Impacts of a flea beetle double-stranded RNA-based insecticide on the ground beetle *Pterostichus melanarius*

by

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A thesis submitted to the Department of Biological Sciences, University of Manitoba,

in partial fulfilment of the requirements for the course

BIOL 4100 (Honours Thesis)

for the degree of

Bachelor of Science (Honours)

Abstract

Flea beetles (*Phyllotreta cruciferae* and *P. striolata*) are the most damaging insect pests of canola, one of Canada's most economically valuable crops. Chemical insecticides are currently the primary method of flea beetle control, but concerns about their impact on non-target organisms highlight the need for safer alternatives. RNA interference (RNAi) has been recognized for its ability to selectively kill insect pests by silencing target genes through sequence-specific exogenous double-stranded RNA (dsRNA). However, sequence similarity between pest and beneficial insects continue to pose risks about potential off-target effects.

In this study, two dsRNAs targeting the *UBE2L3* and *Snf7* gene in *P. cruciferae* were evaluated for off-target effects in the predatory ground beetle *Pterostichus melanarius*. A bioinformatic analysis was used to identify the similarity between the dsRNA sequences and their respective orthologs in *P. melanarius*. Beetles were then injected with the dsRNAs, and relative gene expression and mortality were measured.

Following injection of the *UBE2L3* dsRNA, which shares 83.4% similarity and a 29 bp matching segment with *P. melanarius,* target gene expression was reduced by 69.9%. Conversely, injection of the *Snf7* dsRNA, which only shares 62.6% similarity, did not induce gene knockdown. Despite the knockdown of *UBE2L3*, no significant mortality was observed in any treatment group, including the positive control, suggesting that an insufficient dose of dsRNA was used. These findings demonstrate that sequence similarity influences the off-target effects of dsRNA, which can be predicted using a bioinformatic assessment. Continued sequencing of beneficial insects is required so that dsRNA can be designed to have minimal similarity with other species to reduce off-target effects.

Acknowledgments

I would like to begin by thanking my supervisor, Dr. Steve Whyard, for introducing me to a project that aligned so well with my interests and for his support and guidance throughout the year. I am also thankful to Aditi Singh and Alison Tayler for their assistance with my project and for providing training in laboratory techniques. Lastly, I would like to thank my honours committee members, Dr. Jae-Hyeok Lee and Dr. Mark Belmonte, for their valuable feedback during the development and completion of my project.

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Introduction

Canola is one of Canada's most important crops, second only to wheat in total production, and it contributes $29.9 billion annually to the Canadian economy (LMC International 2020; Statistics Canada 2024). Two flea beetles, the crucifer flea beetle (*Phyllotreta cruciferae)* and the striped flea beetle (*P. striolata;* Coleoptera: Chysomelidae), are the most damaging insect pests of canola (Burgess 1977; Soroka et al. 2018). These pests are responsible for 8-10% in yield loss, equating to >$300 million in annual losses (Lamb and Turnock 1982; Knodel and Olson 2002). Currently, the principal methods to control flea beetles on canola are neonicotinoid seed treatments followed by foliar sprays when feeding damage reaches its economic threshold (Knodel and Olson 2002; Reddy et al. 2014). However, these insecticides have severe shortcomings. Flea beetles have shown the ability to develop resistance to insecticide treatments, and rotating between different insecticide groups is the only recommended strategy for mitigation (Turnock and Turnbull 1994; Mittapelly et al. 2024). Additionally, the use of neonicotinoid insecticides has been found to negatively affect beneficial insects such as pollinators and natural enemies (Cloyd and Bethke 2011; Main et al. 2020). These chemicals can also contaminate the soil and leach into aquatic ecosystems, leading to reduced insect emergence and a shift in community composition (Cavallaro et al. 2019). For this reason, neonicotinoids have been banned in European countries, and growers must now rely on fewer control products (Willis et al. 2020). Therefore, alternative insecticide technologies such as RNAi are being developed as a promising next step for insect control.

RNA interference (RNAi) is the post-transcription gene silencing pathway conserved across eukaryotic cells that, when triggered by exogenous double-stranded RNA (dsRNA), inhibits the expression of target genes (Fire et al. 1998). As this mechanism is conserved among many groups of life, there is a wide range of potential applications. In recent decades, researchers have exploited RNAi for its promising use in medicine, particularly for treating cancer and genetic diseases (Davis et al. 2010; Leachman et al. 2010; Tian et al. 2021). Furthermore, RNAi has emerged as an adaptable, species-specific tool for insect control (Baum et al. 2007; Whyard et al. 2009). RNAi-induced gene knockdown can be achieved in insects through feeding of dsRNA and has been found quite efficacious to those in the order Coleoptera (Baum et al. 2007). Currently, dsRNA-based insecticides are commercially available, such as a foliar spray produced by GreenLight Bioscience that targets the Colorado potato beetle *Leptinotarsa decemlineata* (Rodrigues et al. 2021). However, there are concerns about the potential risk of off-target effects from RNAi-based insecticides (Lundgren and Duan 2013).

In many cases, when an insect ingests dsRNA, it is absorbed by the gut epithelial cells and cleaved into approximately 21 bp small interfering RNAs (siRNA) by the ribonuclease enzyme Dicer (Bernstein et al. 2001). A siRNA duplex is then separated by the Argonaute2 protein and guided to form an RNAi-induced silencing complex (RISC) (Okamura et al. 2004). Using the siRNA as a template, RISC can selectively cleave mRNAs that contain a complementary matching sequence (Hammond et al. 2000). Based on this gene sequence specificity, dsRNA can be designed to target certain pest species while sparing non-target beneficial insects (Whyard et al. 2009).

