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Knockdown of RNA interference pathway genes in western corn rootworm, *Diabrotica virgifera virgifera*, identifies no fitness costs associated with *Argonaute 2* or *Dicer-2*

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Abstract

The use of transgenic crops that induce silencing of essential genes using double-stranded RNA (dsRNA) through RNA interference (RNAi) in western corn rootworm, *Diabrotica virgifera virgifera*, is likely to be an important component of new technologies for the control of this important corn pest. Previous studies have demonstrated that the dsRNA response in *D. v. virgifera* depends on the presence of RNAi pathway genes including *Dicer-2* and *Argonaute 2*, and that down-regulation of these genes limits the lethality of environmental dsRNA. A potential resistance mechanism to lethal dsRNA may involve loss of function of RNAi pathway genes. However, the potential for resistance to evolve may depend on whether these pathway genes have essential functions such that the loss of function of core proteins in the RNAi pathway will have fitness costs in *D. v. virgifera*. Fitness costs associated with potential resistance mechanisms have a central role in determining how resistance can evolve to RNAi technologies in western corn rootworm. We evaluated the effect of dsRNA and microRNA pathway gene knockdown on the development of *D. v. virgifera* larvae through short-term and long-term exposures to dsRNA for *Dicer* and *Argonaute* genes. Downregulation of *Argonaute 2*, *Dicer-2, Dicer-1* did not significantly affect larval survivorship or development through short and long-term exposure to dsRNA. However, downregulation of *Argonaute 1* reduced larval

survivorship and delayed development. The implications of these results as they relate to *D. v. virgifera* resistance to lethal dsRNA are discussed.

Keywords: RNAi, *Diabrotica,* Rootworm, Fitness, Dicer, Argonaute, Drosha

Abbreviations

ANOVA	analysis of variance
dsRNA	double-stranded RNA
F	F statistic
qRT-PCR	quantitative real-time polymerase chain reaction
p	probability value; RQ, relative expression
RNAi	RNA interference
SEM	standard error of the mean

1. Introduction

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is the most devastating pest of corn (*Zea mays* L.) throughout U.S [1,2]. One of the main challenges in managing *D. v. virgifera* is its ability to adapt and evolve resistance to different pest management strategies [1,3,4]. *D. v. virgifera* has evolved resistance to a variety of insecticide classes [5], crop rotation [6], and more recently to some insecticidal proteins from *Bacillus thuringiensis* (Bt) [3,7]. The diversification of management options and implementation of resistance management strategies are critical to maintain the efficacy of current and emerging pest management strategies for *D. v. virgifera* [8–10].

Transgenic crops that induce silencing of essential genes through RNA interference (RNAi) in *D. v. virgifera* represent one of the most promising new technologies for control of this pest [9,11–13]. RNAi is a gene silencing mechanism in eukaryotic cells and essential as a defense mechanism against viral infection and regulation of gene expression [14,15]. RNAi has been widely used to study gene function in numerous insect species and more recently as a potential tool for insect control [9,16–18]. Ingestion of environmental dsRNA in coleopteran species elicits a robust gene silencing response through the RNAi pathway [12,19–24]. *D. v. virgifera* demonstrates the potential to use RNAi as a novel mode of action for pest management [25–28]. Baum et al. [19] showed that dsRNAs delivered *in planta* and in artificial diet cause significant mortality in *D. v. virgifera.* These authors indicate that targeting vacuolar ATPase orthologs A and E, which encode proteins essential to a number of cellular processes, through ingestion of dsRNA caused significant stunting and mortality of *D. v. virgifera* larvae. Further studies reported that dsRNA targeting *Snf7*, a gene involved in membrane trafficking, can be as effective as dsRNA targeting *V-ATPase*

A in controlling *D. v. virgifera* and *Diabrotica undecimpunctata howardi* larvae [12,29]; *V-ATPase C* [22], *snakeskin* (*ssk*) and *mesh* have also successfully provided RNAi-based protection of corn roots from rootworm injury [21].

To successfully suppress the expression of a particular gene, the insect RNAi pathways require synchronized functions of several proteins [28]. Dicer and Argonaute are two of the core protein families with essential roles in the RNAi pathway [28,30,31]. Dicer works in the initial steps of the pathway by dicing dsRNA to produce small RNAs of approximately 22 bp that include small interfering RNAs (siRNAs) or microRNAs (miRNAs) [32]. Insects have two Dicers and their activities have been postulated based on their counterparts in *Drosophila*. The *Drosophila* Dicer-2 is involved in processing dsRNA, and Dicer-1 in biogenesis of miRNA [25,27]. Argonaute proteins provide the catalytic activity to the RNA-induced Silencing Complex (RISC) [34] that facilitates degradation of the target mRNA [30,35–37]. Argonaute 2 is the "Slicer" component of the RISC, responsible for the cleavage of mRNA targets in mammalian cells [35]; its function is the same in *Drosophila* [34,38–40]. On the other hand, Argonaute 1 in *Drosophila* functions primarily to repress miRNA translation [39–41].

Genomic analyses have identified genes encoding these core proteins of the RNAi pathway in numerous insect species [42–45]. Vélez et al. [46] and Miyata et al. [47] identified Dicer-2 and Argonaute 2 in *D. v. virgifera* and characterized their contribution to the dsRNA-mediated RNAi response. Vélez et al. [46] found that the suppression of these pathway genes in *D. v. virgifera* reduced adult mortality and negated gene knockdown after subsequent exposure to lethal concentrations of *V-ATPase A* dsRNA, demonstrating key roles for Dicer-2 and Argonaute 2 in the dsRNA pathway in *D. v. virgifera*.

