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Developing an *in vivo* toxicity assay for RNAi risk assessment in honey bees, *Apis mellifera* L

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Developing an *in vivo* toxicity assay for RNAi risk assessment in honey bees, *Apis mellifera* L

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Highlights

- *D. v. virgifera vATPase-A* dsRNA was evaluated in larval and adult honey bees.
- *Dvv vATPase-A* and *Am vATPase-A* did not affect larval and adult survival.
- Relative *vATPase-A* expression was only affected in adults exposed to *Am vATPase-A*.
- RNAi response in honey bees involves factors other than sequence specificity.
- Results from this study provide guidance for future RNAi risk analyses.

Abstract

Maize plants expressing dsRNA for the management of *Diabrotica virgifera virgifera* are likely to be commercially available by the end of this decade. Honey bees, *Apis mellifera*, can potentially be exposed to pollen from transformed maize expressing dsRNA. Consequently, evaluation of the biological impacts of RNAi in honey bees is a fundamental component for ecological risk assessment. The insecticidal activity of a known lethal dsRNA target for *D. v. virgifera*, the *vATPase subunit A*, was evaluated in larval and adult honey bees. Activity of both *D. v. virgifera* (*Dvv*)- and *A. mellifera* (*Am*)-specific dsRNA was tested by dietary exposure to dsRNA. Larval development, survival, adult eclosion, adult life span and relative gene expression were evaluated. The results of these tests indicated that *Dvv vATPase-A* dsRNA has limited effects on larval and adult honey bee survival. Importantly, no effects were observed upon exposure of *Am vATPase-A* dsRNA suggesting that the lack of response involves factors other than sequence specificity. The results from this study provide guidance for future RNAi risk analyses and for the development of a risk assessment framework that incorporates similar hazard assessments.

Keywords: *vATPase-A,* dsRNA, *Diabrotica virgifera virgifera,* non-target arthropod, *Apis mellifera,* RNAi risk assessment

1. Introduction

Following the discovery of RNAi, a number of authors have suggested a possible role for RNAi for insect pest management (Huvenne and Smagghe, 2010). The successful use of RNAi as a pest management tool depends on the identification of target genes essential for the insect to function (e.g. housekeeping genes), genes with high mRNA turnover coding for proteins with short half-life, stability of dsRNA, and the ability to deliver sufficient amounts of dsRNA (Huvenne and Smagghe, 2010; Scott et al., 2013). The potential applications for RNAi in pest management include oral administration of dsRNA in baits (Zhou et al., 2008), embedded in nanoparticles (Zhang et al., 2010), expressed in *E. coli* and formulated as a biological insecticide (Zhu et al., 2011), or expressed in transgenic plants (Baum et al., 2007; Mao et al., 2007).

Empirical validation of the expression of hairpin RNA in plants targeting insect pests was first reported for the cotton bollworm, *Helicoverpa armigera* (Hübner), (Mao et al., 2007) and the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, 1868 (Baum et al., 2007). *Arabidopsis thaliana* plants were transformed to express cotton bollworm dsRNA targeting a cytochrome P450 monooxygenase gene (*CYP6AE14*) involved with gossypol detoxification. When larvae were fed plant material expressing *CYP6AE14* dsRNA, transcript levels of the P450 gene were reduced in the midgut and larval growth was impeded (Mao et al.). Maize plants expressing *D. v. virgifera* vacuolar *vATPase subunit A* (*vATPase-A*) dsRNA, a universal proton pump in eukaryotes, led to significant mortality of WCR larvae and effectively protected maize plants from root damage (Baum et al., 2007). Studies involving transgenic maize expressing *D. v. virgifera Snf7* dsRNA, a gene crucial to the transport of transmembrane proteins, also resulted in larval mortality and root protection (Bolognesi et al., 2012; Ramaseshadri et al., 2013). The first transgenic RNAi maize for WCR management will combine *D. v. virgifera Snf7* dsRNA with the *Bacillus thuringiensis*

(Bt) toxin Cry3Bb1 and is likely to be released by the end of this decade (Levine et al., 2015).

Prior to the commercial release of plants expressing insecticidal dsRNA, a risk assessment framework to evaluate the effects on nontarget arthropods must be established. The risk assessment framework currently used for Bt crops has been suggested as a starting point to evaluate the potential hazards for RNAi crops (Auer and Frederick, 2009; CERA, 2011; Romeis et al., 2013; EFSA, 2014). This framework tests the risk hypothesis that the stressor (e.g. arthropod-active dsRNA) does not adversely impact the nontarget arthropods in the field (Romeis et al., 2008; EFSA, 2014) and is based on a tiered approach using a series of species that represent key functional groups (e.g. detritivores, predators, parasitoids, pollinators) (USEPA, 1998; Romeis et al., 2008). Tier I assessments use a maximum hazard dose, U.S. EPA suggest a margin of exposure factor of 10x using laboratory procedures with purified proteins in artificial diets (USEPA, 1998; Romeis et al., 2008). Additional laboratory tests, higher-tier, semi-field or field experiments are only required if effects are observed in tier I assessments (USEPA, 1998; Romeis et al., 2008).

