2016

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Elane Fishilevich
Dow AgroSciences, efishilevich2@unl.edu

Ana María Vélez
University of Nebraska-Lincoln, anamaria.velez@gmail.com

Chitvan Khajuria
University of Nebraska-Lincoln, ckhajuria2@unl.edu

Meghan L.F. Frey
Dow AgroSciences

Ronda L. Hamm
Dow AgroSciences

See next page for additional authors

Authors
Elane Fishilevich, Ana María Vélez, Chitvan Khajuria, Meghan L.F. Frey, Ronda L. Hamm, Haichuan Wang, Greg A. Schulenberg, Andrew J. Bowling, Heather E. Pence, Premchand Gandra, Kanika Arora, Nicholas P. Storer, Kenneth E. Narva, and Blair D. Siegfried

Published in *Insect Biochemistry and Molecular Biology* 71 (2016), pp 58–71. doi 10.1016/j.ibmb.2016.02.004

Submitted 8 October 2015; revised 4 February 2016; accepted 4 February 2016; published online 10 February 2016.

Use of chromatin remodeling ATPases as RNAi targets for parental control of western corn rootworm (*Diabrotica virgifera virgifera*) and Neotropical brown stink bug (*Euschistus heros*)

Elane Fishilevich,[1] Ana M. Vélez,[2] Chitvan Khajuria,[2] Meghan L.F. Frey,[1] Ronda L. Hamm,[1] Haichuan Wang,[2] Greg A. Schulenberg,[1] Andrew J. Bowling,[1] Heather E. Pence,[1] Premchand Gandra,[1] Kanika Arora,[1] Nicholas P. Storer,[1] Kenneth E. Narva,[1] and Blair D. Siegfried[3]

1 Dow AgroSciences, 9330 Zionsville Road, Indianapolis, IN
2 University of Nebraska–Lincoln, Department of Entomology, 103 Entomology Hall, Lincoln, NE 68583-0816
3 Entomology and Nematology Department, Charles Steinmetz Hall, University of Florida, P.O. Box 110620, Gainesville, FL 32611-0620

Corresponding author — B. D. Siegfried, email bsiegfried1@ufl.edu

Abstract
RNA interference (RNAi) is a gene silencing mechanism that is present in animals and plants and is triggered by double stranded RNA (dsRNA) or small interfering RNA (siRNA), depending on the organism. In the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), RNAi can be achieved by feeding rootworms dsRNA added to artificial diet or plant tissues transformed to express dsRNA. The effect of RNAi depends on the targeted gene function and can range from an absence of phenotypic response to readily apparent responses, including lethality. Furthermore, RNAi can directly affect individuals that consume dsRNA or the effect may be transferred to the next generation. Our previous work described the potential use of genes involved in embryonic development as a parental RNAi technology for the control of WCR. In this study, we describe the use of chromatin-remodeling ATPases as target genes to achieve parental gene silencing in two insect pests, a coleopteran, WCR, and a hemipteran, the Neotropical brown stink bug, *Euschistus heros* Fabricius (Hemiptera: Pentatomidae). Our results show that dsRNA targeting chromatin-remodeling ATPase transcripts, *brahma*, *mi-2*, and *iswi* strongly reduced the fecundity of the exposed females in both insect species. Additionally, knockdown of *chd1* reduced the fecundity of *E. heros*.

Keywords: *Diabrotica,* stink bug, parental RNAi, chromatin-remodeling ATPase, brahma, Iswi, Chd1, Mi-2

1. Introduction

Corn rootworms (CRW), *Diabrotica* species, are major agricultural pests of maize in the United States. In 2002, USDA estimated corn rootworm damage in the United States at over $1 billion (Marra et al., 2012). The introduction of maize transformed to express *Bacillus thuringiensis* (Bt) proteins for CRWcontrol is believed to have a significant positive impact on yields (Marra et al., 2012), which has been accompanied by as much as an 80% reduction in the use of chemical insecticides between 1995 and 2009 (Coupe and Capel, 2015). Unfortunately, the development of resistance of the western corn rootworm (WCR), *Diabrotica virgifera virgifera,* to a widely-deployed Bt toxin, Cry3Bb1 (Gassmann et al., 2011, 2012), and its rapid adaptation to other management strategies, such as chemical insecticides (Meinke et al., 1998) and crop rotation (Gray et al., 2009; Levine and OloumiSadeghi, 1996) has increased the urgency to discover new insecticidal proteins and/or identification of new modes of action (MOA) for CRW control. The development of RNA interference (RNAi) technology for the control of WCR via transgenic expression of long double-stranded RNA (dsRNA) in maize provides an additional MOA againstWCR (Baum et al., 2007). Previous experiments have shown that RNAi is also effective against adult rootworms (Rangasamy and Siegfried, 2012). Oral sensitivity of adults to RNAi provides an additional option to suppress populations by reducing egg viability.

RNAi offers opportunities for control of other insect pests. The stink bug complex (family Pentatomidae) represents major agricultural pests of cotton and soybeans in the Americas. Stink bugs are

represented by a pest complex that contains more than a dozen species with composition, prevalence, and distribution varying with geography (Koch and Pahs, 2014; Pilkay et al., 2015; Temple et al., 2013). In South America, the stink bug complex is the most destructive pest of soybean crops. In Brazil, a global leader in soybean production, the Neotropical brown stink bug, *Euschistus heros*, is the main target for insecticide applications in soybeans (Sosa- Gomez et al., 2009).

Because there are no known insecticidal proteins with activity against pentatomid stink bugs, RNAi offers a potential transgenic approach to control this insect pest complex. Successes in inducing RNAi in aphids (Hemiptera: Aphididae) by introducing dsRNA in artificial diet and/or via *in planta* expression of dsRNA hairpins to control green peach aphid, *Myzus persicae* (Coleman et al., 2015; Pitino et al., 2011), the pea aphid *Acyrthosiphon pisum* (Mao et al., 2013; Mao and Zeng, 2012), and the grain aphid, *Sitobion avenae* (Wang et al., 2015), bolster the potential for achieving RNAi in stink bugs. Since stink bugs can have multiple generations per year (Pilkay, 2013), parental RNAi-based approaches could be particularly effective in controlling this pest complex. However, oral RNAi delivery to other hemipteran pests such as tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae), has been confounded by high ribonuclease activity in saliva that apparently degrades dsRNA (Allen and Walker, 2012). An initial demonstration of RNAi in pentatomid stink bugs, by injection, would support this insect's capacity to elicit an RNAi response and confirm that stink bugs possess the cellular machinery necessary for RNAi. Injection-based RNAi responses have been described in other families of Heteroptera; Futahashi et al. (2011) describe the role of *Laccase2* in cuticular pigment production of bean bug, *Riptortus pedestris* (Hemiptera: Alydidae), seed bug, *Nysius plebeius* (Hemiptera: Lygaeidae), and a Kudzu Bug relative, Japanese common plataspid stinkbug, *Megacopta punctatissima* (Hemiptera: Plataspididae).

Parental or transgenerational RNAi effects have been observed in both coleopteran (Bucher et al., 2002; Khajuria et al., 2015; Shukla and Palli, 2014) and hemipteran insects (Coleman et al., 2015; Liu and Kaufman, 2004; Mao et al., 2013; Paim et al., 2013). For example, Liu and Kaufman (2004) used pRNAi via injection of dsRNA into the adult female to investigate the developmental role of the *hunchback* gene in the milkweed bug, *Oncopeltus fasciatus* (Hemiptera: Lygaeidae). While in most cases the parental RNAi applications have been carried out via injection, there are observations via oral and plant transgene delivery in the green peach aphid (Coleman et al., 2015). In WCR, parental RNAi (pRNAi) responses have been demonstrated by feeding adult female rootworms dsRNA targeting *brahma* (*brm*) and *hunchback* (Khajuria et al., 2015) genes involved in embryonic development.

To expand the list of pRNAi targets, additional genes that may be critical to genetic reprogramming during gametogenesis and early developmental processes were tested in this study. Gene activation and silencing during this period is largely accomplished through epigenetic modifications of chromatin that involve DNA methylation, histone modification, and the function of ATP-dependent remodeling complexes (de la Serna et al., 2006; Ho and Crabtree, 2010). The ATP-dependent remodeling enzymes are a class of ATPases that contain a SNF2 domain (sucrose non-fermenting, originally identified in *Saccharomyces cerevisiae*) (Durr et al., 2005; Eisen et al., 1995). SNF2-containing ATPases are the catalytic subunits of multi-protein chromatin remodeling complexes that are also known as chromatin remodelers (for review see *e.g.*, Clapier and Cairns (2009)). Chromatin remodelers mobilize nucleosomes and thus change access of the transcriptional machinery to DNA (Cote et al., 1994). Chromatin remodelers may also have broader transcriptional effects on RNA polymerase II (Armstrong et al., 2002). Chromatin-remodeling ATPases are identified by a combination of SNF2 family N-terminal and Helicase conserved C-terminal domains. Most of these proteins have non-redundant functions. Consequently, most of the chromatin-remodeling ATPases are essential for an organism's viability and development. More specifically, chromatin remodelers are needed for activation of specific genes such as homeotic genes that regulate body segmentation during development and may be necessary during gametogenesis (for review, see *e.g.*, Ho and Crabtree (2010)). The ATP-dependent remodeling enzymes are generally divided into families based on functional domains that facilitate histone interactions (*e.g.*, bromodomain, chromodomain, and SANT). The families of chromatin-remodeling ATPases include SWI2/SNF2, ISWI, CHD, and Ino80 (for review see *e.g.*, Bouazoune and Brehm (2006)). In most cases, only one family or subfamily member has been identified in insects further supporting the non-redundant functions for each of these genes. The function of ATP-dependent remodelers in insects during gametogenesis and embryonic development is largely based on mutagenesis studies in *Drosophila* (Bouazoune and Brehm, 2006). However, the phenotypes derived by observing maternal effects using germline clones in *Drosophila* as well as the zygotic effects in mutants can now be explored by RNAi in other insect species.

In a previous study, we observed embryonic lethality in response to knockdown of chromatin remodeling ATPase *brahma* (*brm*), and the gap gene, *hunchback* (*hb*) transcripts via RNAi in adult WCR females (Khajuria et al., 2015). Based on these results, pRNAi was suggested as a potential parental pest management strategy in WCR. In the present study, *E. heros* (Hemiptera: Pentatomidae) was used as a representative stink bug to demonstrate both lethal and parental RNAi effects via injection of dsRNA. Additionally, the list of ATP-dependent chromatin remodelers was expanded to include members of the SWI2/SNF2, ISWI and CHD families. Oral exposure of WCR to dsRNA targeting *brm*-related genes showed that in addition to *hunchback* and *brm*, *mi-2*, *iswi-1*, and *iswi-2* are potent gene targets for pRNAi. In *E. heros,* all dsRNAs tested by injection (*brm, chd1*, *iswi-1*, *iswi-2,* and *mi-2*) produced significant reduction in egg hatching, with *brm* and *mi-2* showing the strongest phenotypes and *iswi-2* the weakest. Our results confirm that chromatin-remodeling ATPases exhibit strong pRNAi phenotypes in WCR and stink bugs, and may provide RNAi targets with potential use for parental pest management of coleopteran and hemipteran pests.

2. Methods

2.1. Transcriptome assembly

A *D. v. virgifera* transcriptome has been previously described by Eyun et al. (2014). Briefly, Illumina paired-end and 454 Titanium sequencing technologies yielded ~700 gigabases of transcript sequence from cDNA prepared from eggs, neonates, and midguts of third instar larvae. *De novo* transcriptome assembly was performed using Trinity (Grabherr et al., 2011) and the pooled assembly resulted in 163,871 contigs.

