., 2021; Kang et al., 2018).

The *in vivo* methods used in this study involve continuous mosquito colony maintenance, primarily due to the nature of *Culex* embryogenesis. *Culex* mosquito eggs become non-viable in a dry environment five hours after egg lay (Vargas et al., 2014). The eggs require daily attention, ensuring that the eggs and subsequent developmental stages (larvae, pupae, and adult mosquitoes) are kept in an environment that fosters complete mosquito development.

By designing a cryopreservation method for *Culex* spp., researchers could reduce labor efforts and costs associated with the upkeep of colonies for *in vivo* studies. Additionally, future transgenic lines could also benefit from an available cryopreservation method (James et al., 2022). Ongoing breeding of transgenic mosquito cell lines may result in genetic or phenotypic changes (Gallichotte et al., 2021), along with accidental cross contamination (Gloria-Soria et al., 2019). Researchers could reduce the expense of rearing many transgenic lines at the same time, keep a low passage number of mosquitoes, and be able to store lines that are not presently in use. Reliable mosquito cryopreservation methods may broaden the area of mosquito biology and, in

turn, might contribute to breakthroughs that lower the burden of mosquito-borne illnesses.

1.1 Mosquitoes as Disease Vectors

Female mosquitoes are disease vectors as a consequence of their reproduction cycle. They require proteins contained in a blood meal for egg maturation (Zhou et al., 2007), which leads them to contact animals that may be infected with a virus or other pathogens. When a virus is acquired through feeding, it replicates in the gut tissue, distributes to the secondary replication sites, such as the salivary glands, and is then eventually delivered into the salivary secretions of the arthropod, where it can be introduced into the host's skin and cutaneous vasculature during the next feeding event (Rückert & Ebel, 2018). Not all mosquito species transmit diseases, although there are some that commonly do (CDC, 2020b).

There are three main vector genera of mosquitoes: *Aedes*, *Anopheles*, and *Culex*. These mosquitoes can transmit viruses and parasites such as Zika virus, chikungunya virus, WNV, dengue virus, yellow fever virus, *Plasmodium* parasites, and filarial worms. Mosquitoes cause more deaths due to pathogen transmission than any other animal, exceeding 700,000 deaths per year (WHO, 2022).

Mosquitoes of the genus *Culex* are vectors of arboviruses and parasitic diseases that cause West Nile fever, Japanese encephalitis, St. Louis encephalitis, filariasis, and avian malaria. In the United States, WNV was prevalent among all states in 2021 (CDC, 2022). WNV is spread to humans and horses in an enzootic cycle via birds and *Culex* mosquitoes (*Cx*. *pipiens and Cx*. *quinquefasciatus*). Infected humans are not able to

acquire high viral levels in the blood, thus do not participate in the cycle, but are dead end hosts (CDC, 2022). Approximately one in five persons will develop mild symptoms and one of 150 experience effects that can be fatal (i.e., encephalitis, meningitis, and acute flaccid paralysis) (CDC, 2022). Currently, vaccines to protect against WNV are not available for humans (Kaiser & Barrett, 2019; Ledgerwood et al., 2011).

Mosquitoes of the genus *Aedes* can be found in tropical, subtropical, and temperate climates. *Aedes aegypti* mosquitoes primarily transmit Zika virus, dengue viruses, and chikungunya virus. This species is more likely to spread these viruses because they live near humans and prefer to feed on them over animals. *Aedes albopictus* mosquitoes also transmit the same viruses but do so to a lesser extent because they prefer to feed on both animals and people (CDC, 2017). Over the last two decades, the number of dengue fever cases reported to the World Health Organization increased from 505,430 cases in 2000 to over 2.4 million cases in 2010, and 5.2 million cases in 2019 (WHO, 2022). From 2000 to 2015, the number of fatalities grew from 960 to 4,032. Most deaths reported were of people under the age of forty. *Aedes*-spread dengue fever is currently the fastest-spreading disease transmitted by mosquitoes in the world ("Dengue Bulletin," 2009). Dengue fever is spreading due to a variety of variables, including globalization, travel, commerce, socioeconomic issues, human habitation, virus evolution, and perhaps climate change (Li & Wu, 2015).

Anopheles mosquitoes transmit *Plasmodium* parasites which cause human malaria, a parasitic infection that kills at least 400,000 people and infects an estimated 200 million each year (WHO, 2016). Malaria primarily affects sub-Saharan Africa.

Children under five years of age are the most impacted group by malaria induced death (Roberts & Matthews, 2016). *Anopheles* mosquitoes only transmit one arbovirus (i.e., o'nyong-nyong virus), in contrast to *Culex* and *Aedes* mosquitoes that primarily transmit arboviruses (Karabatsos & Hygiene, 1985).

The vectors mentioned above are the most well studied species of mosquitoes due to their impact on global health. Most studies attempting to improve vector control target these species. Grand efforts have been made by researchers to reduce vector transmission of mosquitoes via generation of insecticides and environmental maintenance. Some barriers such as insecticide resistance and concern over environmental implications have reduced the effectiveness of these approaches and have led scientists to shift their focus on genetic manipulation strategies (Wilson et al., 2020)

1.2 *Culex* Mosquitoes

The *Culex* genus consists of more than 20 subgenera containing over 1,000 species. Pathogen transmission has been recorded in over 28 species, including viruses and filarial nematodes, such as *Wuchereria bancrofti* (Harbach, 2011). *Culex* spp. have a preference for feeding on birds (González et al., 2020), which are ample hosts for the harboring and amplification of viral particles in the transmission cycle of WNV (Komar et al., 2003). Mosquito-borne avian viruses are primarily transmitted by *Culex* spp. mosquitoes with the exception of Eastern equine encephalitis virus and Highlands J virus. (Pfeffer et al., 2010).

Cx. quinquefasciatus (the Southern house mosquito) originated in Southeast Asia before colonizing Africa and being introduced to the New World via slave ships (Fonseca et al., 2006). *Cx. quinquefasciatus* species are found in almost all regions of the world except in northern regions of the temperate zone.

Compared to their counterparts, *Culex* mosquitoes are understudied (Ruckert et al., 2019). However, information from prior arboviral vector studies can facilitate future *Culex* mosquito investigations. There is a lack of information regarding their antiviral pathway mechanisms, that if elucidated, could assist in the generation of novel vector control strategies (Munawar et al., 2020).

1.3 RNAi

RNAi is an antiviral pathway that works to protect mosquitoes and other organisms from pathogens (Airs & Bartholomay, 2017). RNAi is a major component in the immune system of mosquitoes. RNAi was originally observed in the *Caenorhabditis elegans* worm (Fire et al., 1998). RNAi was later described to be the dominant antiviral response in *Drosophila melanogaster* (*D. melanogaster*) (Li et al., 2002) and mosquitoes (Sánchez-Vargas et al., 2009).

There are three distinct RNAi pathways based on effector RNAs: 1) short interfering (si)RNA, 2) micro (mi)RNA, and 3) PIWI-interacting (pi)RNA (Fig. 1) (Olson & Blair, 2015). The endogenous siRNA pathway is active in both germline and somatic cells in the presence of long dsRNA molecules generated by the cell itself, working to regulate the transcription of transposable elements (TEs) and genes that code for proteins (Blair, 2011). The exogenous siRNA pathway is active in the presence of long dsRNA molecules

that are produced outside the cell. Long dsRNA molecules are generated intermediately upon viral entry into the cell or can be externally provided into the organism or cells to transiently silence target genes (Airs & Bartholomay, 2017).

The nature of the exogenous siRNA pathway allows for the manipulation of target genes by the introduction of synthetic dsRNA. External dsRNA introduced into cells or organisms serves as substrates for cleavage by ribonuclease III-like Dicer proteins, producing 21–25 base pairs of double-stranded small interfering RNAs (siRNAs (Bernstein et al., 2001). Next, siRNAs become single-stranded and integrated into the RNA-induced silencing complex (RISC), where they bind to complementary target RNA molecules. After RISC association, endogenous target mRNAs are cleaved by the action of argonaute (Ago) proteins, which results in translational suppression of the target gene (Hammond et al., 2001). The miRNA pathway functions to manage functions of the cell, including differentiation, and homeostasis (Blair, 2011). The piRNA pathway functions as defense against transposon mobilization and expression, (Olson & Blair, 2015) but has also been implicated in antiviral responses in some mosquitoes (Varjak et al., 2017). In *Ae*. *aegypti*, piRNAs have been generated in virus infected cells (Miesen et al., 2016).

The RNAi pathway in *Culex* mosquitoes is understudied relative to other significant viral vectors such as *Aedes* mosquitoes, with a significant lack in understanding of the piRNA pathway (Ruckert et al., 2019). Many of the proteins in the piRNA pathway have functions that are yet to be determined (Varjak et al., 2017).

