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Research Article

Sublethal Effects of *vATPase-A* and *Snf7* dsRNAs on Biology of Southern Corn Rootworm, *Diabrotica undecimpunctata howardi* Barber

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Abstract

RNA interference is a powerful tool against corn rootworm. Adults and neonates of southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber (Coleoptera: Chrysomelidae), were exposed to the LC_{50} of *vATPase-A* and *Snf7* double-stranded RNAs (dsRNAs), and the effects on female fecundity, egg viability, male fitness as measured by sperm viability and mating capacity, larval recovery along with dry weight, and instar determination 10 d after exposure to dsRNA, were determined. Significant reductions were observed for a number of parameters in dsRNA-exposed rootworms relative to control treatments. Female fecundity and larval recovery were significantly reduced after exposure to both dsRNAs. In addition, larval dry weight and recovery of 2nd and 3rd instars along with dry weight for 3rd instars were significantly reduced after neonate exposure to *vATPase-A* dsRNA. Neither dsRNA affected male capacity to mate or sperm viability after exposure to the respective LC_{50}s. After 10 d of feeding on untreated corn roots, neonates that survived exposure for 2 d to the *vATPase-A* dsRNA LC_{50} exhibited lower dry weight than the control. There was significant gene knockdown in adult males and females after exposure for 5 d to LC_{50} of *vATPase-A* and *Snf7* dsRNAs. The parameters are discussed in terms of fitness and possible outcomes after deployment of corn hybrids expressing dsRNAs.

Key words: RNA interference, resistance management, fitness cost, vATPase-A, *Snf7*

RNA interference (RNAi) is a gene silencing technique that uses double-stranded RNA (dsRNA) to silence specific genes in eukaryotic organisms (Fire et al. 1998). Considered to be an important future pest control technique with a unique mode of action, RNAi is effective against both western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) and southern corn rootworm (SCR), *Diabrotica undecimpunctata howardi* Barber (Coleoptera: Chrysomelidae) larvae (Baum et al. 2007, Bolognesi et al. 2012, Bachman et al. 2013, Koci et al. 2014, Levine et al. 2015, Hu et al. 2016, Pereira et al. 2016) and WCR adults (Rangasamy and Siegfried 2012, Khajuria et al. 2015, Fishilevich et al. 2016, Pereira et al. 2016, Vélez et al. 2016).

The corn rootworm complex includes the WCR and northern corn rootworm (NCR), *Diabrotica barberi* Smith & Laurence (Coleoptera: Chrysomelidae), which are the most important corn insect pests throughout the U. S. Corn Belt. These species collectively have been estimated to cause over $1 billion dollars in control costs and yield loss annually (Krysan 1986, Gray et al. 2009). The SCR is a polyphagous plant pest that occurs mostly in the southern United States and migrates to the Corn Belt during early spring and throughout summer months (Arant 1929, Isely 1929, Krysan 1986). Oviposition of migrating SCR is partitioned over a number of hosts so emergence from field corn in the Corn Belt is often low relative to other *Diabrotica* species, thus causing minimal damage to corn plants (LJ Meinke, personal observation). SCR larvae feed on a wide variety of grasses although corn is the main host. Its host range also includes cucurbits, peanuts, soybeans, cotton seedlings, and dry beans (Isely 1929, Grayson and Poos 1947, Meinke et al. 1985, Krysan 1986).

Induction of RNAi-mediated gene suppression in insects via oral exposure requires efficient uptake of dsRNAs by midgut cells followed by suppression of the target mRNA leading to potential effects on biological and physiological parameters (Bachman et al. 2016). The use of RNAi plants expressing dsRNA pyramided with Bt toxins to control corn rootworm (MON 87411) has recently been registered by the Environmental Protection Agency (EPA 2017) and will be commercially available to corn growers in near future (Ahmad et al. 2016, Bachman et al. 2016, Head et al. 2017). Given

the recent evolution of WCR resistance to different corn hybrids expressing *Bacillus thuringiensis* Berliner traits (Gassmann et al. 2014, 2016; Wangila et al. 2015; Andow et al. 2016; Zukoff et al. 2016b; Ludwick et al. 2017) as well as to pyrethroid insecticides (Pereira et al. 2015, 2017), new technologies with unique mode of actions are urgently needed to mitigate existing resistance and to develop sustainable WCR management programs.

Toxicological studies of sublethal effects of synthetic pesticides have been intensively investigated in nontarget organisms especially pollinators and natural enemies (Desneux et al. 2007, Naranjo 2009). The fitness parameters usually measured in sublethal exposure bioassays involve behavioral and physiological responses, with reproduction and longevity the most widely studied (Desneux et al. 2007). Both larval and adult rootworms can potentially survive after exposure to sublethal concentrations of dsRNA expressed in different plant tissues (Bachman et al. 2016). Therefore, the objectives of this research were to determine if sublethal exposures to dsRNAs targeting *vATPase-A* and *Snf7*, which have previously been identified as lethal target genes and which function in membrane stability and trafficking, respectively (Pereira et al. 2016), affect fitness parameters in SCR that are exposed either as adults or neonates. The reason that SCR and not the most important corn pests in the Corn Belt, WCR or NCR, was used in this research is that its lifecycle is shorter as eggs do not go in obligatory diapause and hatch in 7–8 d compared to 12–14 d in the nondiapausing WCR (no nondiapausing NCR strain was available at the time of this research), SCR females produce twice as many eggs as WCR under laboratory rearing conditions (Jackson 1986), and there is a commercial artificial diet for SCR larvae which was used to expose neonates to both dsRNAs. Importantly, SCR can be reared continuously directly from the field without introgression into the nondiapause strain so that selection can be initiated directly from field populations. Parameters that were measured include: 1) female fitness (i.e., fecundity and egg viability), 2) male fitness (i.e., sperm viability and mating capacity), and 3) larval survival (i.e., recovery from untreated corn plants 10 d after neonate exposure to dsRNA, larval instar determined by measuring head capsule width, and dry weight).