E. heros transcriptome sequences were generated from six selected developmental stages: eggs, 2nd instar, 3rd instar, 4th instar, 5th instar, and adult. Additional samples were prepared using *E. heros* midgut and salivary glands from 10 to 25 mixed sex adults, respectively. Samples were dissected using a stereo microscope and frozen immediately on dry ice. Total RNA was extracted from samples stored at –80 °C and homogenized in 10 volumes of Lysis/Binding buffer in Lysing Matrix-A 2 ml tubes on

a FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA). Total RNA was extracted using a mirVana™ miRNA Isolation Kit (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. RNA sequencing using an Illumina® HiSeq™ system (San Diego, CA) generated a total of approximately 38 gigabases for the eight samples. The reads for each sample were assembled separately using Trinity assembler software (Grabherr et al., 2011). The assembled transcripts were combined to generate a pooled transcriptome; sequences with 100% identify were removed. The *E. heros* pooled transcriptome contains 378,457 contigs (N50 = ~1.1 kb), with an estimated less than 1% of contaminants. Since the purpose of this transcriptome sequencing was to identify related sequences to design dsRNAs, we did not seek to remove redundancy, contaminants, or generate a refined reference transcriptome.

2.2. Chromatin remodeling ATPase ortholog identification

Candidate genes were identified with tBLASTn searches of the *D. v. virgifera* and *E. heros* pooled transcriptomes using sequences of the *Drosophila* Brahma (brm-PA and PC, GenBank Accession numbers: NP_536745 and NP_536746), Mi-2 (Mi-2-PA -PD, GenBank Accession numbers: NP_649154.2, NP_001014591.1, NP_001163476.1, and NP_001262078.1), Iswi (Iswi-PA-PC, GenBank Accession numbers: NP_523719, NP_725203, and NP_725204), and Chd1 (Chd1-PA and PB, GenBank Accession numbers: NP_477197 and NP_001245851) proteins as queries. *D. v. virgifera* and *E. heros brahma*, *mi-2*, *iswi-1*, *iswi-2*, and *chd1* transcripts were identified as candidate parental RNAi target genes. *D. v. virgifera* sequence for *brahma* was previously reported (Accession No. KR152260) (Khajuria et al., 2015). *D. v. virgifera mi-2*, *iswi-1*, *iswi-2* and *chd-1* sequences were deposited into GenBank (GenBank Accession numbers: *mi-2*: KT364639; *iswi-1*: KT364640; *iswi-2*: KT364641; *chd-1*: KT364642), together with *E. heros brahma*, *mi-2*, *iswi-1*, *iswi-2*, and *chd1* (GenBank Accession numbers: *brahma*: KT369801; *mi-2*: KT369802; *iswi-1*: KT369803; *iswi-2*: KT369804; *chd-1*: KT369805). Brown marmorated stink bug, *Halyomorpha halys* deduced-amino acid sequences were obtained from arthropod sequencing i5K Pilot Project — ftp://ftp.hgsc.bcm.edu/I5Kpilot/ Brown_marmorated_stink_bug/ . Domain annotation was performed using the Pfam database (Finn et al., 2014). The protein alignment was performed using MUSCLE (100 iterations) in MEGA 6.06. Bootstrap values (MEGA) support the topology of the ATPdependent remodeler family branches on the maximum likelihood phylogeny tree.

2.3. cDNA preparation and dsRNA synthesis

D. v. virgifera total RNA was isolated from non-diapausing WCR adults (Crop Characteristics Inc., Farmington, MN) using a RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. Total RNA (1 µg) was used to synthesize cDNA using a Quantitech reverse transcription kit (Qiagen, Valencia, CA). DNA templates were amplified using Takara Taq DNA Polymerase (Clontech Laboratories, Inc., Mountain View, CA). Primers were designed using Beacon designer software (Premier Biosoft International, Palo Alto, CA) and T7 promoter sequences were placed at the 50 ends of both forward and reverse primers (Supplemental Table 1). For the negative control, a *green fluorescent* protein (*GFP*) gene was amplified from the pIZT/V5-His expression vector (Invitrogen, Waltham, MA) using gene-specific primers (Supplemental Table 1). For *GFP*, 1 µl of the vector was used as a template. dsRNAs were synthesized using 2 µl of PCR product as the template with a MEGAscript™RNAi kit (Ambion, Life Technologies, Carlsbad, CA) used according to the manufacturer's instructions. The dsRNA was quantified on a NanoDrop™1000 spectrophotometer (Thermo Scientific, Franklin, MA) at 260 nm and analyzed by gel electrophoresis to determine purity.

E. heros RNA that was used for dsRNA synthesis was extracted from a single adult within two days of emergence, using TRIzol® (Life Technologies, Carlsbad CA), following manufacturer's instructions. The RNA pellet was air-dried at room temperature and resuspended in 200 µl of 10 mM Tris–HCl (pH 8.0). cDNA was reverse-transcribed from 5 µg *E. heros* total RNA template and oligo dT primer using a SuperScript III First-Strand Synthesis System™ for RT-PCR (Invitrogen, Waltham, MA), following the supplier's recommended protocol. The final volume was brought to 100 µl with nuclease-free water. Oligonucleotide primers were used to amplify DNA templates for dsRNA transcription (Supplemental Table 1). The DNA templates were amplified using Platinum Taq DNA polymerase (Life Technologies, Carlsbad, CA) using 1 µl of cDNA as the template. Amplified fragments comprised segments of *E. heros* chromatin remodeling ATPases, cytoplasmic and muscle actin genes as lethal gene candidates [GenBank Accession numbers: KT819630 (cytoplasmic actin) and KT369806 (muscle actin)], and a negative control, a segment of *yellow fluorescent protein* gene (*YFP*). All primers contained a T7 promoter sequence at their 50 ends, to enable dsRNA transcription. The dsRNAs were synthesized using 2 µl of PCR product as the template with the MEGAscript™RNAi kit (Ambion, Life Technologies, Carlsbad, CA) used according to the manufacturer's instructions. The dsRNA was quantified on a NanoDrop™ 8000 spectrophotometer at 260 nm and diluted to 1 µg/µl in nuclease-free 0.1X TE buffer (1 mM Tris HCL, 0.1 mM EDTA, pH 7.4).

2.4. D. v. virgifera feeding bioassay

Parental RNAi in *D. v. virgifera* was conducted by feeding dsRNA for chromatin-remodeling ATPases to gravid adult females following the methodology described by (Khajuria et al., 2015). Briefly, non-diapausing *D. v. virgifera* adults were purchased from Crop Characteristics Inc. (Farmington, MN). Adults were kept at 23 ± 1 °C, relative humidity >75%, and 8:16 light:dark photoperiod. Immediately after insect arrival, six virgin males and six virgin females (24–48 h old) were maintained on untreated artificial diet adapted from Branson and Jackson (1988) and allowed to mate for 4 days. On day five, males were removed and the remaining females were provided with seven artificial diet plugs surface-treated with 3 µl at 0.67 µg/µl of gene specific dsRNA (Khajuria et al., 2015). Control treatments consisted of gravid females exposed to diet treated with the same concentration of *GFP* dsRNA or the same volume of nuclease-free water. Freshly treated artificial diet was provided every other day throughout the experiment for a total of six exposures to dsRNA. On day 11, females were transferred to oviposition cages (ShowMan box, Althor Products, Wilton, CT) (Campbell and Meinke, 2010). Females were allowed to oviposit for four days and then removed from the oviposition boxes and flash frozen for qRT-PCR analysis. Eggs were incubated in the soil for 10 days at 27 °C, >80% humidity, 24 hours dark, and thenwashed from the soil with a 60-mesh sieve. Eggs from each treatment were placed in Petri dishes (50 mm Pall Corporation, Port Washington, NY) on moistened filter paper and monitored for 15 days to determine egg viability. Dishes were photographed and egg counting was performed using the cell counter function of ImageJ software (Schneider et al., 2012). Three replications per gene were performed; each replicate included three to six females. Three sets of ten eggs per gene were flash frozen for qRT-PCR.

2.5. E. heros rearing and dsRNA injections

E. heros were reared at 27 °C, 65% relative humidity, and 16:8 light:dark photoperiod. One gram of eggs collected over 2–3 days was seeded in 5 L containers with filter paper at the bottom and covered with #18 mesh for ventilation. Insects were fed fresh green beans three times per week and a sachet of seed mixture containing sunflower seeds, soybeans, and peanuts (3:1:1 by weight ratio) replaced once aweek.Waterwas supplemented in vials with cotton plugs as wicks. After the initial two weeks, insects were transferred to a new container once a week.

A modified *E. heros* artificial diet (Fortes et al., 2006) was used to maintain nymphs after injection. The diet was prepared using lyophilized green beans and raw organic peanuts blended separately in a Magic Bullet® blender. Dry ingredients weight percentages: green beans 35%, peanuts 35%, sucrose 5%, and Vanderzant Vitamin Mixture for insects 0.9% (Sigma–Aldrich, St Louis, MO), were combined in a Magic Bullet® blender. The mixed dry ingredients were then added to a mixing bowl. Water was supplemented with benomyl anti-fungal agent to 50 ppm final concentration and added to the dry ingredient mixture. All ingredients were mixed by hand until the solution was fully blended. The diet was shaped into desired sizes, wrapped loosely in aluminum foil, heated for 4 hours at 60 °C, then cooled and stored at 4 °C.

Injections of *E. heros* were performed using a Nanoject II™ injector (Drummond Scientific, Broomhall, PA), equipped with an injection needle pulled from a Drummond 3.5 inch #3-000-203-G/X glass capillary. Second instar nymphs were immobilized on ice in a Petri dish. Each nymph was injected with 55.2 nl of 500 ng/µl dsRNA solution into the abdomen (i.e., 27.6 ng dsRNA in ~1 mg–1.5 mg insect). A dsRNA targeting 301 nt of a *YFP* transcript was used as a negative control in all *E. heros* experiments. Each trial consisted of 10 insects injected with dsRNA and three trials were repeated on three different days. Injected insects (5 per well) were transferred into 32-well rearing trays (Frontier Agricultural Sciences, Newark, DE) containing a pellet of artificial stink bug artificial diet, and covered with Pull-N-Peel™tabs (Frontier Agricultural Sciences, Newark, DE). Moisture was supplied by means of 1.25 ml water in a 1.5ml microcentrifuge tube with a cotton wick. The trays were incubated at 26.5 °C, 60% humidity, and 16:8 hour light:dark photoperiod. Viability counts and weights were taken on day 7 after the injections.

To evaluate pRNAi effects in *E. heros,* young adults (0–3 days old) were collected and chilled on ice and gender identified based on structural dimorphism of the genitalia (McPherson and McPherson, 2000). The insects were handled with Featherweight entomology forceps and females injected with dsRNA using a Nanoject II™ injector. Twenty females (approximately 90 mg each) per treatment were injected with dsRNA. Each female was injected into the abdomen with 138 nl of dsRNA solution at 1 µg/µl. Each batch of ten females was moved into a 1-quart bin with an opening in the lid covered with #18 mesh for ventilation. Two untreated adult males were added to each bin of ten females. The insects were supplied with a vial of water, green beans, and seeds. Bins were kept at 26.5 °C, 60% humidity, and 16:8 light: dark photoperiod. The number of surviving females, number of eggs oviposited, and number of nymphs recorded on a daily basis starting seven to nine days after injection and continued for up to 15 days. Eggs were removed daily and kept in Petri dishes on a layer of 1% agarose in water. Adult insects were supplied with fresh green beans every other day and surviving insects were transferred into bins with fresh water and food every week.