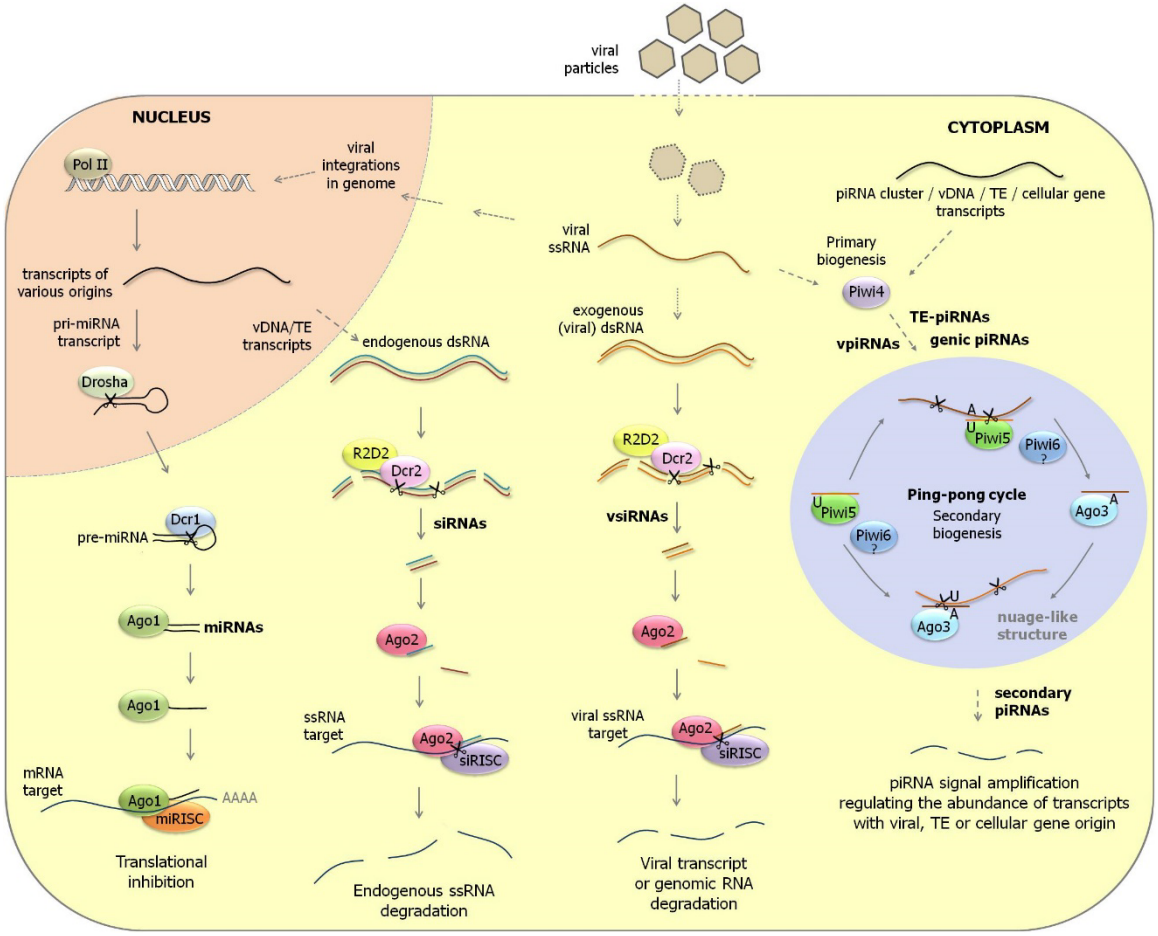


Figure 1. RNAi pathways in *Aedine* mosquitoes (Liu et al., 2019).

The miRNA pathway (on the left). Drosha processes primary miRNA transcripts into pre-miRNAs in the nucleus. Pre-miRNAs are further cleaved by Dicer-1 into mature miRNAs after being transported to the cytoplasm. After hybridization to mRNA targets, miRISC complexes containing Ago-1 control cellular gene expression by inhibiting translation.

The siRNA pathway (middle). Dicer-2 and its co-factor R2D2 cleave long dsRNA precursors with endogenous (transposable elements, viral DNA forms) or exogenous (viral replication intermediates) origins to siRNAs and vsiRNAs. After that, parasite RNA populations (transposon transcripts, viral transcripts and genomic RNAs, RNAs produced from viral integrations in the genome) are scanned and destroyed by siRISC complexes containing Ago-2. The piRNA route (on the right). An increase of PIWI-class Argonaute genes, which are expressed in somatic tissues and are involved in transposon control, antiviral defense, and cellular gene regulation, has been seen in aedine mosquitoes. A Dicer-independent mechanism converts ssRNA precursors from multiple sources (transposable elements, viral mRNAs and genomic RNAs, transcripts from viral DNA forms, cellular gene transcripts) to primary piRNAs. The piRNA ping-pong cycle is thought to be an essential amplification process for controlling the amount of transposon, viral, and cellular transcripts. pri-miRNA is for primary miRNA; pre-miRNA stands for precursor miRNA; vDNA stands for viral DNA; and TE stands for transposable element.

1.4 PiRNA Pathway: PIWI Proteins

In contrast to miRNAs and siRNAs, which depend upon RNase type III enzymes to convert dsRNA precursors into functional small RNAs, mature piRNAs are synthesized from an initial transcript enclosing a piRNA cluster via a unique mechanism (Guida et al., 2016). The PIWI-associated RNA (piRNA) pathway protects the germline from genomic

disruption by regulating transposon activity via the knockdown of transposable elements (Blair, 2019). PIWI family proteins play a large role in the pathway because they process piRNAs that are derived from piRNA clusters in genomic regions with high amounts of transposons (Liu et al., 2019).

The piRNA mechanism was initially described in *D. melanogaster* (Handler et al., 2013). Generation of 24-29 nt piRNAs occurs in a way that is independent of Dicer (ribonuclease). The germline cell expressed proteins, PIWI, Aubergine (Aub), and Argonaute 3 (Ago-3) are heavily involved. The RISC effector complex protein utilizes these three piRNA mechanism proteins. (Miesen et al., 2016) . In *D. melanogaster*, the piRNA pathway is comprised of two subpathways (Liu et al., 2019). The first pathway is an intermediate one. piRNA clusters, which are transposable element remnants, are transcribed into primary piRNAs which are then processed by a nuclease, Zucchini. Subsequently, piRNAs are loaded in effector complexes containing PIWI proteins. This results in nucleic transcriptional gene knockdown of target genes. (Liu et al., 2019). The second pathway takes place in the cytoplasm and is referred to as the ping-pong amplification loop. In this loop, transposon (sense) transcripts undergo cleavage by Aub which is anti-sense piRNA rich. This results in the creation of complementary (sense) piRNAs which then undergo loading in Ago-3 complexes. The newly loaded piRNA-Ago-3 complex next targets anti-sense transposon sequences. Both pathways create the signature amplification loop that generates mature piRNAs that are both antisense and sense and overall function to silence transposons.

PiRNAs produced from viral genomes (vpiRNAs) have been found in *Aedes* spp. cultured cells and mosquitoes post infection with arthropod-borne alpha-, bunya-, and flaviviruses. *Culex quinquefasciatus* mosquitoes and cells less commonly produce vpiRNAs after infection with arboviruses (Goertz et al., 2019; Ruckert et al., 2019). In an initial RNAi study, C6/36 (*Ae. albopictus*) cells were engineered to express DENV2 genome derived dsRNA (Brackney et al., 2010). The cells not only were resistant to infection with DENV2, but they also fundamentally lacked Dicer 2 (Dcr2) activity (because they were infected) which is responsible for processing long dsRNA into exogenous siRNA (Liu et al., 2006). These cells generated more vpiRNA-like sRNA compared to *Ae. aegypti* cells with a functional Dcr2 protein upon arbovirus infection (Brackney et al., 2010; Scott et al., 2010). This finding suggested that the piRNA pathway response compensates, when necessary, but it is less effective than the siRNA pathway (Blair, 2019). According to further studies, *Ae. albopictus* C7-10 (Morazzani et al., 2012) and *Ae. pseudocutellaris* AP-6_1 cells (Goertz et al., 2019) with abnormalities in the exogenous siRNA pathway also exhibited compensatory vpiRNA synthesis after arboviral infection. More studies are currently needed to underline the role of vpiRNAs post viral infection.

Somatic and germline tissue piRNAs are proposed to have an ancestral function for the protection against transposable elements in arthropods (Lewis et al., 2018). *Ago-3* is a singular conserved gene in insects, but the *PIWI* and *aub* genetic lineages exhibit high frequency of duplication events. This phenomenon illustrates the evolutionary arms race against transposons (Lewis et al., 2016). In *D. melanogaster*, a member of the

Diptera order along with mosquitoes, the existence of *PIWI* and *aub* genes illustrates the coupling of a gene found at the base of *Brachycera* (a suborder of Diptera) that is ancestral ("*PIWI/aub*") (Lewis et al., 2016). Instances of gene duplication have been notable, especially in somatic and germline organs of *Ae. aegypti*, *Ae. albopictus* mosquitoes, and *Cx. quinquefasciatus*, three of the most significant vectors of arthropod-borne viruses (arboviruses). Currently, there are seven ("*PIWI/aub*") genes in *Ae*. *aegypti*, nine in *Ae*. *albopictus*, and six in *Cx*. *pipiens quinquefasciatus* (Campbell et al., 2008; Lewis et al., 2016; Wang et al., 2018).

The *Ae. aegypti* genome contains seven PIWI-related genes and one *ago*-3 gene. *PIWI* 4,5,6, and *ago*-3 are expressed in the mosquito's somatic tissue (Miesen et al., 2016; Morazzani et al., 2012). *PIWI*-1-3 expression only occurs in the germline cells (Akbari et al., 2013) and *PIWI*-7 is expressed solely in the early embryonic stage. In *Cx. quinquefasciatus* all PIWI genes are expressed in the ovaries, but *Ago3*, *PIWI2*, *PIWI5*, and *PIWI6* were also highly expressed in midguts and a cell line (Ruckert et al., 2019). The nomenclature of PIWI proteins in *Ae*. *aegypti* and *Culex* mosquitoes is unfortunately not based on relatedness: *PIWI4* in *Ae*. *aegypti* is a direct orthologue of *PIWI2* in *Culex* mosquitoes, while *PIWI2* in *Ae*. *aegypti* seems to be genetically closely related to *Culex PIWI3* (Campbell et al., 2008).

Evidence via an *Ae. aegypti* cell line suggests that *Ae. aegypti* PIWI4 is an important protein involved in the antiviral response (Varjak et al., 2017). It interacts with members of the exogenous siRNA pathway (Ago2 and dcr2) and other PIWI proteins in the piRNA pathway. Small RNAs bound by PIWI4 suggest that the protein is

primarily linked with siRNAs that are virus specific (in Semliki Forest virus-infected cells) and only secondarily associated with vpiRNAs. In a Dcr2 knockout cell line, the authors showed that Ago2 loses its antiviral properties and its siRNA binding ability due to an apparent dependency on Dicer-2 processing of long dsRNA. In contrast, PIWI4 retained its antiviral activity even in the absence of Dicer-2 processing of dsRNA. These combined findings illustrate the dynamic relationship between the siRNA and piRNA pathways in *Ae. aegypti*, while at the same time identifying PIWI4 as an antiviral protein that is seemingly independent of either pathway. In *Cx. quinquefasciatus*, the function of its direct orthologue (PIWI2) has yet to be determined, along with other aspects of the RNAi pathway.

1.5 Gaps in Knowledge in the RNAi Mechanism of *Culex* Mosquitoes

To explore the understudied RNAi pathways in *Culex* spp., a study sequenced small RNA (sRNA) libraries from WNV-infected *Culex* cell lines and tissues (Ruckert et al., 2019). In all the cell lines and tissues, 21-nt virus derived siRNAs were predominately present in the sequencing data, but no larger small RNAs (24-29 nt) with ping-pong signature. These data present that WNV-derived piRNAs are not produced in WNV infected cells. However, sRNA readings were mapped from *Cx. quinquefasciatus* Hsu cells to the Merida virus, an insect-specific rhabdovirus that replicates persistently in these cells, and piRNAs were shown to make up a component of the sRNA response to the Merida virus.