Materials and Methods

Insects

SCR males and females were purchased from Crop Characteristics Inc. (Farmington, MN) and placed in 30 cm^3 BugDorm cages (MegaView Science Co., Ltd., Taichung, Taiwan), provided with agar-based artificial diet (Khajuria et al. 2015) and water in a 250-ml plastic bottle containing a 15-cm long cotton wick (Richmond Dental & Medical, Charlotte, SC) suspended through a hole in the lid, and changed twice per week. For larval experiments, SCR eggs were received from the same vendor and maintained in an incubator at 27°C until hatching.

Exposure of SCR to dsRNA

SCR adult males or females were exposed to previously estimated LC_{50}'s for *vATPase-A* (33.3 ng/cm^2) or *Snf7* (13.2 ng/cm^2) dsRNAs (Pereira et al. 2016) overlaid on artificial diet (Khajuria et al. 2015). Freshly treated diet was provided every other day until day 5; thus, they fed on dsRNA-treated diet for five consecutive days. Control beetles fed on untreated artificial diet only. For larval development assays (see below), SCR neonates were exposed to the LC_{50} of *vATPase-A* (10.0 ng/cm^2) or *Snf7* (3.0 ng/cm^2) dsRNAs for 24, 48, or 72 h overlaid on artificial diet (Frontier Agricultural Sciences, Newark, DE). Control exposures consisted of neonates fed for 3 d on untreated diet.

Larval Development After Neonate Exposure to dsRNA

Bioassays with *Snf7* and *vATPase-A* dsRNA were conducted at different times although methods for both were identical. Neonates (<36 h) were exposed to *vATPase-A* or *Snf7* dsRNAs on surface-treated artificial diet for 24, 48, and 72 h. Control larvae were fed untreated artificial diet for 72 h. After exposure, four larvae were transferred to 50-ml centrifuge tubes (VWR International, Radnor, PA) containing four germinated non-Bt and insecticide-free 'Reid's yellow dent' field corn seeds (Hancock Farm and Seed Co., Inc, Dade City, FL) planted in autoclaved vermiculite (Infinity Fertilizers, Inc., Milan, IL) 4–5 d prior to transfer. The seeds were sterilized in 10% bleach solution for 1 h, stirred every 15 min, rinsed, and dried for 24 h. Dried seeds were then treated with 2 mg of thiabendazole powder/100 g of corn seeds (Sigma-Aldrich Co. LLC., St. Louis, MO) before planting. One milliliter of Peters 20-20-20 soluble fertilizer (J R Peters, Allentown, PA) dissolved in distilled water (48 mg/ml) was placed in the tubes before filling with autoclaved vermiculite. Twenty replicates were used in each treatment for a total of 80 tubes per bioassay. Plants were grown in a chamber at 25°C and 80% relative humidity (RH). Larvae were allowed to feed on corn roots for 10 d and then recovered by removing plants and vermiculite from the tube and sorting on a dark background to estimate larval survival. Head capsule width (mm) was measured using an ocular micrometer to determine the larval instar (Arant 1929). Larvae were dried in a Precision Scientific Thelco 130D laboratory oven (Artisan Technology Group, Champaign, IL) for 24 h at 80°C and dry weight (mg) from each individual larva was recorded using an analytical balance (Sartorius Corporation, Bohemia, NY).

Male Fitness

Sperm Viability

SCR adult males (<48 h) were exposed to *vATPase-A* and *Snf7* dsRNAs for 5 d on surface-treated artificial diet as described previously. Control beetles were maintained on untreated artificial diet only. Males were anesthetized on ice, testes were dissected and placed in 1.5-ml microcentrifuge tubes containing 10 µl of buffer (HEPES 10 mM, NaCl 150 mM, BSA 10%, pH 7.4) and crushed using a 100-µl pipette tip. Sperm viability was immediately assessed using the Live/Dead Sperm Viability kit-L7011 (Molecular Probes, Eugene, OR). One microliter of SYBR 14 (0.1 mM in DMSO, as recommended by manufacturer) was used to stain live sperm and was added to the tube and incubated at room temperature for 10 min, followed by the addition of 1 µl of propidium iodide (2.4 mM) to stain dead sperm followed by a second incubation at room temperature for 10 min. Ten microliters of the sperm-stained solution was transferred to a 75 × 25 × 1 mm green frost micro slide (VWR International LLC, Radnor, PA) and covered with a 25 × 60 mm Fisherbrand cover glass (Fisher Scientific, Pittsburgh, PA) for photography. Images were recorded using an Olympus FV 500 Inverted IX-81 with Confocal Laser Scanning Microscope using 20× Lens Olympus LC Plan FI (Dry). Samples were visualized at 20× with 488 nm excitation, a 505–550 nm band pass for live sperm and 660–700 nm band pass for dead sperm simultaneously. Four males per treatment were dissected, and five digital images were recorded for each individual slide (Fig. 1). Experiments were performed in triplicate using three different cohorts, which resulted in 60 images/treatment. The numbers of live (green color) and dead (red color)