Estimating the ecological risks associated with RNAi poses some unique challenges relative to Bt crops due to the distinct mode of action of dsRNA (USEPA, 2014). The general framework for risk assessments of Bt crops has been suggested as an appropriate model for assessing the risk of RNAi based technologies (CERA, 2011; Scott et al., 2013). However, in contrast to Bt crops, the potential for RNAi to cause adverse effects to non-target organisms is somewhat dependent on the gene, target sequence and the persistence of the effect (USEPA, 2014). Early characterization suggests that the spectrum of activity is expected to be narrow and thus species taxonomically related to the target organism are more likely to be susceptible (Baum et al., 2007; Whyard et al., 2009; Bachman et al., 2013), although more empirical information is necessary (USEPA, 2014). In addition, some orders of insects seem to be inherently resilient to RNAi and are therefore unlikely to be affected (Terenius et al., 2011; Bachman et al., 2013; Scott et al., 2013).

To date, there is limited information on the risk assessment of RNAi on honey bees, *Apis mellifera* L. Evaluation of the biological impacts of non-specific RNAi in honey bees is fundamental given their economic and ecological importance as pollinators worldwide, their use as surrogate species for non-target insect pollinators (Romeis et al., 2008), and the likelihood for bees to be exposed to pollen from transformed maize expressing dsRNA (USEPA, 2014). The objectives of this study were to 1) establish a standardized *in vivo* toxicity assay for *A. mellifera* larvae and adults to dietary RNAi; and 2) evaluate the effects of *D. v. virgifera vATPase-A* dsRNA on larval development, adult eclosion, adult longevity and gene expression of honey bees to a single high dsRNA concentration. The results provided in this study could serve as a basis for future investigations aimed to evaluate the toxicity of other RNAi targets in honey bees and could be used to inform risk assessment for RNAi in pest management.

2. Materials and methods

2.1. Honey bees

Honey bee hives used for bioassays were obtained from an apiary located at the University of Nebraska–Lincoln East Campus. Bioassays were performed from July through September 2013. All colonies were requeened in 2012 with Italian or Carniolan queens obtained from C. F. Koehnen & Sons, Inc., Glenn, CA. Hives were treated 3 times in the spring and one time in the fall with Terramycin™ (Pfizer, New York, NY) for bacterial brood diseases. *Varroa* mites were controlled with Mite Away Quick Strips (NOD Apiary Products, Ontario, Canada) in April 2012 and with Thymol as Apiguard™ (Vita, Basingstoke, United Kingdom) in September 2012 (Dahlgren et al., 2012).

2.2. Preparation for in vivo RNAi toxicity assay

2.2.1. Target region selection

The *D. v. virgifera vATPase-A* sequence was obtained from a *de novo* transcriptome assembly of cDNA prepared from eggs, neonates and midguts of third instar larvae (Eyun et al., 2014). *D. v. virgifera vATPase-A* sequence has been deposited in GenBank, accession number KR024028, and *A. mellifera vATPase-A* sequence was obtained from the GenBank, accession number XM_623492.4.

A 400 nt *vATPase-A* dsRNA was designed based on the region of highest sequence similarity among different non-target species including: the honey bee, *A. mellifera*, the convergent lady beetle, *Hippodamia convergens*, the monarch butterfly, *Danaus plexippus*, and the collembolan, *Folsomia candida*, as well as the target species, *D. v. virgifera*. We chose the region of highest similarity to pose a worst-case scenario of exposure to a dsRNA. Pairwise sequence alignment was conducted between *D. v. virgifera* and each of the surrogate species via MUSCLE (Edgar, 2004). An in-house Perl script was used to determine the number of shared Nmer (e.g., 21 mer) in each pairwise alignment. The script searches for any instances of N continuous positions where there are no gaps in any sequences in the alignment. The Nmer sequence as well as the start and end positions of *D. v. virgifera* and *A. mellifera* are illustrated in Fig. 1. Sequence alignment between the 400 nt *vATPase-A* dsRNA of *D. v. virgifera* and *A. mellifera* is illustrated in Fig. S1.