Although Hsu cells produced piRNAs derived from the Merida virus, the findings suggested that the exogenous siRNA response was the most pertinent sRNA response of

Culex cells and mosquitoes in response to WNV infection. Clearly, different virus-mosquito combinations produce different sRNA responses. Future research involving additional *Culex*-borne viruses may shed more light on how virus-derived piRNAs are produced in *Culex* cells and what role they might play in virus replication control. *Culex* PIWI2 protein function is of interest because it's orthologue protein, PIWI4, that is found to be antiviral in *Ae. aegypti,* suggests that PIWI2 may also be antiviral. Additional research performed in the Rückert lab also suggested that PIWI2 is antiviral, when La Crosse encephalitis virus infected cells that had PIWI2 knocked down produced an increase of viral RNA. In the present study, *in vivo* gene silencing was explored to study the potential antiviral protein PIWI2.

In vivo methods used for this research require continuous labor and maintenance costs. In this thesis, we aimed to eliminate these burdens by developing a cryopreservation protocol of *Culex* mosquitoes. In depth knowledge regarding the eggshell and embryo must be understood when designing a cryopreservation protocol, specifically regarding egg permeability.

1.6 Embryo Desiccation Resistance (EDR) in Mosquitoes

Embryogenesis research has focused mostly on the most well studied genera of mosquitoes: *Aedes*, *Culex*, and *Anopheles*. Many differences in the stages of embryogenesis and the egg laying process are documented between these genera, although they do share some similarities. Insect eggs are classified based on their dependence on water, oxygen, nutrients, or a combination of the three (Hinton, 1981). All mosquito eggs are oxygen and water dependent; they deposit their eggs on water or

near the surface. Soon after egg deposition, water is taken in by the eggs and their size and mass increases (Rezende et al., 2008).

Each species lays eggs in a unique manner. *Aedes aegypti* mosquitoes deposit individual eggs on wet surfaces, whereas *Anopheles aquasalis* mosquitoes lay individual eggs which float on the water surface. *Culex quinquefasciatus* mosquitoes lay eggs on water in rafts, one at a time, where individual eggs (100-300) are stuck together in a highly organized manner (CDC, 2020a). When eggs are laid, the eggshell is transparent and made of the exochorion and endochorion. During the next two hours, melanization occurs, resulting in the darkening of the endochorion and reduction of permeability (Farnesi et al., 2017). Recently laid eggs are sensitive to water loss and shrink when not in a moisturized environment. The degree of this sensitivity varies among species. In all three species, during later phases of embryogenesis, an extracellular matrix formed by the serosa, called the serosal cuticle (SC), is formed. The SC surrounds the embryo and develops into part of the eggshell. The SC contains chitin as one of its main elements and is directly related to EDR, thus being pertinent to egg permeability. The SC influences egg desiccation resistance (EDR) (Farnesi et al., 2015). This trait was previously referred to as, “embryo desiccation resistance” (Vargas et al., 2014) until it was determined that the trait is a quality of the overall egg. Timing of SC development during embryogenesis differs among species.

Aedes aegypti mosquito eggs can withstand dry environments for months, after at least 72 hours of embryogenesis (Farnesi et al., 2017). *Anopheles aquasalis* eggs have a much lower EDR, showing lower viability when left in a dry environment after twenty-

four hours. *Culex quinquefasciatus* embryos are very sensitive to desiccation and can only withstand a dry environment for up to five hours (Farnesi et al., 2017). The EDR trait is reliant upon the water content of the organism, its water loss rate, and dehydration tolerance (the least amount of water in the body prior to death).

Aedes aegypti eggs are more pigmented than *A. aquasalis* eggs, which are more pigmented than *Cx. quinquefasciatus* eggs (Farnesi et al., 2017). This observation led researchers to investigate the correlation between EDR and pigmentation levels of eggs. It was determined that there is a strong correlation between increased egg pigmentation and higher EDR within species. The discovery that pigmentation affects balance of water in the egg is important for understanding insect egg color evolution. In certain circumstances, eggshell and adult cuticle coloration support insect survival, therefore they should be evaluated in terms of species fitness and innovative vector or pest insect management tactics.

The EDR is determined by various qualities of the egg; thickness of the SC, thickness of the endochorion, biochemical makeup of the SC, endochorion sclerotization, melaninization rates, and larval cuticle structures (Gibbs et al., 1997; Gray & Bradley, 2005; Hadley, 1994). Some metabolites found inside the egg that may also be involved in mediating EDR include glycogen, triacylglycerols, glycerol, and trehalose (Gray & Bradley, 2005; Hadley, 1994; Sawabe & Mogi, 1999; T. Sota, 1992). Conversely, the exochorion or egg mass are not factors that influence EDR (Farnesi et al., 2015). The change in egg volume throughout embryogenesis and eggshell surface density is also related to ERD in an inverse manner.

The eggshell is composed of the SC, endochorion, and exochorion. The eggshell is responsible for protecting the embryo from biotic and abiotic stressors and controlling water loss and uptake (Clements, 1992). At the time of egg lay, both endochorion and exochorion structures have been formed (Monnerat et al., 1999). These structures are produced via ovarian follicle cells during the formation of the chorion (Chapman, 1998; Clements, 1992).

A mosquito egg does not have the ability to retain water before it forms the SC, and therefore dies due to lack of water when in dry conditions during embryogenesis. The SC has thus been determined to increase the impermeability of the overall eggshell. The lack of water escaping the eggshell is due to the combined activity of the SC and the endochorion, specifically during melanization and sclerotization (Goltsev et al., 2009; T L Hopkins & Kramer, 1992)

The time in which the EDR trait is acquired has been well studied in these species along with the embryogenesis times. *Aedes aegypti* embryos held at 25 °C complete embryogenesis 77.4 hours after egg lay (HAE). Among different *Aedes* species, embryogenesis completion times vary tremendously according to the literature (Horsfall, 1973; Telford, 1957; Trpis et al., 1973). *Anopheles aquasalis* held at 25 °C completes embryogenesis by 51.3 HAE and that period highly varies among the *Anopheles* species as well (Goltsev et al., 2009). *Culex quinquefasciatus* complete embryogenesis by 34.2 HAE and the overall *Culex* species vary in this aspect as well, but not as highly compared to the other two genera (Rosay, 1959). For these reasons, estimating embryogenesis completion times according to the genus is not sufficient to

decide upon optimal ages for cryopreservation treatments. Embryogenesis time can be impacted by a variety of effects: favorable environmental conditions, spatial distribution, and the number of generations that arise each year (Gillooly, 2000).

For *Ae. aegypti* and *An. aquasalis*, the EDR is acquired at 21% of embryogenesis whereas in *Cx. quinquefasciatus*, the EDR is acquired at 35% of embryogenesis. Due to the difference in the EDR acquirement times and embryonic morphologies, the uprising of the serosa and the EDR happen somewhat independently from embryo development. This finding was confirmed in beetles through inhibition of serosa formation, suggesting a disconnected relationship between embryonic and extraembryonic progressive development (van der Zee et al., 2005). Another interesting finding to note was that in eggs of the lepidopteran species, *Manduca sexta* (tobacco hornworm), water retention is inversely related to gas exchange capacity. Related studies have yet to be done in mosquitoes and could provide more information on the SC (Bernays & Woods, 2000). The formation of the SC is heavily of interest because it causes the increase of impermeability of embryos to water (Rezende et al., 2008) and thus to cryopreservation agents (CPAs) that must be introduced for protection from low intracellular ice formation that may occur during freezing.

1.7 Developing Methods for Cryopreservation of *Culex* spp. Embryos

There are no available protocols for the cryopreservation of *Culex* spp. embryos but related permeabilization methods are available for other insects such as *D. melanogaster (Steponkus et al., 1990; Zhan et al., 2021)*, the New World Screwworm *Cochliomyia hominivorax* (R A Leopold, 2001), and *Anopheles* (Gallichotte et al., 2021;

Valencia et al., 1996) that can potentially be used and optimized for *Culex* spp.. Available protocols commonly begin with the removal of the protein-based chorion (the most outmost layer of the eggshell) using a sodium hypochlorite-based reagent. Next, dehydration and permeabilization of the embryo is implemented by removing water from the embryo and using an alkane for the removal of the waxy lipid layer found beneath the chorion layer of the egg. CPAs such as ethylene glycol (EG) are then inserted into the embryo to ensure vitrification during freezing and to prevent embryo damage due to intracellular ice formation.

1.8 Research Objectives

PIWI2 Knockdown using *in vivo* RNAi methods. Rationale: The first objective was to test whether PIWI2 in *Cx. quinquefasciatus* mosquitoes, an orthologue of an antiviral protein in *Ae. aegypti*, is also antiviral. *In vivo* gene knockdown techniques that utilize RNAi were implemented. Hypothesis: We hypothesized that the chosen *in vivo* knockdown techniques used here (intrathoracic dsRNA microinjection and dsRNA pupae soak) can successfully knock down PIWI2 expression in *Cx. quinquefasciatus* mosquitoes. After dsRNA was introduced, gene expression levels were measured 2-5 days after the introduction of dsRNA for confirmation of gene knockdown.

Optimizing dechorionation, embryo dehydration, and permeabilization methods. Rationale: The second objective was to design a cryopreservation protocol for *Cx. pipiens* embryos that could enhance mosquito biology research by reducing continuous labor, costs, risk of contamination, and genetic or phenotypic effects associated with *in vivo* studies or transgenic cell line maintenance. Hypothesis: We hypothesized that the

cryopreservation methods for *Culex* embryos can be established by optimizing dechorionation, embryo dehydration, and permeabilization methods. We chose sodium hypochlorite as a dechorionation agent and tested soak times for viability effects, along with dehydration times, and an alkane based permeabilization soak. After presumed permeabilization, the embryo was incubated in the CPA (EG) for uptake of EG. Permeabilization to EG was visualized by initial shrinking and subsequent swelling of the embryos after being placed in the solution. Viability was tested through larval counts after hatching.