Based on the demonstrated ability of *D. v. virgifera* to rapidly evolve resistance to different pest management strategies, resistance management for RNAi transgenic technologies is of increased concern. Variation in the phenotypic response after exposure to dsRNA across *D. v. virgifera* populations suggests that genetic and physiological differences can affect the effectiveness of lethal dsRNA in the field [48]. Zhang et al. [49] proposed that low abundance of core enzymes in the RNAi pathway, such as Dicer and Argonaute, might limit the efficacy and lethality of environmental dsRNA. A possible role of RNAi pathway genes in resistance evolution is dependent on whether the reduction in the abundance of core RNAi proteins will adversely affect reproductive fitness in the absence of selection. In *D. v. virgifera*, silencing *Dicer-2* or *Argonaute 2* did not cause significant adult mortality [46] after relatively short exposure. However, it is unknown whether a longer-term reduction in the abundance of these proteins will affect mortality and other life-table parameters in *D. v. virgifera*.

In this study, in addition to *D. v. virgifera* Dicer-2 and Argonaute 2, we included Dicer-1 and Argonaute 1 of the miRNA pathway. In *Drosophila*, Argonaute 2 and Dicer 2 proteins have important functions in controlling viral infections through the RNAi pathway [50,51], while Dicer-1 and Argonaute 1 play roles in the regulation of processes such as embryonic development [41] through the microRNA pathway [25,39,52]. Life-history costs associated with potential mechanisms that insects could use to tolerate lethal dsRNA have a central role in estimating the potential for evolution of resistance to RNAi technologies in *D. v. virgifera*. Additionally, comparison of fitness costs between dsRNA and miRNA may help to delineate the relative roles of these two pathways and identify one that may be less prone to resistance. In this study we evaluated the effect of reducing the gene expression for core dsRNA and pathway genes *Dicer-1, Dicer-2, Argonaute 1*, and *Argonaute* 2 in *D. v. virgifera* larval survivorship and development. We show that of the four interrogated genes, only *Argonaute 1* exhibited life-history costs after both long-term and the short-term knockdown treatments.

2. Methodology

2.1. Double stranded RNA (dsRNA) preparation

Total RNA was isolated from a pooled sample of *D. v. virgifera* adults, larvae and eggs to prepare cDNA. cDNA was made using the cloned AMV first-strand cDNA synthesis kit (cat. no. 12328-032 Invitrogen, CA, USA). A total of 500 ng of RNA from the pooled sample of *D. v. virgifera* was used to prepare cDNA using a high capacity reverse transcription kit (part number 4375575, Applied Biosystems, CA, USA). Sequence-specific primers were used for the cDNA reaction, conjugated to the T7 RNA polymerase promoter to amplify the fragments of *Argonaute 1, Argonaute 2, Dicer-1*, *Dicer-2* and GFP genes that were used later for dsRNA synthesis. Bands of the expected size of ~500 bp were extracted and purified using a Gel Extraction kit (Qiagen, Valencia, California). dsRNA for the five fragments were synthesized from 1 µg of the purified PCR products (500 bp) using the MEGAscript RNAi kit (cat. No. AM1626, Life Technologies, CA, USA). The concentration of dsRNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific Waltham, MA).

2.2. Insect bioassays

Non-diapausing *D. v. virgifera* eggs were purchased from Crop Characteristics Inc. (Farmington, MN). Eggs were incubated at 28 °C, 60% relative humidity, and in darkness for 12 days. Eggs were washed from the soil using

a 60 µm sieve with distilled water and transferred to 40 ml of a 5% MgSO4 solution. Floating eggs were transferred to moistened filter paper in a small Petri dish. Dishes were checked daily for neonates. Insects from three different cohorts were used for each pathway gene bioassay.

To evaluate the effect of pathway genes on *D. v. virgifera* larval survivorship, neonates were exposed to dsRNA for pathway genes on treated artificial diet. Treatments consisted of dsRNA for *Argonaute 1, Argonaute 2, Dicer-1, and Dicer-2*. Experiments of dsRNA for *Argonaute* and Dicer were run together as paired subsets with each subset having its own controls that corresponded to H2O and GFP. Diet pellets of 4mm diameter and 3.5mm height were coated with 4 µl of 200 ng/µl of dsRNA for each gene target. Four pellets were placed in Petri dishes of 47mm diameter, lined with filter paper. To maintain moisture in each experimental unit, filter papers were moistened with 300 µl of sterile distilled water before pellets and larvae were transferred. Approximately 20–30 larvae were transferred to each Petri dish, depending on insect availability.