2.2.2. Treatments

Activity of both *D. v. virgifera* and *A. mellifera* specific dsRNAs were tested by oral ingestion (OEPP/EPPO, 2010; USEPA, 2012). Honey bee larvae and adults were exposed to six treatments: control (untreated diet), *GFP* dsRNA at 10 µg/individual, *D. v. virgifera* (*Dvv*) *vATPase-A* dsRNA at 1 and 10 µg/individual, and *A. mellifera* (*Am*) *vATPase-A* dsRNA at 1 and 10 µg/individual. An LD_{50} for *vATPase-A* dsRNA in WCR larvae was calculated based on the LC50 reported by Baum et al. (2007). Calculations indicated an LD_{50} of 0.00091 µg/WCR larvae of *vATPase-A* dsRNA from a single exposure for 12 d; we tested 1 and 10 µg/individual representing doses that were 100 and 1000-fold greater than the *D. v. virgifera* larval LD_{50}, respectively.

2.2.3. dsRNA synthesis and stability

Total RNA was isolated from the whole bodies of *D. v. virgifera* adults or *A. mellifera* workers using PureLink™ RNA Mini Kit (Ambion, Life Technologies, Carlsbad, CA). Total RNA was used to synthesize first strand cDNA using the Quantitech reverse transcription kit (Qiagen, Valencia, CA). DNA templates were amplified using Takara Taq DNA Polymerase (Clontech Laboratories, Inc., Mountain View, CA) using 1 µl of cDNA as template. Primers were designed using Primer3Plus (Rozen and Skaletsky, 2000) and T7 polymerase promoter sequences were placed in front of both forward and reverse primers (Table 1). For a negative control, a nonspecific *GFP* (green fluorescent protein) gene was amplified from the pIZT/V5-His expression vector (Invitrogen, Carlsbad, CA) using the gene-specific primers given in Table 1. The PCR products amplified for *D. v. virgifera vATPase-A*, *A. mellifera vATPase-A* and *GFP* were used as a template for *in vitro* synthesis of dsRNAs using the MEGAscript transcription kit (Applied Biosystems Inc., Foster City, CA). All the

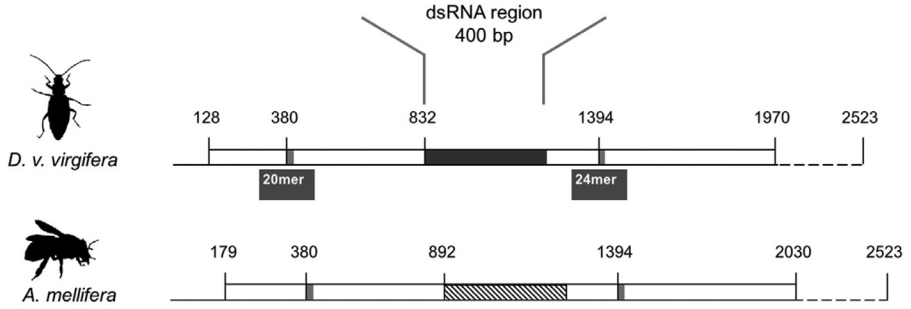

Fig. 1. Schematic representation of the 400 nt *vATPase-A* dsRNA regions tested in *A. mellifera* larvae and adults indicating no 21 nt matches. Sequences were selected based on the region of highest sequence similarity among four non-target species (*A. mellifera*, *Hippodamia convergens*, *Danaus plexippus* and *Folsomia candida*) and the target species *D. v. virgifera*.

synthesized dsRNAs were purified using the RNeasy Mini kit (Qiagen, Valencia, CA). dsRNA preparations were quantified using a Nanodrop™ 1000 spectrophotometer (Thermo Scientific, Franklin, MA) at 260 nm and analyzed by electrophoresis to determine purity.

Stability of dsRNA was evaluated in larval and adult diet to confirm that it was not degraded before and during consumption. For this purpose, 20 µl of diet was collected after 0.5, 8, 12, 24 and 48 h for both larvae and adults. Collected diet was stored at −20 °C until evaluation by electrophoresis. dsRNA from larval diet was extracted by incubation in QG buffer from Qiaquick gel extraction kit (Qiagen, Valencia, CA). One µl of diet was used to visualize dsRNA integrity in a 1% agarose gel.

2.3. Larval bioassays

Larval bioassays were performed using techniques described by Aupinel et al. (2005) with slight modification. Queens from healthy colonies were captured and confined on a frame with a cage made of mesh wire and queen excluder (8 × 8 × 1 cm) for 24 h. Queens were allowed to lay eggs and after 24 h the cage was removed and the queens released. The frame was returned to the hive for egg development and after 3 d, removed and brought to the laboratory (Aupinel et al., 2005). Larvae were 0–3 d old when grafted.