Beneficial insects play an important role in the ecosystem and can positively impact agricultural productivity. Insect pollinators are, to some extent, responsible for pollinating many of the leading global crops and have been found to increase product yield (Klein et al. 2006; Lindström et al. 2016). On the other hand, natural enemies of crop pests, such as parasitoid wasps and predacious ground beetles, can substantially reduce crop damage through their role as biological control agents (Kromp 1999; Hemachandra et al. 2007; Busch et al. 2021). However, in the case of managing flea beetles, attempts made to employ parasitoids were found unsuccessful (Dosdall and Mason 2010). Nevertheless, it is crucial to consider these insects when implementing new control practices to minimize potential off-target effects.

RNAi-based insecticides must also be assessed for potential off-target effects, as multiple species that share conserved orthologs may be affected by the same dsRNA (Whyard et al. 2009). To uncover the limitations of the sequence specificity of RNAi, the red flour beetle *Tribolium castaneum* was tested with dsRNAs of varying matching identity to the target mRNA, and it was found that a perfect match is not required to cause gene knockdown (Chen et al. 2021). Rather, a set of parameters were identified that could be used to predict whether a dsRNA would have off-target effects. According to Chen et al. (2021), dsRNA that share >80% identity or perfectly matched segments of $\geq$16 bp with the target gene could produce siRNAs that trigger significant knockdown. Additionally, dsRNA that share sequences of $\geq$26 bp, containing separated single or double nucleotide mismatches, were also found to be sufficient (Chen et al. 2021). However, in a feeding assay with the bumble bee *Bombus terrestris*, non-target dsRNAs with up to a continuous 20 bp match were tested, and no reduction in transcription levels of the orthologs or other

sequences elsewhere in the genome was observed (Taning et al. 2021). Although these findings provide valuable insight into the potential effects of non-target dsRNA, responses are not the same for all insect species. As very few species have been tested, much remains unknown.

This study aimed to investigate the impact two dsRNAs, designed to target flea beetles, have on a non-target insect that may be exposed to the dsRNAs with its potential application. The ground beetle *Pterostichus melanarius* (Coleoptera: Carabidae) was the beneficial insect chosen for this study. Native to Europe and has since been established in North America, *P. melanarius* is a generalist predator commonly found in canola fields and other agricultural ecosystems (Niemelä and Spence 1999; Busch et al. 2021). Although there are no records of *P. melanarius* feeding on flea beetles, many additional pests, such as other beetles, caterpillars, aphids and slugs, are part of its diet (Bohan et al. 2000; Warner et al. 2008; Alvarez et al. 2013; Unruh et al. 2016). Notably, one study found that *P. melanarius* can be exposed to lethal doses of insecticides by feeding on treated aphids (Mauchline et al. 2004). These traits make *P. melanarius* a suitable model for this study.

DsRNA targeting *P. cruciferae*'s *UBE2L3* and *Snf7* protein-coding mRNA sequences were used in this study. UBE2L3 is a protein involved in the ubiquitination pathway for targeted protein degradation and Snf7 is a vacuolar sorting protein that is part of a complex responsible for trafficking ubiquitinated proteins (Ardley et al. 2001; Vaccari et al. 2009; Zhang et al. 2021). Previous research in the lab has found that these two dsRNAs effectively caused knockdown and mortality in flea beetles (Whyard, unpublished data). An initial BLAST search more than seven years ago found no

sequences with shared 21 bp matches to these dsRNAs. Therefore, it was hypothesized that the *P. cruciferae* dsRNAs would not have an effect on survivability or gene transcript levels in *P. melanarius.* However, as GenBank is far from complete in terms of species representation, a deeper bioinformatic analysis was conducted to assess the species-specificity of the dsRNAs. In addition, these two flea beetle dsRNAs were evaluated for their ability to either knock down the orthologous gene transcripts and/or impact the survivorship in *P. melanarius*. Through these analyses, a better understanding of the potential off-target effects of dsRNA pesticides may be gained, and considerations can be made on how to mitigate this challenge before dsRNA pesticides are deployed in an agricultural ecosystem.

Materials & Methods

P. melanarius maintenance

Adult *P. melanarius* were collected using dry pitfall traps placed outside the Plant Sciences research fields at the University of Manitoba from June to September 2024. Captured beetles were identified with a dichotomous key (Allegro 2021) and then kept in plastic containers filled with approximately 2 cm of moist soil, held at room temperature and exposed to natural daylight. Every second day, beetles were fed lab-reared Queensland fruit fly (*Bactrocera tryoni*) pupae, and water was provided in 15 ml plastic test tubes with the end cut off and plugged with cotton (modified from Ferrante et al. 2017). Water was also frequently dripped sparsely throughout the containers to keep the humidity high. No more than 15 beetles were held in a single container, as cannibalism was regularly observed, unlike what was reported by Ferrante et al. (2017).

Bioinformatics and primer design

The *P. cruciferae* gene sequences for *UBE2L3* and *Snf7* were used to search GenBank using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for orthologous sequences in *P. melanarius* (Table 1). Although the genome of *P. melanarius* has not been sequenced, the incomplete sequences from a partial transcriptomic dataset (GenBank accession no. SRX5590255), along with genomic orthologs from two closely related ground beetle species, *P. madidus* and *P. niger,* were identified (GenBank accession no. OU452315.1 and OX380349.1, respectively). The sequences from *P. madidus* and *P. niger* were directly aligned with those of *P. cruciferae* using the pairwise alignment software in Geneious (version 5.6.3). Degenerate primers, which incorporate the nucleotide differences from the two known beetle sequences, were then designed to PCR-amplify the region in *P. melanarius* that corresponds to the dsRNAs created for *P. cruciferae* (Figure 1). The amplicons from these primers were later sequenced, and *P. melanarius*-specific dsRNAs were synthesized (described below).

Table 1. Sequences of the *UBE2L3* and *Snf7* gene in *P. cruciferae*.

