The effects of topically-applied double-stranded RNA pesticides on *Myzus persicae*

By

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Abstract

The green peach aphid (*Myzus persicae*) is a major agricultural pest, causing billions in crop losses and contributing to global food insecurity. Increasing incidences of insecticide resistance and off-target effects highlight the need for alternative control methods. RNA interference (RNAi), a gene-silencing mechanism triggered by exogenous double-stranded RNA (dsRNA), has emerged as a promising approach to species-specific pest control.

Topically applied dsRNA offers a non-GMO alternative to traditional pesticides, selectively inducing mortality in pests by targeting essential genes. One such gene, *Chitin Synthase* (*CHS*), is crucial for the insect exoskeleton, tracheae, and gut. However, dsRNA penetration through the aphid's hydrophobic exoskeleton remains a challenge, necessitating delivery enhancements like surfactants.

This study tested two dsRNA structures—long linear dsRNA (210 bp) and short paperclip RNA (pcRNA, 25 bp)—targeting CHS, with and without the surfactant Silwet. Third-instar aphids were topically treated with a dsRNA droplet, and their survival and fecundity were monitored over eight days. Several bioassay optimizations were implemented, including stretching diet packets, treating third instars instead of adults, filter-sterilizing diets, and chilling aphids before treatment.

Bioassays demonstrated that dsRNA + Silwet significantly increased aphid mortality, whereas dsRNA alone and all pcRNA treatments had no significant effects. No significant fecundity reduction or CHS gene knockdown was observed. While only a single gene target was assessed, findings suggest RNAi-based pesticides hold potential for

aphid control, and Silwet improves dsRNA efficacy. Further research on optimizing dsRNA formulations could enhance their application as next-generation pesticides.

Acknowledgements

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Finally, my deepest gratitude goes to God Almighty. “You will succeed in whatever you choose to do, and light will shine on the road ahead of you.” (Job 22:28). These words became my strength and lit the fire in me to take every obstacle as a challenge.

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Introduction

Aphids are major agricultural pests that cause significant yield losses of crop plants each year through direct feeding and transmission of plant viruses (Smith and Chuang 2014; Yu et al. 2014). The green peach aphid, *Myzus persicae*, is considered one of the most destructive species, feeding on over 40 plant families, and causing billions of dollars yearly in crop losses (Ali 2022; Ali et al. 2023). Chemical pesticides are routinely used to control aphids and the diseases they spread, but their extensive use has resulted in increasing incidences of insecticide resistance, negative effects on non-target organisms and environmental pollution (Yu et al. 2014; Sparks and Nauen 2015). Together, these issues illustrate a need for new, less environmentally damaging and more pest-specific alternatives to control pest infestations, particularly aphid infestations. One technology that offers the promise of a reduced-risk approach to insect pest control is RNA interference (RNAi). By targeting genes essential for pest insect's growth, development, or reproduction, RNAi could be used selectively to kill pest insects without adversely affecting non-target species (Whyard et al. 2009).

RNAi is a sequence-specific method of suppressing a targeted gene's expression, and because each species is defined by the uniqueness of its genes' sequences, RNAi can potentially be designed in a species-specific manner. RNAi is initiated by the delivery of double-stranded RNA (dsRNA), which can enter eukaryotic cells by dsRNA-specific membrane channels or by endocytosis, to trigger the RNAi pathway (Wytinck et al. 2020). Once inside the cell, the RNase III endonuclease Dicer-2 (Dcr-2), cleaves the dsRNA into short interfering RNAs (siRNAs) which are typically 19-21 nucleotides long (Okamura et al. 2004). The RNA-induced silencing complex (RISC), then unwinds the siRNA, and one

strand (passenger strand), is eliminated (Okamura et al. 2004). Using the retained guide strand, the activated RISC complex scans cellular mRNAs, and an Argonaut protein (Ago2) within RISC cleaves transcripts with complementarity to the siRNA, thus silencing gene expression (Okamura et al. 2004).

Application of RNAi in agriculture, more specifically in pest or pathogen control, can be achieved in different ways. HIGS (Host-induced Gene Silencing) generally refers to transgenic plants that express RNAi transgenes, thereby delivering dsRNA to those pests that consume the plant tissues (Mao and Zeng 2014; Sun et al. 2019). HIGS involves the ingestion of the host-produced dsRNAs by the insect, which is the most common method of dsRNA delivery, largely due to the waterproof exoskeleton of insects (Zheng et al. 2019), which would be impervious to a water-soluble molecule like RNA. However, there have been a few rare examples of where SIGS can be used to deliver the dsRNA by direct topical application. SIGS (Spray-Induced Gene Silencing, also known as topically applied dsRNA) is an alternative dsRNA delivery method that does not rely on the production of genetically modified plants, which are not accepted in many countries (Sikora and Rzymski 2021). Foliar sprays containing the dsRNAs can be applied to crop leaves, which if then consumed by herbivorous insects, can induce RNAi and kill the insect (Andrade and Hunter 2017). The first SIGS-based insecticide, Calantha, has recently been registered for use in the USA to control potato beetles, and many more research groups are actively developing more SIGS-based control products (Pallis et al. 2023). Another application of SIGS is the direct application of the dsRNA to the body of the insects. However, due to the hydrophobic insect exoskeleton of most insects, for some soft-bodied insects, using a formulation of amphiphilic surfactants can enhance dsRNA

uptake, and indeed, this was demonstrated in soybean aphids (Zheng et al. 2019). Various RNA-binding polymers and microcarriers have been observed to improve the delivery of dsRNA to insects, including polycations, polysaccharides (e.g. chitosan), and liposomes(Zhang et al. 2010; Zheng et al. 2019). For topical dsRNAs to be effective insecticides, further research will be required to identify which polymers/microcarriers are the most effective in different target species to assist dsRNA penetration into the insects' bodies.

Although feeding the dsRNA is the most frequently used method to induce RNAi in insects, it still faces some challenges. Once the insect has consumed the dsRNA, the dsRNA must avoid the degradation by nucleases from the insect's salivary glands, midgut, and hemolymph. This has been observed in several insects including the pea aphids (*Acyrthosiphon pisum)* where the dsRNases in their saliva contribute to the lack of silencing response (Cappelle et al. 2016). Additionally, resistance to ingested dsRNA due to reduced uptake of long dsRNA by the gut cells has also been observed in insects (Khajuria et al. 2018). After only 11 generations of increasing dosage selection in the western corn rootworm (*Diabrotica virgifera virgifera*), impaired luminal uptake was observed, rendering them resistant to any dsRNA (Khajuria et al. 2018). Although nanoparticles or liposomes might be used to overcome this form of resistance, these methods can be expensive and microcarriers are not yet appropriate for large-scale applications of dsRNA pesticides (Das et al. 2015; Tayler et al. 2019).

To reduce the susceptibility of the dsRNA to gut nucleases and to overcome the potential resistance to it, our research group developed paperclip RNAs (pcRNAs), which are shorter (~23 bp) in length compared to long dsRNAs (~200 bp) and have both ends of the dsRNA molecule folded over, like a paperclip, to reduce exonuclease degradation

(Abbasi et al. 2020). These pcRNAs were observed to remain intact at least twice as long as conventional linear dsRNAs when exposed to gut juices of several different insects (mosquitoes, flea beetles, tephritid flies; Abbasi et al. 2020 ; Whyard unpublished data). Aside from being more resistant to gut nucleases than conventional dsRNAs, these pcRNAs also exhibited a different mode of entry into insect cells. Most insects examined to date appear to use clathrin-mediated endocytosis to facilitate linear dsRNA uptake (Cappelle et al. 2016). When clathrin was inhibited using either chemical inhibitors or by RNAi-mediated knockdown, pcRNAs could still readily enter mosquito, moth, and Sclerotinia fungus cells (Abbasi et al. 2020; Whyard unpublished data), indicating that the pcRNAs can enter a variety of cells through a clathrin-independent process.

Chitin is the primary polysaccharide of all insects' exoskeleton, including the lining of the trachea, which are the air ducts that enable gas exchange to the interior of the insects (Bansal et al. 2012; Muthukrishnan et al. 2020). Previous studies have explored the RNAi-mediated silencing of the *chitin synthase (CHS)* gene in *Leptinotarsa decemlineata*, resulting in lowered chitin contents in whole body and integument samples, and thinned tracheal taenidia (Shi et al. 2016). Additionally, silencing effects included failure in pupal-adult moulting, significantly reduced foliage consumption, reduced chitin content in the midgut and retarded larval growth (Shi et al. 2016). Moreover, *CHS* was found to be expressed in major tissues such as the gut, fat body and integuments (highest expression levels) in the adult *Apis glycines*, whereas the developing embryos have a 7-fold higher *CHS* expression levels reflecting the embryonic moults in hemimetabolous insects (Bansal et al. 2012). In the presence of a *CHS* inhibitor, *A. glycine* nymphs displayed higher mortality (Bansal et al. 2012). Similarly, RNAi-mediated silencing of

CHS in *A. pisum* resulted in high mortality, reduced moulting, a prolonged period of nymph development and a high deformity rate in nymphs (Ye et al. 2019). Therefore, *CHS* serves as a potential target for RNAi-based aphid pest control. Thus, for my project, I would like to evaluate the effects of topically applied dsRNA on *M. persicae* using two different structures of dsRNA – long linear dsRNA and short paperclip RNA (pcRNA), targeting the *CHS* gene.

While these findings indicate various techniques that can optimize the effects of topically delivered dsRNA in aphids, their effects on *M.persicae*'s survival and fecundity need to be explored. Additionally, to realize the full potential of dsRNA pesticides, the best combination of treatments needs to be explored to create the most effective pesticide. Therefore, my objective is to evaluate and compare the insecticidal activity of long dsRNA and pcRNA targeting the *M. persicae CHS* gene through a topical treatment. It is hypothesized that different structures of both long dsRNA and pcRNA will reduce the survivorship and fecundity in *M. persicae* exposed to the aphid-specific dsRNA to varying degrees but will be unaffected when exposed to non-aphid-specific dsRNA (targeting the *Escherichia coli*'s β-glucuronidase; GUS). Moreover, to facilitate the uptake of the topical dsRNA solution by the hydrophobic body of the aphid, previous efforts in the lab have determined that a surfactant (Silwet) helped the dsRNA spread over the insect. Silwet is also widely used in several pesticide formulations in crops to facilitate uniform distribution onto the sprayed plants by lowering the water surface tension between the pesticide, the droplet and the plant (Chen et al. 2022b; Huynh et al. 2022). I will, therefore, test if formulations with and without the surfactant can induce RNAi-mediated knockdown of the target genes and assess whether the dsRNAs have any insecticidal

activity. Silwet is used at very low concentrations so as not to harm beneficial insects, and in this study, I will endeavour to use a concentration that will not kill the aphids, which was determined to be non-lethal in *M. persicae* by a previous student in our lab (Whyard, unpublished data). It is hypothesized that the surfactant will be more effective than dsRNA alone in knocking down the target genes and inducing higher mortalities and lower fecundities of the insects.