The effect of pathway gene knockdown was evaluated after two-day (short-term) and seven-day (long-term) exposure to dsRNA for individual pathway genes. After each exposure time, five larvae were collected for gene expression analysis and five additional larvae were transferred to untreated corn seedlings four to five days after planting to allow larval growth to later instars. Corn seedlings were grown in vermiculite in 50 ml falcon tubes and placed in an environmental chamber at 28 °C, 60% humidity, 16:8 light: dark conditions. Three seedlings were placed in each tube near the top (40 ml mark). Plants were watered with 2 ml of sterile water every two days. For the short term exposure treatment, larval survivorship was evaluated at the time of transfer to corn seedlings (48 h) and at 12 days after exposure (Fig. 1A). For the long term exposure treatment, survivorship was evaluated at 3, 5, 7, and 14 days after exposure (Fig. 1B). Effects on development were evaluated by establishing the larval instar at seven days after continuous exposure to dsRNA of the pathway genes (N = 12 per treatment). Instar was determined using a dissecting microscope that was fitted with an ocular micrometer at 40× magnification. Following the methodology of Hammack, Ellsbury, Roehrdanz and Pikul Jr [53], larvae with head capsules smaller than 270 µm were classified as first instars, larger than 270 µm were characterized as second instars, and >410 µm as third instars.

In addition to Dicer-1 and Argonaute 1 which are putative components in the microRNA (miRNA) pathway [39,54], an additional experiment was conducted to determine the effect of *Drosha* on larval survivorship. Drosha has also been associated with the miRNA pathway. The experiment was performed under the seven-day exposure methodology described above (Fig. 1B). The effects of *Drosha* were evaluated on two different *D. v. virgifera* cohorts, with three replications per cohort.
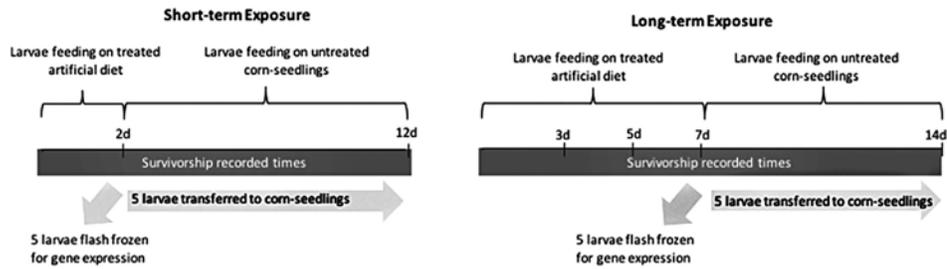
Fig. 1. General bioassay design used to evaluate survivorship and gene expression of RNAi pathway genes in D. v. virgifera larvae. A. short-term exposure larval bioassay. The larvae were kept on artificial diet treated with dsRNA of the pathway gene, GFP or water control for two days, before being collected for transcript level analysis or transferred to maize seedlings for further development. B. Long-term exposure larval bioassay. The larvae were kept on artificial diet treated with dsRNA of the pathway gene, GFP or water control for seven days, before being collected for transcript level analysis or transferred to corn seedlings for further development. Artificial diet treated with dsRNA or control treatments was changed each time survivorship was recorded.

2.3. Real-time PCR

Total RNA was isolated from *D. v. virgifera* larvae using the RNAquous Micro total RNA isolation kit (ThermoFisher Scientific, Waltham, MA USA). 500 ng total RNA was used to synthetize cDNA for qRT-PCR. qRT-PCR analysis was performed in a similar manner as described previously [55]. The Primers used for qRT-PCR were designed via PrimerQuest from Integrated DNA Technologies (Table 1). The primers were validated using standard curves based on serial dilutions of cDNA to determine the primer annealing efficiencies. A no-template control was included in each experiment to check for contamination. The total of five larvae per replication were analyzed with two technical replications. SsoADvanced™ Universal SYBR Green supermix kit (Biorad, Hercules, CA) and a BioRad CFX96 Real time system C100 Touch thermal cycler were used in the qRT-PCR analysis. Relative quantification of the transcripts were calculated using the comparative $2_T^{-\Delta\Delta C}$ method [56] and were normalized to β-actin [24]. The specificity of qRT-PCR was confirmed by melting-curve analyses after each reaction.

Table 1. Sequences and product length for primers used in synthesis of dsRNA and primers for expression analysis by qRT-PCR.

	[a] Primer sequences 5'–3'	Real-time PCR Primer sequences 5'–3' (IDT website, qPCR, 2 primers and intercalating dye) one primer outside dsRNA region, two primers spanning 2 exons.
Dvv Ago1 cDNA	DvvAgo1F (778 bp of cDNA) TAATACGACTCACTATAGGGAG GGTGGTAGAGAAGTCTGGTTTG DvvAgo1R (1284 bp of cDNA) TAATACGACTCACTATAGGGAG CGAAGTCTGCATGTCCGTTA	rtDvvAgo1F2 (1744) AAGTCCACCTTCCAGTCTTTG rtDvvAgo1R2 (1850) TGGCCATTCCTAACACAGTATC
Dvv Ago2 cDNA	DvvAgo2F (1045 bp of cDNA) TAATACGACTCACTATAGGGAG TATCCTCAGATGCCGACACTA DvvAgo2R (1544 bp of cDNA) TAATACGACTCACTATAGGGAG GGTTGTTCTGCTTCACCAATC	rtDvvAgo2F2 (2740) CCGACGTACTATGCCCATTTAG rtDvvAgo2R2 (2593) CTTCTGGATACTGTCCTGGATTT
Dvv Dcr1 cDNA	DvvDcr1F (4645 bp of cDNA) TAATACGACTCACTATAGGGAG AGGCTACCAGATGATGGTTATG DbDcr1R (5143 bp of cDNA) TAATACGACTCACTATAGGGAG TTTCCTCACATTCGGTCTCTAC	rtDvvDcr1F2 (3136) GTTGCTGAGGCTCTCAGATTAG rtDvvDcr1R2 (3250) CTCTTCCTCCCTGGATTTCTTG
Dvv Dcr2 cDNA	vvDcr2F (155 bp of cDNA) TAATACGACTCACTATAGGGAG CATACAGTGAGGGCGGTAAA DvvDcr2R (648 bp of cDNA) TAATACGACTCACTATAGGGAG TTCCTGAGGGTTTGTGGAATAG	rtDvvDcr2F2 (3372) AGTTCAACCAGACGAGAAAGG rtDvvDcr2R2 (3472) GGTTTCCAGACGTTCCAGATTA
Dvv Drosha cDNA	DvvDroF (1594 bp of cDNA) TAATACGACTCACTATAGGGAG TGCTCCCAGTTCCATTTCC DvvDroR (2094 bp of cDNA) TAATACGACTCACTATAGGGAG CACAGTCACGTCTCTCTTCATC	rtDvvDroF3 (1502) TGCGTGAGCTGGAATTGT rtDvvDroR3 (1624) GAATCTCGGCAGGAAATGGA