Prior to larval grafting, 20 µl of treated larval diet (Aupinel et al., 2005) was dispensed in each well of 48-well cell culture plates (Corning Incorporated, Corning, NY) under a laminar-flow hood. Plates were kept in a hermetically sealed plexiglas desiccator containing a saturated K_2SO_4 solution to maintain 96% RH. The desiccator was placed in an incubator set at 34 °C and 24 h darkness (Darwin Chambers Co, St Louis, MO, model H024) (Aupinel et al., 2005). Worker larvae were grafted from the comb to the 48-well cell culture plates (one larva per well) using a sterilized German grafting tool. After transfer, plates were moved to the desiccator and placed back in the incubator. Following 48 h of exposure to dsRNA each well was provided with fresh untreated diet daily as described by Aupinel et al. (2005).

Larvae were exposed to one of the six treatments described above: untreated diet, *GFP* 10 µg/larva, *D. v. virgifera vATPase-A* at 1 and 10 µg/larva, and *A. mellifera vATPase-A* at 1 and 10 µg/larva dsRNA. Three hives with three replications per hive were tested; each replication included 8 larvae per treatment for a total of 24 larvae per treatment per hive. Larval mortality was first recorded at 48 h after grafting and checked daily until day eight. One larva per treatment was flash frozen for qRT-PCR at 48 and 96 h after exposure. On day eight, larvae were transferred to 24-well cell culture plates (Corning Incorporated, Corning, NY) for pupation. Wells were lined with a 2 × 3 cm piece of a Kimwipe (Kimberly–Clark, Irvin, TX). Plates were held in a different plexiglas desiccator with a saturated KCl solution to maintain 88% RH and kept at 34 °C. Adult emergence was evaluated 21 d after dsRNA exposure (Aupinel et al., 2005).

2.4. Adult bioassays

Adult bioassays were performed using methods described by Dahlgren et al. (2012). Briefly, late-stage capped brood frames were collected and placed in an incubator at 34 °C, 90% RH and 24 h darkness. Newly emerged adult bees were brushed from frames daily into screened wooden cages (9 × 10 × 18 cm), provided with a 1:1 sugar-water solution and returned to the incubator (Dahlgren et al., 2012). Three to four day old adult workers were anesthetized with carbon dioxide and

Table 1. Primer pairs used for synthesis of dsRNA and for expression analysis using qRT-PCR.

Gene name	Primer sequences for dsRNA synthesis	Product length (bp)
D. v. virgifera vATPase-A	Forward: TAATACGACTCACTATAGGGAGAGCTCTTTTCCCATGTGTA Reverse: TAATACGACTCACTATAGGGAGAGCATTTCAGCCAAACG	400
A. mellifera vATPase-A	Forward: TAATACGACTCACTATAGGGAGATCACTTTTTCCATGCGT Reverse: TAATACGACTCACTATAGGGAGAGCATTTCAGCTAATCGACC	400
GFP	Forward: TAATACGACTCACTATAGGGGGTGATGCTACATACGGAAAG Reverse: TAATACGACTCACTATAGGGTTGTTTGTCTGCCGTGAT	370

Gene name	Primer sequences for qRT-PCR	Product length (bp)	Slope	R^2	Primer efficiency (%)
A. mellifera vATPase-A	Forward: TGTATGAGTTGGTTAGAGTAG Reverse: GTATAGTAGCCATATCACCTT	78	3.361	0.995	98.49
A. mellifera β-actin	Forward: TGCCAACACTGTCCTTTCTG Reverse: AGAATTGACCCACCAATCCA	156	−3.1	0.919	109.2

Underlined sequence corresponds to T7 promoter. R^2: Correlation coefficient.

20 bees per treatment per replication were placed in wax-coated paper cups (177 cm^3; Solo S306, Highland Park, IL). Cups were covered with cotton cheesecloth and secured with rubber bands (Dahlgren et al., 2012). Adults were exposed to the same treatments as larvae: untreated diet, *GFP* 10 μg/adult, *D. v. virgifera vATPase-A* at 1 and 10 μg/adult, and *A. mellifera vATPase-A* at 1 and 10 μg/adult dsRNA. Three hives with three replications per hive were evaluated; each replication included 20 adult bees for a total of 60 bees per treatment per hive.