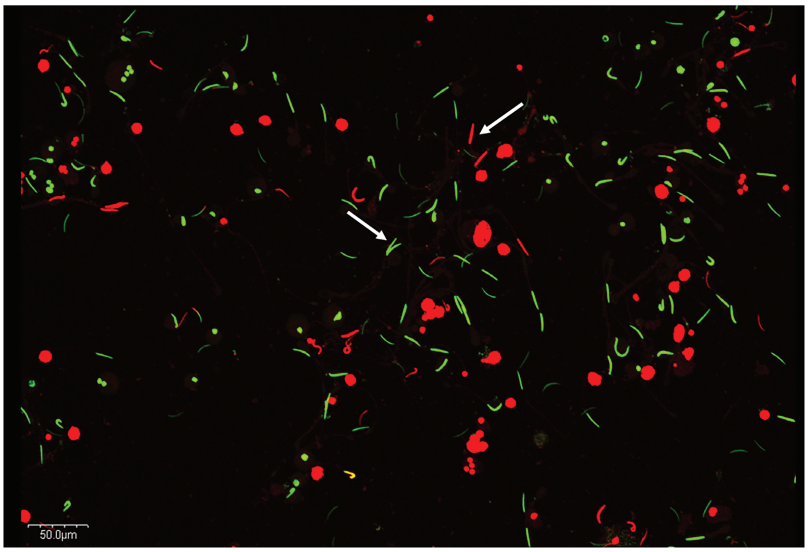

Fig. 1. Picture of SCR sperm taken on Olympus FV 500 Inverted IX-81 as described in Materials and Methods. Arrows show live (green) and dead (red) sperm cells.

sperm were counted (Fig. 1) using ImageJ software (Schneider et al. 2012; Johnson et al. 2013a,b).

Female Fecundity After Mating With Exposed Males

Bioassays with *Snf7* or *vATPase-A* dsRNA were conducted separately at different times using identical methods. Virgin SCR males were exposed to LC_{50}s for *vATPase-A* and *Snf7* dsRNAs, and 12–15 survivors were held in cages with 10 virgin females (<48 h after emergence) and allowed to mate for 1 wk. The cages consisted of 6.0 × 6.0 × 8.0 cm clear plastic boxes (Althor Products LLC, Windsor Locks, CT) lined with moistened gauze (12.8 cm × 6.0 cm) as oviposition substrate (Surgitube, Derma Sciences Inc., Princeton, NJ). Eggs were washed from the gauze weekly using a # 30-mesh sieve (500 µm) on top of a # 60-mesh sieve (250 µm) (Hogentogler & Co. Inc., Columbia, MD) using tap water at room temperature. Beetles were transferred to clean cages with new gauze weekly, and fresh food provided every other day. Each treatment was replicated 8–10 times.

Female Fitness

SCR Female Fecundity After Exposure to dsRNA

To investigate the effects of sublethal exposures to dsRNA in adult females, two different developmental stages were tested. Virgin females were exposed to the LC_{50} of both dsRNA molecules for 5 d. The exposed females (10 per cage) were then paired with 15 unexposed males and allowed to mate for 7 d after which time the males were removed. For the other stage tested, 26-d-old mated females (during peak oviposition) maintained on untreated artificial diet were exposed to the same LC_{50} for *vATPase-A* or *Snf7* dsRNAs for 5 d. Control females for both stages were maintained on untreated artificial diet.

The treated and untreated (control) adult females were maintained in oviposition cages for weekly recordings with methods identical to those previously described in male fitness (female fecundity). To determine egg viability from each treatment, six egg samples each containing 120–150 eggs from weekly collections were placed in 50.0 × 9.0 mm petri dishes (# 7242 PALL Corporation, Port Washington, NY). Eggs were incubated at 27°C and checked daily until no further hatching was observed for three consecutive days. Not all treatments produced enough eggs for three replicates.

Quantitative Real-Time PCR

Adults were exposed to LC_{50}'s of *vATPase-A* or *Snf7* dsRNAs for 5 d before being flash frozen at −80°C. For controls, beetles were allowed to feed on artificial diet treated with RNAse free water and 33.3 ng/cm^2 or 13.3 ng/cm^2 of Green Fluorescent Protein (GFP) dsRNA which corresponds the LC_{50} of *vATPase-A* and *Snf7* dsRNAs, respectively. RNA was extracted with RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's protocol and stored at −80°C. Total RNA (500 ng) was used to synthesize first strand cDNA using the Quantitech Reverse Transcription kit (Qiagen, Valencia, CA). Reactions were performed in duplicate using three samples of one beetle each. Master mix containing RNA, *vATPase-A* or *Snf7* primers, and SYBR Green (30 µl) was replicated in three wells of 96-well plates using 10 µl/well. The quantity of extracted RNA was estimated on a NanoDrop1000 (Thermo-Fisher scientific, Wilmington, DE) and quality evaluated using 1% denaturing agarose gel electrophoresis. Relative quantification of the transcripts was calculated using the comparative 2-ddCt method (Livak and Schmittgen 2001) and were normalized to *β-actin* (Rangasamy and Siegfried 2012).