Chapter 2: PIWI2 knockdown using *in vivo* RNAi methods

2.1 Background

The exogenous siRNA pathway can be induced by introducing synthetic dsRNA into cells or organisms to cause gene knockdown. *In vivo* intrathoracic microinjection of dsRNA into adult mosquitoes has been successful for transiently knocking down genes in *Cx. quinquefaciatus* (Puglise et al., 2015; Xiao et al., 2015) and *Ae. aegypti* (Kang et al., 2018; Londono-Renteria et al., 2015). *In vivo* dsRNA pupae soak is another *in vivo* method that has been successful in transiently knocking down genes in *Ae. aegypti* (Arshad et al., 2021). In this study, we utilized these *in vivo* techniques to knockdown PIWI2 in *Cx. quinquefasciatus* mosquitoes. PIWI2 is suspected to be an antiviral protein due to the determined antiviral role of its orthologue in *Ae. aegypti*, PIWI4 (Varjak et al., 2017) and unpublished data from the Rückert lab involving *in vitro* experiments using La-Crosse infected cells. To investigate if PIWI2 is antiviral, after its successful

knockdown, we would then infect the mosquitoes with virus, and then evaluate viral RNA levels.

2.2 RNAi Techniques to Investigate PIWI2

DsRNA may be introduced into insects in a variety of ways to perform gene knockdown. The design strategy of dsRNA for RNAi technology is relatively simple, only requiring the target gene sequence. Some challenges include enzymes that degrade dsRNA, cell absorption efficiency, expression of core RNAi components, target gene nature, concentration and permanence of dsRNA, and the specific feeding method of each insect (Santos-Ortega & Flynt, 2022). Intrathoracic microinjection of dsRNA is a commonly used RNAi method for knocking down specific genes in adult mosquitoes (Balakrishna Pillai et al., 2017). For example, in one study, researchers were able to successfully knockdown two *Ae*. *aegypti* genes: cysteine desulfurylase (*Nfs1*) and short-chain dehydrogenase (*SDH*) (Kang et al., 2018). Results indicated that the *Nfs1* gene was more readily silenced than the *SDH* gene, demonstrating that knock down efficiency almost certainly varies among genes. Further results demonstrated that in this species, 500 ng of dsRNA was sufficient to silence readily silenced genes such as *Nfs1* for up to 3 weeks post intrathoracic injection. This technique, with only some modifications, was applied to *Cx. quinquefasciatus* adult mosquitoes in attempt to transiently silence the gene that encodes for the potentially antiviral protein PIWI2.

RNAi studies typically involve the introduction of dsRNA to adult mosquitoes (Munawar et al., 2020) with only a few studies targeting mosquitoes at earlier developmental life stages such as larvae (Singh et al., 2013) or pupae (Arshad et al.,

2021). In *Ae*. *aegypti* pupae, researchers introduced dsRNA through a water-based soak. This method allows for a feasible way to knockdown genes in a large population of mosquitoes that is less time consuming compared to intrathoracic microinjections. Additionally, unlike larval RNAi studies, the pupae dsRNA soak does not require a carrier molecule and presents a lower osmotic challenge due to the higher permeability of pupae. This method, with some alterations, was used to attempt to reduce the expression of the potential antiviral protein PIWI2 in *Cx. quinquefasciatus*.

2.3 Methods

Mosquitoes

Culex quinquefasciatus mosquitoes were reared at 25 °C in an insectary at 40-55% relative humidity. Eggs were hatched overnight in small plastic water cups. Eggs were then transferred to a container with water. First instar larvae were then placed in a container with water and provided with a powdered fish food diet (Product No. 77101, Tetramin© Tropical Flakes, VA). Adults were provided with a generic white sugar cube and water cup.

Generation of dsRNA for Pupae Soak and Intrathoracic Microinjection

First strand complementary DNAs (cDNAs) were synthesized from *Cx. quinquefasciatus* mosquito total RNA via a reverse transcription kit according to the manufacturer's instructions (Cat# 4368814; Applied Biosystems™). Random primers included in the kit were used. Next, gene-specific primers for PIWI2, containing the T7 promoter sequence on both the forward and reverse primer, were used to amplify respective target cDNAs

of about 500 bp in length (Table 1). A Green Fluorescent Protein (GFP) vector plasmid was used to produce a DNA template by PCR with primers containing T7 promoters. The PCR conditions were as follows: 98 °C for 1 min. and 10 sec., 68 °C for 1 min. followed by 39 cycles of denaturation at 98 °C for 10 sec, and extension at 72 °C for 5 min. The PIWI2 and Green Flourescent Protein (GFP) products incorporated the T7 promoter sequence in both sense and antisense orientations, permitting synchronized transcription of target RNA strands. *In vitro* transcription was accomplished using a MEGAScript™ RNAi kit according to the manufacturer's instructions, with an exception in the purification protocol (Cat# AM1626M; Invitrogen™). The high concentrations of dsRNA required for the experiment were achieved by scaling up the *in vitro* transcription reaction. Three *in vitro* transcription reactions were carried out per gene and pooled to reach at least a concentration of 5000 ng/µL. The incubation time was also increased to 16 hrs. The purification of dsRNA was performed per the Zamanian lab protocol (http://www.zamanianlab.org/ZamanianLabDocs/protocols/Molecular_Biology/RNAi_dsRNA_Synthesis/RNAi_dsRNA_Synthesis/). Briefly, for each 50 µL of dsRNA from the digestion reaction, 150 µL of nuclease-free H_2O, 100 µL of phenol, and 100 µL of chloroform were added. The reaction was shaken for 15 sec., with caution taken to not produce foam. The mixture was centrifuged at 15,000 *x g* for 10 min. at 4 ºC. The top phase (aqueous) was transferred to a new RNase-free tube. Care was taken to avoid the transfer of any interphase or bottom phase. To aqueous dsRNA, 500 µL of isopropanol was added. The tube was placed at 80 ºC for 30 min. to precipitate RNA. The tube was

centrifuged at 15,000 *x g* for 15 min. at 4 ºC. A pellet was seen after centrifugation (gelatinous blob) and the supernatant was then removed. Next, 600 µL of cold 70% ethanol was added, and the pellet was gently pipetted up and down. The tube was then centrifuged at 5,000 *x g* for 5 min. at 4 ºC. If the pellet was pale white, a second wash with 70% ethanol was implemented to clean out the salt. The supernatant (500 µL) was then removed, and the remaining pellet was briefly spun in the minicentrifuge. The p20 pipette was used to remove as much of the supernatant possible. The pellet was then again briefly spun in minicentrifuge. The p2 pipette was then used to remove the remaining supernatant. The p2 tip was used to move the gelatinous pellet up the side of the tube to facilitate drying and collect any remaining supernatant that is in tube. The pellet was air dried in the fume hood for 5-10 min. and resuspended in a minimal amount of RNase-free water. To help the pellet dissolve, the tube was placed in a 4 °C refrigerator. A 1:10 dilution of the original product was made, and the concentration and purity was then measured with the Nanodrop (Cat#13400525; Thermo Scientific™). The dsRNA was stored in -80 ºC until use.

Table 1: Primer Sequences for dsRNA Synthesis

Primer Name	Forward (F) and Reverse (R) Primer Sequences (5’ to 3’)
PIWI2	F: TAATACGACTCACTATAGGGGGACGTAATCTGTTCGACGC R: TAATACGACTCACTATAGGGGTCATCTGGCAAAGTTCCGG

GFP	F: TAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGAGCTGTTC R: TAATACGACTCACTATAGGGCTGGGTGCTCAGGTAGTGGTTGTCGGGC

(*T7 promoter sequence is underlined)

Table 2: Primer Sequences for qRT-PCR and Semi q-PCR Assays

Primer Name	Forward (F) and Reverse (R) Primer Sequences (5' to 3')
PIWI2	F: TTTCCAACTACCTCCCGATCAAC R: CGCCATCACGGTAGAAGATGATAC
Actin 5	F: CAACTGCCCAAATCGAATGAC R: CGACGCACTCTCGGAATAAA

DsRNA Pupae Soak

In the morning of the experiment, all pupae were collected from their container. Newly molted pupae (molted within 4 h) were then collected every hour and transferred to a cylindrical glass bottle (5.8 cm x 2 cm, Glass Storage Tubes, Bead Landing™) containing 500 µL of nuclease-free deionized (DI) water with five micrograms of PIWI2 of GFP dsRNA. The final concentration of the dsRNA used was (1 µg/µL). To avoid any harm to the newly molted, delicate pupae, a paintbrush was used to remove them from the container and transfer them to the glass container. The glass bottle was then taped and placed to the bottom of a cage.

DsRNA Intrathoracic Microinjection

DsRNAs targeting endogenous mRNAs were diluted in water and prepared at the concentration: 5 µg/µl. A volume of 0.1 or 0.2 µl was injected for each dsRNA solution into the thorax of cold-anesthetized three to six-day old females using a microinjector

(Nanoject III, Drummond Scientific Company, PA). Glass capillaries included with the microinjector were used as injection needles (3.5" Drummond #3-000-203-G/X) (Drummond Scientific Company, PA). The needles were pulled (Model P-1000 Flaming/Brown Micropipette Puller, Butter Instrument, U.S.) at the following conditions: 430 heat, 50 pull, 75 velocity, 999 pressure. After injections, the resulting mosquitoes were placed in small plastic cylindrical containers containing a small, moistened cloth.

DsRNA Cell Transfection for Testing dsRNA Integrity

The *Cx. quinquefasciatus* ovary-derived cell line Hsu (Hsu et al., 1970) was maintained at 28 °C in DMEM supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin) in a humidified atmosphere of 5% CO_2 in air. Cells were seeded in a 24-well plate in 1 mL of culture media. Three replicate wells of cells were treated with either PIWI2 dsRNA or GFP dsRNA (1000 ng/well). The transfection was performed according to the manufacturer's instructions (Lipofectamine® RNAiMAX, Life Technologies).

RNA Extraction for Pupae Soak, Intrathoracic Microinjection, and Hsu dsRNA Cell Transfection

Total RNA was extracted via Direct-zol RNA Mini Prep Kit (Cat# R2050; Zymo Research Corporation) at either 2- or 5-days post introduction of dsRNA for the pupae soak and microinjection experiments. RNA was extracted three days after the dsRNA cell transfection. RNA extraction was performed according to the manufacturer's instructions.