The dsRNA was prepared in a 1:1 sugar-water solution made with nuclease free water and 1 ml of solution was provided to each cup in a 1.5 ml microcentrifuge tube with two small holes on the bottom. Cups with adult worker bees were maintained in an environmental chamber at 34 °C in constant darkness. Treated diet was provided in the initial feeding and after treated diet was completely consumed (24–48 h), fresh untreated sucrose solution was provided daily (OEPP/EPPO, 2010). Adult bees mortality was evaluated for approximately 25 days or until all individuals were dead. Four adults per treatment were flash frozen for qRT-PCR at 48 and 96 h after exposure.

2.5. D. v. virgifera reciprocal test

A reciprocal test was performed with *D. v. virgifera* adults to confirm the activity of dsRNA preparations. *D. v. virgifera* artificial diet was treated with solutions of honey bee larval and adult diet containing dsRNA. Beetles were exposed to the following treatments: control (untreated honey bee diet), *GFP* 1 μg/beetle, *D. v. virgifera vATPase-A* 1 μg/beetle, and *A. mellifera* 1 μg/beetle dsRNA. Three replications with ten WCR adults per treatment were performed for larval and adult honey bee diet.

Bioassays were performed as described by Khajuria et al. (2015). Briefly, newly emerged non-diapausing WCR adults were purchased from Crop Characteristics Inc. (Farmington, MN). Artificial diet adapted from Branson and Jackson (1988) was poured into a sterile polystyrene petri dish (Fisher Scientific, Waltham, MA). After solidification diet plugs (ca. 0.4 cm in diameter) were cut with a #1 cork borer. Ten diet plugs were provided per treatment and placed in a well of 16-well trays (5.1 cm long × 3.8 cm wide × 2.9 cm high). Diet plugs were surface treated with 1 μg dsRNA embedded in 3 μl of larval or adult honey bee diet. Trays were covered with vented lids and ten WCR adults were transferred to each well. Treated diet was provided for 48 h after which untreated diet was provided every other day. Mortality was recorded for 15 d.

2.6. Transcriptional validation using quantitative real time PCR

Relative transcript levels of *A. mellifera vATPase-A* were evaluated using quantitative real time PCR (qRT-PCR). Total RNA isolation and cDNA synthesis was performed as previously described. cDNA was used as template for qRT-PCR and primers were designed using Primer3Plus (Rozen and Skaletsky, 2000) (Table 1). The efficiencies of primer pairs were evaluated using 5-fold serial dilutions (1:1/5:1/25:1/125:1/625) in triplicate. All primer combinations showed a linear correlation between the amount of cDNA template and the amount of PCR product. The 7500 Fast System SDS v2.0.6 Software (Applied Biosystems Inc., Foster City, CA) was used to determine the slope, correlation coefficients, and efficiencies (Table 1). Amplification efficiencies were higher than 98% and correlation coefficients were larger than 0.92 for all the qRT-PCR primer pairs used in this study (Table 1). Four individuals per treatment per hive each with two technical replications were used for qRT-PCR analysis. qRT-PCR was performed using SYBR green (Applied Biosystems Inc., Foster City, CA) and the 7500 Fast System real-time PCR detection system following the supplier's protocol (Applied Biosystems Inc., Foster City, CA). Relative quantification of the transcripts were calculated using the comparative 2- ddCT method (Livak and Schmittgen, 2001) and were normalized to *A. mellifera β*-actin (Antonio et al., 2008).

2.7. Statistical analysis

Analyses were performed using SAS software (SAS-Institute, 2011). Honey bee adult emergence and gene expression for both larvae and adult bioassays were analyzed with an analysis of variance using PROC GLIMMIX with the least square estimated means procedure to determine differences between treatments. Honey bee larval and adult mortality, and *D. v. virgifera* mortality from the reciprocal tests were analyzed with a repeated measures analysis using PROC GLIMMIX with the least-square estimated means procedure to determine differences between treatments over time.

3. Results

3.1. Larval bioassays

The analysis performed to evaluate larval mortality indicated significant mortality among treatments ($F_{5, 19} = 2.83$, $p = 0.045$) and over time ($F_{5, 19} = 4.7$, $p = 0.0083$) (Fig. 2A). The highest mortality was observed in larvae fed with *Am vATPase-A* dsRNA 10 μg (16.7% ± 2.3) and the lowest with *Am vATPase-A* dsRNA 1 μg (2% ± 0.6). Pairwise comparisons revealed significant differences between *Am vATPase-A* dsRNA 10 μg with the control ($t_{19} = -2.34$, $p = 0.03$) and *GFP* ($t_{19} = -3.05$, $p = 0.007$). No significant effects of *A. mellifera* and *D. v. virgifera* specific dsRNAs were observed on the percent of adult eclosion ($F_{5, 48} = 0.997$, $p = 0.997$) (Fig. 2B). The control had the lowest percent adult eclosion (63.2% ± 7.5) and larvae treated with *Am vATPase-A* dsRNA 1 μg had the highest (80.2% ± 4.8), although the pairwise comparison revealed no significant differences between these treatments ($t_{48} = 0.5$, $p = 0.62$).