Gene	Sequence
UBE2L3	ATGGCAGCCACCAGACGATTGCAAAAAGAACTAGGAGACATTAGAAAT TCTGGATTAAAATCATTCCGTGATATTCAAGTGGACGAAACTAATATTC TAACTTGGCAAGGTCTTATAGTTCCGGATAATCCGCCTTACAACAAAGG AGCTTTTAAAATAGAAATTAATTTCCCCGCAGAATATCCTTTTAAGCCG CCAAAAATAAATTTTAAGACGAAAATTTATCATCCTAATATAGATGAAA AGGGCCAGGTATGTCTGCCCATTATAAGTGCCGAAAATTGGAAACCCG CCACTAAAACGGAACAAGTGATCCAAGCATTGGTTGCTCTTGTGAACGA GCCAGAACCGGAACATCCATTGCGCGGAGACCTAGCTGAAGAATACCT CAAAGATAAGAAAAAATTTGCGAAAAATGCCGAAGATTATACGAAAAA ACACAGTGAAAAGAGACCAGCAGACTAG
Snf7	ACAATGAGTTTCTTTAGTAAAATATTCGGAGGAAAAGAAAAGGATGTG GGCCCCACGCCAGGCGAAGCCATCCAAAAGCTCAGGGAAACCGAGGAC ATGTTATATAAAAAACAAGAGTTTTTAGAAAAGAAAATAGAGGAATAT ACACTAGTAGCCAAAAAAAATGCATCAAAGAATAAGAGAGTCGCGCTG CAGGCGCTCAAAAAGAAAAAACGATTGGAAAAAACTCAATTACAAATC GATGGCACACTCACTACAATCGAAATGCAAAGGGAGGCCCTCGAAGGA GCCAGCACCAACACAGCCGTCTTAGATTCAATGAAGAATGCAGCTGAA GCTCTCCAAAAAGCCCACAAAAACTTGAATGTCGATGATGTCCACGAC ATCATGGATGACATAGCTGAACAGCATGACATTGCCAACGAGATTACT ACTGCTATTAGCCAGCCGGTAGGCTTCGCTGATGATTTGGACGAGGATG AATTAGAAAGCGAACTAGAAAAGCTCGAACAAGAAGGATTAGAGGAA GATTTGCTGAAAGTTCCAGGCCCAACTGAACTACCTGCTGTACCTACAG GAGCTATTGCCAAGCCCGTTAAACCTGCTGCTAAAAAAGTGGAGGATG ATGATGACATGAAAGAATTGGAAGCTTGGGCTTCATAA

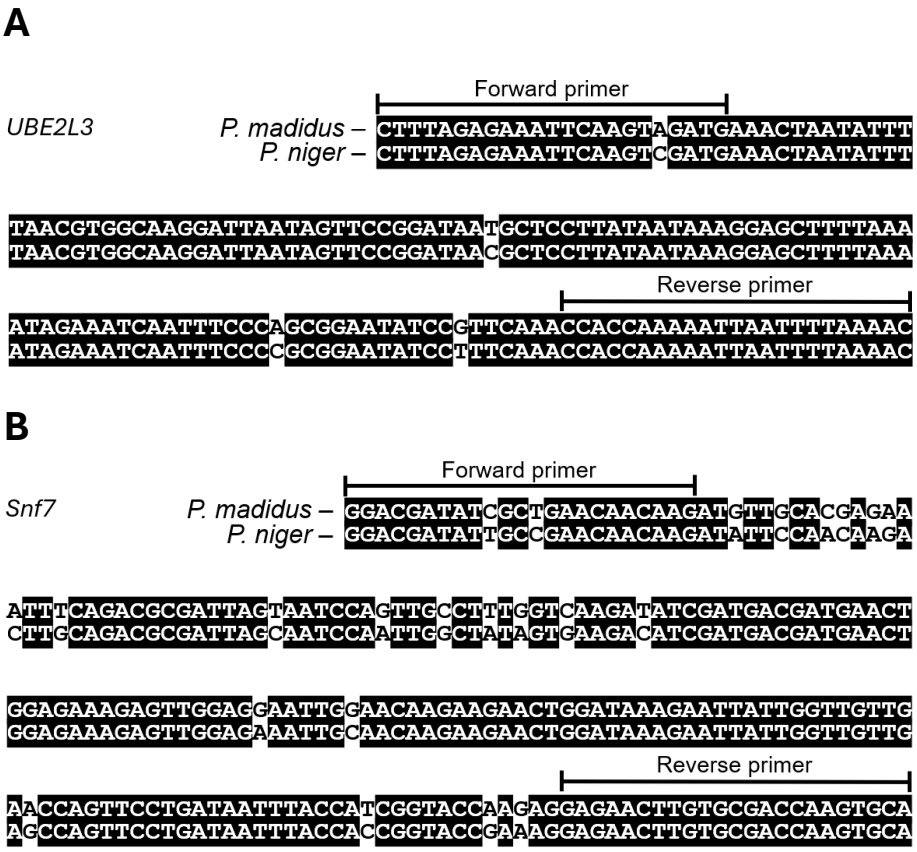

Figure 1. Sequence alignments of select regions of the (**A**) *UBE2L3* and (**B**) *Snf7* gene from two *Pterostichus* ground beetles, *P. madidus* and *P. niger*. Degenerate primers were designed so that the target amplicon could be PCR-amplified from the closely related *P. melanarius*. Letters highlighted in black indicate matching nucleotides between the two sequences. Alignments were done using Geneious (version 5.6.3)

A preliminary alignment of the *P. cruciferae* dsRNA sequences to the sequenced fragments of *P. melanarius* were created. However, as the fragments were not designed to the exact length and location of the dsRNAs, much of the alignment was still unknown. Furthermore, designing quantitative reverse transcriptase PCR (qRT-PCR) primers for later measurement of mRNA expression was not possible, as they require specific design parameters that were not able to be met using the two related beetle species sequences. Therefore, longer sequences of *P. melanarius*'s *UBE2L3* and *Snf7* genes were required, and a deeper search was conducted. Using the identified DNA sequences, a BLAST search of the partial transcriptomic dataset revealed a collection of matching sequences that were approximately 100 bp in length. Certain sequences overlapped with the ends of the search template, allowing for an extension of the total sequence. Repeated BLAST searches were performed to identify end reads, and extended sequences of genes were constructed (Table 2). Complete pairwise alignments were performed between the *P. cruciferae*-specific dsRNA sequences and the constructed orthologs of *P. melanarius* to assess whether the dsRNAs would induce knockdown. Predictions were made based on the parameters identified in *T. castaneum* by Chen et al. (2021). If any of the parameters were satisfied in either sequence alignment, it could be predicted that some level of gene knockdown would be triggered from that dsRNA.