Quantitative real-time PCR analysis (qRT-PCR)

cDNAs were synthesized from all RNA samples via a reverse transcription kit according to the manufacturer's instructions (Cat# 4368814; Applied Biosystems™). Random primers included in kit were used. All reactions were performed in a total volume of 20 µl, containing 10 µl of SYBR Green PCR Master Mix (Cat# 1708882; BioRad, CA), 200 nM of each primer and cDNA as a template under the following condition: 95°C for 3 min., then 60°C for one min., followed by 60 cycles of denaturation at 95 °C for 10 sec., annealing and extension at 65 °C for 1 min. qRT-PCR and melting curve analyses were performed using CFX 96 RT-PCR-cycler (BioRad, CA). The PCR data was normalized to the actin 5 gene as an internal control and target mRNA relative abundances were displayed in comparison to the control mosquitoes that were injected with GFP (see Table 2 for primer sequences). The equation 2^ (actin cq-piwi2 cq) was used for gene expression normalization. This formula provided the ratio of PIWI2 RNA copies per actin copy. Data was then further normalized to the GFP control to determine the change in PIWI2 expression between treatments. This is the method commonly used in our lab.

Statistical Analysis

GraphPad Prism 9 software (La Jolla, CA, USA) was used to analyze data. Normalized values of PIWI2 and GFP fold change were compared using unpaired-t tests.

2.4.1 DsRNA Pupae Soak Results

There was no statistically significant knockdown of PIWI2 after soaking pupae in dsRNA. Figure 2A-C represents the three experimental attempts in sequential order. The mean PIWI2 fold changes compared to the GFP treated control group were 4.8 (Fig. 2A),

1.2 (Fig. 2B), and 1.1 Fig. 2C). In the first experiment (Fig. 2A), one of the GFP control samples presented with a fold change of more than one standard deviation greater than the mean. In the PIWI2 dsRNA treated samples, one sample also displayed a fold change of over one standard deviation greater than the mean. These values suggest high variability between samples. Although conditions were attempted to be kept consistent throughout the experiments, there must have been some lack of consistency in the experimental procedures. In the second replicate experiment (Fig. 2B), samples from each treatment group either showed a high (>1.5) or low fold change (0.5>), again suggest high sample variability. For the 3rd replicate experiment (Fig. 2C), one of the nine GFP treated replicates produced a value over one standard deviation from the mean and two of the nine PIWI2 treated replicates produced a value over one standard deviation from the mean in comparison to the GFP treated group. While the PIWI2 treated replicate values were closer to 1 and there was less overall variability between samples compared to the previous panels, there was still no significant knockdown ($p = 0.947$).

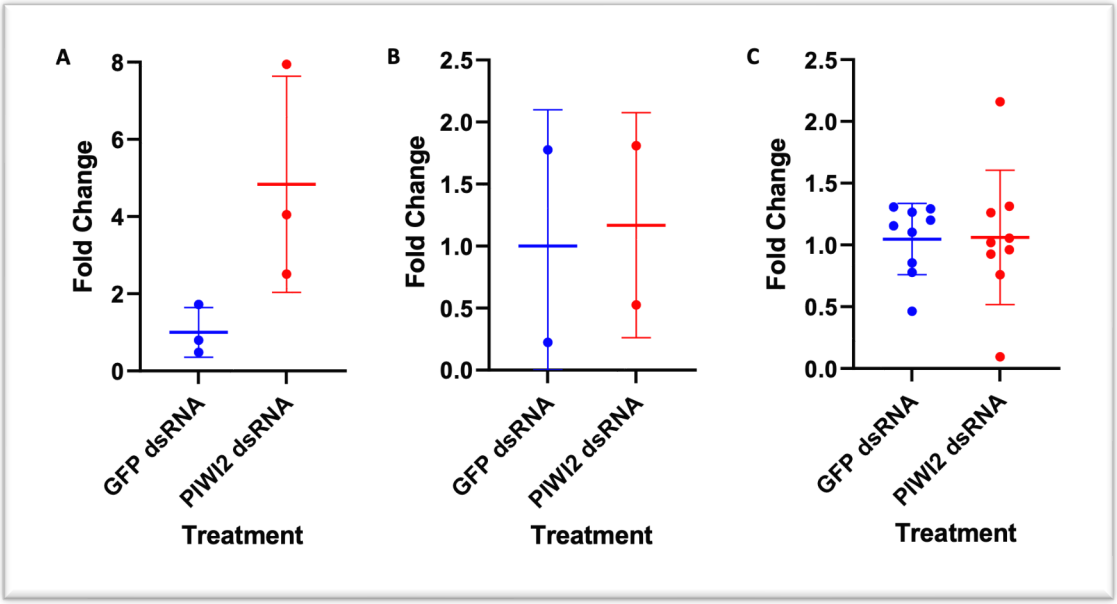


Figure 2. **PIWI2 dsRNA pupae soak (whole body RNA extraction, 2 days post treatment).**
Mosquito pupae no older than 4 hrs were treated with either 5 µg of PIWI2 or GFP dsRNA containing DI water soak. Amount of mRNA was analyzed by real-time PCR. Expression was normalized to actin5 housekeeping gene and expression levels in pupae treated with 5 µg of GFP dsRNA were set to 1. RNA was extracted from adult mosquito bodies two days after the treatment. Each panel represents one independent experiment. The bars represent one standard deviation from the mean. In Figure 2C, *n* = 9 for each treatment. Data was analyzed by unpaired t-test and no significant knockdown of PIWI2 was observed ($p>0.05$).

Since viral replication initiates in the midgut, we were particularly interested in the PIWI2 expression levels in the midguts after the dsRNA treatment. Midgut RNA was extracted from parallel mosquitoes of one of the experiments (Fig. 2C) two days after

dsRNA pupae soak (Fig. 3). PIWI2 expression apparently increased in treatments in comparison to the GFP treated control group, but not significantly as variation was extremely high (Fig. 3.)

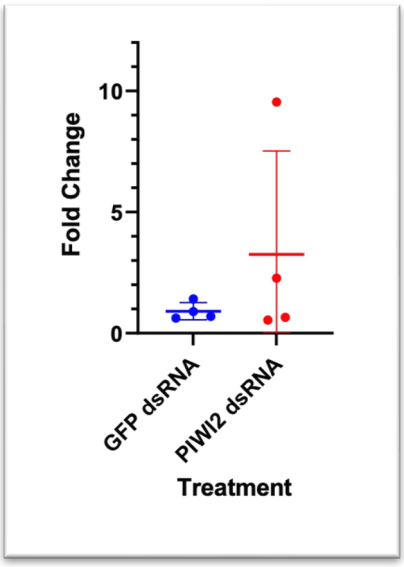


Figure 3. PIWI2 dsRNA pupae soak (RNA extraction from midgut 2 days post treatment).
Mosquito pupae no older than 4 hrs were treated with either 5 µg of PIWI2 or GFP dsRNA containing DI water soak. Amount of mRNA was analyzed by real-time PCR. Expression was normalized to actin5 housekeeping gene and expression levels in pupae treated with 5 µg of GFP dsRNA were set to 1. RNA was extracted from full adult mosquito midguts two days after the treatment. The bars represent one standard deviation from the mean. Data was analyzed by unpaired t-test (n=3) and no significant knockdown of PIWI2 was observed ($p>0.05$).

Total body RNA was extracted five days after the dsRNA pupae soak to see if additional time was needed for the dsRNA to cause a gene knockdown. There was no significant knockdown in total body RNA that was extracted five days after the dsRNA

pupae soak (Fig. 4), although PIWI2 expression was generally decreased (mean fold change = 0.5) in comparison to the GFP treated control group.

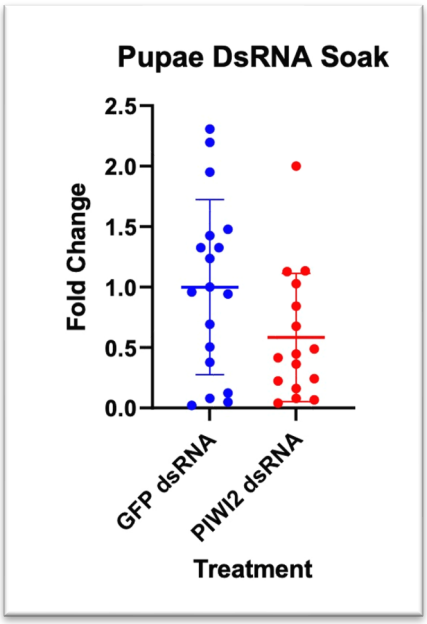


Figure 4. PIWI2 dsRNA pupae soak (whole body RNA extraction, 5 days post treatment).

Mosquito pupae no older than 4 hrs were treated with either 5 µg of PIWI2 (n=16) or GFP (n=18) dsRNA containing water soak. Amount of mRNA was analyzed by real-time PCR. Expression was normalized to actin5 housekeeping gene and expression levels in mosquitoes treated with 5 µg of GFP dsRNA were set to 1. RNA was extracted from full adult mosquito bodies five days after the treatment. The bars represent one standard deviation from the mean. Data was analyzed by unpaired t-test and no significant knockdown of PIWI2 was observed ($p>0.05$).

2.4.2 DsRNA Intrathoracic Microinjection Results

Mosquitoes subjected to intrathoracic microinjection also did not show significant knockdown of PIWI2 (Fig. 5). The mean PIW2 fold change compared to the

GFP treated control was 0.57. One out of six replicates in the GFP dsRNA treated samples report fold change values over one standard deviation from the mean. Two out of six replicates in the PIWI2 dsRNA treated samples report fold change values over one standard deviation from the mean compared to the GFP treated control.

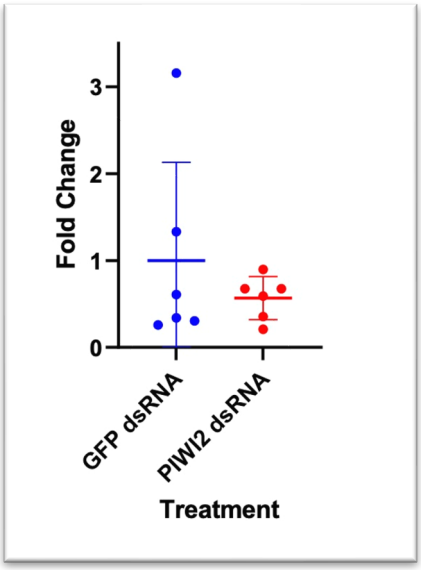


Figure 5. PIWI2 dsRNA intrathoracic microinjection full body RNA extraction. Adult mosquitoes were injected intrathoracically with either PIWI2 or GFP dsRNA. Amount of mRNA was analyzed by real-time PCR. Expression was normalized to actin5 housekeeping gene and expression levels in mosquitoes treated with 500 ng of GFP dsRNA were set to 1. RNA was extracted from full adult mosquito bodies five days after the treatment. Data was analyzed by unpaired t-test (n=6) and no significant knockdown of PIWI2 was observed ($p>0.05$).