No significant treatment effects were observed in the relative expression of *A. mellifera vATPase-A* in larvae collected after 48 h ($F_{5, 53} = 1.1$, $p = 0.373$) (Fig. 2C). Relative *vATPase-A* expression in larvae collected after 96 h revealed significant treatment effects ($F_{5, 55} = 3.61$, $p = 0.007$) (Fig. 2C). However, there was no indication of significant knockdown in any of the treatments.

3.2. Adult bioassays

The repeated measures analysis performed to evaluate adult longevity indicated significant mortality over time ($F_{5, 233} = 7381.1$, $p < 0.0001$) but no differences among treatments ($F_{5, 39} = 0.79$, $p = 0.566$) (Fig. 3A). Relative expression in adults collected after 48 h showed significant differences among treatments ($F_{5, 52} = 4.06$, $p = 0.004$) (Fig. 3B). Compared to the larval expression analyses, pairwise comparisons indicated a significant knockdown (38%) in adults fed with 10 μg of *Am vATPase-A* dsRNA at 48 h relative to adults feed with untreated diet ($t_{52} = 4.06$, $p = 0.0002$) and 10 μg of *GFP* dsRNA ($t_{52} = -2.88$, $p = 0.006$). Significant reduced expression (29%) was also observed between the control and *Am vATPase-A* dsRNA 1 μg ($t_{52} = 3.09$, $p = 0.003$), although no differences were observed with adults fed with *GFP* dsRNA. The relative *vATPase-A* expression in adults collected after 96 h displayed no significant differences among treatments ($F_{5, 55} = 1.01$, $p = 0.422$), suggesting that the reduced gene expression observed at 48 h for *Am vATPase-A* dsRNA was transient (Fig. 3B). In addition, there was no significant effect from either concentration of *Dvv vATPase A* at 48 and 96 h.

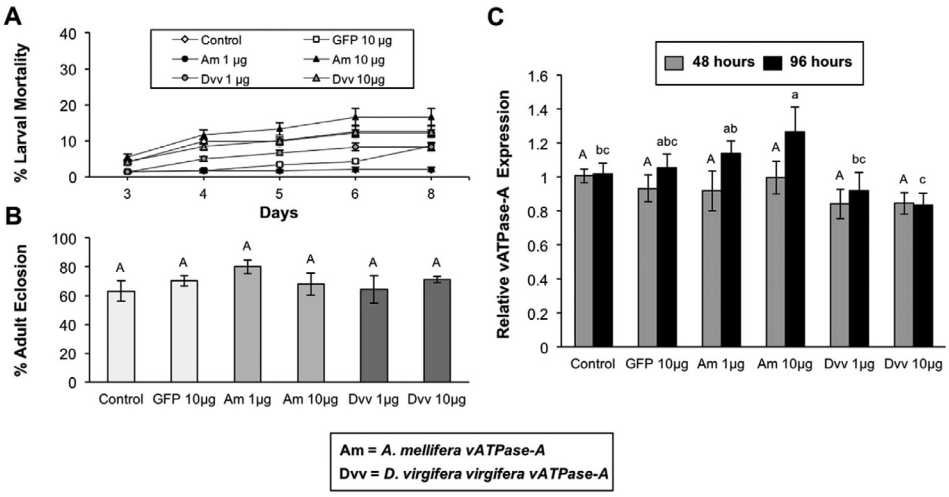

Fig. 2. *A. mellifera* larval bioassays results. Larvae with less than 24 h of emergence were exposed to two doses, 1 µg and 10 µg per larvae, of *A. mellifera* and *D. v. virgifera vATPase-A* dsRNA. Three hives with three replications were tested. **(A)** Percent larval mortality evaluated every other day from day 3 to 8 (N = 12–18 individuals/replication/hive). **(B)** Percent adult eclosion evaluated at 18–21 d after treatment (N = 12–18 individuals/replication/hive). **(C)** Relative *vATPase-A* expression at day 3 and 5 after exposure (N = 3–4 biological replications/hive with 2 technical replications/sample). Error bars indicate the standard errors of the mean. Different letters represent significant differences at *p-value* < 0.05.