Table 2. Sequences of the *UBE2L3* and *Snf7* gene in *P. melanarius*. Letters highlighted in black are the identified sequences PCR-amplified with the degenerate primers.

Gene	Sequence
UBE2L3	ATGACTGATATTTAATTCCTTATCAACGTTTTGTATGAAATACTTTAACA TCAAATTTTGAAATTTGAGAAAGGTAGATGCGTGAAAAATCCAGTCAGT GAACACAGAAGTCAGATTAGTTCCACATTTGCTAGTTAGGAAATAGTAG GAACAACTGTTTTCCTGAAAGAATCGTGCTTGACAGATATTTTTTCTGG GTTTAAAGTGATGAATATATAAATTTTACGTGCTTTTTTGTATATTAGCT TTAATTTCCTGAATTATTTGTTTATTTTAAACAATATTATTGTCTAAATC CATTTGTCATATTGCAAAATGGCCGCAACAAGAAGATTACAAAAGGAA CTGGGGGATATCCGCAATTCAGGATTGAGATCCTTTAGAGAAATTCAAG TAGATGAAACTAATATTTTAACGTGGCAAGGATTAATAGTTCCGGATAA CGCTCCATACAACAAAGGAGCTTTTAAAATAGAAATAAATTTCCCTGCA GAATATCCCTTCAAACCACCAAAAATTAATTTTAAAACCAAGATATATC ATCCTAATATCGATGAGAAAGGGCAAGTGTGTTTACCTATTATTAGTGC TGAAAATTGGAAGCCGGCTACCAAAACTGATCAAGGTAATATTTATTTC AAC
Snf7	CGGACAATGACTCATAAAAGTTAATTACATGAAATTTTGAACTTTACTG TCCAATAAACACGCGCAAAAATTATAAACATTCTTGACTGCACTTGGTC GCACAAGTTCCCCTTTTGGTACCGATGGTAAATTATCAGGAACTGGTTC AACAACCAATAATTCTTTATCCAGTTCTTCTTGTTCCAATTCCTCCAACT CTTTCTCCAGTTCATCTTCGTCAATATCTTGACCAAAGGCCACCGGATTA CTAATCGCGTCCGAAATTTCTCTTGCAACATCTTGTTGTTCAGCGATATC GTCCATCATGTCATGAACCTGGTCGACGTCCCTGTTAAAATAGGTTTTT AGAAAGGTATTAATTTGGAAAGGAGTAAATAAAAGTACATATGTTTGT GAGCAGCTTTAAGGGCATCGGCAGCATTCTTCATAGTAGTGAGAACGG CAGTGTTGGTATTAGCACCCTCCAGAGCTTCTCTCTGCATTTCGATGGTG CTGAGGGTGCCATCGATTTGCTGCAGCTGTTTTTCATATCTCTTCTTGCG CTTTAAAGCCTGTATTGCCGCTAAAGTTATCATTAATCATCAATCAAAG CTAGATAAAAA

Using the Integrated DNA Technologies PrimerQuest tool (specified to an amplicon size of ~100 bp and a Tm of 60 ºC), primers for qRT-PCR were designed for both genes adjacent to the target region of the dsRNAs (Table 3). The *Actin* gene was selected as the reference gene, and primers were designed to the specifications above. However, as *Actin* is very conserved among species, the primers could be designed from the two beetle sequences because they were nearly identical (GenBank accession no. OU452307.1, *P. madidus*; OX380338.1, *P. niger*).

Table 3. Primers designed for use in qRT-PCR to determine relative gene expression in *P. melanarius*.

Gene	Primer sequence
UBE2L3	Forward: TCCAGTCAGTGAACACAGAAGT
	Reverse: CTGTCAAGCACGATTCTTTCAGG
Snf7	Forward: GTCATGAACCTGGTCGACGT
	Reverse: TGCCCTTAAAGCTGCTCACA
Actin	Forward: TACAGGGAGAGTACGGCCTG
	Reverse: GCTCCCCTCAACCCCAAG

Preparation of dsRNA

The excised posterior three abdominal segments of adult *P. melanarius* were homogenized in a lysis buffer solution prior to total RNA extraction using a GeneJET RNA purification kit (Thermo) according to the manufacturer's instructions. RNA (1 µg) was purified of genomic DNA contamination with TURBO DNase (Ambion), and cDNA was synthesized with qScript SuperMix (Quanta) according to the manufacturer's specifications. PCR was performed using Phusion DNA polymerase (Thermo), according to the manufacturer's specifications, with 1 ng of cDNA as the template and 0.5 µM of the forward and reverse degenerate primers. The PCR products were isolated by DNA fragment length using 1.5% agarose gel electrophoresis stained with ethidium bromide, and bands of the expected length were purified with an E.Z.N.A gel extraction kit (Quanta). Using a CloneJET PCR cloning kit (Thermo), purified PCR products were ligated into pJET plasmids and then transfected into DH5α *E. coli*. Colonies were cultured on LB-ampicillin agar plates, and successful transformants were selected. Single colonies were isolated, and plasmid DNA was extracted from the *E. coli* culture using an E.Z.N.A plasmid DNA mini kit (Quanta), and the DNA fragments were sequenced (shown in Table 2). With the DNA sequence identity confirmed, the previously used primers were

redesigned to incorporate T7 promoter sequences. These T7 primers were used in a PCR to amplify the DNA fragment within pJET, where the *in vitro* transcription of dsRNA was performed using a MEGAScript RNAi Kit (Invitrogen) according to the manufacturer's instructions. The concentration of dsRNA was measured using a Synergy H1 microplate reader (BioTek).