E. heros ovarian development was observed in females 9 and 14 days after injection of *brm* and *mi-2* dsRNA as described above. *E. heros* ovaries were dissected in 1X PBS under stereo microscope and then fixed in 4% paraformaldehyde/1X PBS solution for 2 hours on ice. Trachea surrounding the ovaries was removed with #5 biology forceps. Images of three to four sets of ovaries for each treatment were captured with a Leica M205 FA stereo microscope (Wetzlar, Germany).

2.6. Quantitative real-time PCR (qRT-PCR)

D. v. virgifera qRT-PCR was performed using SYBR green and a 7500 Fast System real-time PCR detection system (Applied Biosystems, Grand Island, NY). Total RNA isolation and cDNA preparation was performed as described in the *D. v. virgifera* cDNA preparation section. cDNA was diluted 50 fold for use as template. Primers used for qRT-PCR analysis are described in Supplemental Table 3. Primer pair efficiencies were evaluated using 5-fold serial dilutions (1: 1/5: 1/25: 1/125: 1:625) in triplicate. Primer sets with amplification efficiencies between 90 and 110% and correlation coefficients larger than 0.99 were used for all qRT-PCR experiments (Supplemental Table 3). qRT-PCR analysis was performed with three to six biological replicates, each biological replicate had two technical replications. qRT-PCR cycling parameters were performed as described in the supplier's protocol. At the end of each PCR reaction, a melting curve was generated to confirm single peaks and rule out the possibility of primer-dimer and nonspecific product formation. Relative quantification of the transcripts were calculated using the comparative 2°ΔΔCq method (Livak and Schmittgen, 2001) and were normalized to b-actin (Rangasamy and Siegfried, 2012).

E. heros tissues for qRT-PCR were collected from zero to three day-old females injected with dsRNA as described above. After seven days, female ovaries were dissected under a stereo microscope in nuclease-free 1X PBS (pH 7.4) and frozen individually on dry ice in collection microtubes. Tissue disruption was performed with the RL lysis buffer and the Klecko™ tissue pulverizer (Garcia Manufacturing, Visalia, CA). Following tissue maceration, the total RNAwas isolated in high throughput format using the Norgen Total RNA Purification 96-well kit (Norgen BioteK Corp, Ontario, Canada) following the manufacturer's protocol using Turbo™ DNase (Life Technologies, Carlsbad, CA) for 1 hour at 37 °C on the elutant. cDNA synthesis was performed using the high capacity cDNA RT kit (Life technologies, Carlsbad, CA) according to the manufacturer's protocol with the following modifications. Total RNA was adjusted to 50 ng/µl with nuclease-free water. RNA samples were heated to 70 °C for 10 min and cooled to 4 °C. Half reactions were initiated by addition of 5 µl of 2X mix. The primer mix, which is supplied solely as random primers, was first spiked with custom synthesized T20VN oligo (Integrated DNA Technologies, Coralville, IA) to a final concentration of 2 µM, in order to improve the sensitivity of 30UTR based assays. Following first strand synthesis, the samples were diluted 1:3 with nuclease free.

E. heros qRT-PCR primers and hydrolysis probes were designed using LightCycler Probe Design Software 2.0 (Roche, Basel, Switzerland) for the reference gene and Primer Express® Software Version 3.0 (Applied Biosystems, Grand Island, NY) for the target genes (Supplemental Table 2). Non-injected insects were used as controls. *E. heros* muscle actin was used as the reference gene. Probes were labeled with FAM (6-Carboxy Fluorescein Amidite). The final primer concentrations were 0.4 µM and probe concentrations were 0.2 µM, in 10 µl reaction volumes. Relative transcript levels were analyzed by probe hydrolysis qRT-PCR using Roche LightCycler480 (Roche, Basel, Switzerland). All assays included negative controls of no-template (mix only). For the standard curves, a blank was included in the source plate to check for sample cross-contamination. PCR cycling conditions included a 10 min target

activation incubation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 seconds, anneal/extend at 60 °C for 40 seconds, and FAM acquisition at 72 °C for 1 second. The reaction was followed by a 10 second cool-down at 40 °C. We were not able to detect *E. heros iswi-2* reliably both in the negative controls and dsRNA exposed females, therefore *iswi-2* data was omitted from the final results. The data was analyzed using LightCycler™ Software v1.5 and relative changes in expression were calculated using $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

Means comparisons of oviposition, percent hatch, and relative transcript expression levels ($2^{-\Delta\Delta Cq}$) were corrected for multiple comparisons and were performed in JMP® Pro 11 with a control using Dunnett's Method (JMP®, 2007). Water treatments were used as controls for *D. v. virgifera* and *YFP* dsRNA injections for *E. heros*. Individual comparisons of transcript levels within *E. heros* ovaries, using non-injected insects as controls, were performed using Student's *t*-test.

3. Results

3.1. Domain conservation in chromatin remodeling ATPases of D. v. virgifera and E. heros

Sequence alignments, protein domain identification (Figure 1), and phylogenetic tree construction (Figure 2) enabled classification of WCR and *E. heros* Brahma-related proteins. Previous analysis of predicted WCR Brahma proteins describes domain conservation of this protein with *Tribolium castaneum* and *Drosophila melanogaster* Brahma proteins (Khajuria et al., 2015). *Brahma* of the SWI2/SNF2 family of the ATP-dependent remodeling enzymes contains a bromodomain, which binds acetylated histones (Hassan et al., 2002). Similar domain conservation was observed for *E. heros* (Figure 1A). In addition to the presence of Brahma BRK and bromodomains, the relative positions of these domains are conserved across three insect orders (Figure 1A). *Brahma*-related genes were identified in WCR and *E. heros* based on sequence similarity to *Drosophila* and WCR *brahma*. All full-length and most partial peptide sequences contained a combination of SNF2 family N- and Helicase conserved Cterminal domains, which are characteristic of chromatin remodeling ATPases (Figure 1).

The identified chromatin-remodeling ATPase proteins were further segregated into known families of chromatin remodelers. One group was classified as ISWI (Imitation SWI) family proteins (Figure 2). Although *Drosophila* has only one Iswi (Elfring et al., 1994), we identified one full-length and one partial Iswi coding sequences in *E. heros* and one full and one partial Iswi coding sequences in WCR (Figure 1B). All predicted polypeptides with identified C-termini contained the HAND-SANT-SLIDE (or HAND-SLIDE) domain (Grune et al., 2003). The partial sequence of WCR Iswi-2 consists of the SNF2 domain only (Figure 1B). The WCR Iswi protein sequences share 94.5% identity over the aligned region. At the transcript level, WCR *iswi* transcripts share 47.6% nucleotide identify with no consecutive 21-mers. The partial sequence

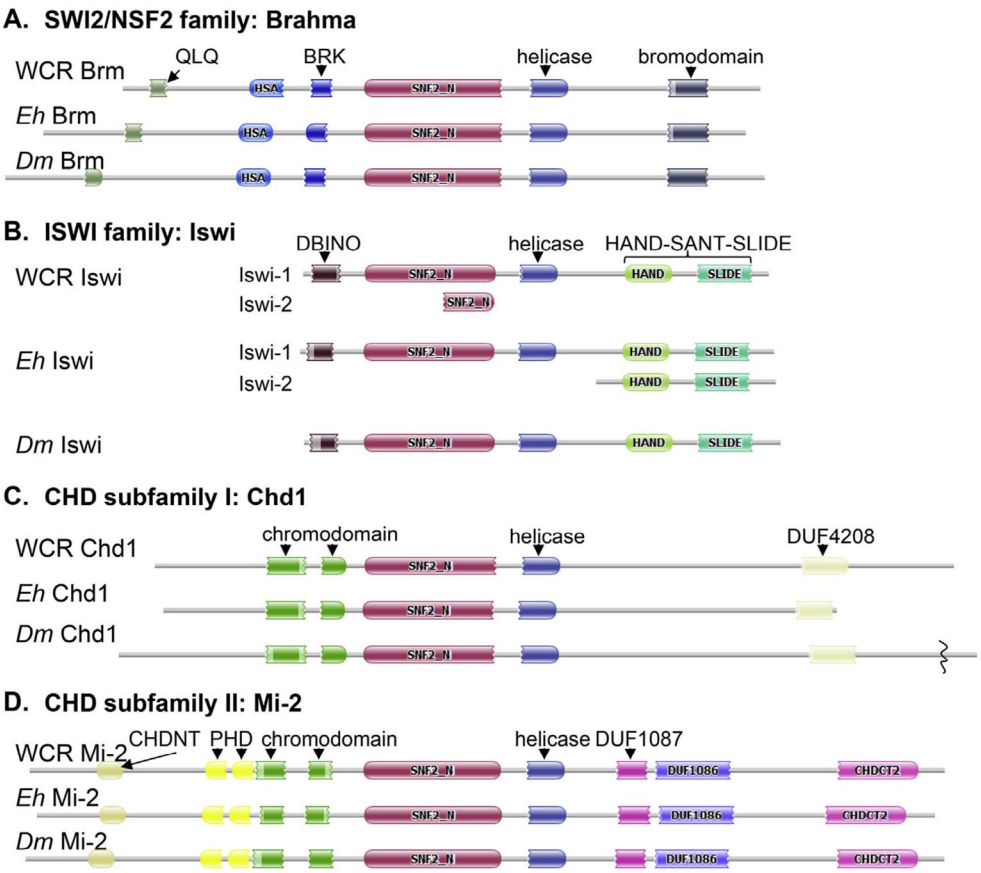

Figure 1. Domain conservation of chromatin remodeling ATPases in *D. v. virgifera* (WCR), E. heros (Eh), and Drosophila melanogaster (Dm)**.** Pfam database identified the following domains: SNF2 family N-terminal (PF00176) and Helicase conserved C-terminal (PF00271) domains, QLQ (PF08880), HSA (PF07529), BRK (PF07533), bromodomain (PF00439), DBINO DNA-binding domain (PF13892), HAND (PF09110), SLIDE (PF09111), chromodomain (PF00385), CHDNT (PF08073), PHD fingers (PF00628), CHDCT2 [NUC038] domain (PF08074), and domains of unknown function DUF1087 (PF06465), DUF4208 (PF13907), and DUF1086 (PF06461).

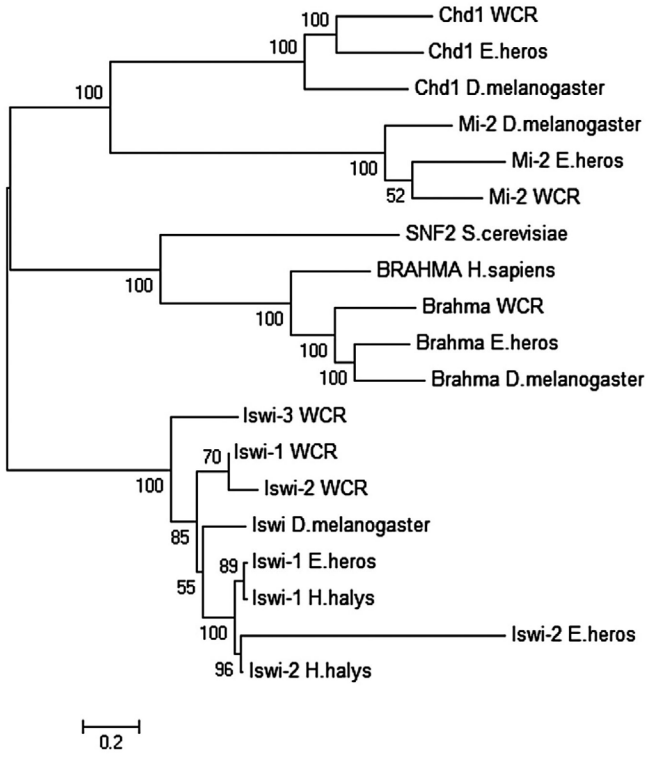

Figure 2. Phylogenetic tree of ATP-dependent remodelers from *D. v. virgifera* (WCR), *E. heros,* and *Drosophila melanogaster***.** For comparison, the tree also contains human BRAHMA, *Saccharomyces cerevisiae* SNF2, and Iswi homologs from the brown marmorated stink bug, *Halyomorpha halys*.