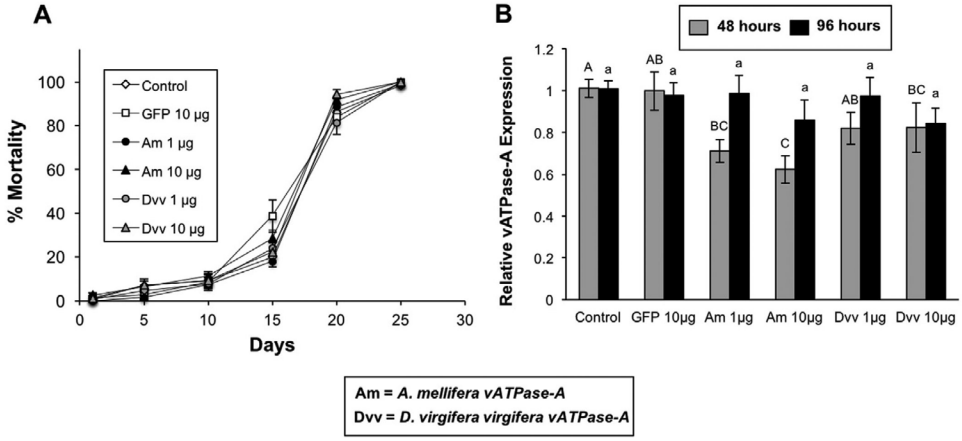

Fig. 3. *A. mellifera* adult bioassays results. Three to four day adult workers were exposed to two doses, 1 µg and 10 µg per individual, of *A. mellifera* and *D. v. virgifera vATPase-A* dsRNA. Three hives with three replications of 20 bees were tested. **(A)** Percent adult mortality evaluated daily (N = 3 replications of 20 bees/hive). **(B)** Relative *vATPase-A* expression at day 3 and 5 after exposure (N = 3–4 biological replications/hive with 2 technical replications/sample). Error bars indicate the standard errors of the mean. Different letters represent significant differences at *p-value* < 0.05.

3.3. Stability of dsRNA on diet

The stability of dsRNA in larval diet was difficult to evaluate due to interference in migration of the dsRNA from components of the royal jelly, and additional purification was necessary to allow movement of dsRNA through the agarose gel. The dsRNA embedded in larval (Fig. 4A) and adult (Fig. 4B) diet did not degrade after 48 h, indicating that the environment and salivary enzymes did not have an effect on the stability of dsRNA.

3.4. D. v. virgifera reciprocal test

Results from the *D. v. virgifera* reciprocal tests used to confirm activity of the dsRNA indicated that *A. mellifera vATPase-A* dsRNA did not have an effect on the mortality of WCR adults compared to the control when fed honey bee larval (t_{48} = −0.28, p = 0.778) and adult diet (t_{48} = −1.31, p = 0.196) (Fig. 5A and B). In contrast, *D. v. virgifera vATPase-A* dsRNA provided in adult honey bee diet generated significant mortality of WCR adults (100% by day 9) compared to the controls (10%) (t_{48} = −15.49, p < 0.0001) confirming the activity of the dsRNA tested (Fig. 5B). However, *D. v. virgifera vATPase-A* dsRNA embedded in larval diet did not generate significant mortality of WCR compared to the controls (t_{48} = 1.14, p = 0.261) (Fig. 5A). This result is consistent with the assessment of dsRNA stability in larval diet where the dsRNA was difficult to evaluate by gel electrophoresis and may indicate that components of the larval diet interact with dsRNA preventing uptake from the gut lumen.

4. Discussion

The present study evaluated the toxicity of dsRNA targeting *D. v. virgifera* on honey bees using oral exposure to a single high concentration (OEPP/EPPO, 2010; USEPA, 2012). The European Food Safety Authority (EFSA, 2014) suggests that exposures used for toxicity studies with non-target arthropods should be high compared with the maximum amount of dsRNA expected to be available in the environment. We tested a single exposure of two concentrations, 1 and 10 µg per individual, of both *D. v. virgifera* and *A. mellifera* specific *vATPase-A* dsRNA in larvae and adult honey bees corresponding to an exposure 100 and 1000 times higher than the LC_{50} for *vATPase-A* dsRNA