Injection bioassays

Four dsRNAs were injected into *P. melanarius*: *P. cruciferae*-specific *UBE2L3* and *Snf7* dsRNAs, *P. melanarius*-specific *UBE2L3* dsRNA, and green fluorescent protein dsRNA (referred to as *Pc UBE2L3*, *Pc Snf7*, *Pm UBE2L3*, and *gfp*, respectively). The *Pc UBE2L3*, *Pc Snf7*, and *gfp* dsRNAs were previously prepared in the lab. The *Pm UBE2L3* dsRNA served to validate the RNAi response in *P. melanarius* and to demonstrate the level of knockdown triggered by a perfect sequence match. *Pm Snf7* dsRNA was not used in the experiment to maintain a sufficient sample size, as there was a limited number of beetles. The *gfp* dsRNA, which has no target sequence in insects, was used as a negative control. Lastly, insects were injected with phosphate-buffered saline (PBS) to demonstrate mortality caused by injection as well as normal mortality throughout the bioassay.

Prior to injections, beetles were held on ice for up to 15 minutes, where they became motionless and easy to handle. Between 14 and 18 beetles were used for each treatment. Using 1 mL syringes and 26.5-gauge needles (Becton, Dickinson & Co., Franklin Lakes, USA), beetles were injected with 2 µg of dsRNA (10 µL diluted to a concentration of 200 ng/µL in PBS) into the ventral abdomen between the 3rd and 4th sterna. The beetles were then kept for seven days in separate 150 mL ventilated plastic containers prepared to similar maintenance conditions described above. Two days post-

injections, 9 beetles from each treatment were frozen at -80 ºC for later qRT-PCR analysis, while the survival of the remaining beetles was monitored for the following five days. Beetles that died within the first 24 hours after injection were excluded from the analysis, as their deaths were likely caused by injection-related injuries.

qRT-PCR

In preparation for qRT-PCR, total RNA extraction and cDNA synthesis were conducted on the frozen beetles, as described above. qRT-PCR was performed using a CFX Connect real-time system (BioRad) with reactions containing 7.5 µL of SsoFast master mix (BioRad), 1 µL of the forward and reverse qRT-PCR primers, 0.25 µL of the synthesized cDNA, and 5.25 µL of nuclease-free water. Melt curve analyses were performed at 46-95ºC for 5 seconds each step to verify that only a single PCR product was amplified for each primer pair. Three technical replicates were performed for each biological sample, and then an average Cq value was calculated for each sample. The relative expression of the targeted mRNAs for each treatment were calculated using the $2^{-\Delta Ct}$ method.

Statistical Analyses

Statistical analyses were conducted using RStudio (version 2024.12.0+467). p-values < 0.05 were considered significant. Welch's ANOVA was used to analyze *UBE2L3* expression data (3 groups), while Welch's t-test was used for *Snf7* expression data (2 groups). Dunnett's T3 post hoc tests were used to identify significant differences between groups. Values were presented as mean $\pm$ SE.

Results

Sequence alignment analysis

Alignments of *P. cruciferae UBE2L3* and *Snf7* dsRNA sequences with the corresponding orthologs in *P. melanarius* were performed to predict potential off-target knockdown from shared siRNAs. The alignment of the *UBE2L3* sequences showed an overall pairwise identity of 84.3%, along with a conserved contiguous sequence that is 29 bp long, which exceeds the minimal length required to produce fully matching siRNAs (Figure 2A). Of additional interest, multiple long matching sequences ($\geq$26 bp) were discovered, which were interrupted by fewer than three nucleotide mismatches. The longest identified was 50 bp (including the 29 bp sequence) with just two mismatches interspersed (Figure 2A). Conversely, analysis of the *Snf7* sequence alignment revealed a pairwise identity of 62.6%, with the longest conserved sequence being only 9 bp (Figure 2B). No further regions of interest were identified in the *Snf7* sequence alignment.