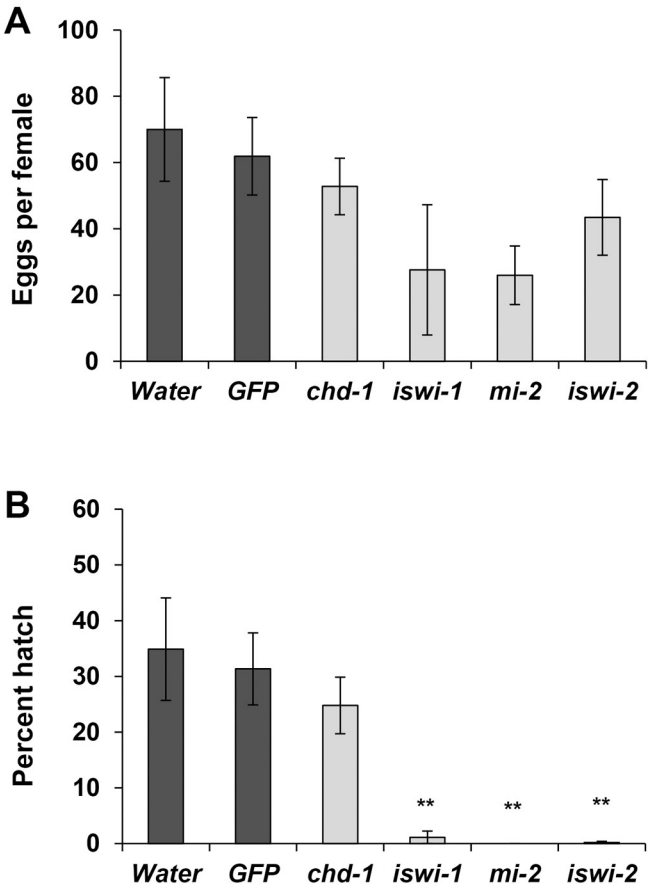

Figure 3. *D. v. virgifera* oviposition and percent egg hatch rates following knockdown of chromatin remodeling ATPases. Females were fed with diet treated with 0.67 µg/µl dsRNA 6 times; the diet was provided every other day for 12 days. **A.** Oviposition: eggs collected from dsRNA-fed females after last feeding exposure. **B.** Percent egg hatch: egg hatch rate was calculated based on eggs oviposited in **A.** Means comparisons were performed with water as control using Dunnett's Method in JMP, $^{**}p < 0.05$.

of Iswi-2 protein from *E. heros* contains only the HAND-SANT-SLIDE domain (Figure 1B). Since *E. heros* Iswi-2 does not contain the first half of the expected Iswi protein sequence, it is likely that our *E. heros* transcriptome includes an incomplete *iswi-2*. A BLAST search of publically available brown marmorated stink bug, *H. halys,* proteins also identified two Iswi homologs (HHAL009893-PA and HHAL007873-PA), which paired with *E. heros* homologs (Figure 2). Thus, the phylogenetic tree supports the presence of two ISWI family members in *E. heros*. The *E. heros* Iswi sequences share 95.3% amino acid identity over the aligned region. At the nucleotide level, the *E. heros iswi-1* and *iswi-2* share 76.2% identity over the aligned region. The dsRNA targeting *iswi-1* of *E. heros* shares no consecutive 21-mers with the identified *iswi-2* transcript. However, dsRNA targeting *E. heros iswi-2* has a single 24 nt match to the *iswi-1* transcript. Consequently, the RNAi effects of *E. heros iswi-2* dsRNA may be attributed to the depletion of the Iswi-2 protein or to the ability of *iswi-2*-targeted dsRNA to knockdown *iswi-1* (Figure 2).

Multiple WCR and *E. heros* SNF2 + Helicase-containing proteins clustered with the CHD (chromodomain helicase DNA-binding) family (Figure 2). This family of ATP-dependent remodeling enzymes contains two amino-terminal chromodomains [chromatin organization modifier] (Figure 1C–D). The CHD family is further subdivided into three subfamilies (Marfella and Imbalzano, 2007), here referred to as subfamilies I, II, and III. The *Drosophila* CHD proteins include Chd1, Mi-2, Chd3, and Kismet. The *Drosophila* Chd1 belongs to CHD subfamily I, which comprises a C-terminal DNA-binding domain (Figure 1C, Pfam family: DUF4208). WCR and *E. heros* Chd1 homologs share the same domain architecture as their *Drosophila* counterpart (Figure 1C). CHD subfamily II carries no DNA-binding domain but has Zn-finger-like domains called PHD (plant homeodomain) fingers; *Drosophila* Mi-2 and Chd3 belong to this subfamily (Marfella and Imbalzano, 2007). We identified WCR and *E. heros* proteins that most likely represent Mi-2 orthologs in these insects (Figure 2). The WCR and *E. heros* Mi-2 proteins mirror the *Drosophila* domain arrangement, which includes the SNF2 ATPase/helicase domain, the double chromodomain, PHD fingers, the CHDNT domain that is associated with PHD finger-containing chromodomain helicases, and two other conserved domains of unknown functions, DUF1087 and DUF1086 (Figure 1D).

3.2. Parental RNAi effects of chromatin-remodeling ATPases in D. v. virgifera

dsRNA targeting remodeling ATPase transcripts *iswi-1*, *iswi-2*, *chd1*, and *mi-2* were fed to gravid WCR females via surface application of dsRNA to artificial diet. There were no significant differences in female mortality between dsRNAs targeting chromatin remodeling ATPases and the controls; mortality in all treatments ranged between 0% and 13%. Oral application ofWCR *mi-2* and *iswi- 1* dsRNAs to gravid females resulted in somewhat lower numbers of eggs per female (average eggs per female were 26 and 29, respectively) compared to females in the water and *GFP*

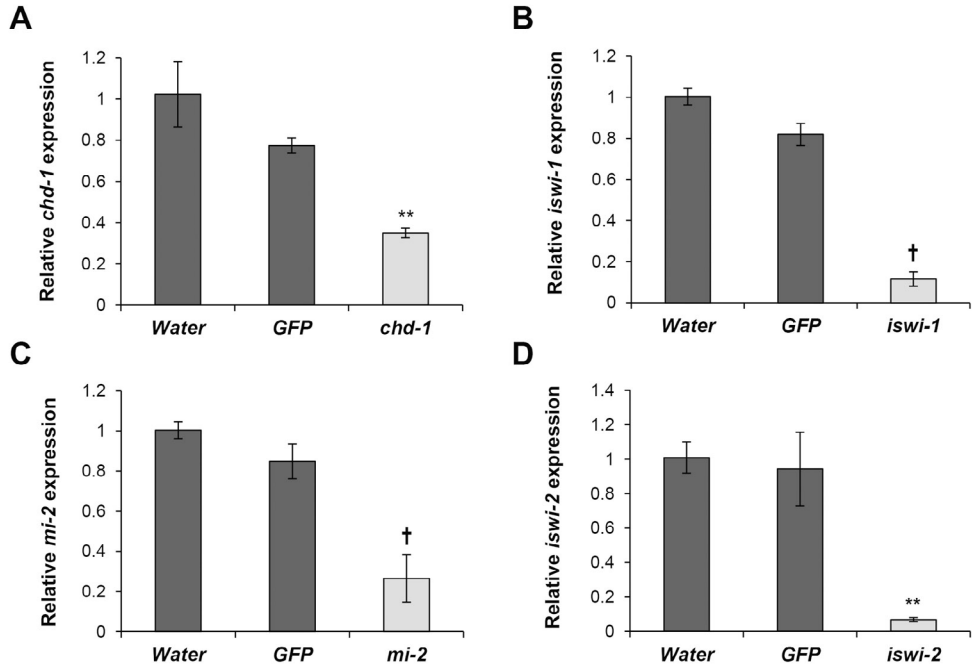

Figure 4. Percent knockdown of chromatin remodeling ATPases in *D. v. virgifera* females. Relative expression was normalized to *β-actin*. Females were fed with diet treated with 0.67 μg/μl dsRNA 6 times, diet was provided every other day for 12 days. Females were collected two days after the last dsRNA feeding. **A.** Relative *chd-1* transcript expression. **B.** Relative *iswi-1* transcript expression. **C.** Relative *mi-2* transcript expression. **D.** Relative *iswi-2* transcript expression. Means comparisons were performed with water as control using Dunnett's Method in JMP, † $p < 0.001$, ** $p < 0.05$. $N = 3$ to 6 individual females.

dsRNA controls (average eggs per female were 70 and 62 respectively; Figure 3A, also see Supplemental Figure 2A). Moreover, the percent of larvae hatching from females fed with *mi-2*, *iswi-1*, and *iswi-2* dsRNAs was <2% for all treatments; these hatch rates were significantly reduced compared to the 35% percent hatching in water-treated and 31% in *GFP* dsRNA-treated females (Figure 3B, also see Supplemental Figure 2B). The results presented in Figure 3 indicate a low rate of hatching in the control which may be the result of seasonal variation in egg viability that we have observed among commercially available lab populations of WCR. To verify the results, the experiment was repeated with *mi-2* and *iswi-1* dsRNAs. The second experiment produced similar results (0.15% hatch rate for *iswi-1* dsRNA and no eggs hatched for *mi-2* dsRNA), with higher egg hatch rates in the controls (74% for water and for 78% *GFP* dsRNA) (Supplemental Figure 2). Dissections of unhatched eggs revealed an absence of embryonic development.

A decrease of relative transcript levels (>60%) was observed in WCR females exposed to *chd1* (Figure 4A), *iswi-1* (Figure 4B), *mi-2* (Figure 4C), and *iswi-2* dsRNA (Figure 4D). A similar trend was observed with the eggs produced by the females exposed to chromatin remodeling ATPase dsRNA. The relative expression levels of *iswi-1* (Figure 5B), *mi-2* (Figure 5C), and *iswi-2* (Figure 5D) were lower compared to the control eggs. However, the transcript levels of *chd1* in eggs (Figure 5A) were not statistically different from the controls. Thus, it is possible that the lack of pRNAi phenotype ofWCR *chd1* (Figure 3A and B) is due to incomplete knockdown in eggs rather than *chd1* being non-essential for embryo development. For WCR genes that showed strong effects on hatching rates (*iswi-1, mi-2,* and *iswi-2*), egg dissections revealed a lack of embryonic development. Overall, these results indicate that in addition to *brahma* (Khajuria et al., 2015), chromatin remodelers belonging to the ISWI and CHD subfamily II (*i.e.*, *mi-2*) can serve as effective parental RNAi targets for parental control of WCR.