a. T7 sequences (TAATACGACTCACTATAGGGAG) added to 5' end of oligonucleotide sequence for dsRNA amplification and transcription as described in the manufacturer's protocol (MEGAScript, Ambion).

2.4. Data analysis

Data on survivorship, development, and gene expression after exposure to each pathway gene was analyzed using an ANOVA with a generalized linear mixed model. Random variables include the replication per insect cohort. Means of treatments were compared by the Fisher's least significant difference test. All statistical analyses were performed using the statistical package SAS/STAT software version 9.1.3 (SAS Institute Inc., Cary NC 2004).

3. Results

3.1. Impact of Dicer and Argonaute knockdown on larval survivorship after short-term exposure to dsRNA

Relative gene expression in *D. v. virgifera* larvae after 48 h of exposure to dsRNA showed a significant reduction of all pathway genes relative to control larvae ($p < .0001$) (Fig. 2). These results confirm that expression of the respective genes was reduced when larvae were transferred to seedling corn to monitor further survivorship. Knockdown levels for *Argonaute 1, Argonaute 2* and *Dicer-2* were 66%, 71%, and 70%, respectively after 48 h of exposure to dsRNA for each gene (Fig. 2A, B and D). *Dicer-1* knockdown was only 45% after 48 h of exposure (Fig. 2C).
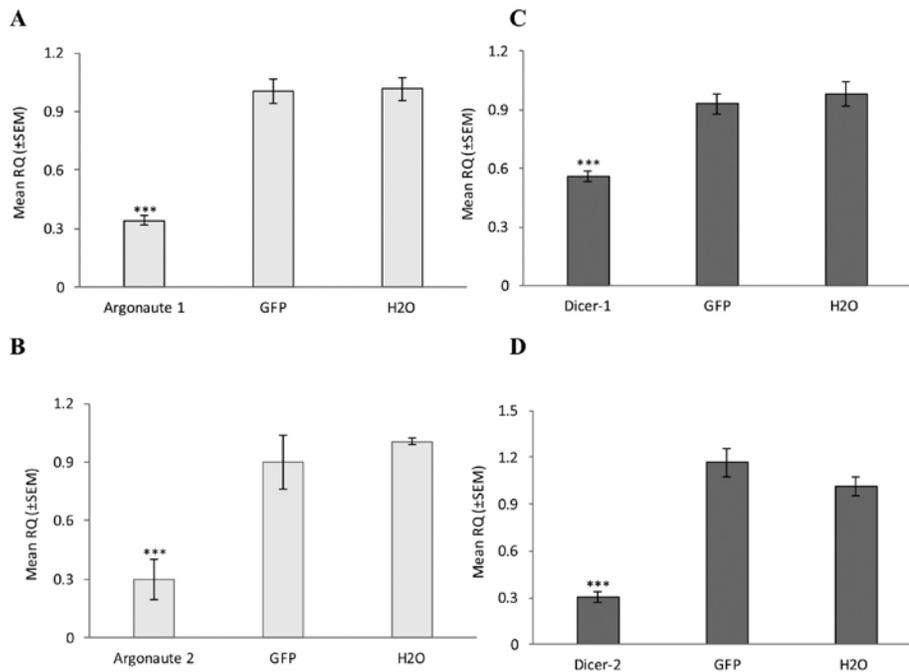

Fig. 2. Relative gene expression (RQ) of RNAi pathway genes in *D. v. virgifera* larvae, following dsRNA treatment after 48 h of exposure. Newly emerged larvae were fed with 4 μl of 200 ng/μl dsRNA per diet pellet, or same volume of water as a control. At 48 h, larvae were collected and flash frozen on dry ice and were used for expression analysis using qRT-PCR. **A.** RQ of *Argonaute 1*; F = 56.03; $p < .0001$. **B.** RQ of *Argonaute 2*; F = 14.67; $p < .0001$. **C.** RQ of *Dicer-1;* F = 12.05; $p < .0001$. **D.** RQ of *Dicer-2*; F = 35.13; $p < .0001$. (***: $p \leq .001$; insignificant differences are not annotated ($p > .05$). Error bars represent the standard error of the mean (SEM).