Statistical Analysis

All experiments, but sperm viability, were performed under a completely randomized design. Sperm viability was performed in triplicate, with each time considered a block using different cohort. The values for live, dead, and total sperm recorded from five pictures (pseudo replicates) were averaged to produce one value for each individual adult. Means of adult and larval head capsule width and dry weight, weekly and cumulative number of eggs (female fecundity, when females are exposed to dsRNAs or unexposed females are mated with exposed males), and sperm count were compared by analysis of variance or pairwise comparison (two means) using least square means in a generalized linear mixed model (PROC GLIMMIX) in SAS 9.4 (SAS Institute, Cary, SC) at $\alpha = 0.05$. Percentage data such as egg hatch, larval recovery, and larval instar had binomial distributions with all response variables considered as random effects. Number of larvae that were recovered from larval development bioassays were unbalanced and presented different degrees of freedom for each treatment. The data were compared

with analysis of variance using least square means in generalized linear mixed model (PROC GLIMMIX) in SAS 9.4 (SAS Institute, Cary, SC) at $\alpha = 0.05$.

Results

Larval Development (10 d) After Neonate Exposure to dsRNAs

Significantly more larvae were recovered from control treatments relative to the dsRNA treatments for both *vATPase-A* dsRNA ($F = 11.5$; df = 3, 79; $P < 0.0001$) and for *Snf7* dsRNA ($F = 16.3$; df = 3, 78; $P < 0.0001$) 10 d after neonate exposure for all durations (24, 48, and 72 h) (Table 1). Percentages of larval survival as neonates declined from 93 to 45% after exposure to *vATPase-A* dsRNA and from 82 to 13% after exposure to *Snf7* dsRNA (Table 1) with increasing duration of exposure, suggesting that survival decreased with increased exposure time for both dsRNAs.

Larvae recovered from control and 24-h exposure to *vATPase-A* dsRNA weighed significantly more than larvae recovered from the 48- and 72-h treatments ($F = 8.87$; df = 3, 203; $P < 0.0001$) (Table 1) after feeding for 10 d on corn roots. In contrast, no significant differences among treatments were observed for *Snf7* dsRNA bioassay ($F = 1.53$; df = 3, 130; $P = 0.2094$) (Table 1), indicating that surviving larvae recovered and grew normally. Most of the larvae recovered from the corn roots were 3rd instars for all treatments (Fig. 2). For *vATPase-A* dsRNA exposure, 6% of the larvae were still 1st instar 10 d after exposure for 72 h to dsRNA (Fig. 2). There were significantly more 3rd instars in the control when compared to 48- and 72-h exposure to *vATPase-A* dsRNA ($F = 3.65$; df = 3, 79; $P = 0.0161$) and significantly more 2nd instars in the 48- and 72-h exposure to *vATPase-A* dsRNA ($F = 3.86$; df= 3, 56; $P = 0.0140$), when compared to control (Fig. 2). In contrast, there were no significant differences between control and *Snf7* dsRNA treatments for 2nd and 3rd instars (Fig. 2).

When larval dry weight was compared among 3rd instars within each treatment for each gene, controls exhibited significantly greater dry weight (1.67 mg) ($F = 5.87$; df= 3, 168; $P = 0.0008$) than those exposed as neonates for 48 h to *vATPase-A* dsRNA (0.91 mg) (Table 1). No significant differences ($F = 1.39$; df = 3, 107; $P = 0.2496$) were observed for dry weight

Table 1. Percentage of SCR larval recovery and larval dry weight recovered 10 d after neonate exposure to LC_{50} of *vATPase-A* or *Snf7* dsRNAs for 24, 48, and 72 h

Treatments	N*	Larval recovery (%)	Dry weight (mg)		
			Total	2nd instar (N*)	3rd instar (N*)
			vATPase-A		
Control	73	93 ± 3.0a	1.67 ± 0.11a	1.51 ± 0.70a (2)	1.67 ± 0.11a (71)
24 h	49	63 ± 5.4b	1.31 ± 0.13a	0.71 ± 0.44a (5)	1.41 ± 0.13ab (44)
48 h	44	54 ± 5.7bc	0.91 ± 0.14b	0.89 ± 0.30a (10)	0.91 ± 0.16b (34)
72 h	38	45 ± 5.1c	1.03 ± 0.13b	0.69 ± 0.30a (10)	1.17 ± 0.16ab (26)
	N*		*Snf7*		
Control	73	82 ± 5.2a	1.56 ± 0.14a	0.46 ± 0.05a (8)	1.70 ± 0.16a (65)
24 h	27	29 ± 6.3b	1.04 ± 0.22a	0.47 ± 0.05a (5)	1.20 ± 0.15a (22)
48 h	17	18 ± 5.0bc	1.62 ± 0.27a	0.56 ± 0.06a (5)	2.0 ± 0.37a (12)
72 h	14	13 ± 4.1c	1.43 ± 0.31a	0.37 ± 0.01a (3)	1.72 ± 0.42a (11)

Means (±SE) followed by the same letter are not significantly different when compared with least squares means using PROC GLIMMIX in SAS 9.4, at $\alpha = 0.05$.
*Number of larvae recovered from each treatment/each instar.