2.4.3 DsRNA Cell Transfection Results

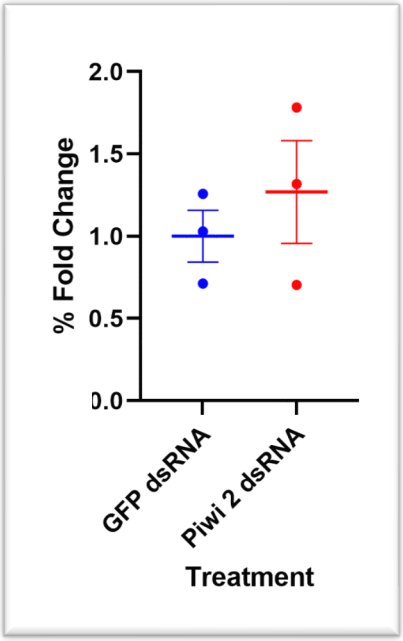


Figure 6. dsRNA did not produce sequence specific gene silencing in Hsu *Cx. quinquefasciatus* cells.

Hsu cells were transfected with PIWI2 dsRNA (1000 ng/well). Amount of mRNA was analyzed by real-time PCR. Expression was normalized to actin5 housekeeping gene and expression levels in cells transfected with 1000 ng of GFP dsRNA were set to 1. RNA was extracted three days after the treatment. The bars represent a standard error of the mean (SEM) (*n* = 3). Data was analyzed by unpaired t-test and no significant knockdown of PIWI2 was observed ($p>0.05$).

Since the dsRNA was not efficient for knocking down PIWI2 using *in vivo* methods, we tested the dsRNA for knockdown ability *in vitro*. Previous knockdown of PIWI2 has been determined in *Cx. quinquefasciatus* Hsu cells using dsRNA by other lab members of the Rückert lab. However, there was no significant knockdown of PIWI2 observed in the *Cx. quinquefasciatus* Hsu cells (Fig. 6).

2.5 Discussion

No significant knockdown of PIWI2 was observed in any treatments attempted. Because of the high variability in individual data points, results are likely to be inaccurate due to a lack of experience with these methods, potentially leading to pipetting errors during RNA extraction or qRT-PCR. Another possible explanation is that the dsRNA may not be effective due to some unknown reason. A dsRNA transfection experiment in Hsu *Cx. quinquefasciatus* cells was performed to verify whether the produced dsRNA would result in PIWI2 gene silencing using well-established laboratory protocols. No significant knockdown was observed. Previously, other lab members have successfully knocked down PIWI2 by following the same transfection protocol used in this experiment. The only difference made in the method for the generation of dsRNA was the purification process. Prior lab members purified dsRNA via the spin columns provided with the MEGAScript RNAi kit, while phenol-chloroform purification was used here to achieve further dsRNA concentration. Evidence in literature does not imply that purifying dsRNA using a phenol-chloroform method would affect dsRNA integrity, which is why it is difficult to find a reasonable explanation for the lack of knockdown in the cells. Testing previous dsRNA used in successful knockdown *in vitro* experiments in addition to the phenol-chloroform purified dsRNA would have been helpful to determine if it really was the dsRNA that was inefficient for knockdown instead of issues in following the transfection protocol.

Nonetheless, the dsRNA produced for this study may have been less effective at gene silencing than other dsRNA stocks used in the lab.

The quantity of dsRNA used in the microinjection experiments (500 ng delivered in 100 nL) was estimated to be sufficient to cause a gene knockdown based on prior studies (Kang et al., 2018). In one study, in attempt to knockdown *Cx. quinquefasciatus* Say *IAP1* homolog (*Cqu IAP1*), they delivered two micrograms of dsRNA in 152 nL of solution into adult mosquitoes and assessed for gene knockdown 2 and 3 days after injection (Puglise et al., 2015). A significant knockdown was observed. In another study using the microinjection delivery method, they delivered one microgram of dsRNA in 300 nL to perform the gene knockdown of *Cx. pipiens pallens Hig* (*CpHig*) (Xiao et al., 2015). They assessed and confirmed significant gene knockdown six days after microinjections.

Off target effects of dsRNA generation are possible (Powell et al., 2017), and for this reason the use of siRNA injection may be suggested, although this method does not always work *in vivo* in *Ae. aegypti* (McFarlane et al., 2021). Some genes are easier to silence than others, and thus may require more dsRNA (Kang et al., 2018) due to gene-specific expression of tissues and/or feedback mechanisms (McFarlane et al., 2021). It could be that *PIWI2* is harder to knock down than other genes used in other studies, and the quantity used was not sufficient for knockdown.

2.6 Conclusion

DsRNA intrathoracic microinjection and dsRNA pupae soak are two RNAi methods that have the potential to aid in the investigation of protein function in *Ae.*

aegypti. This study attempted to investigate the protein function of the potentially antiviral *Culex* PIWI2 using these methods. In this study, it was not determined if either method is effective in *Culex* species to knockdown PIWI2 due to lack of precision and observation of knockdown in the data, along with data that suggested the inefficiency of dsRNA used in the experiment. It might be useful for researchers attempting to use these methods to further verify the integrity and functionality of dsRNA to be used before attempting experiments using *in vivo* studies.

Chapter 3. Establishment of *Culex* Mosquito Cryopreservation Methods

3.1 Background

Studying arbovirus mosquito interactions typically involves long term rearing of mosquito colonies, requiring intensive labor for upkeep (Gallichotte et al., 2021). There is also a risk of colony cross-contamination and loss of valuable mosquito lines. New methods for the generation of transgenic mosquitoes (Ito et al., 2002; Moreira et al., 2002) are enabling the generation of valuable new mosquito lines in need of constant maintenance. There is an urgent need to develop reliable protocols for long-term storage of mosquitoes. In cell culture systems, valuable lines are commonly preserved by cryopreservation in liquid nitrogen tanks (Vyletova et al., 2016). Reliable protocols exist that protect cells during the freezing process, but successful *in vivo* cryopreservation strategies are rare. There are a few successful protocols to cryopreserve the embryos of *D. melanogaster* (Mazur et al., 1992; Steponkus et al., 1990; Zhan et al., 2021) and the New World Screwworm *Cochliomyia hominivorax* (R A Leopold, 2001). Partial success in protocol development also involved other species,

such as late stage embryos of *Spodoptera exigua* (Luo et al., 2006) and *Anopheles* (*An.*) *gambiae* (Valencia et al., 1996). *Anopheles gambiae* mosquitoes have been successfully permeabilized. These methods can likely be adjusted and optimized for cryopreservation of *Cx. pipiens* embryos and other mosquito species. The goal of this research was to test, adjust, and optimize individual steps within these protocols developed for other insect species to define a method for the cryopreservation of *Cx. pipiens* mosquito embryos.

3.2 Strategies for Insect Cryopreservation

Currently, no cryopreservation protocol has been universally adopted for any mosquito species. A cryopreservation protocol has been available for *D. melanogaster* (Steponkus et al., 1990), but failed to become universally adopted due to the complexity of the steps, reproducibility, and equipment use, although an improved protocol has been recently developed (Zhan et al., 2021).

The steps required for designing a cryopreservation method are similar for most insects. The removal of the chorion must first be achieved for the purpose of removing water and the waxy layer underneath the chorion, and for the insertion of vitrified CPAs that allow for the embryo to freeze in a glass like manner, in sub-zero temperatures. CPAs must be vitrified to reduce injury to the embryo, resulting in the need for a vitrification step that involves rapid cooling and warming rates of the CPA.

The dechorionation step commonly utilizes solutions with sodium hypochlorite (Monnerat et al., 1999; R A Leopold, 2001; Steponkus et al., 1990; Valencia et al., 1996; Zhan et al., 2021). After the dechorionation step, a dehydration step is implemented for

the removal of extra embryonic water that can cause intracellular ice formation during freezing (Valencia et al., 1996). Following the dehydration step, an alkane or alkane-alcohol solution is usually used to dissolve the waxy lipid layer and vitelline membrane found on most insect embryos (Steponkus et al., 1990). These first two steps are designed to result in a permeable egg that can take up a CPA. The optimal CPA chemical must be selected and introduced into the embryo to protect the embryo from intracellular ice formation that may occur in subzero temperatures. EG and dimethyl sulfoxide (DMSO) are commonly used CPAs (Zhan et al., 2021). A mixture of permeable and non-permeable CPAs may be used to decrease CPA-induced toxicity (Zhan et al., 2021). Vitrification techniques employ CPA concentrations high enough to prevent ice from crystallizing (Fahy & Wowk, 2015) and are implemented next in pair with CPA loading. After vitrification treatments, insect embryos are then placed in liquid nitrogen. Upon removal from storage, eggs are placed in a nutrient-rich heat treatment before they are left to hatch (Steponkus et al., 1990; Valencia et al., 1996; Zhan et al., 2021).

In 2021, a research team developed a novel procedure that may be universally adopted for *D. melanogaster* cryopreservation (Zhan et al., 2021). The authors used 50% household bleach to dechorionate the embryos, as in previous *D. melanogaster* protocols (Steponkus et al., 1990). Following dechorionation, a 10 sec. soak of embryos in a mixture of D-limonene and heptane was used for successful permeabilization. EG was found to be the least toxic CPA, especially when used in combination with a non-permeable CPA (39% wt EG with 9% sorbitol). Adding a non-permeable CPA reduced toxicity and increased survival after cryopreservation. For the vitrification procedure,

first a low concentration of EG was introduced to the embryo, followed by EG at a high concentration with rapid cooling down to 4°C. A higher concentration was used to increase the protection of the embryo from intracellular ice formation during cooling or warming but was kept low enough to minimize toxicity at sub-zero temperatures. Due to osmolarity changes, the embryos initially release water and shrink when placed in CPA solutions before returning to their normal volume, when the CPA has entered. This dehydration time, which is visualized by shrinkage, is also a variable that affects the vitrification process. In this study, they used a 9 min dehydration time. In the next step, they placed 200-600 embryos on a custom-generated cryomesh (2cm x 2cm nylon mesh connected to a thin polystyrene holder), removed residual CPA, and then quickly placed the eggs in liquid nitrogen. The careful and complete removal of the CPA from the cryomesh resulted in a higher warming rate, which resulted in increased survival rates. To unload intraembryonic CPA, embryos were treated with 15% w/v sucrose before treatment with a cryobuffer (isotonic saline) used to prevent lethal osmotic shock. Leaving embryos to hatch on schneider medium resulted in an increased survival rate compared to other methods. This medium works as an aqueous environment for CPA unloading intra-embryonically while providing embryos with nutrients to continue their age progression. Their method resulted in a hatch rate of 52.9 ± 6.3 % and an adult rate of 31.8 ± 5.3 %, in contrast to 97% and 89%, respectively, for embryos that were not treated.