Effect of *Dicer* and *Argonaute* knockdown on survivorship of *D. v. virgifera* larvae after 48 h of exposure was not significantly different form the control treatments (H_2O or GFP) ($p > .05$) (Fig. 3A and B). However, after ten additional days of feeding on seedling corn roots, neonates exposed to *Argonaute 1* dsRNA exhibited significantly reduced survival compared to the controls and other dsRNA treatments (Fig. 3C), suggesting that *Argonaute 1* dsRNA-induced mortality may be associated with later stages of development. *Argonaute 2, Dicer-1,* or *Dicer-2* dsRNA treatments did not decrease the survivorship, even at twelve days of dsRNA exposure when compared to H_2O and GFP controls (Fig. 3C and D).
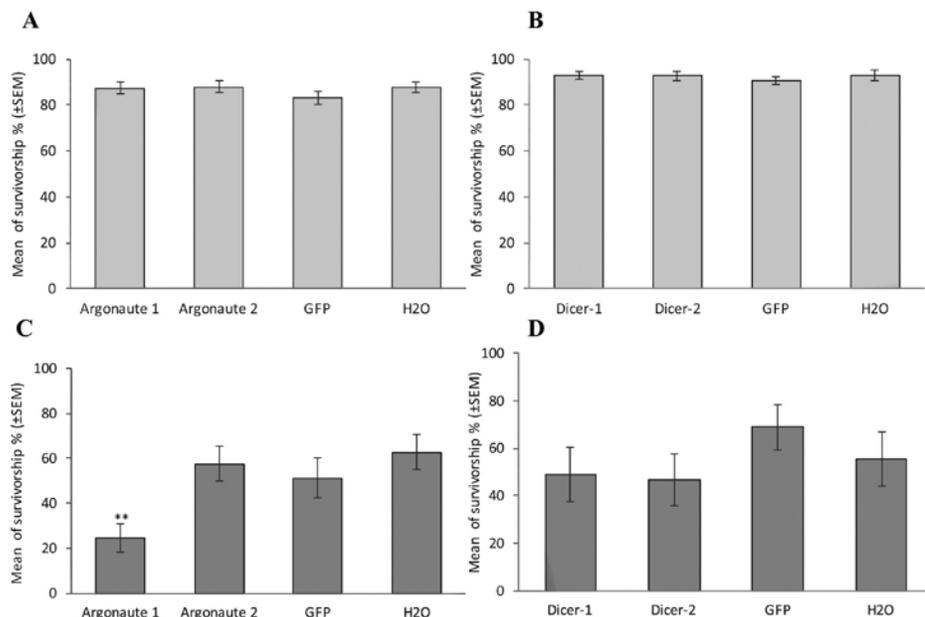

Fig. 3. Mean survivorship of *D. v. virgifera* larvae after 48 h of oral exposure (short-term exposure) to Argonaute and Dicer RNAi pathway genes. Error bars represent the standard error of the mean. **A.** and **B.** represent survivorship of *D. v. virgifera* larvae after 48 h of exposure in artificial diet. **C.** and **D.** represent survivorship of *D. v. virgifera* larvae at twelve days in corn seedlings after the initial 48 h of exposure to dsRNA. All analyses were performed using a generalized linear mixed model with binomial distribution. ANOVA and F-test for mean treatment comparison. (Differences representations **: $p \leq .01$; no significant differences have no asterisk representation $p > .05$). **A:** F = 0.85; $p = .475$. **B:** F = 0.27; $p = .84$; **C:** F = 4.39; $p = . 0083$. **D:** F = 0.91; $p = .448$.

3.2. Effect of the knockdown of Dicer and Argonaute on larval survivorship and development after long-term exposure to dsRNA

After seven days of continuous exposure to dsRNA on artificial diet, qRT PCR analysis indicated a significant reduction in expression of all targeted pathway genes relative to larvae on control diets (Fig. 4) Knockdown levels for *Argonaute 1* and *Argonaute 2* were 75% and 94%, respectively (Fig. 4A and B), while knockdown levels of *Dicer-1* and *Dicer-2* were 69% and 68%, respectively (Fig. 4C and D). These results suggest that the additional exposure time resulted in increased levels of knockdown (Figs. 2 and 4) but that the increases were relatively small.