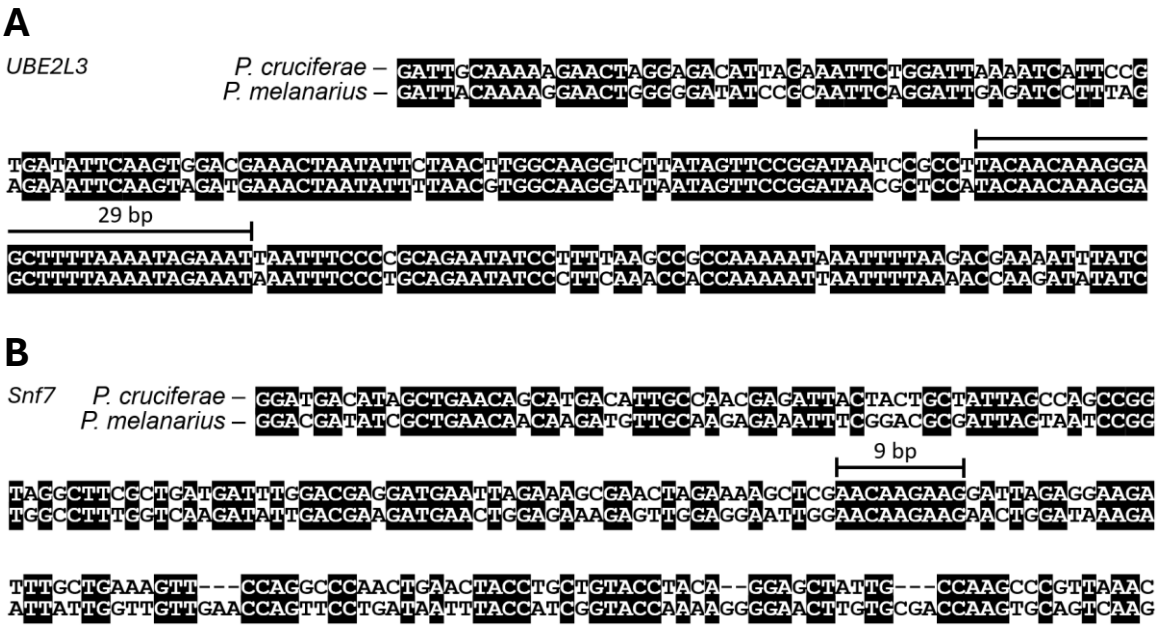

Figure 2. Sequence alignments of (**A**) *P. cruciferae UBE2L3* dsRNA (210 bp) and, (**B**) *P. cruciferae Snf7* dsRNA (212 bp) to the respective orthologs of *P. melanarius*. Letters highlighted in black indicate matching nucleotides between the two sequences. The longest continuous matching sequences are labelled (A, 29 bp; B, 9 bp). Alignments were done using Geneious (version 5.6.3).

Relative expression levels of target mRNA

To determine whether knockdown occurred in *P. melanarius* following the injection of the dsRNAs, the expression levels of the target genes, *UBE2L3* and *Snf7*, were measured relative to the fully expressed *Actin* gene (Figure 3). The level of transcripts of the targeted mRNAs in *gfp* dsRNA-treated insects were normalized to a value of 1.0, and all other treatments were subsequently compared to that value. The positive control *Pm UBE2L3* dsRNA, which shares 100% identity with the target gene, induced significant knockdown, reducing transcript levels by 92.3% ($p < 0.05$, ANOVA, Figure 3A). Injection of *Pc UBE2L3* dsRNA, which shares a high sequence identity (84.3%) and a 29 bp continuous match with *P. melanarius*'s *UBE2L3* sequence, resulted in a 69.9% transcript knockdown ($p < 0.05$, ANOVA, Figure 3A). In contrast, no significant difference in expression level was observed between the *Pc Snf7* dsRNA, which shares a lower identity (62.6%) and no siRNA-length sequences with *P. melanarius*'s *Snf7* gene, and the *gfp* dsRNA ($p > 0.1$, Welch's t-test, Figure 3B).

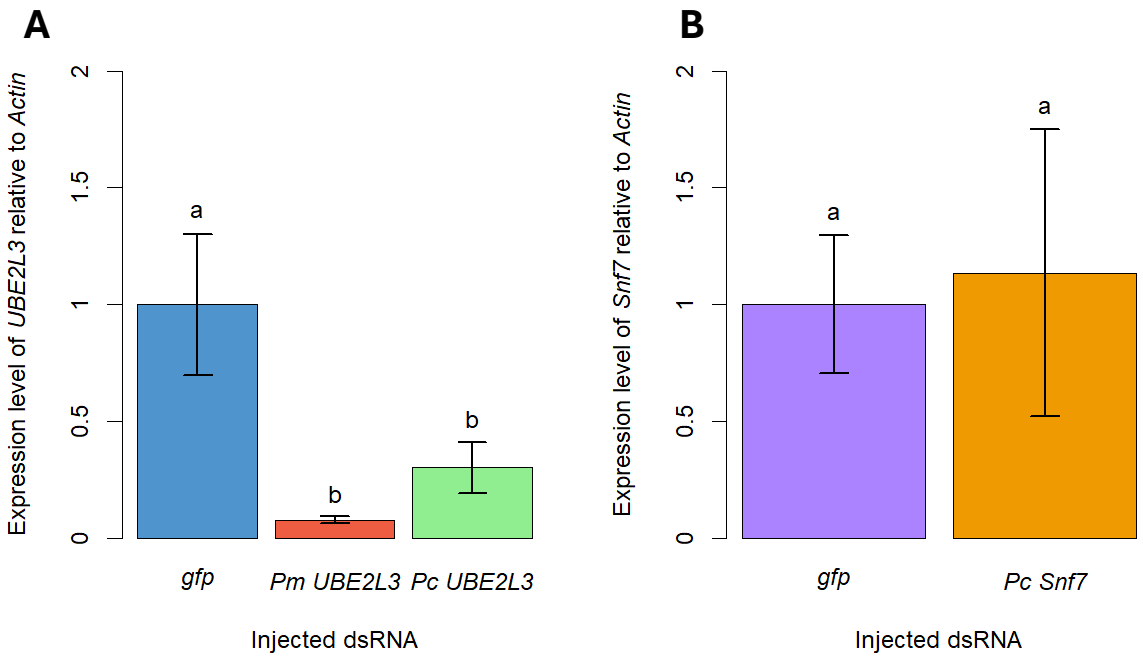

Figure 3. Expression levels of *UBE2L3* and *Snf7* transcripts relative to the reference gene (*Actin)* in *P. melanarius* 48h post-injection of dsRNA. (**A**) Relative *UBE2L3* transcript levels from *Pm UBE2L3* and *Pc UBE2L3* dsRNA, and (**B**) Relative *Snf7* transcript levels from *Pc Snf7* dsRNA (*Pm*, *P. melanarius*; *Pc*, *P. cruciferae*). Expression levels are compared to the normal levels of the control (*gfp*). Different letters (a, b) indicated values that are significantly different from one another (Dunnett's T3 post hoc test). Data are presented as the mean of nine replicates ($n = 9$), with error bars indicating ± standard error.