In mosquitoes, no successful cryopreservation protocol exists. However, components of the original *D. melanogaster* cryopreservation protocol (Steponkus et al., 1990) have been tested and optimized for use in the permeabilization of *Anopheles* embryos (Valencia et al., 1996). The authors were able to successfully permeabilize *Anopheles* embryos using 10% lab grade sodium hypochlorite, monitored air drying, and heptane exposure for 30 sec. (Valencia et al., 1996). Surficial embryonic water must be removed if heptane is to make close interaction with the vitelline membrane, due to its immiscibility to water (Valencia et al., 1996). In this study, (Valencia et al., 1996) the authors performed the drying step using a vacuum chamber that passed through the bottom of a polycarbonate membrane filter, while the embryos were laid on top. This drying step increased survival of the embryos. We took factors described above into consideration when designing our methods.

In this thesis, we designed the methods by first aiming to permeabilize the embryos, which we were able to do, although permeabilization resulted in complete lethality. We then worked to optimize each individual step based on the conditions that were used to permeabilize the Culex embryos.

3.3 Methods

A general method for the cryopreservation of *Culex* mosquitoes was designed based on previous insect cryopreservation and related protocols (Fig. 7). Mosquitoes must first undergo a force lay to provide 6-9 hr old embryos, ensuring that the SC has not formed which can prevent permeabilization.

Collected embryos were then dechorionated with sodium hypochlorite, dehydrated, permeabilized with an alkane, and then loaded with CPAs and vitrification solutions before being placed in liquid nitrogen for storage. Once embryos were removed from liquid nitrogen storage, they underwent a nutrient heat treatment and then be left to hatch and develop into adult mosquitoes.

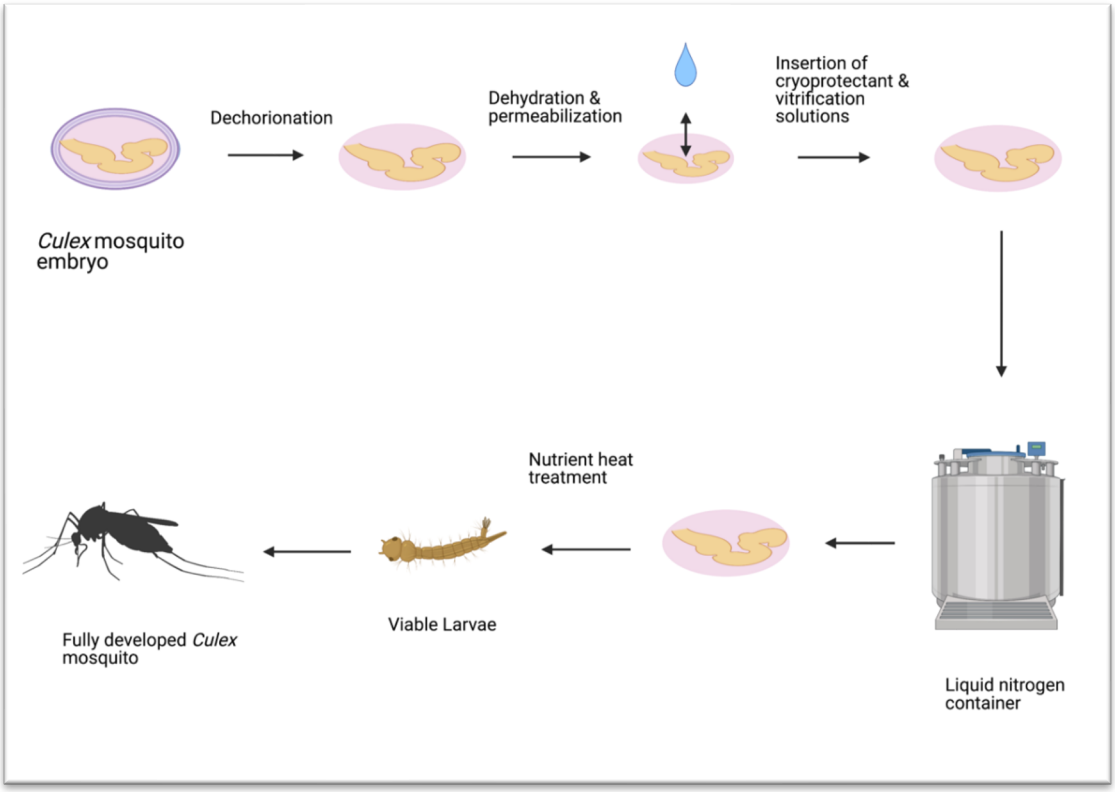


Figure 7. Schematic overview of a proposed cryopreservation protocol for Culex mosquito embryos.

6–9-hr. old *Culex* embryos will undergo the steps described in the schematic overview for cryopreservation.

Forced Egg Lay

A colony of blood fed *Cx. pipiens* females was anaesthetized in a freezer five to six days after a blood meal. Once anaesthetized, mosquitoes were moved to 8.5 cm diameter

Petri dishes and placed on a small piece of parafilm. This parafilm was big enough to place the mosquitoes while they recovered. Water was then added to fill half of the petri dish. The petri dish was then placed in the dark and left untouched for at least one hour. The embryos used ranged from 6-9 hrs old to ensure the SC is not present, which prevented permeabilization.

Dechorionation

Cell strainers (Biologix Research Company, 15-1040, 40 μm mesh) were used to transfer embryos between treatments. Egg rafts were carefully placed on each cell strainer using a paintbrush and were then handled together in each cell strainer. Embryos were placed in 10% lab grade sodium hypochlorite, which was determined to be safe for use in *An. gambiae* (Valencia et al., 1996). Various dechorionation soak times were tested. Embryos were then rinsed with 125 mL of DI water. The cell strainer containing egg rafts was then blotted onto a Kimwipe to remove excess water if they were to be treated with heptane next. If not, embryos were left to hatch in water and once hatched, fed with fish food (Product No. 77101, Tetramin© Tropical Flakes, VA).

Permeabilization

Next, embryos were air-dried until shrinkage was visualized and then treated with a lab-grade heptane soak. They were then air-dried for 10 sec. Various heptane soak times were tested. Embryos were then placed in water to assess hatching.

Controls

Two control groups were used for this experiment. In the first control group (no handling), embryos were left untouched in their egg container (plastic cup with water) after being counted under the microscope. In the other control group (with handling), embryos were also transferred onto cell strainers and treated in the same manner as the treated embryos but using DI water instead of reagents.

3.4.1 Dechorionation Results

A variety of dechorionation soak times were tested using 10% lab grade sodium hypochlorite. The impact of embryo handling on survival was also analyzed. We observed a strong association of survival/lethality and handling technique between experiments (Figures 8a, 8b, and 9). Specifically, handling caused significant lethality of embryos depending on the day of the experiment. In figure 8a, dechorionation soak times of 15-25 min were tested and compared to the control groups. In figure 8b, dechorionation soak times of 4-8 min were tested and compared to a control (with handling) group. In one experiment (Figure 8a) survival in the control (no handling) group ranged from 38. 7-45.3% and that range dropped to 6.5-22.5% in the control (with handling) group. However, in another experiment (Figure 8b), the control (with handling) group resulted in a much higher survival rate (57-58%) than observed previously (Figure 8a). No control without handling was included here, making a direct comparison difficult.

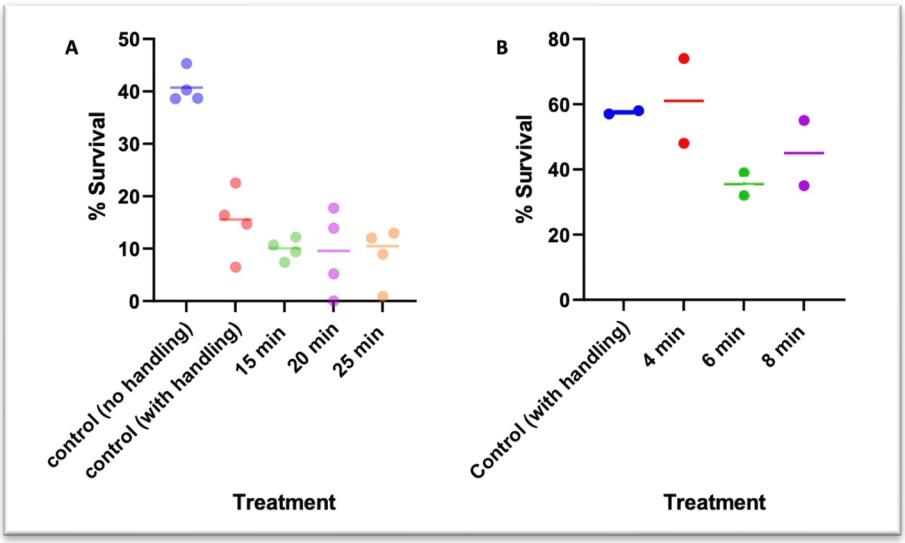


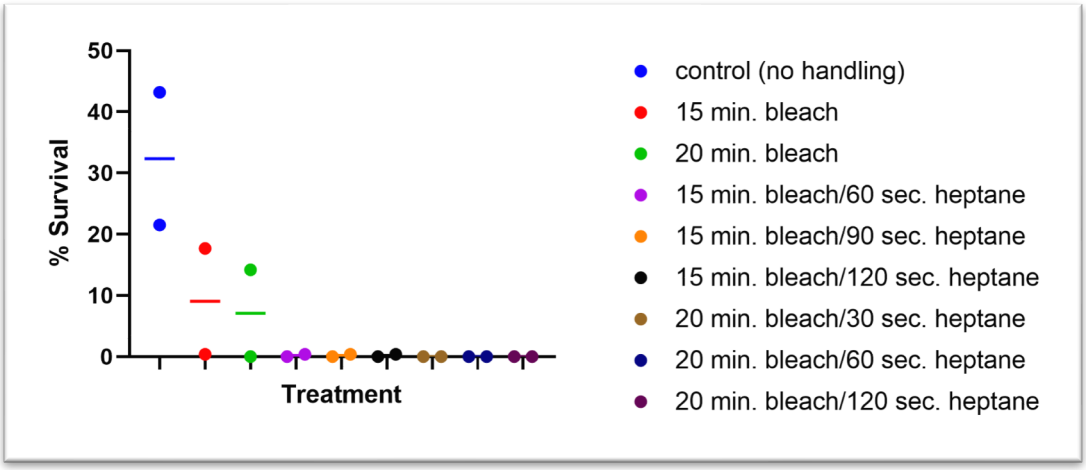
Figure 8. Dechorionation survival.
Dechorionation soak times with 10% lab grade sodium hypochlorite were tested for 15-25 min (A) and 4-8 min (B). Three egg rafts were used per treatment. Survival was calculated as the number of embryos that developed into larvae out of all treated eggs per replicate. The mean % survival is indicated in the figures (*n* = one egg raft (~250 eggs))