Methods

Maintenance of Myzus persicae

A colony of *M. persicae* aphids was maintained in a cage inside an incubator, maintained at a temperature of 22^{o} C with a 12:12 light: dark cycle. The aphids lived and fed on *Brassica napus* (Canola) plants. Plants undergoing chlorosis or that had wilted were replaced with fresh ones, usually before flowering. A heavily aphid-infested leaf was placed onto the new plant to transfer a subset of the aphid population. Humidity levels were maintained between 80% and 95% by placing a water-filled container in the incubator and was tracked using the Govee Home humidity tracking device.

Synthesis of dsRNA

DNA templates for long dsRNA (209 bp for *GUS* and 300 bp for *CHS*) and short pcRNA (23 bp for *GUS* and 25 bp for *CHS*) targeting the *GUS* gene and the *CHS* gene in *M. persicae* were obtained from Integrated DNA technologies (Tables 1 & 2). To anneal the pcRNA templates, two ultramer DNA oligonucleotides were heated to 95^{O} C for 2 minutes, followed by incrementally decreasing the temperature to 22^{O} C (Figure 2). All DNA templates were flanked by T7 promotor sequences, which enabled *in-vitro*

transcription using the T7 RNA polymerase in the MEGAscript RNAi kit (Invitrogen). The resulting sense and antisense strands were incrementally cooled from 75^{O}C to 22^{O}C to anneal the segments into the desired double-stranded structures (Figure 1). Long dsRNA was purified by centrifugation with spin columns from the MEGAscript RNAi kit (Invitrogen), while ethanol precipitation was used to purify pcRNA. Following purification, both constructs' concentrations in solution were measured using a BioTek Take3 spectrophotometer (Table 3). The quality of structures was determined by resolving a small aliquot on an ethidium bromide-stained 1.5% agarose gel and checking for the presence of a single distinct band.

Table 1. Sequences of *M. persicae* long dsRNA target and *E.coli GUS* negative control

Gene	**Target**
CHS	GTGATGAAAACGATGATTGGAATCAAGTGAATCGATTCGTAAA GCTTTTAGTAAGTACAATCGACGAAGCAGCAACTCACGTG CACGAAACGAATATACGCATCAAACCACCGACTAAATATCCT TCTCCGTACGGTGGCCGTTTAGTGTGGACGTTACCTGGAAA AACTAAGCTCACTGTACACATCAAAGACAAATCCAAAATC CGACACAGAAAACGATGGAGTCAGGTCATGTACATGTACT ACTTACTTGGTCACCGATTAATGGAACTACCGATTTCCGTT GAGCGGAAAGAAG
GUS	GCTGAAGAGATGCTCGACTGGGCAGATGAACATGGCATCGT GGTGATTGATGAAACTGCTGCTGTCGGCTTTAACCTCTCTTT AGGCATTGGTTTCGAAGCGGGCAACAAGCCGAAAGAACTG TACAGCGAAGAGGCAGTCAACGGGGAAACTCAGCAAGCGC ACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAAAA CCACCC

Table 2. Sequences of *M. persicae* siRNAs and pcRNAs for *CHS* and *GUS* negative control

Gene	siRNA structure	Sequence
CHS		
	siRNA	GACGUUACCUGGAAAAACUAAGCUC
	pcRNA top strand	ACCTCATAATACGACTCACTATAGAGCTTAGTT TTTCCAGGTAACGTCAAAAAAAAAGACGTTA CCTGGAAAAACTAAGCTCACCATGAAAAAAA AACATG
	pcRNA bottom strand	CATGTTTTTTTTTCATGGTGAGCTTAGTTTTTCC AGGTAACGTCTTTTTTTTTGACGTTACCTGGAA AAACTAAGCTCTATAGTGAGTCGTATTATGAGGT
	Resulting pcRNA	GAGCTTAGTTTTTCCAGGTAACGTCAAAAAAAA GACGTTACCTGGAAAAACTAAGCTCAACCATGA AAAAAAAACATG
GUS		
	siRNA	GATGCTCGACTGGGCAGATGAAC
	pcRNA top strand	ACCTCATAATACGACTCACTATAGATGCTCGACT GGGCAGATGAACAAAAAAAAAGTTCATCTGCCC AGTCGAGCATCTCCATGAAAAAAAAACATG
	pcRNA bottom strand	CATGTTTTTTTTTCATGGAGATGCTCGACTGGGC AGATGAACTTTTTTTTTGTTCATCTGCCCAG TCGAGCATCTATAGTGAGTCGTATTATGAGGT
	Resulting pcRNA	GTTCATCTGCCCAGTCGAGCATCTCAAAAAAAA AGATGCTCGACTGGGCAGATGAACATCATGAAA AAAAAACATG

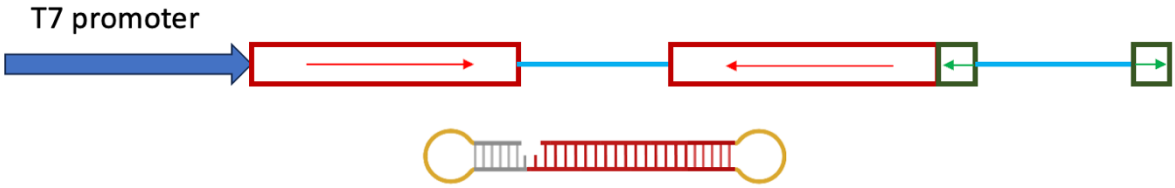

Figure 1. Graphic representation of the formation of pcRNA from T7-annealed double-stranded structure. The top image represents the T7-annealed double-stranded structure, and the bottom image represents the pcRNA. The T7-annealed double-stranded structure consists of the T7 promotor as the blue arrow and two red boxes with arrows running in opposite directions, representing the sense and antisense siRNA sequences. The blue lines represent the two loops, and the green boxes represent the sense and antisense oligonucleotide strands that will be adjacent to the 2 nucleotide-break.

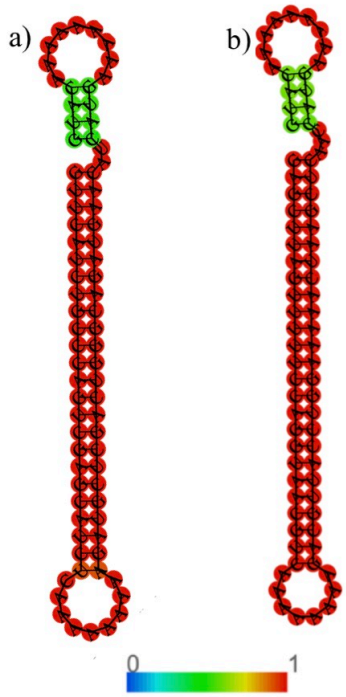

Figure 2. Predicted minimum free energy secondary structures of pcRNA. a) *GUS*, b) *CHS* pcRNA structures were predicted with the RNAfold server. The probability of the predicted structure is colour-coded, with the highest likelihood of structure denoted in red. The green portions of the molecules indicate that the short (4 bp) region had a lower likelihood of confirmed structure relative to the rest of the molecule (Gruber et al. 2008).

Table 3. Concentrations of *GUS* and *CHS* dsRNA

dsRNA structure	**Concentration (ng/ µL)**
GUS long dsRNA	1180
GUS pcRNA	1327.3
CHS long dsRNA	940
CHS pcRNA	1608.53

Topical treatment bioassays

An artificial diet for the aphids was prepared using the guidelines described in van Emden & Wilde, 2020[24]. Parafilm was used to make feeding packets containing 150 µL of the artificial diet. The diet packets were secured at the bottom of 1-ounce plastic cups by stretching the outer corners of the parafilm. Aphids were transferred from an infested canola plant to the feeding cups. Five aphids were carefully transferred into each cup using a fine-tipped paintbrush, making sure their proboscis was intact. Eight treatment sets were tested: no treatment, Silwet-only treatment, long dsRNA *CHS*, pcRNA *CHS*, long dsRNA *GUS* + Silwet negative control, pcRNA *GUS* negative control, long dsRNA *CHS* + Silwet and pcRNA + Silwet with five replicates each. The topical solutions were prepared at a concentration of 200 ng/µL, and Silwet solutions contained 0.005% Silwet, which was a non-lethal dosage determined by the previous efforts in the lab (Whyard unpublished data). Each aphid received a 0.5 µL droplet of its respective treatment solution, ensuring 100 ng of the respective dsRNA, applied to its dorsal surface. The droplets were allowed to dry off by leaving the lid open for 5-7 minutes before closing the lid and putting the cups back in the box. The cups will be monitored daily to look for nymphs and to track mortality for eight days post-treatment. Dead aphids, exoskeletons and nymphs were removed daily with fine-hair paintbrushes sprayed with ethanol. To measure gene knockdown levels caused by RNAi in aphids, the assay was set up similarly, but only three replicates were made due to time constraints, and the samples were collected on day three post-treatment and were directly transferred to lysis buffer and stored in -80^{O} C until RNA extraction. For both bioassays, the aphids were maintained at a temperature range between 20-22^{O} C and at a humidity range between 80-

95%, which were monitored using the Govee Home humidity tracking device. They were exposed to a 12:12 light: dark cycle using a timed lamp while humidity levels were maintained by placing a wet paper towel under the cups.

Optimizing the topical bioassays

Although the bioassay was curated to be ideal for the experiment, there were several challenges faced, such as (i) poor survival of the aphids once transferred into the cups, (ii) fungal growth in the feeding packets as early as three days after they were prepared, (iii) aphids' constant movement during the topical treatments and. (iv) no initial mortality/ gene knockdown observed. There were optimizations made to the protocol to improve the longevity of the bioassay. (i) To address the poor survival of the aphids post-transfer onto the feeding packets, the parafilm was stretched until its breaking point, and while placing it in the cup, it was secured by stretching it onto the base of the cup, ensuring that it was taut as this will help replicate the cell turgidity of the plant cells. This was done while ensuring that the aphids had their proboscis intact. (ii) Fungal growth was delayed by filter sterilizing the aphid diet. For this, a 10 mL sterile plastic syringe from Fisher Scientific, along with a Nalgene sterile syringe filter (25 mm diameter) with SFCA membrane (0.2 μm) from Thermo Scientific, was used according to the manufacturer's instructions. (iii) To reduce the constant movement of aphids due to the application of the topical solution, the cups were left at -20^{O} C for 15 minutes, which seemed to slow the aphids down and even made them stationary during the treatments. (iv) The first bioassay did not show any significant mortality in any of the treatment groups. To ensure sufficient uptake of the dsRNA solutions and higher susceptibility, nymphs were transferred into each cup and were allowed to grow into their third instar for 2-3 days, after which they

were exposed to the topical treatments. This led to significant mortalities observed in response to specific treatments.