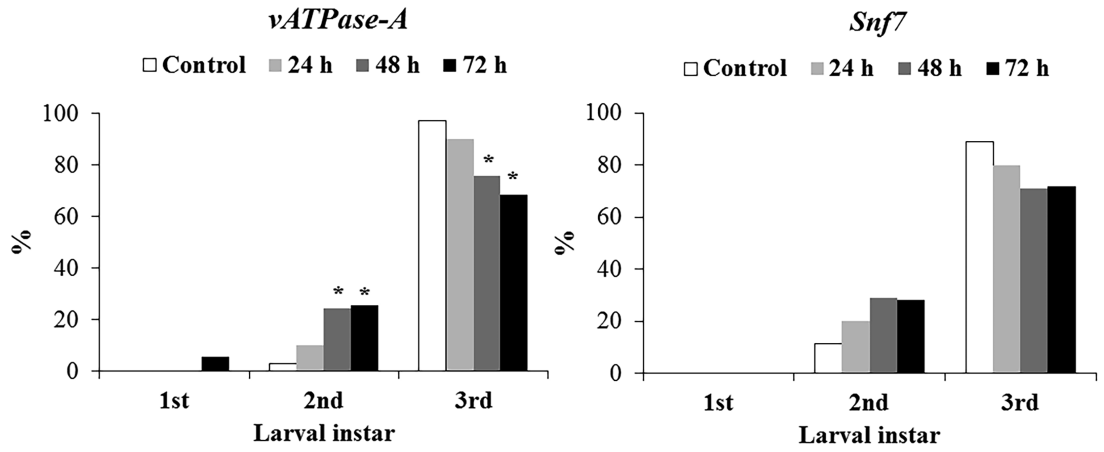

Fig. 2. Percentage of SCR larval instar 10 d after neonate exposure to LC_{50} of vATPase-A and Snf7 dsRNAs for 24, 48, and 72 h. *Significantly different when compared to respective control in Dunnet's test using PROC GLIMMIX in SAS 9.4, at α=0.05.

among *Snf7* treatments (Table 1). For 2nd instars, the sample size was small as most of the larvae were 3rd instar. Therefore, no significant differences in dry weight were observed among treatments for *vATPase-A* dsRNA (F = 0.43; df = 3, 23; P = 0.7347) and *Snf7* (F = 1.45; df = 3, 19; P = 0.2598) dsRNAs, though there was numerically greater dry weight for larvae exposed for 48 h to *Snf7* dsRNA, when compared to control (Table 1).

In a separate bioassay similar to larval development, we evaluated the adult emergence after exposure of neonates for 3 d to LC_{50}s of *vATPase-A* and *Snf7* before transferring larvae to plants in 50-ml tubes. Fewer beetles emerged from those tubes in which larvae were exposed to dsRNAs when compared to control (data not shown). However, very few larvae completed development to adult, and statistical analysis was not possible.

It is known that exposure of neonates for 3 d to dsRNAs overlaid on artificial diet caused significant gene knockdown (Pereira et al. 2016). Therefore, larval development and adult emergence assays were performed with 3-d exposure to dsRNAs to be similar in methodology, besides to avoid possible contamination of the artificial diet.

Table 2. Mean (±SE) number of total, live, and dead SCR sperm cells in males exposed to the LC_{50} of *vATPase-A* or *Snf7* dsRNAs

Treatments	Total sperm	Live sperm	Dead sperm
Water	193 ± 19.3a	157 ± 15.1a	36 ± 5.0a
vATPase-A	215 ± 16.2a	179 ± 14.1a	36 ± 4.6a
Snf7	214 ± 14.3a	180 ± 12.6a	34 ± 3.7a

Means (±SE) refer to the average of sperm counted in 60 pictures taken from 12 adults per each treatment, five pictures per each adult (see details in Materials and Methods). Means followed by the same letters within the columns are not significantly different compared in least squares means using PROC GLIMMIX in SAS 9.4, at α= 0.05.

Male Fitness

Sperm Viability

No significant differences were observed in the number of live (F = 0.19; df = 3, 6; P = 0.8966), dead (F = 0.22; df = 3, 6; P = 0.8770), and the total number of sperm (live + dead) (F = 0.14; df = 3, 6; P = 0.9351) between control and dsRNA treatments (Table 2).

Female Fecundity After Mating With Exposed Males

The cumulative number of eggs produced by females mated with treated males was not significantly different from control females mated with unexposed males, with egg production of 1,455 and 1,122 eggs/female for control and *vATPase-A* dsRNA treatments, respectively (Fig. 3A) (t = 2.54; df = 1, 18; P = 0.1286), and 2,108 and 1,965 eggs/female for control and *Snf7* dsRNA treatments, respectively (Fig. 3B) (t = 0.14; df = 1, 14; P = 0.5575). However, those females that mated with males exposed to *vATPase-A* (Fig. 3A) produced significantly fewer eggs for the first 4 wk (F = 18.14; df = 1, 166; P < 0.0001) and exposed to *Snf7* (Fig. 3B) produced significantly fewer eggs for the first 3 wk (F = 13.11; df = 11, 149; P < 0.0001) than females that mated with unexposed males.