A higher dechorionation soak time, in addition to the handling of the embryos, caused further lethality as seen in the difference in survival rates of embryos that underwent longer versus shorter dechorionation soak times. No vast difference in survival percentage were observed among groups of embryos soaked for 15, 20, and 25 min with dechorionation agent (Figure 8a). The average survival of embryos treated with 15-, 20-, and 25-min dechorionation soak times were 9.9, 9.2, 8.6%, respectively. The survival of embryos that were treated with dechorionation agent in this experiment ranged from 0-17.8%.

Shorter dechorionation soak times of 4,6, and 8 min were used to treat embryos (Figure 8b). The average survival of embryos treated with 4,6-, and 8-min dechorionation soak times were 61, 35.5, and 45%, respectively. The survival percentages of embryos that were treated with dechorionation agent (4-8 min) ranged from 32-74%.

3.4.2 Dechorionation and Permeabilization Results

We tested heptane soak times for permeabilization of the embryos in addition to the dechorionation treatment (Figure 9). The control (no handling) groups ranged in survival from 21.5-43.2%. In this experiment, dechorionation soak times of 15 and 20 min were used to treat embryos. In two replicates of 15 and 20 min respectively, 0% survival was reported. The other two survival percentages were 17.7 for the 15 min dechorionation soak and 14.2 for the 20 min. dechorionation soak. These data along with the previous data discussed imply that dechorionation treatment and handling effects cause lethality. All heptane soak times (in addition to the dechorionation



treatment) resulted in 0% survival of the embryos.

Figure 9. Dechorionation & permeabilization survival.
A variety of dechorionation soak times (15-20 min) were tested using 10% lab grade sodium hypochlorite along with heptane soak times (90-120 sec.). Three egg rafts were used per treatment. The survival represents the percentage of embryos that developed into larvae out of the total number of eggs treated. The mean percent survival is indicated. (*n* = one egg raft (~250 eggs))

3.4.3 Permeabilization Results

Dehydration was visualized by the wrinkling of the embryos soon after placing them in 1 M EG (Fig. 10). Permeabilization was visualized by the swelling of the embryos, and reduction in wrinkling. No embryos survived this procedure.

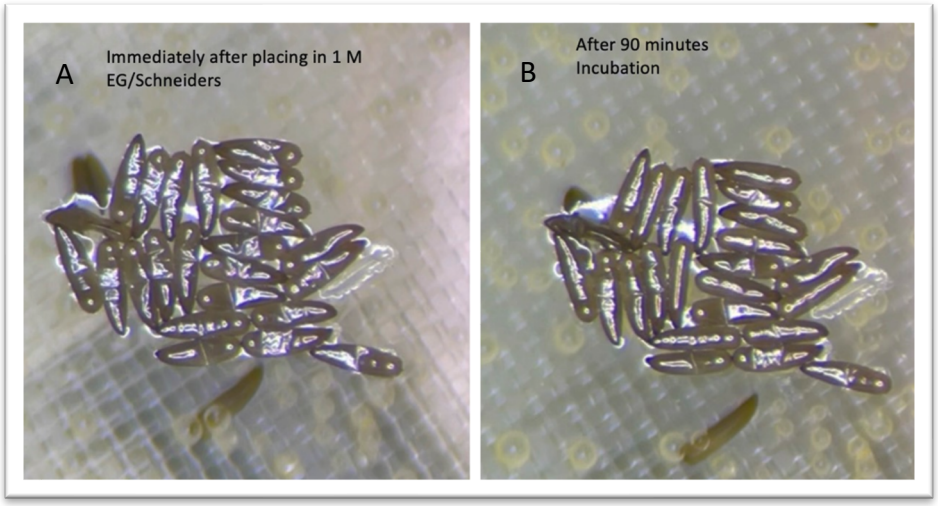


Figure 10. Permeabilization of Culex embryos.
Culex pipiens embryos were treated with 10% sodium hypochlorite for thirty min, air dried until shrinkage was viable, soaked in heptane for 120 sec, and transferred into 1M

EG in Schneider's culture media to show initial shrinkage/dehydration of eggs (A) and uptake of EG solution visualized as swelling after 90 min. (B).

3.5 Discussion

When comparing all experiments together, the control (no handling) groups ranged in survival from 21.5-45.3%. These data indicate that survival percentages in the control (no handling) groups are much lower than anticipated and vary with each batch of egg rafts. These egg rafts were not handled; therefore, some eggs might not be as biologically fit as other eggs, resulting in them not hatching and developing into larvae. Younger embryos might be more sensitive to the handling technique and therefore are more likely to perish after the procedures. The use of older embryos (~9 hrs) might have improved the survival percentages seen in figure 8B. Some changes to the egg laying procedure could be implemented to ensure embryos of only one specific age are used for the purpose of minimizing that variable in the experiment, which could be affecting their ability to sustain the procedures enough to survive. Approximately 60 mosquitoes were forced to lay eggs for every experiment, allowing for the collection of 6–9-hr old embryos. The collection of embryos of 6-9 hrs old allowed for the gathering of embryos without the SC. If more mosquitoes were force laid, it would be possible to ensure that only nine-hour old embryos are used, by collecting all egg rafts after one hour of forced lay, instead of three. At nine hours old, it is possible they are most likely less sensitive than six-hour old embryos, and still have not developed the SC. At least 120 mosquitoes should be used when force laying *Culex* egg rafts. Force laying ~60 mosquitoes did not

allow for the collection of strictly nine-hour old embryos, because it took at least three hours for mosquitoes to produce enough rafts for experiments.

The control (with handling) groups ranged in survival from 6.5-58%. The handling techniques must be altered for a higher survival rate of the embryos. The current technique that involves the use of a squirt bottle to rinse off the sodium hypochlorite may be inflicting too much pressure onto the fragile, freshly laid embryos. We also noted high experimental variability, possibly due to the exact batch of egg rafts or due to the difference in the person performing the handling of the experiment, since some experiments were performed by an undergraduate researcher. In the dechorionation step, instead of using a squirt bottle to rinse off the sodium hypochlorite, a water soak could be implemented instead. One way to perform this is to transfer post-dechorionated embryos in cell strainers to fresh cups of DI water, multiple times, after a 30 sec. soak. This would dilute the sodium hypochlorite sequentially until it is completely rinsed off. This new method was implemented in the procedure towards the end of the study to assess if embryo damage was occurring due to the water pressure from the squirt bottle. These studied did indicate some increased survival (not shown here). In summary, the handling effects usually cause significant lethality, but can be dependent on the embryo fitness, embryo age, handling technique, and the person performing the handling.

While 10% sodium hypochlorite reduced survival of *Culex* embryos compared to a water control, this reagent was deemed safe for *Anopheles* mosquitoes (Valencia et al., 1996) and a much harsher chemical was used in the successful *D. melanogaster*

cryopreservation procedure (50% household Clorox) (Zhan et al., 2021). While sodium hypochlorite was the most obvious chemical to test for the development of a dechorionation protocol based on previous literature, other chemicals may need to be tested for *Culex* mosquito embryos to improve embryo survival.

Heptane in addition to the dechorionation treatment was completely toxic to *Cx. pipiens* embryos in our study. In the *Anopheles* embryos, permeabilization to water and EG has been previously assessed in hyperosmotic sucrose and EG solutions in D-20 (Valencia et al., 1996). Using these additional reagents in contrast to solely using 1M EG in Schneider's medium may provide higher survival for *Culex* embryos undergoing permeabilization.

3.6 Conclusion

Visualization of the permeabilization procedure (Fig. 9) showed that a 10% sodium hypochlorite dechorionation soak time of 30 min and 120 sec heptane treatment makes embryos permeable to 1 M EG in Schneider's medium. In this study, we attempted to optimize this procedure to make it so embryos could survive permeabilization. No embryos survived permeabilization, which indicated that more optimization is needed to achieve this such as using a water rinse instead of a squirt bottle to rinse off the dechorionation agent, force egg laying of 150 or more mosquitoes to ensure embryos are within one hour in age, and test other permeabilization reagents.

4. Thesis Conclusion

Adult *Cx. quinquefasciatus* mosquitoes and pupae were treated with dsRNA via intrathoracic injection and a soak, respectively, to knockdown the potential antiviral

protein, PIWI2. The results showed that neither dsRNA delivery technique resulted in a substantial knockdown of *PIWI2*. The dsRNA employed in our investigations might not have been effective, according to an analysis of dsRNA knockdown effectiveness in *Cx. quinquefasciatus* Hsu cells. Further *in-vivo* testing using a different dsRNA generation process and other volumes and concentrations of dsRNA would be the next step in continuing this research.

In-vivo experiments require continuous and labor-intensive care of *Culex* eggs. To address this issue, we attempted to develop a cryopreservation technique for *Culex* embryos to cut down on labor expenditures and to safely store mosquito lines without risk of cross-contamination or colony loss. Survival of embryos was examined after dechorionation, permeabilization, and handling procedures. Results indicated that lab-grade sodium hypochlorite and heptane could be used to permeabilize *Culex* embryos to the CPA, EG, but the currently employed procedure was deadly. More research is required to determine the optimal reagents, times, and handling techniques for the permeabilization and cryopreservation of *Culex* embryos.

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