Assessing gene-knockdown levels

All five aphids in each cup were pooled together for RNA extractions. The aphids were homogenized in lysis buffer (1 mL lysis buffer supplemented with 10 µL β-mercaptoethanol) in 1.5 mL Safe-Lock microcentrifuge tube (Eppendorf), and RNA was extracted using the RNeasy kit (Qiagen) following the manufacturer's protocol, followed by a DNase 1 (Thermo) treatment to remove all genomic DNA. Initially, all RNA samples were treated with 1 µL of DNase 1 and incubated for 45 minutes, but when subjected to an end-point PCR and resolved on the electrophoretic gel with ethidium bromide, there seemed to be genomic DNA remaining in the sample (Figure 3). To ensure the complete removal of genomic DNA from the samples, the addition and incubation with 1 µL of DNase 1 was repeated, which seemed to remove genomic DNA from the samples (Figure 4). Fresh RNA samples were treated with DNase twice (Figure 5) for the samples that still showed genomic DNA contamination, marked with an asterisk in Figure 4. cDNA was synthesized using a qScript cDNA Synthesis Kit (Quanta). After cDNA synthesis, quantitative reverse transcriptase PCR (qRT-PCR) was performed on a BioRad iQ5 Real-Time PCR Detection System using a master mix containing SsoFast™ master mix (BioRad), nuclease-free water, and primers designed to anneal to the region outside of the dsRNA target region for *Actin* (reference gene) and *CHS* (table 4). Conditions for cycling and curve analysis were completed as per the manufacturer's instructions.

Table 4. qRT-PCR primers for *Actin* and *CHS*

Gene	**Primer set**	**Sequence**	**Amplicon size (bp)**
Actin	Forward primer	TTGTTGACGCTCGATGGTGA	104
	Reverse primer	ATTCTACCACAAGCGGCTCC	
CHS	Forward primer	GGTGTCTCCCACACAGTACC	142
	Reverse primer	CAGCGGTGGTGGTGAAGCTG	

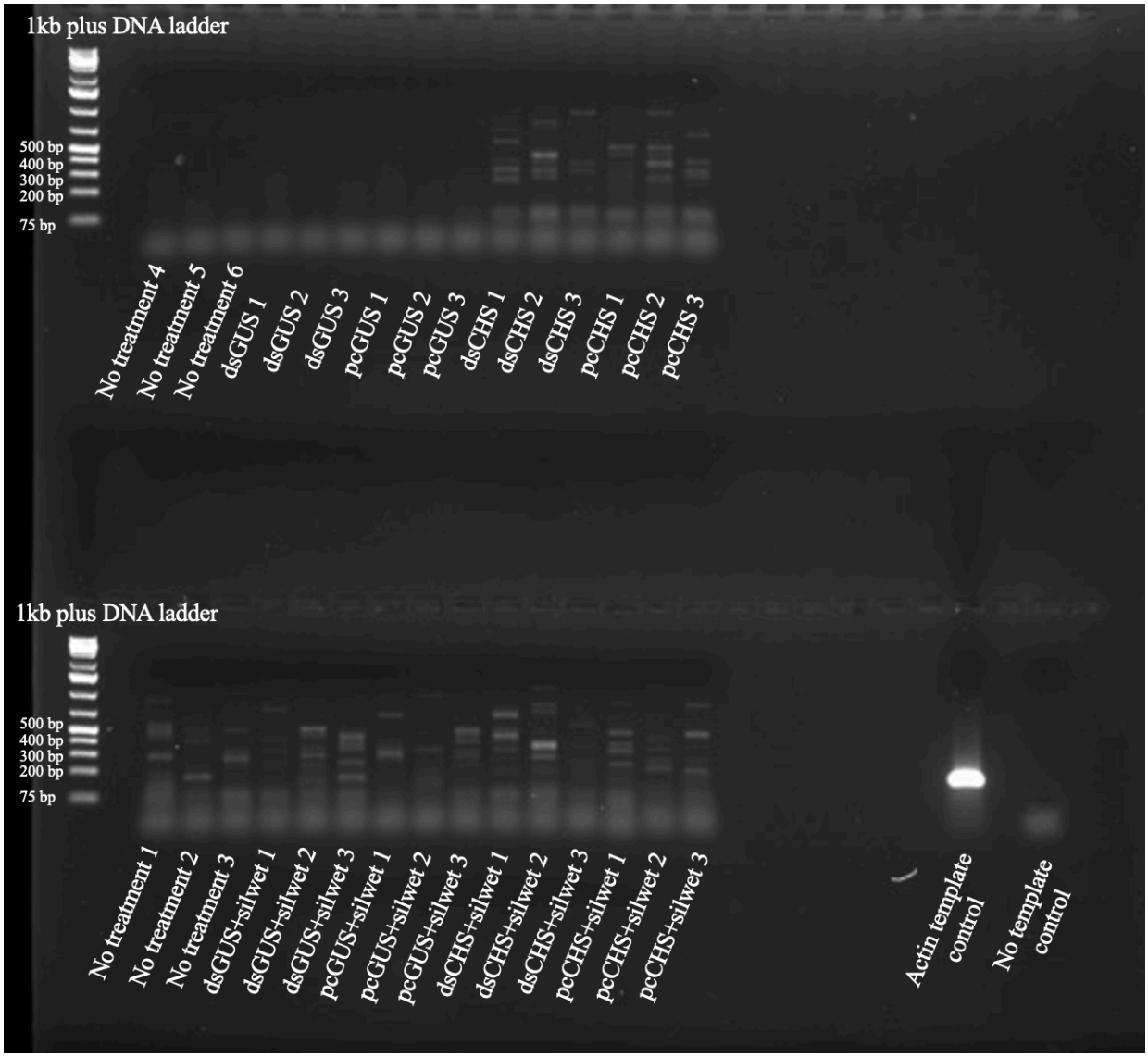

Figure 3. Resolution of end-point PCR products of the DNase-treated RNA samples with *M. persicae Actin* primers (amplicon size = 104 bp) on an agarose gel. 1.5% ethidium bromide-stained agarose gel was submerged in 1x TRIS Borate EDTA (TBE) buffer and resolved at 130V for 30 minutes. 1 Kb plus DNA ladder was loaded in the first lane in both rows. The last two rows are the *Actin* template control and the no-template control. Ideally, none of the wells should have any bands, but the presence of bands denotes genomic DNA contamination.

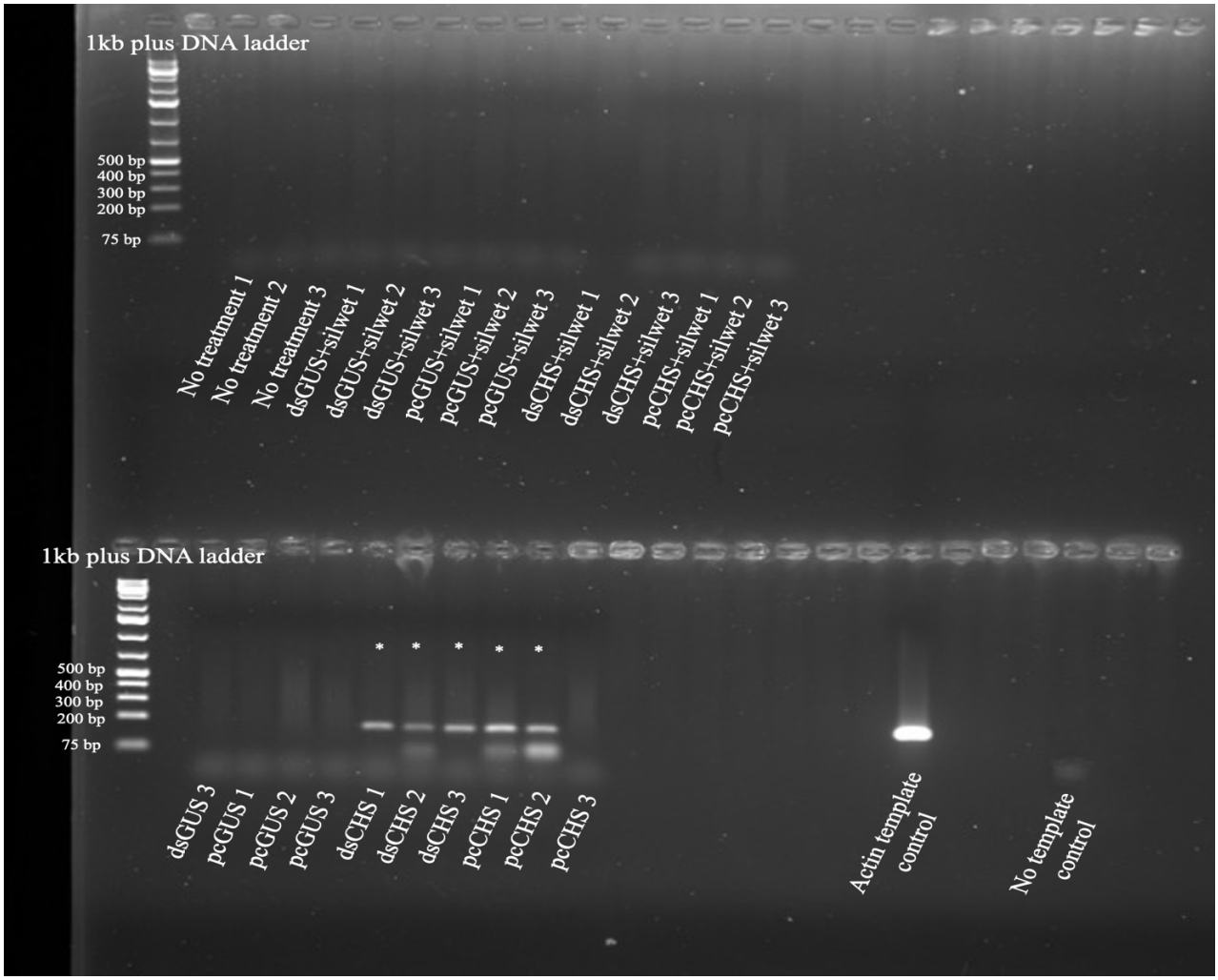

Figure 4. Resolution of end-point PCR products of the DNase-treated RNA samples, subject to a second DNase treatment, with *M. persicae Actin* primers (amplicon size = 104 bp) on an agarose gel. 1.5% ethidium bromide-stained agarose gel was submerged in 1x TRIS Borate EDTA (TBE) buffer and resolved at 130V for 30 minutes. 1 Kb plus DNA ladder was loaded in the first lane in both rows. The last two rows are the *Actin* template control and the no-template control. Ideally, none of the wells should have any bands, but the presence of bands denotes genomic DNA contamination. The wells with asterisks '*' denote samples with genomic DNA contamination.