Female Fitness

Female Fecundity After Exposure to dsRNA

Female fecundity was significantly reduced when both virgin and 26-d-old females were fed with artificial diet treated with the LC_{50} of *vATPase-A* and *Snf7* dsRNAs when compared to respective controls (Fig. 4). Females exposed prior to mating appeared to recover and started to lay a few eggs approximately 8 wk after exposure to *vATPase-A* dsRNA (Fig. 4). However, the cumulative number of eggs over the entire observation period was greatly reduced when compared to control females that fed on untreated diet (F = 47.88; df = 4, 39; P < 0.0001) (Fig. 4). The overall percentage egg hatch was

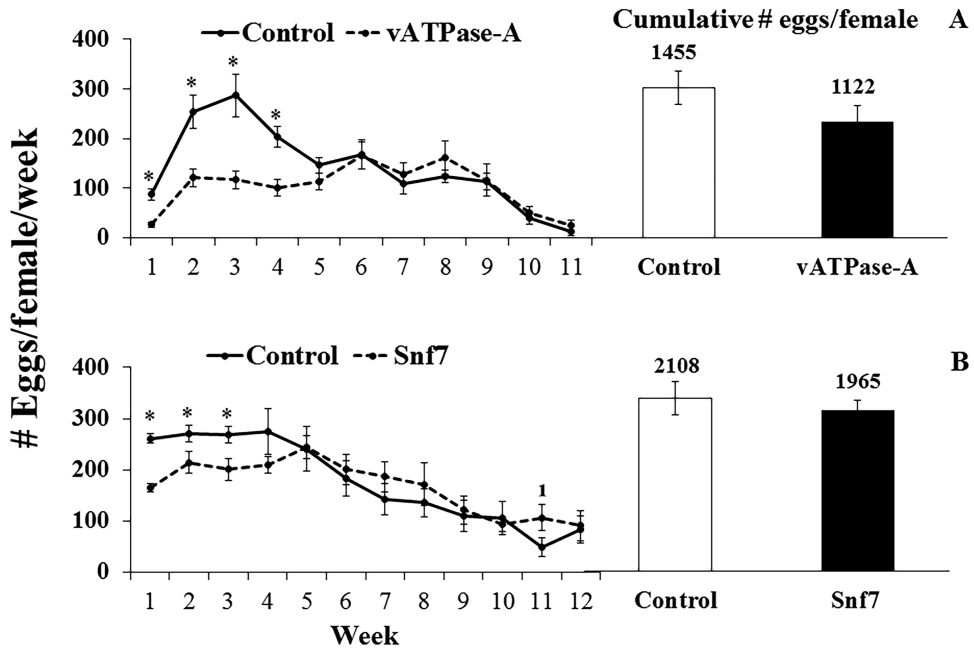

Fig. 3. Weekly and cumulative number of SCR eggs/female during lifetime, after females were mated with males exposed for 5 d to LC_{50} of vATPase-A (A) and Snf7 (B) dsRNAs. *Means (±SE) significantly different between control and dsRNA treatments in least square means using PROC GLIMMIX in SAS 9.4, at α = 0.05. [1]Means significantly different at α = 0.1. Cumulative number of eggs not statistically different between control and dsRNA treatments in least square means using PROC GLIMMIX in SAS 9.4, at α = 0.05.

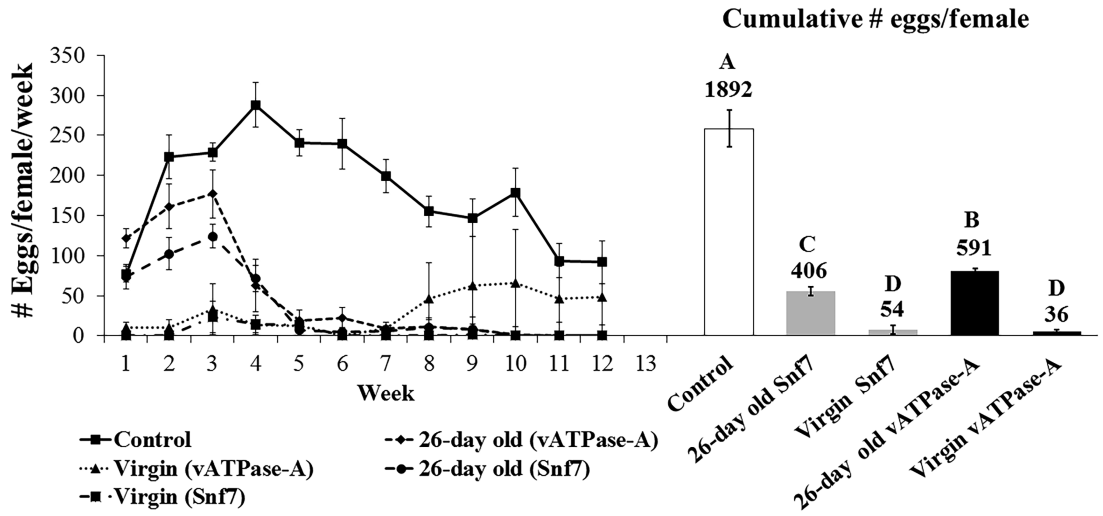

Fig. 4. Weekly and cumulative number of SCR eggs/female during lifetime after exposure of SCR females for 5 d to LC_{50} of vATPase-A and Snf7 dsRNAs before mating (virgin) and at peak of egg production (approximately 26 d). Females and unexposed males were allowed to mate for 1 wk after which males were removed from cages. Data points represent means (± SE) of eight cages containing up to 10 females for each treatment. Means (± SE) followed by different capital letters are significantly different when compared on least squares means in PROC GLIMMINX in SAS 9.4, at α=0.05.

Table 3. Overall mean (±SE) percentage hatch of eggs collected from SCR females exposed to LC_{50} of *vATPase-A* or *Snf7* dsRNAs prior to mating and 26 d after mating

Treatments	% Egg hatch (±SE)
Control	24.4 ± 5.4a
26-d-old *vATPase-A*	20.6 ± 6.3a
Virgin *vATPase-A*	26.3 ± 5.7a
26-d-old *Snf7*	17.8 ± 3.1a
Virgin *Snf7*	20.3 ± 6.6a

Means (±SE) are average of six 5-cm petri dishes containing 120–150 SCR eggs from weekly egg collections. Means (± SE) followed by the same letters are not significantly different when compared with least squares means using PROC GLIMMIX in SAS 9.4, at α =0.05.

not significantly different (F = 0.15; df = 4, 80; P = 0.9633) between control and dsRNA treatments, which ranged between 17.8% (26-day old exposed to *Snf7*) and 26.3% (virgin females exposed to *vATPase-A*) (Table 3).