Mortality of P. melanarius

The mortality of *P. melanarius* was monitored for seven days following the injection of PBS or one of the four dsRNAs. Deaths that occurred within the first 24 hours post-injection were deemed to be due to injection-related injuries, whereas those that died after the first 24 hours were regarded as possible mortalities due to transcript knockdown. Among the 9 beetles that died during the bioassay, 7 succumbed to injection injuries, while only 2 died within the time frame to be considered due to transcript knockdown (Figure 4). Of those 2 beetles, one was injected with *Pc UBE2L3* dsRNA, and the other with PBS. Surprisingly, no beetles injected with the positive control *Pm UBE2L3* dsRNA or the two other treatments, *gfp* and *Pc Snf7* dsRNA, died after the first 24 hours. Given the small sample size for this experiment, with the majority of deaths attributed to just the injection process itself, it is not possible to determine whether the dsRNA injections had any significant impacts on the insects' survival.

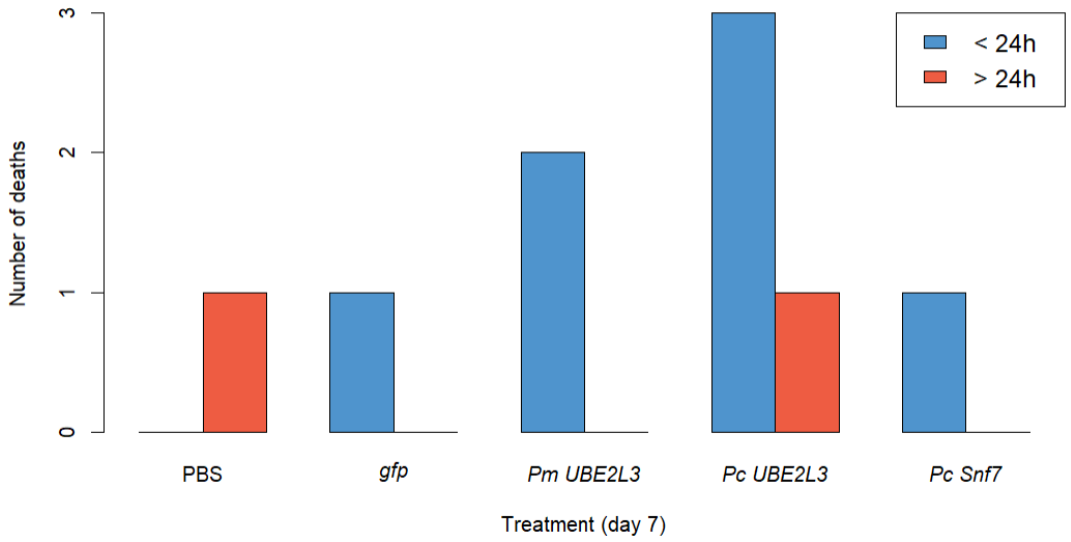

Figure 4. Number of *P. melanarius* that died less than 24h (blue) or greater than 24h (red) post-injection of dsRNA or PBS over a 7-day period. Values are presented as the raw number of deaths for each treatment (PBS, n = 5; *gfp*, n = 5; *Pm UBE2L3*, n = 5; *Pc UBE2L3*, n = 6; *Pc Snf7*, n = 6; *Pm*, *P. melanarius*; *Pc*, *P. cruciferae*). Beetles that died within 24h of injection were excluded from analysis.

Discussion

Environmentally safe pest control is a key part of an integrated pest management strategy, which aims to reduce the effects on non-target species (Barzman et al. 2015). RNAi has been proposed as a species-specific pest control method due to its ability to target exact gene sequences (Whyard et al. 2009). However, the extent of this specificity in real-world applications remains unclear, particularly regarding potential off-target effects on beneficial insects. In this study, the effects of two dsRNAs being considered for flea beetle control were tested on the predatory ground beetle *P. melanarius* which shares the same habitat. Initially, it was hypothesized that neither *P. cruciferae UBE2L3* nor *Snf7* dsRNA would induce gene knockdown or mortality in *P. melanarius*. However, a bioinformatic analysis revealed that the *Pc UBE2L3* dsRNA had a high level of sequence similarity to the orthologous gene in *P. melanarius*, while the *Pc Snf7* dsRNA exhibited little similarity. Based on this new information, the hypothesis was revised to predict that *Pc UBE2L3* dsRNA might cause a change in gene expression and mortality in *P. melanarius*, whereas *Pc Snf7* dsRNA would not. Significant gene knockdown was observed in beetles treated with *Pc UBE2L3* dsRNA, while those treated with *Pc Snf7* dsRNA showed no knockdown, which supports the revised hypothesis. However, neither dsRNA treatment resulted in significant mortality. These results highlight the importance of sequence identity in predicting off-target effects, demonstrating that a dsRNA does not require a perfect match to cause some degree of off-target gene knockdown.

To assess the RNAi response in *P. melanarius*, a dsRNA targeting the beetle's own *UBE2L3* gene was used as a positive control, resulting in a 92.3% reduction in transcript levels (Figure 3A). When *P. melanarius* was injected with *Pc UBE2L3* dsRNA, gene

expression was reduced by 69.9% (Figure 3A). This partial knockdown was likely due to the high sequence identity and contiguous 29 bp match shared between the *Pc UBE2L3* dsRNA and the *P. melanarius* ortholog (Figure 2A). Thus, the ribonuclease Dicer, a key enzyme in the RNAi pathway, was able to cleave segments of perfect or nearly perfect 21 bp siRNAs from the conserved region of the dsRNA (Bernstein et al. 2001). However, since the full sequence of *Pc UBE2L3* dsRNA does not perfectly match the *P. melanarius* ortholog, the level of knockdown observed was lower than that induced by the positive control. In contrast, *Pc Snf7* dsRNA did not cause significant gene knockdown, which was expected based on the low sequence similarity between the *Pc Snf7* dsRNA and the *P. melanarius* ortholog (Figure 2B, 3B).