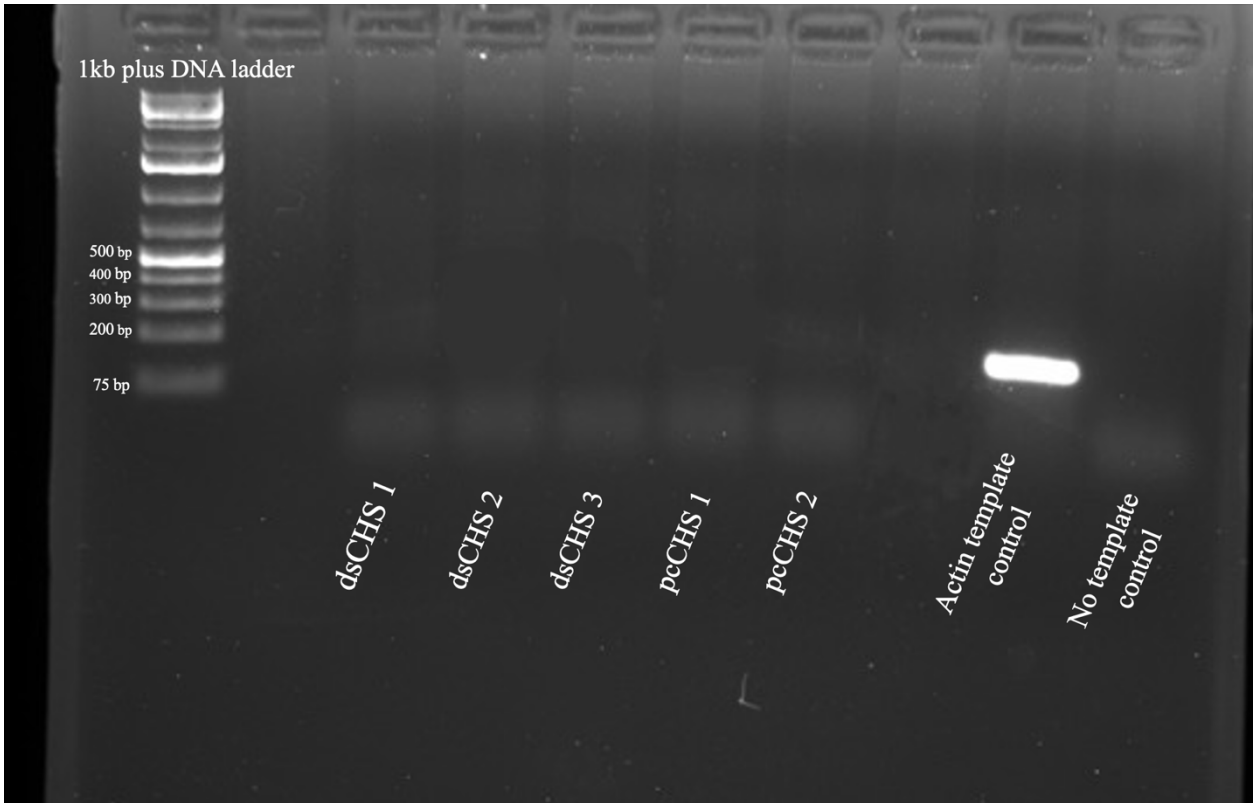

Figure 5. Resolution of end-point PCR products of the newly double DNase-treated RNA samples due to the presence of genomic DNA in the samples, when treated with a double DNase treatment, with the rest of the samples with *M. persicae Actin* primers (amplicon size = 104 bp) on an agarose gel. 1.5% ethidium bromide-stained agarose gel was submerged in 1x TRIS Borate EDTA (TBE) buffer and resolved at 130V for 30 minutes. 1 Kb plus DNA ladder was loaded in the first lane in both rows. The last two rows are the *Actin* template control and the no-template control. Ideally, none of the wells should have any bands, but the presence of bands denotes genomic DNA contamination.

Statistical Analysis

The mortality data were represented as a Kaplan-Meier survival graph with all five replicates combined (n=25). The Log-rank Mantel-Cox analysis was used to assess whether there were significant differences between groups, as the data represents time-to-event data. The reduced mobility of aphids, the effect of treating third instars over adults and the fecundity data were expressed as means ± standard errors for the number of stationary aphids per total number of aphids present in the sample, the number of surviving aphids on day eight post-treatment and the number of nymphs produced per living adult per day respectively, keeping the replicates separate (n=6,3 & 5 respectively). The one-way ANOVA test followed by t-test: Two-Sample Assuming Equal Variances post hoc tests with Bonferroni correction were used to detect significant differences in the reduced mobility of aphids between the groups while the two-way ANOVA tests followed by Tukey's post hoc tests were used to detect significant differences in the mortalities caused when third instars were treated vs adults. The one-way ANOVAs (3 or more comparisons) followed by Tukey's post hoc test were used to detect significant differences in the daily fecundity between groups as well as the total fecundity between groups, as normality was fulfilled. Statistical analyses were conducted using GraphPad Prism version 9 (GraphPad Software, CA, USA). p-values ≤ 0.05 were considered significant.

Results

Improved feeding packets and filter-sterilized aphid diet

To ensure the effectiveness of the bioassays and minimize the impacts of confounding variables (like a spoiled aphid diet containing fungi, often observed by day 3, and the aphids' inability to feed properly from the packet), specific methods needed to be optimized. The feeding packets were prepared by stretching the parafilm as much as possible and securing the diet packet to the bottom of the cup, ensuring it was tight. Additionally, the aphid diet was filter sterilized using a 10 mL sterile plastic syringe from Fisher Scientific, along with a Nalgene sterile syringe filter (25 mm diameter) with an SFCA membrane (0.2 μm) from Thermo Scientific. Stretching the diet packets tightly prevented aphids from getting stuck underneath enabled immediate feeding through the membrane, and reduced aphid mortality on the first day post-transfer (pre-treatment) from 34% to 0%. Meanwhile, filter-sterilizing the aphid diet prevented fungal growth for the entire 9 days of the bioassay.

Immobilising the aphids to facilitate the topical treatments

To effectively deliver the topical treatment to the aphids, it was important to control their mobility, as their constant movement made it difficult to apply the droplet to the back of the aphid. When each sample (a cup containing five aphids) was placed at -20^{o}C for 15 minutes, their mobility was significantly reduced (Figure 6) during the treatment, compared to aphids treated at room temperature ($p < 0.001$, using a t-test with a post hoc Bonferroni correction). The number of stationary aphids increased dramatically, from 6.67% to 70% of the aphids remaining stationary during the topical treatments.

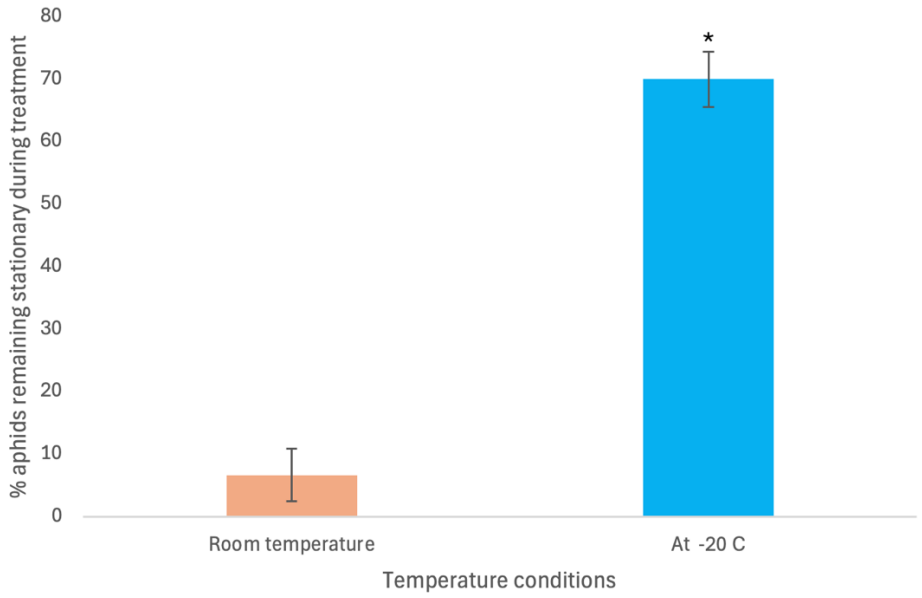

Figure 6. Pre-chilling aphids reduced their mobility, thereby improving dsRNA delivery. The percentage of stationary aphids during the topical dsRNA treatments increased when aphids were first chilled at -20ºC. DsRNA formulations were applied to the backs of aphids that were either held at room temperature (~ 21ºC) or had been pre-chilled for 15 minutes at -20ºC before being returned to room temperature. Each treatment cup had five aphids, and the values represent the mean ± SE for 6 replicate cups. A t-test with a post hoc Bonferroni correction was performed, and the asterisk indicates a significant difference (when $p \leq 0.001$) in the two treatments.

Using third instar nymphs instead of adults

Treatments of adult aphids with different dsRNA formulations failed to show any significant impacts on aphid survival. Still, a preliminary trial using 3-day-old nymphs suggested that the younger insects were sensitive to some of the formulations (Figure 7). Three replicates for each treatment group were tested to check for mortality. Significant mortalities were calculated by a two-way ANOVA with Tukey's post hoc and observed in the third instars treated with long dsRNA *CHS* + 0.005% Silwet relative to no treatment controls in adult and third instars as well as adults treated with the same formulation ($p<0.001$, $p<0.05$ and $p<0.01$ respectively; Figure 6). No such significant differences were observed in pcRNA *CHS* and pcRNA *CHS* + 0.005% Silwet treatments.

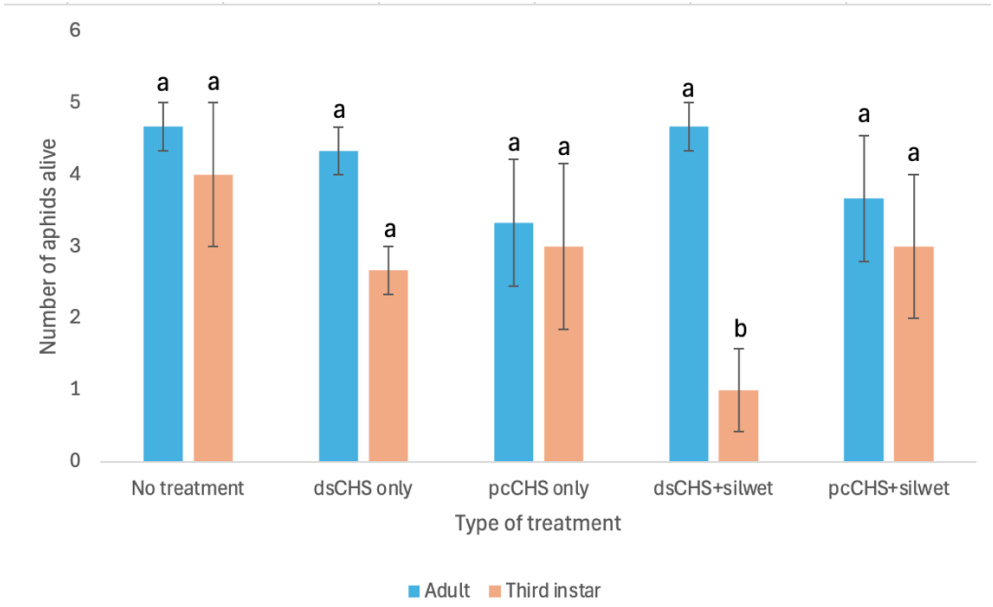

Figure 7. The difference in the surviving adults on day 8 post-treatment was observed in the adult bioassays vs the third-instar bioassay. The difference in comparison between no treatment negative control, long dsRNA *CHS* alone, pcRNA *CHS* alone, long dsRNA *CHS* + 0.005% Silwet and pcRNA *CHS* + 0.005% Silwet treatments administered to adults vs third instars. Each treatment cup had five aphids, representing the mean ± SE for 3 replicate cups. A two-way ANOVA with Tukey's post hoc was performed, and the 'a' indicates no significant difference among the treatment groups while 'b' represents a significant difference (when $p \leq 0.05$) relative to all the other treatment groups.