Relative Gene Expression

Gene expression was significantly reduced after SCR males and females fed on LC_{50}'s of *vATPase-A* (Fig. 5A) (F = 13.92; df = 2, 9; P = 0.0018) and *Snf7* (Fig. 5B) (F = 11.05; df = 2, 9; P = 0.0029) dsRNAs for 5 d, with gene knockdown of 91–92% for *vATPase-A* and 61–63% for *Snf7* in females, and 89% for *vATPase-A* and 57–66% for *Snf7* in males, when compared to the water and GFP controls (Fig. 5A and B).

Discussion

Results of this study indicate that sublethal exposure to dsRNAs targeting housekeeping genes negatively affected several fitness parameters in both SCR adults and larvae. Many of the effects related to fitness, especially for adults, will potentially occur under field conditions after corn hybrids expressing RNAi traits are commercialized. The specific outcomes will depend on dose and duration of exposure experienced while feeding on dsRNA expressing corn plants. For adults, the expression of dsRNA in the above ground tissues will be especially important since adults are less susceptible than larvae (Pereira et al. 2016) and are more likely to encounter sublethal concentrations of dsRNA in above ground plant structures (Bachman et al. 2016).

It is also important to note that sublethal exposure to dsRNA as larvae could cause a delay in larval development, and therefore, it is likely that adult emergence would be delayed as a consequence. This delay in adult emergence could affect random mating between potentially resistant individuals emerging from corn plants expressing dsRNA and susceptible individuals emerging from refuge plants, which are required for current Insect resistance management (IRM) plans (Andow et al. 2016). In a previous study, Pereira (2016) reported that SCR neonates surviving exposure to concentrations of *vATPase-A* dsRNA that varied between LC_{50} and LC_{75}, delayed adult emergence up to 8 d when compared to unexposed neonates. Additional greenhouse and field research using transgenic plants that express RNAi traits will be necessary to clearly address questions related to larval development and adult emergence after sublethal exposure.

The comparison of dry weight for 2nd and 3rd instars among treatments after exposure as neonate larvae indicated differences in response of larvae exposed to the two dsRNAs. For *vATPase-A*, larvae from control treatments exhibited significantly greater dry weight as 3rd instars with a similar trend for 2nd instars, although the differences were not significant. However, for *Snf7* both 2nd and 3rd instars were equal in weight after neonate exposure, suggesting that larvae recover without long-term effects.

The cumulative lifetime number of eggs produced by SCR females was dramatically reduced after exposure to dsRNAs for both genes, especially in virgin females where a 50-fold reduction for *vATPase-A* and a 35-fold reduction for *Snf7* was observed relative to control treatments. Interestingly, egg production declined to zero for females exposed to dsRNA 26 d after emergence and during active oviposition. We also observed a 3- and 5-fold reduction in cumulative number of eggs relative to controls in 26-d-old females for *vATPase-A* and *Snf7*, respectively.

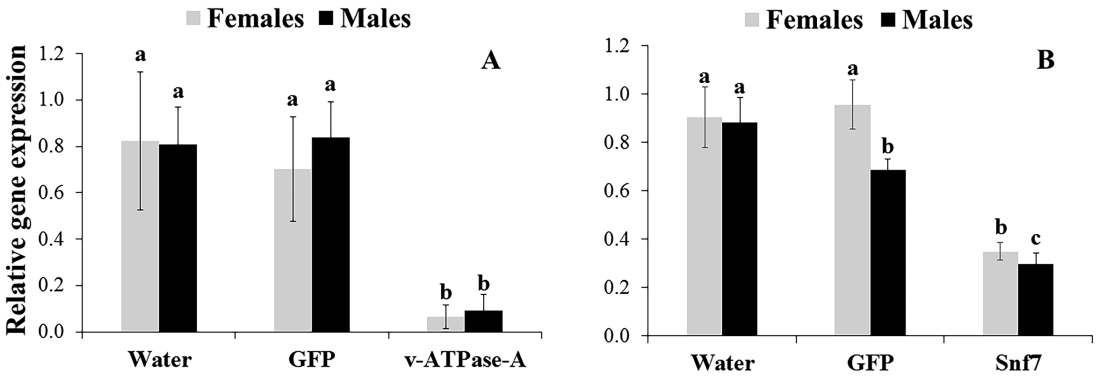

Fig. 5. Relative gene expression of SCR males and females after exposure for 5 d to LC_{50} of vATPase-A (A) and Snf7 (B) dsRNAs. Means (± SE) followed by the same letter within male or female category were not significantly different when compared in least squares means using PROC GLIMMIX in SAS 9.4, at α= 0.05.