Survivorship of *D. v. virgifera* larvae over the seven days of continuous exposure to *Argonaute 2*, *Dicer-1*, or *Dicer-2* dsRNA followed by ten days on seedling corn did not differ significantly from that of the H_2O and GFP
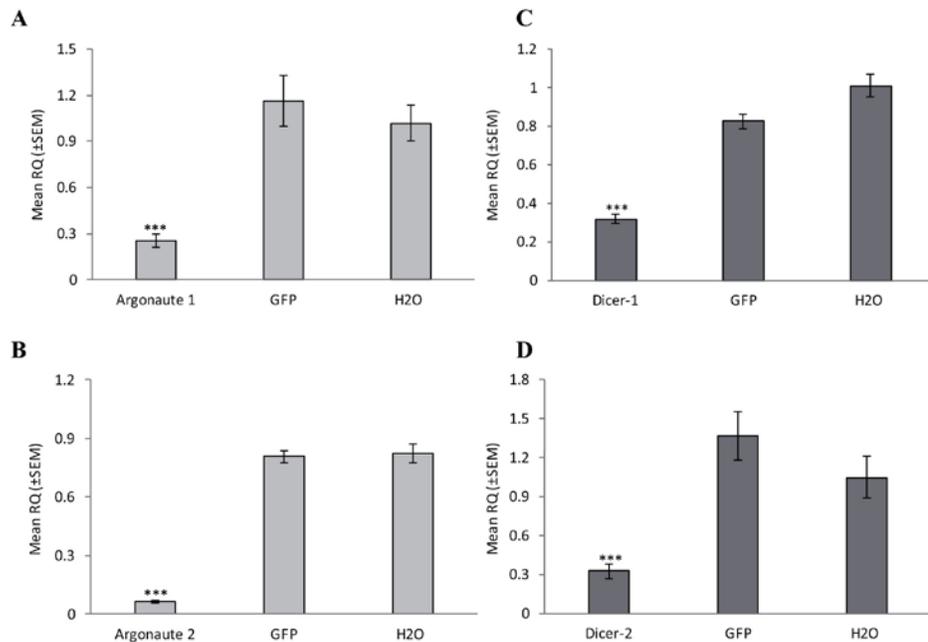

Fig. 4. Relative expression (RQ) of RNAi pathway genes in *D. v. virgifera* larvae, following dsRNA treatment after seven days of exposure. Newly emerged larvae were fed with 4 µl of 200 ng/µl dsRNA per diet pellet, or same volume of water as a control. At 48 h, larvae were collected and flash frozen on dry ice and were used for expression analysis using qRT-PCR. **A.** Relative expression of *Argonaute 1*; F = 56.03; $p < .0001$. **B.** Relative expression of *Argonaute 2*; F = 14.67; $p < .0001$. **C.** Relative expression of *Dicer-1*; F = 12.05; $p < .0001$. **D.** Relative expression of *Dicer-2*; F = 35.13; $p < .0001$. (***: $p \leq .001$; insignificant differences are not annotated ($p > .05$)). Error bars represent the standard error of the mean (SEM).

control treatments (Fig. 5). However, larvae exposed to *Argonaute 1* dsRNA showed a significant reduction in larval survivorship. No larvae in the *Argonaute 1* treatment survived the additional seven days on seedling corn, whereas survivorship in the other treatments was > 50% (Fig. 5A).

Larvae at seven days after continuous exposure to dsRNA targeting RNAi pathway genes began to change from first to second instar (Fig. 6). There were no significant differences in the instar rating between *Argonaute 2*, *Dicer-2*, *Dicer-1,* and the control treatments of GFP and H_2O (Fig. 6). However, larval development in the *Argonaute 1* dsRNA treatments was significantly slower than the control treatments (Fig. 6A).

3.3. Effect of the knockdown of Drosha on larval survivorship

To further probe differential phenotypes observed for the miRNA pathway genes *Argonaute 1* and *Dicer-1*, we examined fitness costs associated with *Drosha*, the product of which cleaves miRNA primary transcript (pri-miRNA)
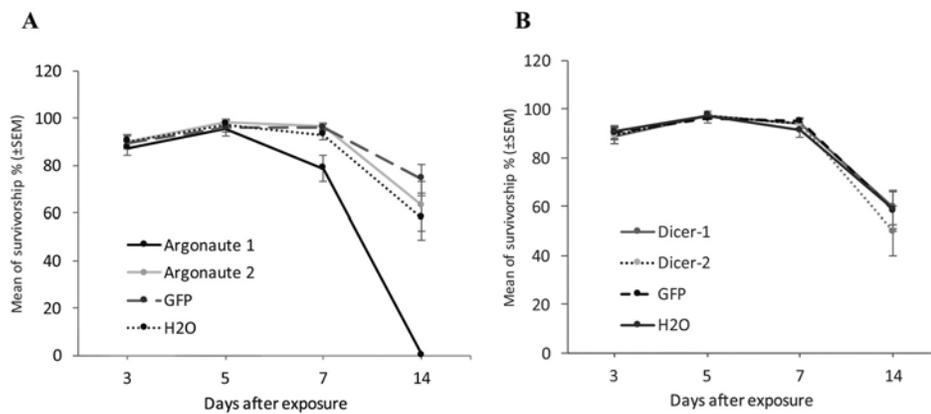