Assessing mortality of M. persicae nymphs using different dsRNA formulations

The mortality of *M. persicae* nymphs was assessed every day throughout an 8-day bioassay, and significant changes in survivorship were evaluated using Log-rank (Mantel-Cox) tests (GraphPad Prism 4, GraphPad Software). The mortality observed in aphids treated with long dsRNA *CHS* + 0.005% Silwet was significantly higher ($p<0.01$) than the mortality observed in untreated aphids ("no-treatment condition"; Figure 8). Additionally, there were no significant differences ($p=0.13$) between the mortalities observed in no-treatment controls relative to the mortalities observed in aphids treated with 0.005% Silwet alone (diluted in nuclease-free water; Figures 8 & 9). Moreover, there were no significant differences observed between mortalities observed in dsRNA *GUS* + 0.005% Silwet ($p=0.76$; Figure 8) or pcRNA *GUS* + 0.005% Silwet ($p=0.5$; Figure 9) relative to the no-treatment controls. Unexpectedly, there were no significant differences between the mortality observed in aphids treated with pcRNA *CHS* alone ($p=0.33$) or pcRNA *CHS* + 0.005% Silwet ($p=0.31$) relative to the no-treatment controls.

There were no significant differences observed between the long dsRNA and pcRNA treatment groups except with the addition of Silwet. As Figures 8 and 9 display survivorship curves that overlap one another and as none of the graphs compare the mortalities caused by dsRNA and pcRNA together, the data were re-represented, just comparing the long vs the pcRNA treatments, with and without Silwet, to help visualize the data more clearly (Figure 10). Long dsRNA *CHS* alone caused 40% mortality, while pcRNA *CHS* caused only about 24% mortality, which was not statistically significant ($p=0.36$). In contrast, dsRNA CHS + 0.005% Silwet caused about 56% mortality (highest

out of all treatment groups), while pcRNA *CHS* + 0.005% Silwet only caused about 24% mortality, which is statistically significant($p=0.05$).

Although dsRNA *CHS* + 0.005% Silwet treatment caused the highest mortality, there weren't any significant differences ($p=0.33$) when compared to mortalities caused by dsRNA *CHS* alone, even though the addition of Silwet seems to increase the mortality from 40% to 56% in long dsRNA treatments (Figure 10). Similarly, there weren't any significant differences ($p=0.81$) in mortalities caused by pcRNA *CHS* + 0.005% Silwet compared to those caused by pcRNA alone (Figure 10).

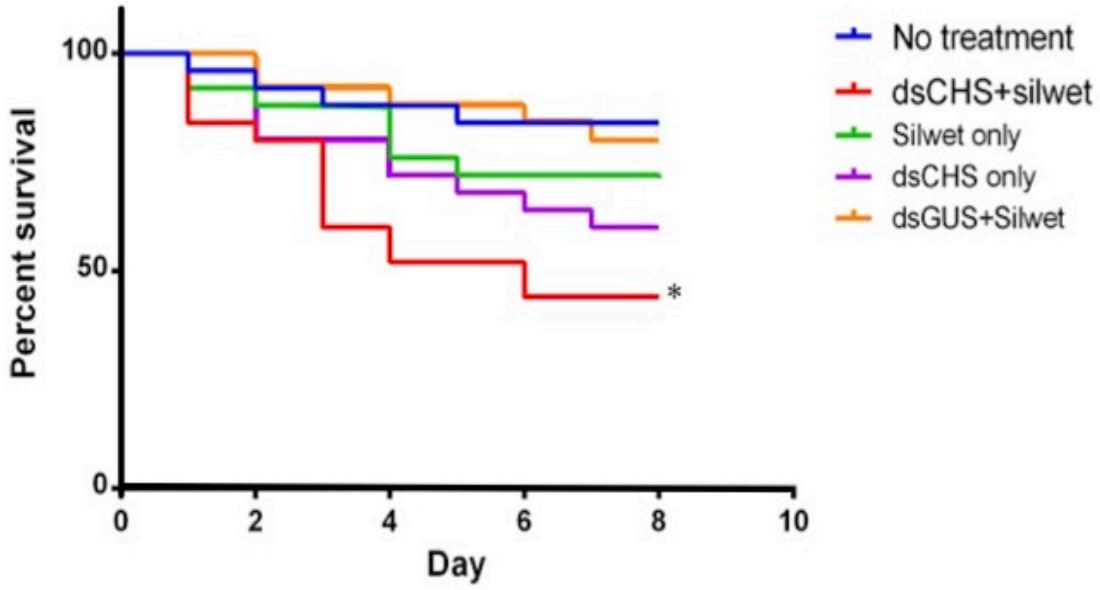

Figure 8. Survival of aphids over an 8-day bioassay following treatments with different long dsRNA formulations. Kaplan-Meier survival analysis of aphids treated with long dsRNA *CHS* alone (purple), long dsRNA *CHS* + 0.005% Silwet (red), 0.005% Silwet solution (green), dsRNA *GUS* + 0.005% Silwet (orange) and the 'no-treatment control' (blue). The data is representative of n=25 as all five replicates were pooled together for the analysis. The dsCHS + Silwet was the only treatment that was significantly different from that of the other treatments ($p \leq 0.05$, using the Log-rank Mantel-Cox test) and is denoted with an asterisk (*).

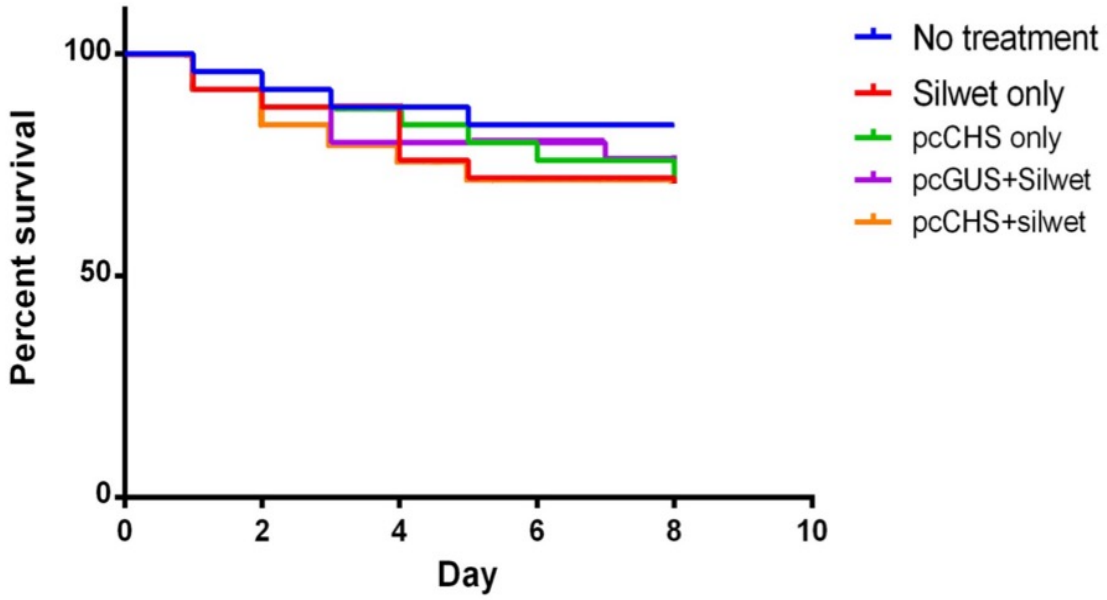

Figure 9. Survival of aphids over an 8-day bioassay following treatments with different short pcRNA formulations. Kaplan-Meier survival analysis of aphids treated with pcRNA *CHS* alone (green), pcRNA *CHS* + 0.005% Silwet (orange), 0.005% Silwet solution (red), pcRNA *GUS* + 0.005% Silwet (purple) and the 'no-treatment control' (blue). The data is representative of n=25 as all five replicates were pooled together for the analysis. No significant differences ($p \leq 0.05$) were observed across the treatments using the Log-rank (Mantel-Cox) test.

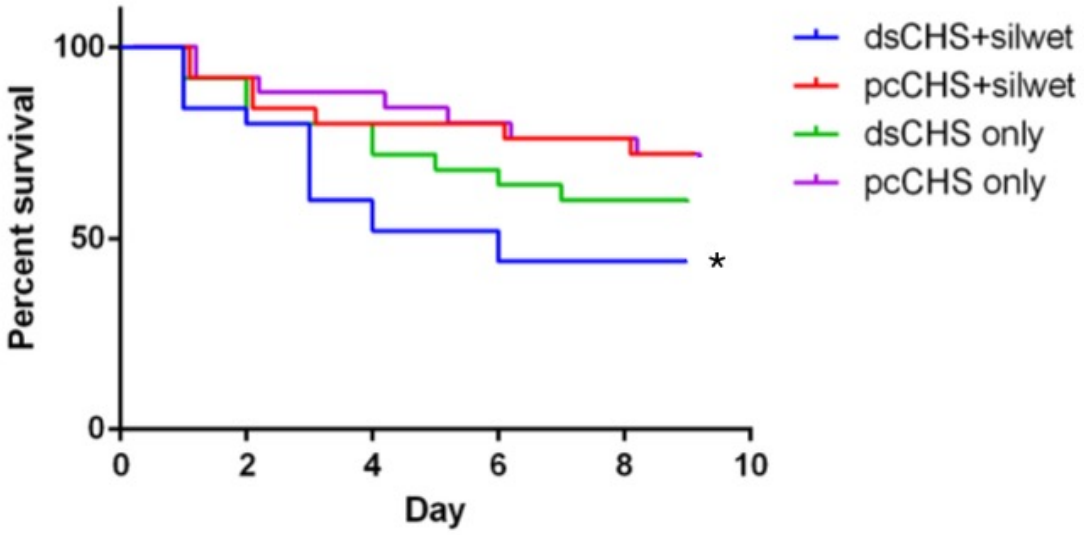

Figure 10. Kaplan-Meier survival analysis of aphids treated with long dsRNA *CHS* alone (green), long dsRNA *CHS* + 0.005% Silwet (blue), pcRNA *CHS* + 0.005% Silwet (red) and the pcRNA *CHS* alone (purple). The data is representative of n=25 as all five replicates were pooled together for the analysis. Significant differences ($p \leq 0.05$) were determined using the Log-rank (Mantel-Cox) test and are represented with asterisks (*) for significant differences between pcRNA *CHS* + 0.005% Silwet and long dsRNA *CHS* + 0.005% Silwet.