3.3. Lethal RNAi effects in E. heros nymphs injected with dsRNA

To establish whether *E. heros* are susceptible to RNAi, 2nd instar nymphs were injected with dsRNA targeting essential gene transcripts. Two dsRNAs targeting *E. heros* transcripts that likely represent cytoplasmic and muscle actins were used. *Act-1* dsRNA targets *E. heros* cytoplasmic actin, which shares 100% amino acid identify with the *Drosophila Actin5C* and 99.47% with the *Drosophila Actin42A*, while *act-2* dsRNA targets *E. heros* muscle actin, which shares 98.40% identity *with the Drosophila Actin87E* and 97.87% identity *Actin57B*. The two *E. heros* actins share 97.34% identity. High-dose injection of both *act-1* and *act-2* dsRNA resulted in 100% mortality, while the negative controls (non-injected and *YFP* dsRNA injected) showed mortality under 10% (Table 1). To probe the range of stink bug sensitivity to RNAi, we carried out serial dilutions (10X, 100X, and 1000X) of *actin* dsRNAs. In this experiment, even 1000X dilution (0.0276 ng of dsRNA or 18.4 ng/g to 27.6 ng/g body weight) led to over 80% mortality (Supplemental Figure 1). Further, there was no significant difference between *YFP* dsRNA injected and noninjected insects. Overall, it appears that *E. heros* is highly sensitivity to injected dsRNA targeting essential genes. Based on the observed lethal response to dsRNA targeting *actin* transcripts, we tested whether *brahma* dsRNA confers lethality in 2nd instar *E. heros* nymphs. Similar to results with WCR, mortality for *E. heros brm* dsRNA was not significantly different from that observed with insects exposed to the same amount of *YFP* dsRNA (Table 1).

3.4. Parental RNAi phenotypes in E. heros

Zero to three day-old *E. heros* females were injected to verify that *YFP* dsRNA injection could be used as a negative control in pRNAi experiments. Oviposition was compared between females injected with *E. heros brm* dsRNA and those not injected. *YFP* control

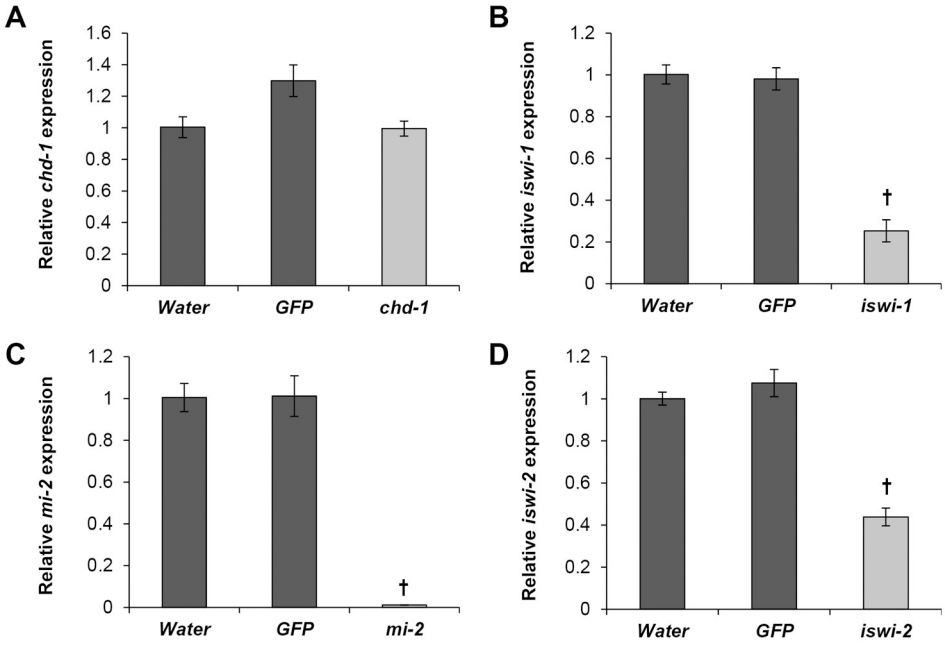

Figure 5. Percent knockdown of chromatin remodeling ATPases in *D. v. virgifera* eggs produced by dsRNA-fed females. Relative expression was normalized to *β-actin*. **A.** Relative *chd-1* transcript expression. **B.** Relative *iswi-1* transcript expression. **C.** Relative *mi-2* transcript expression. **D.** Relative *iswi-2* transcript expression. Means comparisons were performed with water as control using Dunnett's Method in JMP, † $p < 0.001$, ** $p < 0.05$. N = 3 sets of 10 eggs.

females did not produce statistically different numbers of eggs from not-injected females (Table 2). Based on this observation, only a single control (*YFP* dsRNA) was used in all bioassay experiments. These data also show that while the *YFP* dsRNA and non-treated female groups produced over a thousand eggs, females injected with *brm* dsRNA did not oviposit.

For parental RNAi experiments with *E. heros*, egg hatch rates, and female viability in response to injections of dsRNAs targeting *brm*, *chd1*, *iswi-1*, *iswi-2*, and *mi-2* was recorded. Higher mortality was observed in females injected with *brahma* and *mi-2* dsRNA compared to the other dsRNA applications (Figure 6A). Stink bug females molt into adults with undeveloped ovaries, and under laboratory conditions the pre-oviposition period in *E. heros* lasts approximately 8 days (Silva et al., 2011). To allow for a preoviposition period, oviposition in *E. heros* was monitored after dsRNA injection and the eggs were collected on a daily basis after the negative control insects initiated oviposition. To visualize any trend in oviposition and hatch, we binned the data on a weekly basis for the first two weeks of egg collection (Figure 6B and C). Injection of dsRNA for all chromatin remodeling ATPases except *E. heros iswi-2*, greatly decreased oviposition compared to the *YFP* dsRNA negative control or eliminated oviposition altogether (Table 3, Figure 6B).We observed a reduction in oviposition for most of the chromatin remodelers in the second week of egg collection relative to the control treatment. It is possible that a timedependent decrease in oviposition for the chromatin remodeler parental effect represents a delay in the parental RNAi effect. The delay in pRNAi response may reflect the rate at which RNAi effect spreads in insects, protein turnover rate, or other factors that may influence the general onset of the RNAi response. The decrease in oviposition can also be gleaned both from the total number of eggs oviposited within the fifteen-day egg collection period and from the average numbers of eggs produced per female per day (Table 3). When daily oviposition rates were compared over the entire egg

Table 1. Viability of 2nd instar *E. heros* nymphs injected with *actin* and *brm* dsRNA. Percent mortality was scored seven days after injection. Mortality was evaluated in three trials, with ten insects injected for each dsRNA. Means comparisons were performed with *YFP* dsRNA as control, using a Student *t*-test with Dunnett's adjustment in JMP. N = number of trials; SEM = standard error of the mean; * significantly different from *YFP* dsRNA p < 0.05.

Treatment	Average % Mortality	SEM	N Trials	*p*-Value
YFP dsRNA	3.3	3.3	3	
not injected	6.7	3.3	3	0.6438
E. heros act-1	100	0.00	3	<0.0001*
E. heros act-2	100	0.00	3	<0.0001*
YFP dsRNA	10	5.5	3	
not injected	13	3.3	3	0.9384
E. heros brm	27	12.0	3	0.3039

Table 2. Oviposition by *E. heros* females in response to *brahma* dsRNA injections. Ten females were injected with 138 ng of dsRNA targeting *E. heros brahma* or the negative control, *YFP* dsRNA. Egg counts were recorded starting on day 7 post injection for 15 consecutive days. For the *brahma* treatment, the collection was stopped at 13 days due to female mortality. Means comparisons were performed on average numbers of eggs produced by females, using daily oviposition values. *YFP* dsRNA was used as control for Student *t*test with Dunnett's adjustment in JMP. N = number of days; SEM = standard error of the mean; * significantly different from *YFP* dsRNA p < 0.05.

dsRNA	Total # of eggs produced in 15 days	Average # of eggs/day/female	SEM	N Days	p-Value
YFP	1280	10.12	0.53	15	
Not injected	1429	9.53	0.74	15	0.6697
E. heros brm	0	0.00	0.00	13	<0.0001*

Table 3. Oviposition by *E. heros* females injected with chromatin remodelers dsRNA. Total numbers of eggs produced in 15 days and average numbers of eggs per female injected with negative control *YFP* dsRNA or chromatin remodeling ATPase dsRNAs. Twenty females were injected with each dsRNA. Egg counts started on day 9 post-injection and continued for 15 consecutive days. The N number of days during which eggs were collected varied between treatments due to female mortality in *brm* and *mi-2* treatments. Means comparisons were performed on average numbers of eggs produced by females, using daily oviposition values. *YFP* dsRNA was used as control for Student t-test with Dunnett's adjustment in JMP. N = number of days; SEM = standard error of the mean; *significantly different from *YFP* dsRNA p < 0.05.

dsRNA	Total # of eggs in 15 days	Average # of eggs/day/female	SEM	*N* Days	*p*-Value
YFP	1629	6.75	0.357	15	
E. heros brm	0 0.00		0.000	10	<0.0001*
E. heros chd1	496	2.65	0.338	15	<0.0001*
E. heros iswi-1	209	0.84	0.142	15	<0.0001*
E. heros iswi-2	1097	5.54	0.433	15	0.0171*
E. heros mi-2	42	0.22	0.085	13	<0.0001*

collection period, all the evaluated chromatin-remodeling ATPase treatments produced significantly fewer eggs than the *YFP* dsRNA control (Student *t*-test, p < 0.05) (Table 3).

Eggs produced within each day of the pRNAi experiment hatched within four to six days. We recorded egg hatch rates with respect to the date of oviposition (*i.e.*, day 1 hatch represents total eggs that hatched from eggs that were produced on day 1 of collection). Both the total numbers of eggs hatched and the hatch rates indicated that the number of offspring produced by *E. heros* females injected with dsRNAs for all chromatin remodelers were significantly lower compared to the controls (Figure 6C and Table 4). Interestingly there was a decrease in the *iswi-2* phenotype over time suggesting that the pRNAi response decreases after acute exposure and should be studied in greater detail to better predict pRNAi outcomes in potential field applications of this technology.

To confirm the knockdown of chromatin remodeling genes, we performed qRT-PCR using cDNA generated from ovary tissues of *E. heros* treated with dsRNA. All genes, with the exception of *iswi-2* (not shown, see Methods), showed > 70% knockdown relative to the non-injected control (Figure 7). Our inability to measure *iswi-2* knockdown may reflect the low endogenous expression level of this gene. Low gene expression of *E. heros iswi-2* was also consistent with the weakest pRNAi phenotype. Alternatively, our inability to detect *iswi*-2 may also be an effect of poor primer/probe performance in qRT-PCR (Supplemental Table 2).

3.5. Ovarian development phenotypes in E. heros

Based on the complete lack of oviposition in *E. heros* in response to *brahma* dsRNA and severe inhibition of oviposition in response to *mi-2* dsRNA, we investigated the state of oocyte and ovary development in parent females. The females were examined 9 and 14 days post injection. By day nine after injection, control females began oviposition. Since *brm* dsRNA injections led to lethality within about two weeks, day 14 was chosen to capture phenotypes from the last surviving females (Figure 6A). Mature eggs and developing oocytes were observed in *YFP* dsRNA-injected females (Figure 6C and D). *Brahma* and *mi-2* dsRNA-injected females showed lack of ovary development and ovariole elongation (Figure 8). These insects showed no maturing oocytes or mature eggs (Figure 8E, G, and H), or oocytes that were in a state of decay (Figure 8F).