These findings demonstrate that a perfect sequence match is not necessary for dsRNA to induce gene knockdown and that the degree of sequence identity can influence its extent. Previous studies have shown that in *T. castaneum*, RNAi can be triggered by dsRNAs with >80% sequence identity or by shorter segments with perfect (≥16 bp) or nearly perfect (≥26 bp) matches to the target gene (Chen et al. 2021; 2024). Similarly, in the western corn rootworm *Diabrotica virgifera virgifera*, significant gene knockdown was observed from non-target dsRNAs containing 21 bp matches (Bolognesi et al. 2012). RNAi has generally been most effective in Coleoptera (Baum et al. 2007). However, reports of the non-target effects vary across other insect orders. For instance, in Hymenoptera, no detectable knockdown was observed in *Bombus terrestris* or *Apis mellifera* when exposed to dsRNAs containing perfect 20 bp and 22 bp matches, respectively (Vélez et al. 2016; Taning et al. 2021). In contrast, off-target effects were reported in the Dipteran *Bactrocera minax* and the Lepidopteran *Manduca*

quinquemaculata when exposed to dsRNAs sharing 93% and 96% identity, respectively, with their target genes (Chen et al. 2015; Poreddy et al. 2017). There is a lack of consistency among studies on how sequence similarity is measured. However, these findings indicate that specific criteria for each insect group may be necessary to assess the off-target effects of dsRNA.

Within the seven-day bioassays, no significant mortality was observed, even in beetles injected with the positive control, which experienced 92.3% gene knockdown (Figure 4). This outcome suggests that the dose of dsRNA used may have been insufficient to induce lethal effects. Since the level of knockdown increases with a higher dose of dsRNA, one possible explanation for the lack of mortality is that the dose used was too low (Tomizawa and Noda 2013; Chen et al. 2024). The amount of dsRNA injected in this study (2 µg) was intended to represent a worst-case scenario, exceeding what insects would likely encounter in the field (Romeis et al. 2013). However, when considering the large body size of the beetle, this equates to approximately 0.01 µg dsRNA/mg body weight. By comparison, a previous study found that injecting 0.2 µg dsRNA/mg body weight into the larvae of the smaller beetle *Cylas brunneus* resulted in mortality (Christiaens et al. 2016). Another possible explanation for the lack of mortality is that the beetles were able to recover gene expression after the dsRNA was eventually degraded (Niu et al. 2024). If this is the case, prolonged exposure to dsRNA, either through repeated injections or a feeding assay, could have sustained knockdown and potentially led to significant mortality.

Rather than representing a worst-case scenario, the dsRNA dose used in this study may be more comparable to the small amount *P. melanarius* would be exposed to in a

field setting. For exposure to occur, *P. melanarius* would likely need to consume an insect that had already ingested the dsRNA. While it has been found that topically applied dsRNA can enter insects through the cuticle or inter-segmental membranes (Romeis and Widmer 2020), it is unlikely that dsRNA can sufficiently penetrate the thickened cuticle of beetles. Since flea beetles primarily feed on canola leaves, *P. melanarius*, which hunts on the ground, would have few opportunities to encounter a flea beetle that has ingested dsRNA. Instead, the exposure would most likely occur if a treated flea beetle died and fell to the ground, requiring the dsRNA to persist in the insect long enough for *P. melanarius* to ingest it. One study found that dsRNA can persist in dead planthoppers (*Laodelphax striatellus*) with a half-life of 1.5 hours (Zhang et al. 2022). It was also reported that gene knockdown could occur in the spider *Pardosa pseudoannulata* after it consumed *L. striatellus* containing dsRNA (Zhang et al. 2022). Additionally, research found that dsRNA fed to *A. mellifera* could be transferred to the parasitic mite *Varroa destructor*, further demonstrating that dsRNA can be exchanged between insects (Garbian et al. 2012). Many technologies are being developed to improve dsRNA stability, which may enhance the likelihood of secondary exposure by allowing the molecule to persist longer (Silver et al. 2021). Furthermore, *P. melanarius* may also prey on other canola pests that have ingested dsRNA, such as the diamondback moth *Plutella xylostella*, providing an additional route of transfer. However, it remains unclear how many treated insects *P. melanarius* would need to consume to receive a significant dose of dsRNA. A future study could investigate this question by feeding *P. melanarius* insects that have ingested dsRNA or been injected with a measured quantity of dsRNA.

Although mortality is the primary goal of insecticidal treatments, many sublethal effects often go unmeasured (Roberts et al. 2020). These effects, which include changes in development, fecundity, and behaviour, can be difficult to assess because they require information specific to each species (Roberts et al. 2020). In this study, reduced expression of the *UBE2L3* gene was observed in *P. melanarius*, but it did not result in mortality. Furthermore, potential sublethal effects were not evaluated. Since ubiquitination is crucial for cellular function, even a small reduction in efficiency could have serious effects over a long period of time. For example, in *Drosophila melanogaster*, reduced expression of a related protein in the ubiquitination pathway was found to disrupt cell signalling essential for development (Zhang et al. 2021). Therefore, *P. melanarius* may experience sublethal effects if continuously exposed to small doses of *Pc UBE2L3* dsRNA.

Overall, this study demonstrated that while dsRNA can induce gene knockdown in non-target insects, its effects vary depending on sequence similarity. Significant gene knockdown was induced by the *P. cruciferae UBE2L3* dsRNA when injected into *P. melanarius*, but no mortality occurred, and potential sublethal effects remain unknown. The results suggest that *Pc Snf7* dsRNA is a more suitable choice for flea beetle control because it did not induce knockdown in *P. melanarius*. However, the *Pc UBE2L3* dsRNA could potentially be redesigned to target a less conserved region of the gene, reducing the risk of off-target effects. Additionally, further genome sequencing of beneficial insects is needed to improve the specificity of designed dsRNAs. Furthermore, this study confirmed that a bioinformatic analysis can be used to predict potential off-target effects of dsRNA, ensuring species-specificity and minimizing the impact on beneficial insects.

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