Fecundity of M. persicae following treatments with dsRNA formulations

The average daily fecundity of *M. persicae* was assessed throughout the 8-day bioassay, and changes in nymph production were evaluated using a one-way ANOVA test followed by Tukey's post hoc test (GraphPad Prism 4, GraphPad Software). The fecundity data was represented as the mean ± SE for the total number of nymphs produced across treatment classes (Figure 11). Thus, to identify trends in fecundity reduction due to treatments and to account for the adults dying during the bioassay, graphs were generated to examine nymph production per living aphid per day (Figures 12 &13). Significant differences were observed only on day six post-treatment.

No significant differences were observed in daily fecundity for any of the treatments, except on day six, where, unexpectedly, the nymphs per adult aphid were significantly higher ($p=0.03$) in aphids treated with long dsRNA *CHS* relative to the no-treatment controls (Figure 12). The nymphs per adult aphids were significantly lower ($p=0.04$) in aphids treated with pcRNA *CHS* relative to the nymphs per adult treated with long dsRNA *CHS*.

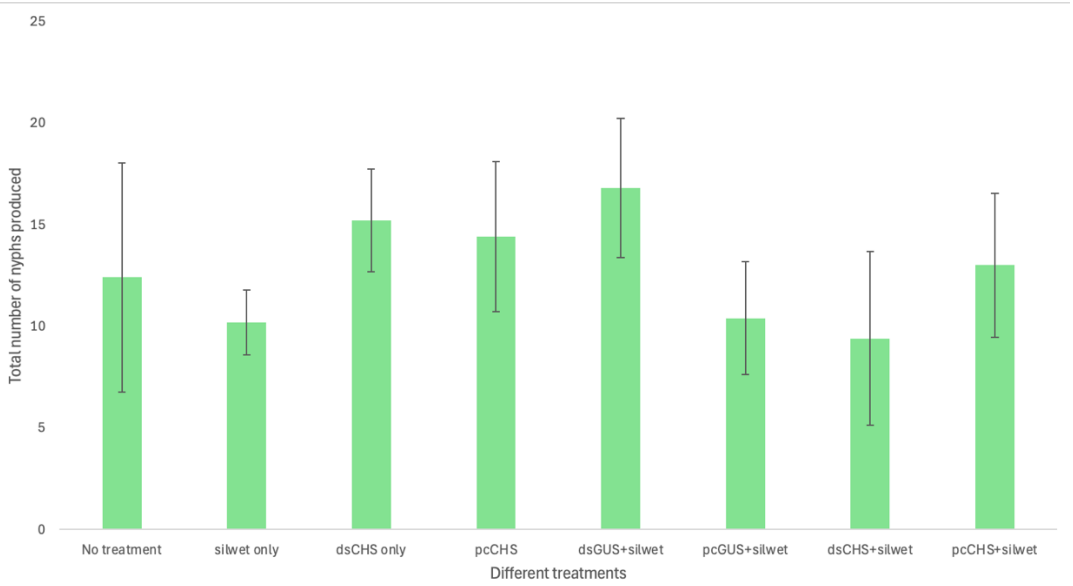

Figure 11. Graph representing the total nymph production across treatment classes. *M. persicae* fecundity values for all replicates (n=5) were averaged for eight days post-treatment. The bar graph is plotted as mean ± SE for the duration of the entire assay. One-way ANOVA with Tukey's post hoc tests were conducted to determine significance. No significance was recorded.

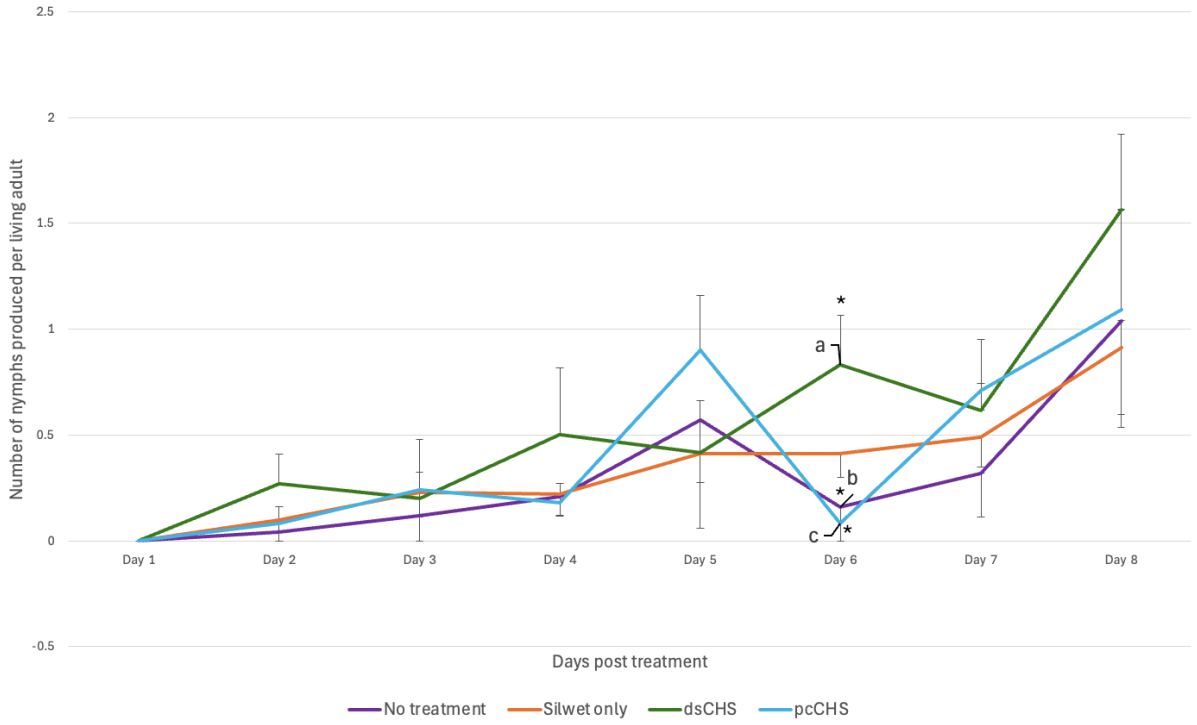

Figure 12. Average nymphs produced per living aphid as a measure of fecundity. *M. persicae* fecundity values for all replicates (n=5) were averaged every day for eight days post-treatment. The line graph was plotted for fecundity values as mean ± SE for each day. One-way ANOVA with Tukey's post hoc tests were conducted to determine significance. The following symbols denote significance when $P \leq 0.05$: (*) significant difference in dsRNA *CHS* (a) relative to the no-treatment denoted by (b) and (*) significant difference in pcRNA *CHS* (c) relative to dsRNA *CHS* (a).

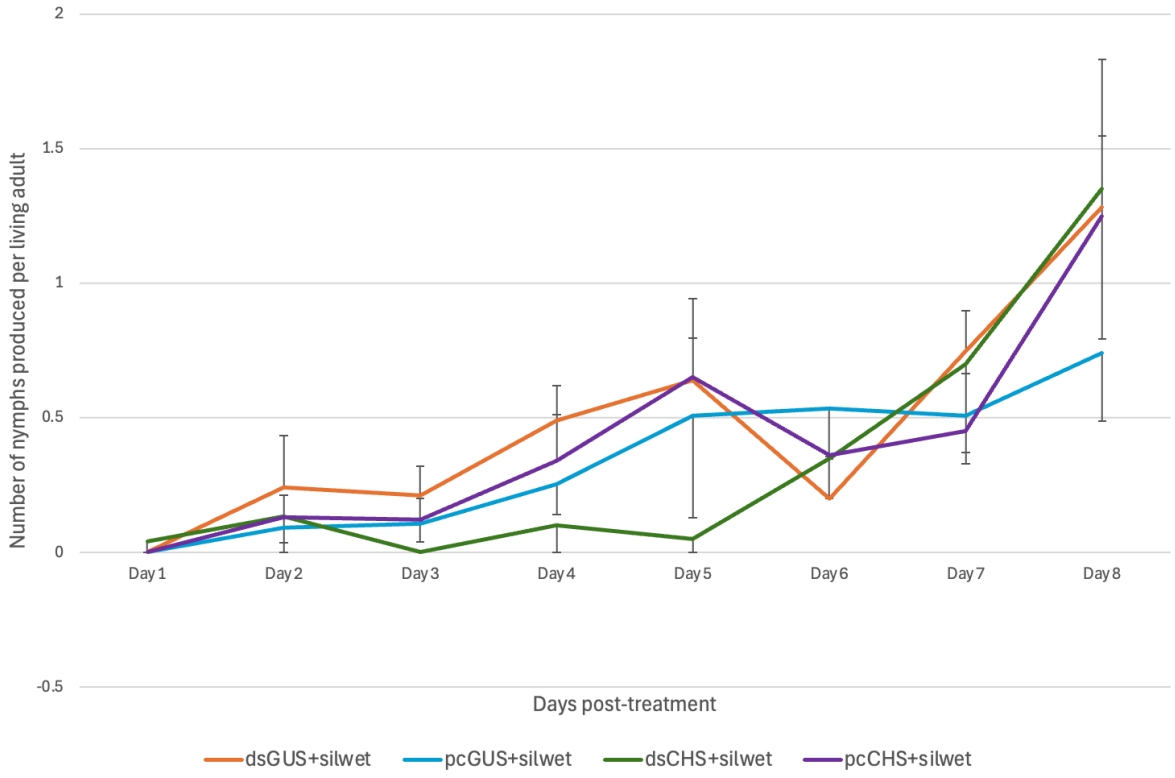

Figure 13. Average nymphs produced per living aphid as a measure of fecundity. *M. persicae* fecundity values for all replicates (n=5) were averaged every day for eight days post-treatment. The line graph was plotted for fecundity values as mean ± SE for each day. One-way ANOVA with Tukey's post hoc tests were conducted to determine significance.