4. Discussion

The experiments presented in this study explore the possible use of chromatin-remodeling ATPases as gene targets for parental RNAi. Results from WCR support the parental RNAi effect for the *brahma*-related genes *mi-2* and two *iswi* homologs, *iswi-1* and *iswi- 2*. The effects observed for these genes including reduced rates of egg hatching are similar to the phenotype reported for

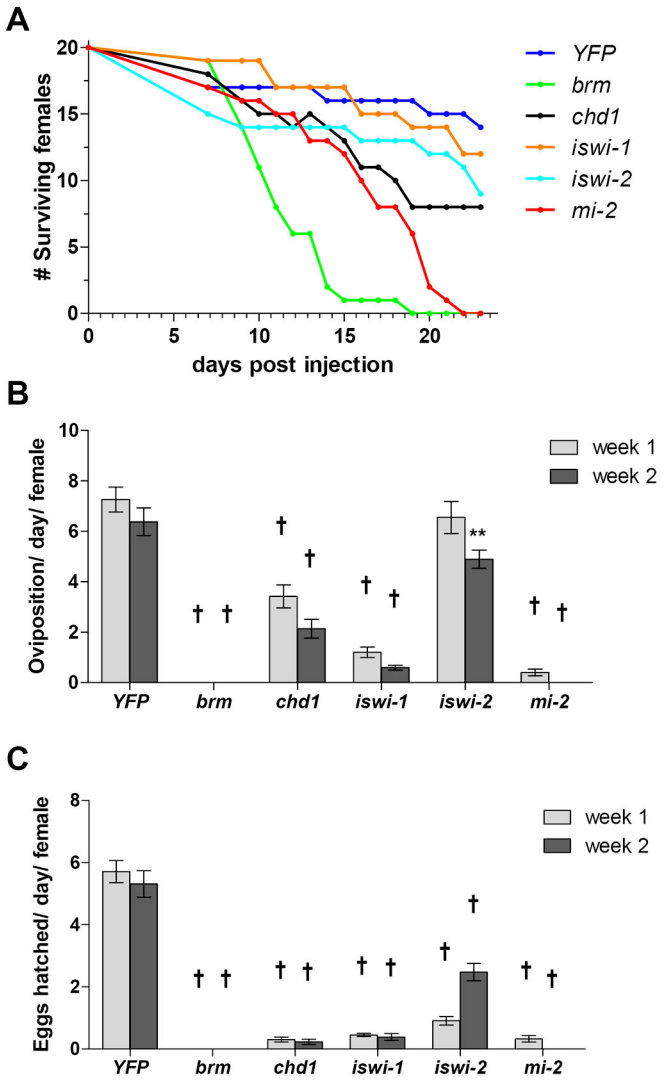

Figure 6. *E. heros* adult female survival, oviposition, and egg hatch rates following dsRNA injections that target chromatin remodeling ATPases. Females were injected with dsRNA at 0–2 days post adult molt. **A.** Female survival: twenty females were injected with each dsRNA and survival rate was monitored for 23 days. **B.** Oviposition: eggs collected from dsRNA-injected females starting at 9 days post-injection. The oviposition rates plotted are per day per female, based on each week of collection. **C.** Egg hatching: eggs hatched based on the numbers oviposited in B. Means comparisons were performed with *YFP* dsRNA as the control, using Dunnett's Method in JMP, † $p < 0.001$, ** $p < 0.05$.

Table 4. Total and average numbers of eggs hatched from *E. heros* females injected with chromatin remodelers dsRNA. Total numbers of eggs hatched in 15-day collection and average number of eggs hatched per female per day of oviposition, from females injected with negative control *YFP* dsRNA or chromatin remodeling ATPase dsRNAs. Twenty females were injected with each dsRNA. Nymph emergence was evaluated from eggs oviposited on day 9 post-injection for 15 consecutive days. Means comparisons were performed on numbers of eggs hatched each day per female, using daily values. *YFP* dsRNAwas used as control for Student-t test with Dunnett's adjustment in JMP. SEM = standard error of the mean; *significantly different from *YFP* dsRNA p < 0.05.

dsRNA	Total # of eggs hatched from 15-day collection	Average # of eggs hatched/female/day	SEM	*p*-Value
YFP	1321	5.47	0.257	
E. heros brm	0	0.00	0.000	<0.0001*
E. heros chd-1	51	0.28	0.054	<0.0001*
E. heros iswi-1	93	0.39	0.062	<0.0001*
E. heros iswi-2	312	1.63	0.253	<0.0001*
E. heros mi-2	34	0.17	0.067	<0.0001*

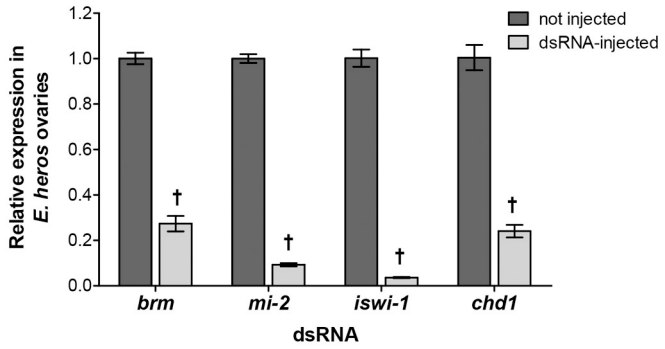

Figure 7. Percent knockdown of chromatin remodeling ATPases in *E. heros* ovaries. *E. heros* muscle actin transcript was used as a reference gene and ovaries from noninjected females as a negative controls. Four sets of ovaries were used in each qRTPCR experiment. Means comparisons were performed using Student's *t*-test in JMP; † p < 0.001.

brahma (Khajuria et al., 2015). In *E. heros*, in addition to *brahma*, *mi-2*, and *iswi* homologs, we observed parental RNAi phenotypes for *chd1*. In the *E. heros* experiments, we observed a reduction in the total number of eggs produced in response to *brm*, *chd-1* and *mi-2* knockdowns, and a reduction in eggs hatching for all the chromatin-remodeling ATPases evaluated. Our results also show no dsRNA-mediated mortality or delayed mortality for chromatinremodeling ATPases in adult WCR and *E. heros*. A parental RNAi phenotype is only detected when the parental insect survives and the lethality or phenotype is observed in the next generation. However, parental control may not exclude lethality; the fact that dsRNA targeting chromatin ATPases do not cause lethality enables initial characterization of the parental RNAi response and future studies of the potential benefits of pRNAi traits. The above results, taken together with our assumptions about pRNAi traits, support the utility of chromatin remodeling genes as RNAi targets for parental control of multiple pest insect species.

Identifying pRNAi gene targets that deliver a broad response is important to optimize the efficacy of a pRNAi insect resistance trait. Epigenetic effects conferred by chromatin remodeling ATPases and other chromatin modifying enzymes make them attractive targets for multi-generational/parental RNAi. Brahma-related proteins and SNF2-type chromatin-remodeling ATPases are subunits of chromatin remodeling complexes that play global roles in mobilizing nucleosomes. These proteins stably change chromatin accessibility and alter gene expression during critical stages of development (Bouazoune and Brehm, 2006; Ho and Crabtree, 2010). Thus, a short-term RNAi exposure that targets a chromatin-remodeling ATPase can potentially lead to long-lasting effects.

In addition to epigenetic effects, characteristics of RNAi target genes that may impact the efficacy of pRNAi include whether the gene is essential for oogenesis and/or development. In terms of pRNAi response, an oogenesis effect may result in lack of oviposition by females (a fertility defect), while a developmental defect may result in a decrease in egg hatch or an increase in larval mortality (decrease in fecundity). In this study we observed both fertility and fecundity defects. More specifically, in WCR we observed no significant decreases in oviposition for *mi-2*, *iswi-1* and *iswi-2* dsRNA treatments (Figure 3A and Supplemental Figure 2A). Whereas in *E. heros*, all chromatin-remodeling ATPase dsRNA treatments led to significant decreases or a complete absence (*brahma*) of oviposition (Table 3). In interpreting these results, it is likely that an oogenesis effect will also impact egg hatch and early embryonic development and therefore overall fecundity of the insects. However, there is also a possibility that the expression of the genes studied here is necessary during both oogenesis and embryogenesis; a requirement at both of these stages would likely increase the reliability of pRNAi phenotype and the durability of a trait.

Most of the knowledge of chromatin-remodeling ATPases in insects is based on studies in *D. melanogaster* (Bouazoune and Brehm, 2006). In *Drosophila*, chromatin remodeling ATPases are known to have roles in both oogenesis and development. The *Drosophila* chromatin remodeling ATPases that are essential for oogenesis include Iswi (Deuring et al., 2000), Chd1 (McDaniel et al., 2008), Mi-2 (Kehle et al., 1998), and possibly Brm (He et al., 2014). In addition to oviposition defects, it was reported that the lack of maternal *iswi* leads to developmental arrest in an early stage of oogenesis (Deuring et al., 2000). It is also known that the *Drosophila chd1* is required for gametogenesis in both sexes (McDaniel et al., 2008). Based on the complexity of chromatin-remodeling ATPase phenotypes in *Drosophila,* it is clear that more studies will be necessary to determine clear roles of these genes in oogenesis in both Coleoptera and Hemiptera. It is also possible that chromatinremodeling ATPases are involved in male gametogenesis in these insects.

After oogenesis is complete, the function of developmental genes can be contributed maternally (as mRNA or protein) or expressed zygotically. A maternal vs. zygotic effect may be difficult to assess in pest insects due to genetic intractability (*e.g.*, transgenic approaches that would enable tissue-specific gene knock-downs). Nevertheless, the impact of maternal contribution can be very significant. Frequently, homozygous mutant offspring of a heterozygous mother can survive to adulthood using the mother's deposit of RNA or protein in the egg; in fruit flies the essential function of such genes can be assessed by germline mutant clones (Perrimon, 1998). Since it is believed that the RNAi effect is not amplified in insects (Roignant et al., 2003; Tomoyasu et al., 2008), pRNAi may more effectively target the finite mRNA load from the mother. Hence, genes that have essential maternal contribution of mRNA are likely to offer more sensitive pRNAi targets. In *Drosophila*, for example, the maternal contribution of *brahma* is needed for early embryogenesis, while the zygotic *brahma* expression is necessary