Fig. 5. Survivorship of *D. v. virgifera* larvae after seven days of oral exposure (long-term exposure) to Argonaute 1, Argonaute 2, Dicer-1, and Dicer-2 dsRNA. Panel **A** represents survivorship of *D. v. virgifera* larvae after continuous exposure to *Argonaute 1* and *Argonaute 2*. Panel (Treatment * Day: F = 14.91 *p* < .0001; At Day 7: *Argonaute 1* vs *Argonaute 2 p* = .0002, Ago1 vs GFP *p* = .0003, *Argonaute* 1 vs H_2O *p* = .004; At day 14: *Argonaute 1* vs *Argonaute 2 p* **Fig. 7.** Fitness parameters of *D. v. virgifera* larvae in response to reduced expression of Drosha after seven days of exposure to dsRNA. Panel **A** represents survivorship of larvae at seven days after treatment F = 1.08; *p* = .38. Panel **B** represents the relative gene expression of *Drosha* in larvae at eight days after continuous exposure to dsRNA F = 24.65; *p* = .001. Panel **C** represents instar at seven days after treatment F = 2.56; *p* = .096. Error bars represent the standard error of the mean (SEM). Insignificant differences are not annotated (*p* > .05).
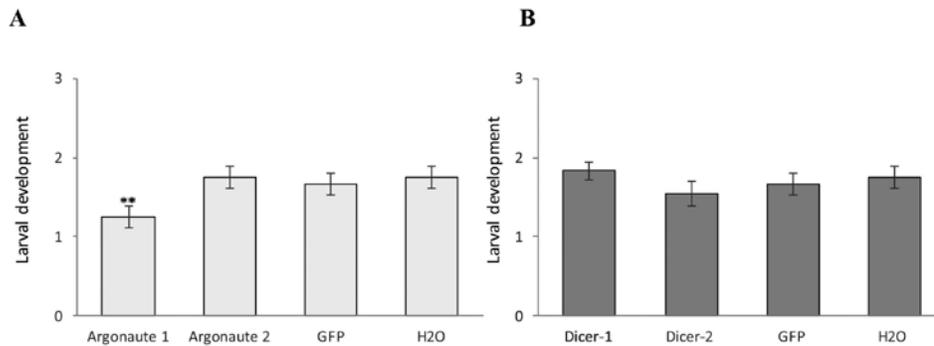
Fig. 6. Larval development of *D. v. virgifera* at seven days after exposure to pathway genes at 200 ng/µl with 4 µl per larval diet pellet. Head capsules of twelve larvae were measured to determine instar [53]. A. Larval development under *Argonaute 1* and *2* dsRNA exposure. F = 3.29; *p* = .0319. B. Larval development under *Dicer-1* and *-2* dsRNA exposure. F = 0.89; *p* = .4525 (***: *p* ≤ .001; insignificant differences are not annotated (*p* > .05). Differences of Treatment Least Squares Means *Argonaute 1* vs Dicer-1: *P* = .0006, *Argonaute 1* vs Dicer-2: *P* = .05. Error bars represent standard error of the mean (SEM).

to produce miRNA precursors (pre-miRNA), which are then then processed into mature miRNAs by Dicer-1 [57,58]. Relative gene expression levels of *Drosha* show significant transcript reduction in larvae treated with dsRNA for this pathway gene, when compared to the control treatments of GFP and H_2O (*p* = .0013). No significant differences were observed in survivorship or larval development between insects feeding on dsRNA for *Drosha* and H_2O or GFP (Fig. 7).

4. Discussion

Understanding the potential mechanisms of resistance to RNAi-based technologies provides important information for the development of insect resistance management (IRM) strategies that promote sustainability of this technology for the control of *D. v. virgifera*. Mechanisms of resistance with minimal fitness costs are anticipated to evolve more rapidly than those where there is a clear impact on reproductive success.

In earlier work, we showed that knockdown of *Argonaute 2* or *Dicer-2* decreases the efficacy of the RNAi response, evidenced by decreased knockdown of the reporter gene, *V-ATPase A,* and decreased levels of lethality [46]. Results from the present study indicate that downregulation of *Argonaute 2* and *Dicer-2* did not show a significant effect on larval
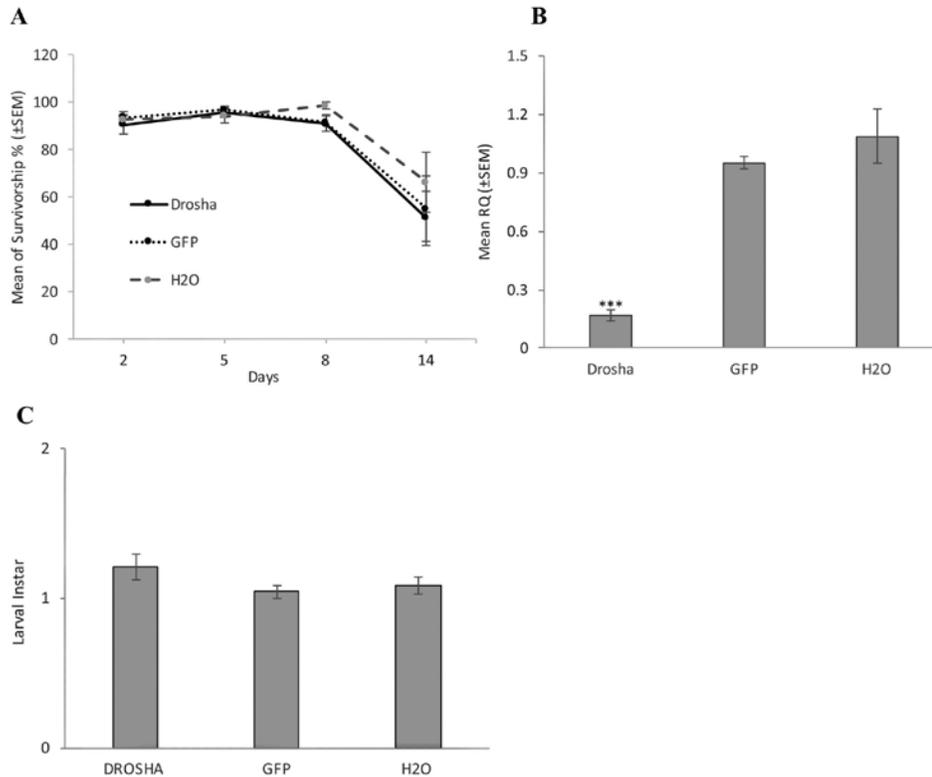
Fig. 7. Fitness parameters of *D. v. virgifera* larvae in response to reduced expression of Drosha after seven days of exposure to dsRNA. Panel **A** represents survivorship of larvae at seven days after treatment F = 1.08; p = .38. Panel **B** represents the relative gene expression of *Drosha* in larvae at eight days after continuous exposure to dsRNA F = 24.65; p = .001. Panel **C** represents instar at seven days after treatment F = 2.56; p = .096. Error bars represent the standard error of the mean (SEM). Insignificant differences are not annotated (p > .05).