Assessing CHS gene knockdown levels

To evaluate RNAi-mediated knockdown in the treated aphids, RNA was extracted from pooled groups of 5 aphids, and the RNA was treated twice with DNase. The second DNase treatment was deemed necessary, as all samples, after a single DNase treatment, produced PCR amplicons in the "no reverse transcriptase control" samples. After a second DNase treatment, all samples appeared free of contaminating genomic DNA (Figures 4 & 5). The cDNA's quality in each sample was then assessed by attempting to PCR-amplify an Actin cDNA fragment using endpoint PCR. Regrettably, only 12 of the 25 cDNA samples produced the expected 104 bp band (Figure 14). Those twelve samples were used for qRT-PCR analysis to measure the *CHS* transcript levels. *Actin* was used as a reference gene to calculate the relative level of *CHS* transcripts in each sample, and a pc*GUS* sample was the only negative control sample available to calculate *CHS* transcript knockdown for the remaining samples The knockdown values are shown in Figure 15. As there are insufficient replicates of any samples, including negative control treatments, the knockdown values, while seeming high for most samples, cannot be considered reliable indicators of whether RNAi was achieved in any of the treatments.

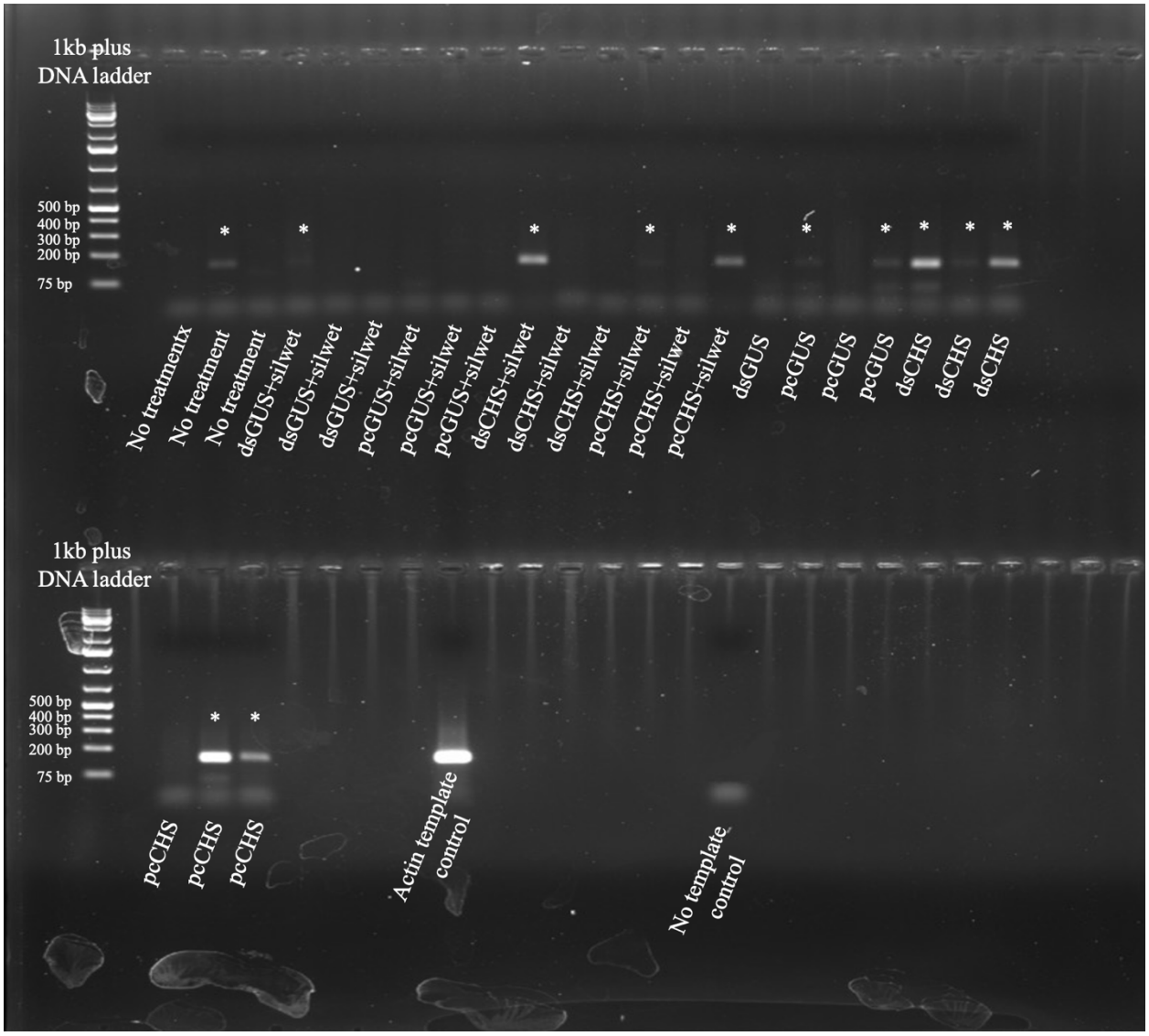

Figure 14. Resolution of end-point PCR products of cDNA samples with *M. persicae* *Actin* primers (amplicon size = 104 bp) on an agarose gel. 1.5% ethidium bromide-stained agarose gel was submerged in 1X TRIS Borate EDTA (TBE) buffer and resolved at 130V for 30 minutes. Most of the cDNA samples failed to produce a visible band, but there were 12 samples produced visible amplicons of the expected size (marked with a *). These 12 samples were used for subsequent qRT-PCR. 1 Kb plus DNA ladder was loaded in the first lane in both rows. The last two rows are *Actin* template control and the no template control.

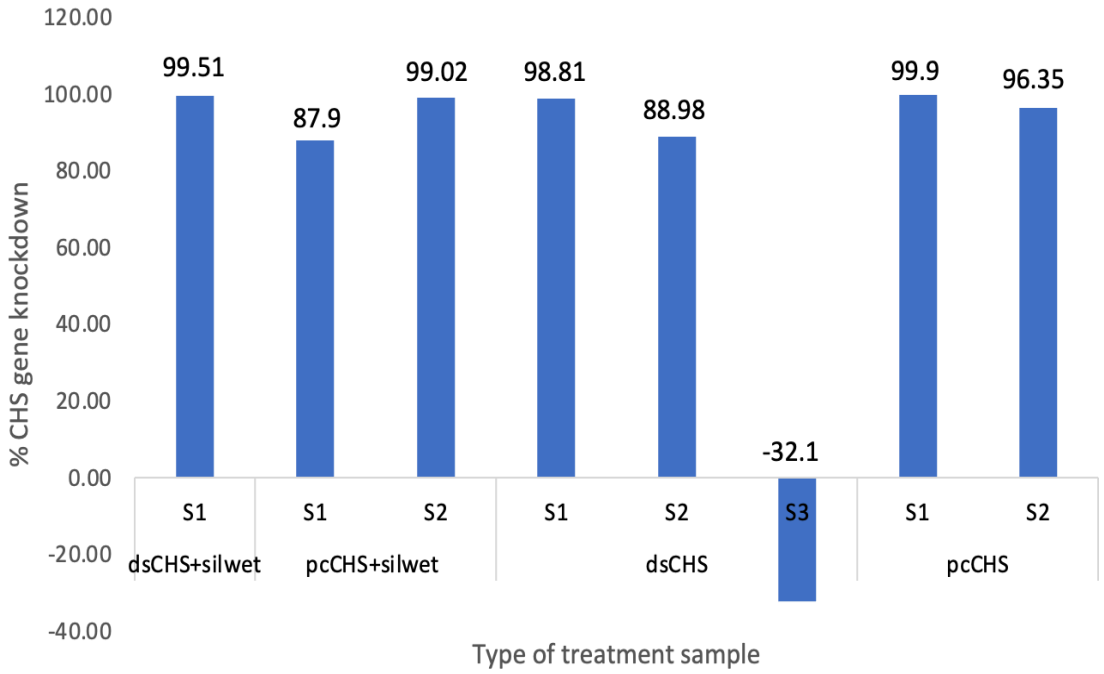

Figure 15. The percent knockdown of the *M. persicae CHS* gene in the selected treatment samples relative to the pc*GUS*-treated control. The replicate ID is represented as 'S1', 'S2' and so on. The qPCR was done on 12/25 samples chosen, as shown in Figure 14.

Discussion

Effective control of *M. persicae* (the green peach aphid) is crucial for reducing significant crop losses and ensuring global food security. With increasing concerns about the adverse environmental effects of many chemical pesticides, along with rising instances of resistance due to the continuous use of current insecticides, there is a need for new pest management strategies (Yu et al. 2014; Sparks and Nauen 2015). RNAi-based pesticides are being developed to control a variety of crop pests. These pesticides show promise because they are potentially species-specific, offering an environmentally safer way to protect crops (Killiny et al. 2014; Yan et al. 2020; Chen et al. 2022a). Since chitin is the primary polysaccharide present in insect exoskeletons, including the lining of their trachea, gut and fat bodies (Bansal et al. 2012; Shi et al. 2016), targeting the *CHS* gene should produce sub-lethal to lethal effects in aphids and reduce their fecundity (Bansal et al. 2012; Ye et al. 2019). In this study, different dsRNA structures were tested to see how effectively they could kill aphids and reduce the fecundity. Additionally, the effect of adding Silwet (a surfactant) was tested for its ability to improve the uptake of dsRNA by the insects. After optimizing the bioassay, the combination of dsRNA targeting *CHS* with 0.005% Silwet resulted in the highest mortality rate in the aphids. However, no clear trends were observed regarding the effect on fecundity. Although this study only tested four treatment combinations for a single gene target, it provides valuable evidence on the effectiveness of certain dsRNA pesticide combinations and highlights key factors that influence the potency of such treatments and the design bioassays for aphids.

Optimizations of the bioassay

At the start of this study, I encountered several challenges, including maintaining the viability of the aphids on an artificial diet, fungal contamination in the diet, and the constant movement of the aphids impeding the delivery of dsRNA droplets to the back of aphids. However, through optimization, I successfully overcame these issues. Since *M. persicae* have shorter proboscises than many other aphids, they typically feed on thinner plant parts, such as the underside of the leaves, the midrib, and the side vein (Dixon et al. 1995; Ahmed et al. 2022). To address this, I stretched and thereby thinned the parafilm used for the feeding packet, which reduced the distance needed to reach the liquid diet. This, in turn, enhanced their feeding response to the sucrose present in the solution (Emden and Wild 2020). Furthermore, I filter-sterilized the aphid diet to prevent fungal growth, which would otherwise harm the aphids. According to the manufacturer's guidelines, the filter effectively removes filamentous fungi from cell cultures and other biological samples while preserving the bulk of the proteins in the solution (ThermoFisher Nalgene syringe filters green fact sheet).