Virgin females that survived the initial exposure appeared to recover egg-laying ability and were able to produce up to 50 eggs/week (Fig. 4). Because males were present in the cages with females for 1 week, the results suggest that females were able to mate, store sperm, and achieve fertilization after recovery from the exposure. It is possible that some of those females affected by the dsRNAs did not mate or did not have the spermatophore transferred during copulation. In a preliminary study to verify if exposed and unexposed males would be able to transfer spermatophores to unexposed females after mating for 24 and 48 h, we dissected and observed low percentages (up to 36%) of females that had the spermatophore for both dsRNA and control in both times (data not shown). However, it has been reported that SCR females usually mate with up to 15 males before allowing the transfer of a single spermatophore (Tallamy et al. 2000). In our study, healthy males were kept with females for 1 wk and it was assumed that all or most of the females were mated. In WCR, spermatophores are degraded and disappear in 5–7 d in mated females (Lew and Ball 1980). Therefore, we did not evaluate the presence of spermatophores in SCR females mated after 1 wk.

Percentage of egg hatch was not significantly different among treatments, suggesting that the concentrations of dsRNA provided to the females did not have a parental effect. Significant cannibalization of unhatched eggs by SCR neonates was frequently observed and likely contributed to the relatively low hatch rate that was observed (Jackson 1986). For cumulative egg production, the difference among treatments, especially between virgin females and control, was several fold suggesting that exposure of SCR adults to dsRNAs could have an impact on field populations. Such effects in the case of high and continuous exposure and depending on physiological stage of the beetles, could also affect potential selection for resistance over multiple generations.

Adult males that survived the sublethal exposures to dsRNAs were also able to mate with females, which later produced eggs. These females initially produced significantly fewer eggs (weeks 1–4) when compared to females that mated with healthy unexposed males (Fig. 3). However, oviposition increased and some weeks even exceeded the control, resulting in similar cumulative egg production relative to the control treatment. It seems unlikely that the sperm quality was affected in the dsRNA treatments because sperm viability was not different among treatments (Table 2). In addition, the *vATPase-A* and *Snf7* genes are not known to be related to reproduction. It is possible that substances transferred to the females during copulation that affect survival of spermatozoa as well as to promote ovulation and oviposition (Cordero 1995) were adversely affected in exposed males, thus reducing fertilization and oviposition in the first 4–5 wk.

Toxicological studies performed with other insects have shown the effect of sublethal exposures to dsRNAs especially on fecundity and fertility. Wu and Hoy (2014) documented the loss of egg production in the predatory mite *Metaseiulus occidentalis* Muma after feeding on dsRNA targeting ribosomal protein genes (*RpL11, RpS2, RpL8,* or *Pros26.4*). Estep et al. (2016) reported 12- and 38-fold lower fecundity after injection of female adults with sublethal doses of dsRNA targeting two ribosomal protein genes (*RPS6* and *RPL26*) in the mosquito *Aedes aegypti* L. (Diptera: Culicidae). Li et al. (2011) documented the knockdown with dsRNA of the *noa* gene responsible for fatty acid elongase and involved in sperm development in the testes of the Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) 2 d after feeding dsRNA to adults in artificial diet. The authors also documented a 32% drop in egg production after the fruit flies fed on *noa* dsRNA for 7 d, when compared to flies fed on *GFP* dsRNA, although the reduction was not statistically different (Li et al. 2011). Whyard et al. (2015) reported great reduction in male fertility in *A. aegypti*, after larvae fed on dsRNA targeting testis genes. In a study to document parental effects of dsRNAs targeting a chromatin remodeling gene (*brahma*) and a gap gene (*hunchback*) on sperm viability, fecundity, and egg hatch in WCR, Vélez et al. (2017) found significant reduction in egg hatch after exposure of females to both genes. Importantly, these studies used dsRNA targeting reproductive function and embryonic development such that effects on reproduction are not unexpected.

In the present study, gene expression was significantly reduced in SCR male and female adults after exposure to the LC_{50} of lethal dsRNAs for 5 d when compared to the controls water and GFP, which is in agreement with other studies in WCR for both genes (Bolognesi et al. 2012, Rangasamy and Siegfried 2012, Ramaseshadri et al. 2013, Ivashuta et al. 2015, Li et al. 2015, Pereira et al. 2016). Reduced knockdown of *Snf7* gene for adults was observed when compared to *vATPase-A*, which is similar to results found for WCR and SCR adults in a previous study (Pereira et al. 2016) that were exposed to LC_{50} dsRNAs for 8 d.

In conclusion, this research shows multiple adverse effects on SCR fitness after exposure to two different dsRNAs targeting lethal genes in SCR larvae and adults. These studies suggest that the level of damage caused by dsRNA in the insect is variable and the fitness effects seem to be dependent on the target gene. *Snf7* dsRNA seemed to have a greater impact on larval survival but appeared to cause fewer adverse effects on those fewer larvae that survived exposure as neonates when compared to *vATPase-A* dsRNA. Sublethal

exposure of SCR adults and/or larvae to transgenic maize expressing dsRNAs (Bachman et al. 2016) can possibly take place in the field, especially in situations where seed blend (larval movement between transgenic and nontransgenic plants) or refuge (beetle movement between transgenic and nontransgenic fields) are adopted. Several studies have shown movement of larvae between transgenic and nontransgenic plants and have suggested that if larvae survive sublethal exposure to Bt toxin or come in contact with Bt plants in later states (i.e., 2nd instar), resistance evolution can potentially happen over time (Zukoff et al. 2012, 2016a, Head et al. 2014). Greenhouse and field studies of sublethal exposures of WCR, and perhaps NCR, adults and larvae to dsRNA expressed in corn hybrids should be performed to understand effects on fitness parameters as well as possible selection for resistance to this new technology.

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