survivorship and development through short- and long-term exposure to dsRNA. The absence of an effect of *Argonaute 2* and *Dicer-2* knockdown on fitness parameters in *D. v. virgifera* larvae suggests that a mutation affecting the expression or function of these pathway genes could confer resistance without major fitness costs. However, to date there is no evidence suggesting resistance to dsRNA will evolve in this manner. Given that the RNAi pathway plays an important role in the antiviral response in insects (reviewed in [59]), downregulation of these pathway genes might increase the susceptibility of *D. v. virgifera* to viral infection. Future studies examining downregulation of RNAi pathway genes in the presence of known rootworm viruses should clarify possible fitness costs that may accompany the loss of function of *Dicer* and *Argonaute* proteins. Moreover, because protein expression was not examined in the current investigation,

it is uncertain if the lack of fitness response to downregulation of *Dicer-2, Dicer-1* and *Argonaute 2* is caused by the maintenance of residual protein in the insect after exposure to dsRNA.

In contrast to the results observed for *Dicer-2, Dicer-1,* and *Argonaute 2*, exposure to dsRNA for *Argonaute 1* resulted in significantly reduced larval survivorship and development under both short- and long-term exposure conditions. The lethal effect of *Argonaute 1* knockdown may be associated with its key role in regulation of miRNAs essential for insect survival and development. In *Drosophila,* miRNAs reduce the stability and suppress the translation of mRNAs, lead to mRNA degradation [60–62], or target cleavage through association with Argonaute 2 [39]. MiRNAs are required for a wide range of functions including metabolic homeostasis, cell death, cellular proliferation and differentiation, oogenesis and embryonic development, and maintenance of germline stem cells [41,63–67]. Given the essential role of *Argonaute 1* in the miRNA pathway, regulation of essential metabolic and developmental processes by miRNA could be affected by downregulation of its expression.

In insects, deleterious effects on embryonic development have been observed in loss-of-function *Dicer-1* and *Argonaute 1* mutants [68]. However, in this study we did not observe any deleterious effects on larval survival and development after long- and short-term exposures to *Dicer-1* dsRNA. It is uncertain why the knockdown of *Dicer-1* does not phenocopy *Argonaute 1* knockdown. Similar results were observed with *D. v. virgifera* adults where knockdown of *Argonaute 1* caused significant mortality, and reduced oviposition and egg viability while adults exposed to *Dicer-1* dsRNA were unaffected [69]. A trivial explanation may be that residual Dicer-1 protein masks the life-history costs explored in this study. To this point, gene expression of *Dicer-1* was least affected by short- and long-term exposures to dsRNA, which can influence the level of the protein in the insect. There is also some overlap of function between Dicer-1 and Dicer-2 in regulating miRNAs [25]. Another possibility for discrepancy in *Argonaute 1* and *Dicer-1* knockdown phenotypes in *D. v. virgifera* larvae is that *Argonaute 1* plays a broader role in *D. v. virgifera* biology than *Dicer-1.* This idea is supported by recent research that identified Argonaute functions independent of Dicer-1 or miRNA [70]. For example, *Drosophila* Argonaute 1 can associate with and repress the translation of *nanos* in a miRNA-independent manner [71]. Consistent with the possibility that different proteins of the miRNA pathway may lead to different levels of fitness cost, our knockdown experiments with *Drosha* also showed no effect on the evaluated fitness parameters for *D. v. virgifera* larvae.

5. Conclusions

Overall, our work identified no life-history costs, as measured by larval survivorship and rate of development associated with the depletion of the key dsRNA pathway genes *Argonaute 2* and *Dicer-2*. Our data suggest the potential for *D. v. virgifera* to develop resistance to lethal dsRNAs through down-regulation or loss-of-function mutations of these pathway genes because no apparent fitness costs were associated with their knockdown. However, it is impossible to say whether this mode of resistance is likely to occur in the field until additional potential fitness costs are examined, including exposure to viral pathogens. Furthermore, it will be important to understand the variability and distribution of *Argonaute 2* and *Dicer-2* expression alleles. Swevers et al. [72] hypothesized that the genes in the RNAi pathway responsible for defense against invading dsRNA are dispensable. High maintenance costs (*e. g.,* production transgene-triggered siRNA targeting essential genes) could select for mutant or down-regulated RNAi pathway genes. Our results showing lack of fitness costs associated with the knockdown of *Arognaute 2*, *Dicer-1* or *Dicer-2* genes are consistent with this possibility. In contrast to *Arognaute 2*, *Dicer-1* and *Dicer-2*, our results indicate that *Argonaute 1* is essential in *D. v. virgifera* larvae.

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