Placing the aphids at -20ºC for 15 minutes reduced aphid mobility significantly during the application of the topical solutions compared to aphids at room temperature. Aphids, being ectotherms, rely on the ambient temperature to regulate their metabolic rates (MacMillan et al. 2012). Placing them at -20 C induces a cold shock response, which leads to a slowing down of their metabolic rate and limits activity to conserve energy to acclimatize to the ambient temperature (Durak and Durak 2021). This caused temporary immobility, which made it easier to apply topical solutions on their backs. Moreover, treating third instars with topical dsRNA solutions caused significantly higher mortalities

when treated with dsRNA *CHS* alone and with dsRNA *CHS* +0.005% Silwet relative to their adult counterparts. Insects undergo a process soon after each nymphal moult, known as cuticular sclerotization, whereby the exoskeleton's cuticle becomes increasingly hardened and stabilized through the incorporation of greater amounts of phenolic compounds (Andersen 2010). The hardened exoskeleton of the adult aphid provides the necessary rigidity for muscle attachment and movement while offering maximal physical protection (Andersen 1974, 2010). Due to their thicker exoskeletons, entry of the long dsRNA and the pcRNA through the cuticle was seemingly hindered in the adults, despite the addition of the surfactant, whereas the younger nymphs succumbed more easily to the dsRNA treatments.

Mortality and fecundity of M. persicae

One treatment combination, long dsRNA *CHS* + 0.005% Silwet, caused a 56% increase in aphid mortality by day 8, compared to untreated aphids, with a statistical significance of $p<0.01$. In contrast, dsRNA *CHS* alone caused 44% mortality. When *A. pisum* nymphs were injected with dsRNA *ApisCHS* alone, 56.7% mortality was observed by day 3 (Ye et al. 2019). This difference in earlier mortality between injection and topical treatments can be attributed to the mode of delivery and the amount of dsRNA administered. Injecting dsRNA ensures that the full dose is delivered directly inside the aphid without it being degraded or lost to the environment, which is a risk in topical treatments (Christiaens et al. 2020). In that study, 300 ng of dsRNA was used for nymphs, which was increased to 600 ng for adults to achieve similar results (Ye et al. 2019). In contrast, my study used only 100 ng of dsRNA for third-instar aphids, which may not have been fully internalized, and yet a similar mortality was observed, albeit 5 days later.

Although the mortality caused by dsRNA *CHS* alone was not statistically significant, relative to the negative controls, adding 0.005% Silwet to the treatment enhanced the mortality, which appeared to help overcome some barriers associated with the topical treatment. Since the treatment with Silwet alone caused 36% mortality, it wasn't significantly different from the deaths that occurred in no-treatment controls. Thus, it can be concluded that a 0.005% concentration of Silwet is a non-lethal dose, and the increase in mortality is mostly attributed to its role as a surfactant, enabling the dsRNA to spread over the cuticle, and likely entering the insects through their spiracles, and into the trachea, where the cuticle thins to enable gas exchanges to all interior tissues within the insect (Bossen et al. 2023).

Contrary to my predictions, pcRNA treatments, with or without Silwet, weren't successful in causing any significant mortalities when compared to the no-treatment condition or pcRNA *GUS* (Figure 9). A possible explanation for the lack of efficacy of the pcRNA is that the siRNA contained within the pcRNA could not bind effectively to the target CHS mRNA to mediate RNAi-induced transcript knockdown (Abbasi et al. 2020). Not all siRNAs are effective at facilitating RNAi-mediated knockdown (Smith 2006). To design the pcRNA, I used the Integrated DNA Technologies (IDT) design software tool, which predicts effective siRNAs based on an algorithm that evaluates the strength of the siRNA binding to an mRNA based on nucleotide complementarity scores (Owczarzy et al. 2008). Although the design algorithm is regularly updated as more models are published, permitting more effective siRNAs to be predicted, we cannot be certain that the resulting siRNA will produce an efficient RNAi response (Smith 2006; Owczarzy et al. 2008). One factor that can contribute to differences in siRNA efficacy that is not considered by the

IDT algorithms is the secondary and tertiary structure of each mRNA (Smith 2006). Our lab has very recently confirmed that the structure of the mRNA can greatly influence the ability of the siRNA to bind to its target and facilitate Argonaute-mediated cleavage of the mRNA; that is, regions with higher entropy scores or reduced secondary structures, such as single-stranded loops, are better targets for siRNAs and result in greater levels of transcript knockdown (Lu and Mathews 2008 & Whyard unpublished data). Figure 16 depicts the entropy score of the siRNA binding region in the *M. persicae CHS* mRNA, which was estimated to have a relative positional entropy value of 24.1. In a recent analysis of 15 different siRNAs tested in mosquitoes, siRNAs with relative position entropy scores under 25 failed to induce detectable transcript knockdown (Whyard, unpublished data). Unfortunately, I had already conducted my experiments before these data were available, and it is clear that more pcRNAs with higher positional entropy scores should be tested to better evaluate their ability to act as effective insecticides in aphid topical treatment bioassays.

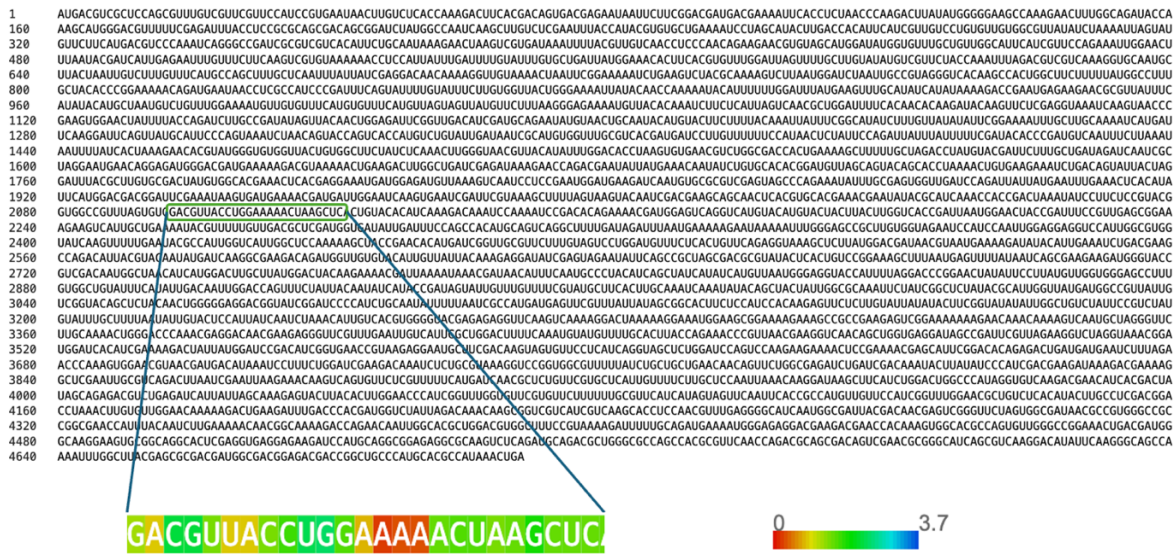

Figure 16. The predicted positional entropy score of the CHS pcRNA used in this study was calculated using the heat-map colourized siRNA derived from the RNAfold server. The positional entropy of each nucleotide of the siRNA is colour-coded, with the highest positional entropy denoted in blue and the lowest in red(Gruber et al. 2008). Using the heat map scale, this siRNA had an overall positional entropy score of 24.1, which is considered a poor-quality siRNA (Whyard, unpublished data).

The fecundity data collected in this study did not reveal any significant findings. While there were some small, yet statistically significant differences observed on day 6 post-treatment, all other days showed no difference in fecundity, and the relative fecundity rates for the entire 8-day period were not affected by any of the treatments. Although I anticipated that the dsRNA could have adversely affected the fecundity of the aphids, this hypothesis was not supported by the data. The overall fecundity of the aphids during this bioassay was surprisingly low, even for the untreated aphids. Typically, under optimal laboratory conditions, *Myzus persicae* adult females produce between 2 to 6 offspring per

day (Gao et al. 2021), but in my assays, the average number of progeny in the untreated controls was only 0.33 nymphs/day/female. In all likelihood, the rearing conditions were suboptimal for normal reproductive cycles, as the bioassays were conducted on the lab bench, where the room temperatures dropped well below optimal rearing conditions, and the assays were conducted with ambient photoperiod cues during the winter months, when this insect does not typically breed. In addition, the aphids were gradually dying due in part to the consumption of the dsRNA, leaving fewer and fewer adults to produce nymphs, which further reduced the progeny production. Taken together, there were too many factors that were limiting fecundity for me to detect any RNAi-mediated impacts, and it is clear that future bioassays should be conducted in incubators with controlled environmental conditions that are optimized for aphid reproduction, and more aphids needed to be used in the assay to generate meaningful data. Furthermore, another study that explored the effect of RNAi-mediated silencing in aphid nymphs focused on phenotypes such as deformity rate in newborn nymphs, reduction in the size of embryos as well as newborn nymphs and extended developmental duration for nymphs (Ye et al. 2019). Therefore, tracking each aphid for the number of nymphs it produces, as well as tracking additional nymph phenotypes, can help understand the effects of RNAi-mediated silencing of *CHS* in embryo/ larval development.

Assessment of gene knockdown levels

To examine whether the dsRNA-based pesticides caused transcript knockdown in aphids, qRT-PCR analyses were attempted to measure the *CHS* transcript levels. Unfortunately, due to an insufficient number of replicates, I am not able to conclude that transcript knockdown occurred in any of the dsRNA-treated samples. Chief among the

problems that I encountered was low yields of RNA from the pooled aphids, with concentrations ranging between 30 ng/ µL to 80 ng/ µL. Moreover, following repeated DNase treatments, the concentrations dropped even further, typically 10-fold lower, such that I had limited amounts of RNA to use for cDNA synthesis. Some of this loss was likely attributed to repeated freeze thaws, which can degrade RNA, but the increased risk of introducing ribonucleases with each nuclease treatment also likely contributed to some loss of RNA. Additionally, divalent cations (such as Mg^{2+}) present in the RNA samples mainly from the aphid tissues (Maguire and Cowan 2002) from the RNA extraction kits can lead to RNA hydrolysis when the sample is heated during the DNase treatment (Koculi et al. 2007). In short, I found I had limited amounts of RNA available, which is likely the main reason that most samples failed to detect *CHS* or *Actin* transcripts. To overcome these difficulties, future analyses will require a greater number of aphids treated per replicate, to ensure a higher concentration of RNA extracted per sample

Conclusion

In summary, it is essential to develop new, sustainable pest management strategies to mitigate crop losses caused by green peach aphids, which inflict significant damage to many of our crops. The widespread use of chemical pesticides has led to increased resistance to pests and harmful environmental effects on non-target organisms, making it crucial to explore and develop biosafe alternatives (Pistorius et al. 2009; Bartling et al. 2019). RNAi-based pesticides show considerable promise, and in this study, the addition of Silwet as a surfactant to the dsRNA offers a step towards creating effective dsRNA-based topical pesticides. Although this study focused solely on targeting the *CHS* gene, the findings provide valuable insights into the potential of RNAi-based pesticides with alternative dsRNA formulations, for controlling insect pests that have significant economic impacts.

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