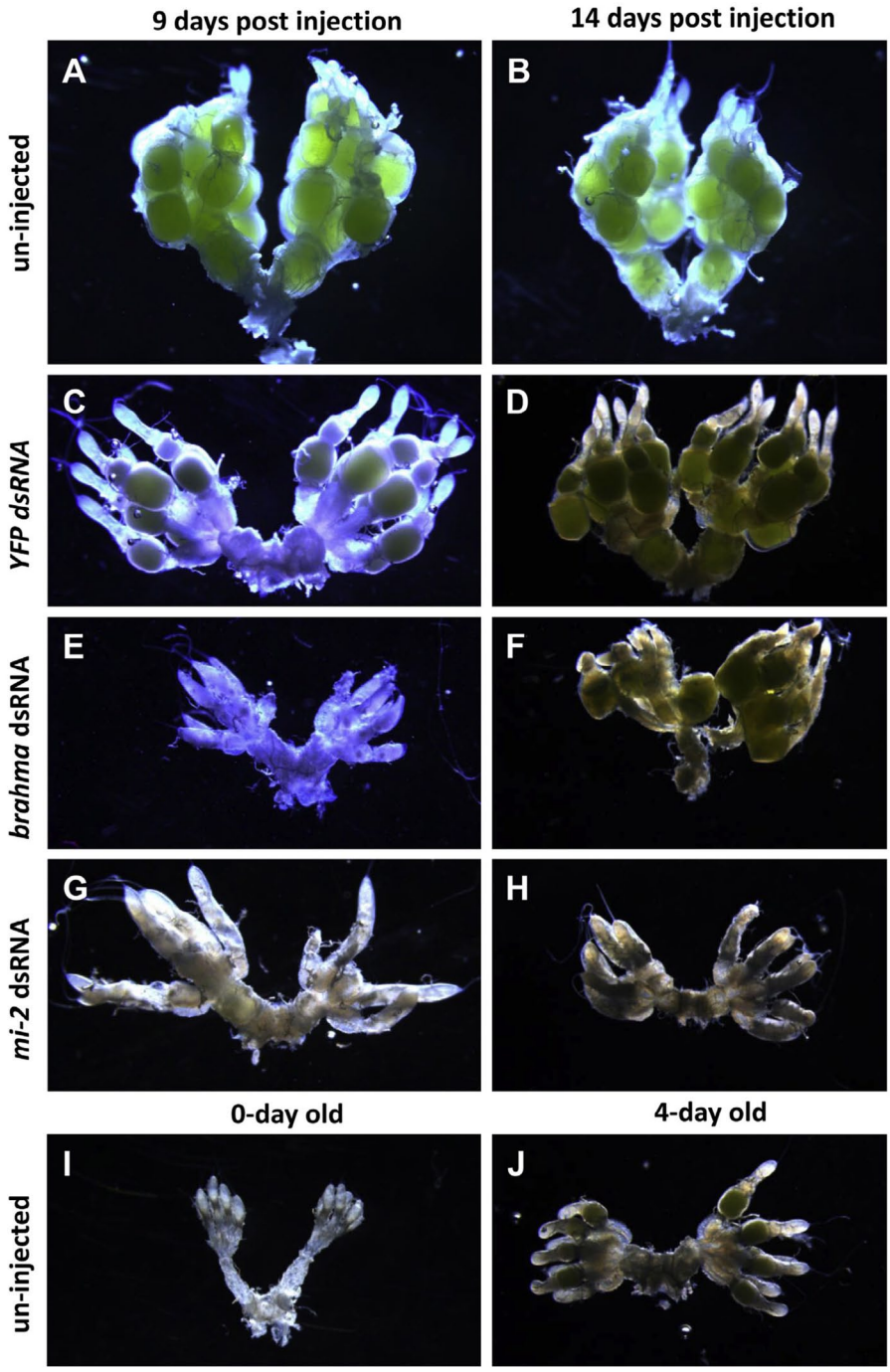

Figure 8. Ovaries of *E. heros* females injected with *brm* or *mi-2* dsRNA. A – B. Ovaries of non-injected 9 and 14 day-old adult *E. heros* females. **C** – **D.** Ovaries of females injected with *YFP* dsRNA, **E** – **F.** Ovaries of females injected with *brm* dsRNA at 9 and 14 days post injection. **E.** Shows lack of ovariole elongation and lack oocyte development, and **F.** shows decaying oocytes. **G** – **H.** *mi-2* dsRNA at 9 and 14 days post injection. **H.** Shows lack of ovariole elongation and **G.** shows somewhat elongated ovaries with no mature oocytes. **I** – **J.** Ovaries of non-injected *E. heros* females at zero and four days after adult molt, provided for developmental comparison.

for late embryonic development (Brizuela et al., 1994). For zygotically expressed genes, the dsRNA loaded into the oocyte must target newly expressed and replenishable transcripts. Therefore, it is possible that gene products with high zygotic expression will present more challenging targets for pRNAi.

The pRNAi phenotypes observed inWCR and *E. heros* are largely consistent with mutant phenotypes described in *Drosophila*. Our observations of parental effects in coleopteran and hemipteran pests also generally agree with each other. The main differences between *E. heros* and WCR involved significant decreases in oviposition in response to *brm*, *mi-2*, and *iswi-1* dsRNA in *E. heros*, but no significant decreases in oviposition rates for WCR. Further, *E. heros* ovarian development was severely stunted in response to *brm* and *mi-2* dsRNA (Figure 8). Since injection-based dsRNA delivery is expected to be far more effective than oral delivery, the differences observed in responses between species were likely due to delivery method. The differences in oogenesis are less likely to be the cause of phenotypic differences in

pRNAi response, since both Coleoptera and Hemiptera have telotrophic meroistic ovarioles (Klowden, 2008). Newly molted stinkbug females have undeveloped ovaries, and the ovary development seems to proceed at the same rate regardless of whether the female has mated or not (Fortes et al., 2011). The pre-mating and pre-oviposition periods seem to vary based on the species, with the pre-mating period lasting up to ten days with pre-oviposition periods of up to two weeks (Fortes et al., 2011; Silva et al., 2011). Similarly in WCR, the pre-oviposition period lasts between 12 and 15 days (Branson and Johnson, 1973; Hill, 1975). The fact that in both species develop ovaries during adulthood suggests that early exposure of females to dsRNA targeting chromatin remodeling genes may have a greater effect on genes that have functions in oogenesis. In addition to the method of application, the differences observed in pRNAi phenotypes are likely a combination of the factors such as individual gene functions, their functions in the egg or in the ovary, their maternal or zygotic functions, mRNA stability, and protein turnover.

The use of parental RNAi as a pest management tool for WCR was initially suggested for the gap gene *hunchback* and the chromatin-remodeling ATPase *brahma*; both genes prevented egg hatching (Khajuria et al., 2015). Here we described a family of WCR chromatin-remodeling genes, *mi-2*, *iswi-1* and *iswi-2*, that lead to pRNAi effects similar to those reported for *brahma* (Khajuria et al., 2015). Moreover, we demonstrated that pentatomid stink bugs also exhibit robust lethal and parental RNAi responses.

A successful RNAi response depends on the sensitivity of the insect, the efficacy of the RNAi target gene, and the delivery method. The current study examined the sensitivity of efficacy of pRNAi genes in WCR and *E. heros*. In terms of sensitivity to RNAi, WCR has previously shown a strong pRNAi response (Khajuria et al., 2015), our current results indicate that *E. heros* are highly sensitive to dsRNA by injection. A dose of ~20 ng of dsRNA per gram of the insect body weight (ng/g) or 20 parts per billion (ppb) for an essential gene (*actin*), led to approximately 80% mortality. Baum and Roberts (2014) compiled a comprehensive set of RNAi targets and concentrations tested in insects which indicates that Coleoptera are some of the most sensitive insects to orally administered dsRNA, with LC50 for multiple gene targets in WCR ranging from 1 to 10 ppb. While in Hemiptera, the LC50 ranged between 3 and 5000 ppm for ingested dsRNA with a number of species exhibiting no detectable response at the highest concentration tested by feeding. In the desert locust, *Schistocerca gregaria*, doses of dsRNA as low as 1 ng/g of body weight (1 ppb) lead to significant decreases in transcript levels (Wynant et al., 2012). In other orders such as Diptera, generally higher doses are needed to achieve an effective RNAi response. For example, in the African malaria mosquito, *Anopheles gambiae*, doses on the order of 1 µg/g to 10 µg/g are necessary to achieve an effective RNAi response (Boisson et al., 2006); and Lepidoptera needs a vast range of concentrations that span from <0.01 µg/g to > 1 µg/g depending on the species (Terenius et al., 2011). *Drosophila* lack systemic RNAi (Roignant et al., 2003), and therefore are not as sensitive to injection or oral administration of dsRNAs (*e.g.*, most RNAi assays in *D. melanogaster* are performed with transgenic flies designed to express hairpin RNAs (Dietzl et al., 2007)). However, observations that dsRNA incorporated into cationic liposomes leads to oral response in *Drosophila* (Whyard et al., 2009), suggest that dsRNA must be protected by formulation to be efficacious. Therefore, a functional RNAi response in the insect and potent lethal RNAi or pRNAi gene targets are among of the critical milestones to delivering RNAi to pest insects.

In terms of the efficacy of RNAi targets, our results demonstrate a robust parental RNAi response for several chromatin-remodeling ATPases that greatly decreases or eliminates oviposition and/or development of the embryos. The delivery of dsRNA to trigger an RNAi response has been demonstrated for WCR larval control via expression of long dsRNA hairpins in maize (Baum et al., 2007). The parental phenotypes via *in planta* expression are yet to be documented. For pentatomid stink bugs, field-relevant enablement of pRNAi, or RNAi in general, may present additional challenges. Based on observations in *L. lineolaris* (Allen and Walker, 2012), it is likely that *E. heros* has salivary ribonuclease activity, this implies that some form of protection of dsRNA may be necessary to achieve oral activity. While we have documented that *E. heros* exhibits sensitivity to injected dsRNA, it will be necessary to develop an oral dsRNA delivery system for stink bug management for either lethal and parental RNAi targets. In addition to formulations, a potential strategy to delay the effect of ribonucleases in saliva would be the expression of dsRNA in chloroplasts instead of expressing dsRNA in the cytoplasm. This strategy has been tested in Coleoptera (Zhang et al., 2015) and further studies are necessary to determine if this can be an effective way to deliver dsRNA for stink bugs. Our observations of pRNAi phenotypes taken together with known roles of chromatin-remodeling ATPases in oogenesis and essential embryonic maternal and zygotic functions support their utility in parental management of coleopteran and hemipteran pests. To fully evaluate the utility of pRNAi as a pest management tool more studies are necessary to identify the dose, timing, and duration of exposure necessary to achieve pRNAi and the longevity of the pRNAi response for both WCR and *E. heros*.

Parental RNAi has the potential to augment existing refuge-based insect resistance management (IRM) strategies for insect protected crops when expressed in combination with one or more insecticidal substances, such as Bt proteins, that protect from the same pest populations. This benefit arises when insects that are resistant to a Bt protein occur as a higher proportion of the population in the transgenic crop than in the refuge crop that does not produce Bt proteins. If only adults that carry resistance to a Bt protein feed on pRNAi crops (because they developed in the crop that expresses the Bt protein) and susceptible adults from refuge areas are not exposed to pRNAi through feeding, the ratio of Btresistance alleles to Bt-susceptible alleles that are passed on to the next generation will be lower in the next generation. In this way, the co-expression of pRNAi and a Bt protein can help to improve the effectiveness of the refuge in delaying Bt resistance. On the other hand, if the pRNAi affects adults from both the transgenic and the refuge crops (for example if the refuge is presented as a seed blend with the transgenic crop or if there are high levels of adult dispersal between refuge and transgenic crop patches prior to feeding), this benefit of delayed resistance development is reduced. To identify the IRM benefits of pRNAi, information on the effects of temporal exposure to pRNAi relative to pest dispersal and feeding behavior would be needed. Further, more research is needed to understand the mode of action and interaction(s) of pRNAi with insecticides, Bt toxins, other RNAi targets, and it's efficacy on populations resistant to other means of insect control.

Acknowledgments — This research was supported through the University of Nebraska-Lincoln, Life Sciences Industry Partnership Grant Program with Dow AgroSciences, Research Agreement No. 2014.0479. The authors acknowledge the dedicated efforts of Natalie Matz and Albina Divizinskaya who assisted with most of the data collection associated rootworm experiments. The authors also thank Dow AgroSciences scientists Shirnivasrao Mane for bioinformatics support and Abhilash Balachandran for insect husbandry.

Appendix A. Supplementary data Supplementary data related to this article follows the References.

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SUPPLEMENTAL TABLES

Supplemental Table 1. Primer pairs used to amplify DNA templates for dsRNA transcription. *E. heros* T7 Promoter: TTAATACGACTCACTATAGGGAGA; *D. v. virgifera* T7 Promoter: TAATACGACTCACTATAGGG; product size excludes T7 sequence.

Gene (Region)	Primer ID	Sequence	Product Length (bp)
brahma	*E. heros_brm*-1-F	T7 + GATGATGAAGAAGATGCAAGTAC	499
	E. heros_brm-1-R	T7 + CTCCACTCCCTCGGGTC	
mi-2	*E. heros_mi-2*-1-F	T7 + GACTACCTCGAGGGTGAAGG	496
	E. heros_mi-2-1-R	T7 + GTAATTCTTCAACAGCTTTATCGTC	
iswi-1	*E. heros_Iswi-1*-1-F	T7 + CAAAAATTGAAACTGACCGTTCTAG	481
	E. heros_iswi-1-1-R	T7 + GCTAATGTTGATTTTGGTACGATG	
iswi-2	*E. heros_iswi-2*-1-F	T7 + GTTCAAGATTTCCAATTTTTCCCAC	490
	E. heros_iswi-2-1-R	T7 + GAAACGGTGCTCTATATCGACTC	
chd1	*E. heros_chd1*-1-F	T7 + CAGCTGGAACCATATATTCTACGAC	496
	E. heros_chd1-1-R	T7 + GTGAATTTTCAGCATTGAAATGATCG	
act-1	*E. heros_act*-1-F	T7 + GACTGAAGCACCTCTTAACCC	488
	E. heros_act-1-R	T7 + CAAGGAATGAAGGCTGGAAAAG	
act-2	*E. heros_act*-2-F	T7 + GATGACCCAGATCATGTTTGAGAC	462
	E. heros_act-2-R	T7 + CAAGATTCCATACCCAAGAAGGAAG	
YFP	*YFP*_F	T7 + GCATCTGGAGCACTTCTCTTTCA	301
	*YFP*_R	T7 + CCATCTCCTTCAAAGGTGATTG	
mi-2	*D. v. virgifera_mi-2*-F	T7 + AAGAAGGCATAGAACAGA	319
	D. v. virgifera_mi-2-R	T7 + TCAGAATGGTAATCAGAGA	
iswi-1	*D. v. virgifera_iswi-1*-F	T7 + TGAATCAGTCTACCAATT	357
	D. v. virgifera_iswi-1-R	T7 + GGTTCTGACTCATCTATT	
iswi-2	*D. v. virgifera_iswi-2*-F	T7 + TTGCTCAATCCTACATACA	270
	D. v. virgifera_iswi-2-R	T7 + GAATACCAACAGGCTACT	
chd1	*D. v. virgifera_chd1*-F	T7 + TTTGCTTCCTTCTTTCAA	315
	D. v. virgifera_chd1-R	T7 + CTTCTTTGTTAAACGGATT	
kis	*D. v. virgifera_ksmt*-F	T7 + GATCAAATTCAAGCAACT	341
	D. v. virgifera_ksmt-R	T7 + TTCTTCCTAAACCATGTT	

etl1	*D. v. virgifera_etl1*-F	T7 + ACTTATCTAAAGGGATGCTA	345
	D. v. virgifera_etl1-R	T7 + GTAGAGAGTCGTCTTCTG	
GFP	*GFP*_F	T7 + GTGATGCTACATACGGAAAG	376
	*GFP*_R	T7 + TTGTTTGTCTGCCGTGAT	

Supplemental Table 2. Oligonucleotides and probes used for *E. heros* probe hydrolysis qRT-PCR assay and primer efficacy results. MGB = Minor Groove Binder probes from Applied Biosystems.

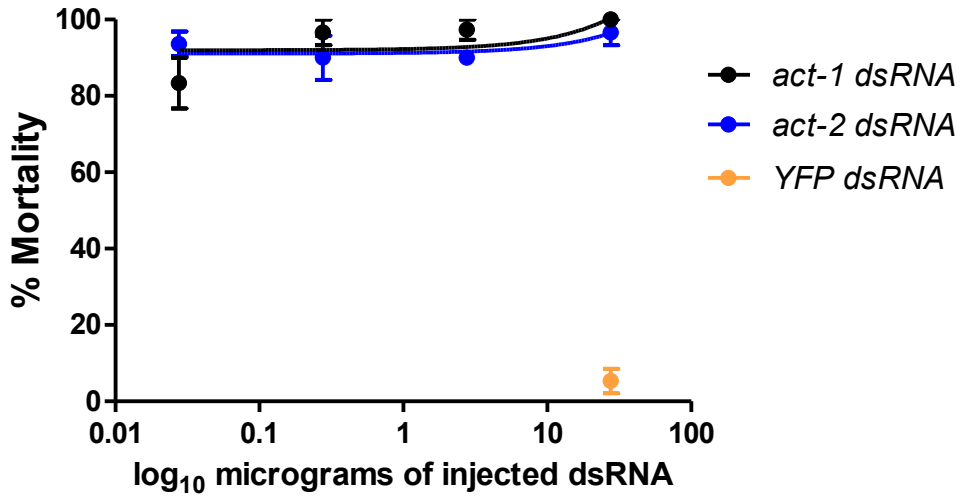
Reference GENE	**NAME**	**SEQUENCE**	**Product Length (bp)**	**Slope**	**Primer Efficiency (%)**
Actin, muscle	*Act*-F	TCAAGGAAAAACTGTGCTATGT	120	-3.77	92
Actin, muscle	*Act*-R	TACCGATGGTGATGACCTGA			
Actin, muscle	*Act*-FAM	ACCGCCGCTGCC			
Target GENE	**NAME**	**SEQUENCE**			
brahma	*brm*-F	TCATCAAGGACAAGGCAGT	205	-3.54	93.5
brahma	*brm*-R	GACGGGAGGAGAAAGTTTAGA			
brahma	*brm*-FAM	CGACGAGGGACACAGGATG			
mi-2	*mi-2*-F	GATGAGGGCTTGCTGTT	149	-3.55	95.5
mi-2	*mi-2*-R	GAGGCGGGAAGTATTGAC			
mi-2	*mi-2*-FAM	ATGAGGAAGGAAGCAGAAGTGC			
iswi-1	*iswi-1*-F	GAGTTCAACGAAGAAGACAGTAA	155	-3.67	94.5
iswi-1	*iswi*-R	CGATGAGCACGATCCATAG			
iswi-1	*iswi-1*-FAM	TTAGCCACCGCAGATGTAGTCA			
iswi-2	*iswi-2*-F_MGB	ACGTAAGGGAGATGGATCTATTTCA	65	-3.96	89
iswi-2	*iswi-2*-R_MGB	CAGGGCTGCTTTTATCACTCTGT			

iswi-2	*iswi-2*-FAM_MGB	CTCCACCTGTCTCTG			
chd1	*chd1*-F	CAACAGTGGCTGGTCCTTCA	68	-3.71	93
chd1	*chd1*-R	ACCAACTTGTGACATTGACGAAA			
chd1	*chd1*-FAM	TCTGGTTTCAGCTCTT			

Supplemental Table 3. Oligonucleotides used for *D. v. virgifera* qRT-PCR assay and primer efficacy results. *β-Actin* reference is also described in Khajuria, *et. al.,* 2015.

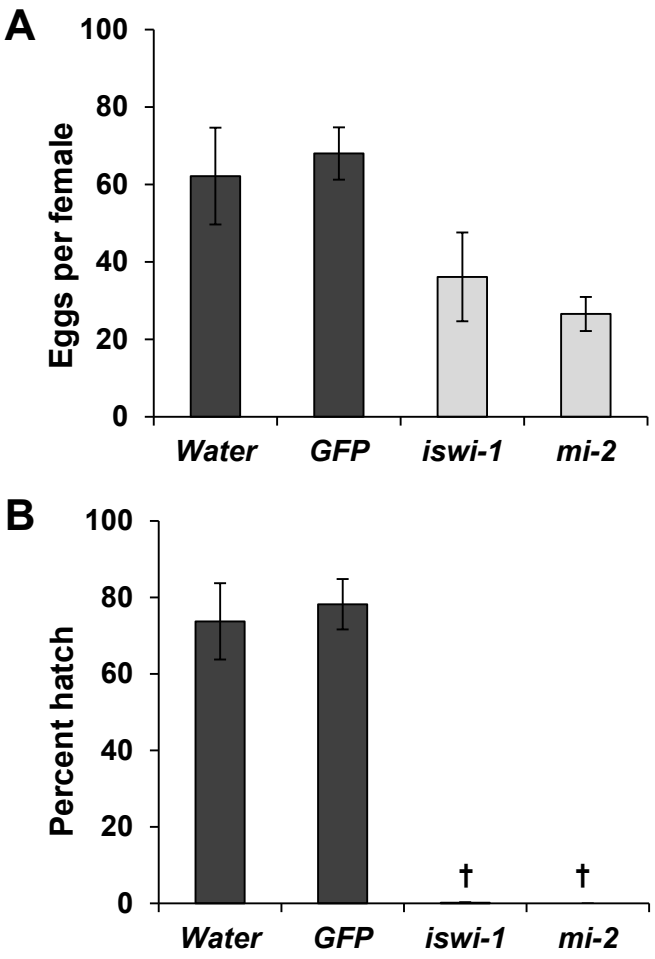
Reference GENE	**NAME**	**SEQUENCE**	**Product Length (bp)**	**Slope**	**R^2**	**Primer Efficiency (%)**
β-Actin	*β-Actin*-F	TCCAGGCTGTACTCTCCTTG	134	-3.419	0.999	96.1
	β-Actin-R	CAAGTCCAAACGAAGGATTG				
Target GENE	**NAME**	**SEQUENCE**	**Product Length (bp)**	**Slope**	**R^2**	**Primer Efficiency (%)**
mi-2	*mi-2*-F	AGAGTGAGGAAACAGGTT	101	-3.331	0.999	99.6
	mi-2-R	AAGTCAGAATGGTAATCAGAG				
iswi-1	*iswi-1*-F	TCACAGTCGAAACACCCACT	126	-3.526	0.999	92.2
	iswi-1-R	TGGCCTTCCTTTCTCTTTTG				
iswi-2	*iswi-2*-F	GCAGTAAGAAGTTGAGAAGA	84	-3.414	0.999	96.3
	iswi-2-R	AGAATACCAACAGGCTACT				
chd-1	*chd1*-F	TTATAGGTTAGTTACTGCTAGATC	101	-3.236	0.996	103.7
	chd1-R	TCGTGTCCATTCTCTGAA				
kis	*ksmt*-F	CACGAAGGACATTGGAAA	92	-3.38	0.999	97.6
	ksmt-R	GCACACCCTCAATCTTTC				
etl1	*etl1*-F	TGATATTTGTGATGCCGAAT	92	-3.534	0.998	91.9
	etl1-R	AGAGTCGTCTTCTGCTTT				

Supplemental Figure 1



Supplemental Figure 1. Concentration response of 2nd instar E. heros nymphs to actin dsRNA. Mortality of E. heros nymphs injected with 10X dilutions of cytoplasmic actin (act-1) and muscle actin (act-2) dsRNAs seven days after injection. Ten insects injected per replicate for each dsRNA and three replicates were performed at each dose. YFP dsRNA was injected only at the highest dose (27.6 µg). Error bars indicate the standard error of the mean. Linear regression curve is plotted on logarithmic scale.

Supplemental Figure 2



Supplemental Figure 2. Additional experiment; D. v. virgifera females fed with iswi-1 and mi-2 dsRNA targeting chromatin remodeling ATPases. Females were fed with diet treated with 0.67 µg/µl dsRNA 6 times; the diet was provided every other day for 12 days. A. Oviposition: eggs collected from dsRNA-fed females after last feeding exposure. B. Percent egg hatch: egg hatch rate was calculated based on eggs oviposited in A. Means comparisons were performed with water as control using Dunnett's Method, † $p < 0.001$.