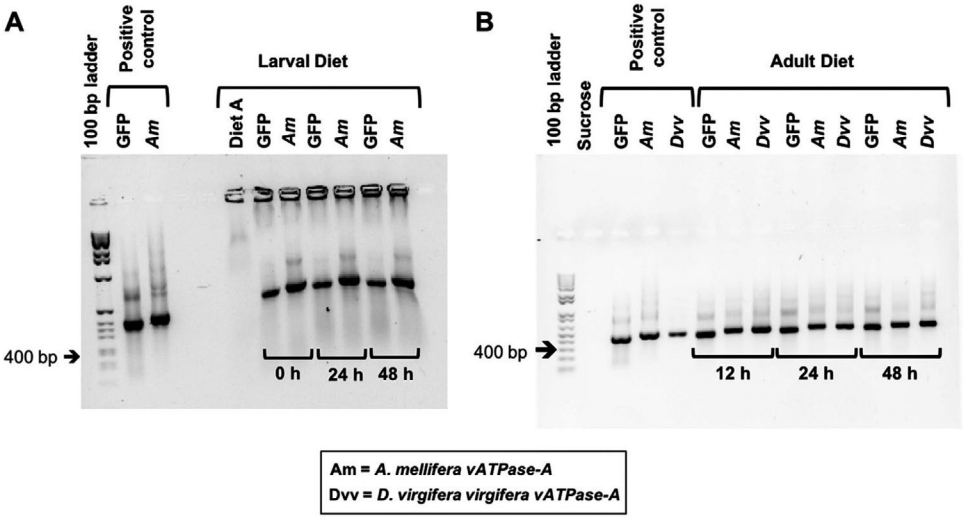

Fig. 4. Stability of dsRNA incorporated on *A. mellifera* larvae and adult diet at a concentration of 10 µg per individual. Positive controls consisted of dsRNAs eluted in water at the same concentration. **(A)** dsRNA on larval diet evaluated at 24 and 48 h **(B)** dsRNA on adult diet evaluated at 12, 24 and 48 h.

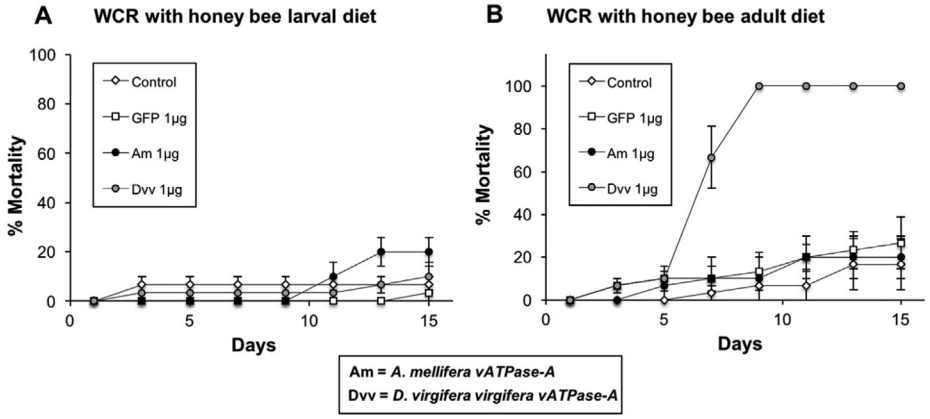

Fig. 5. *D. v. virgifera* reciprocal test results. Newly emerged WCR adults were fed artificial WCR diet treated with solutions of honey bee larval and adult diet containing 1 µg/individual of *A. mellifera* and *D. v. virgifera vATPase-A* dsRNA. **(A)** Percent mortality of WCR adults fed artificial WCR diet treated with honey bee larval diet containing dsRNA (N = 30). **(B)** Percent mortality of WCR adults fed artificial WCR diet treated with honey bee adult diet containing dsRNA (N = 30). Error bars indicate the standard errors of the mean. Different letters represent significant differences at *p-value* < 0.05.

reported for *D. v. virgifera* larvae (Baum et al., 2007). Currently, there is limited information on expression of dsRNA by transgenic plants although Tan et al. (2015) reported that *D. v. virgifera Snf7* dsRNA expression in maize pollen ranges between 0.056 and 0.224 ng/g Crailsheim et al. (1992) estimated that a worker bee consumes 3.4–4.3 mg of maize pollen per day, while Babendreier et al. (2004) estimated that 1.52–2.04 mg of pollen could be consumed during larval development. Based on the reported amounts of dsRNA expressed in maize pollen and the average consumption of pollen by honey bee larvae and adults, the concentrations evaluated in this study were 200,000 and 2,000,000 times higher than estimated daily field exposures.

Our results indicate that honey bee larval development (Fig. 2A), adult eclosion (Fig. 2B) and adult survival (Fig. 3A) were unaffected by both *D. v. virgifera* as well as *A. mellifera* dsRNA, suggesting that honey bees are insensitive to *vATPase-A* dsRNA. Although significant reduction of *A. mellifera vATPase-A* expression was observed after 48 h in adults exposed to the highest concentration of *Am vATPase-A* dsRNA, the effect was transient (Fig. 3B) and did not affect adult longevity. Unlike adults, we did not see any evidence of reduced expression in larvae exposed to *A. mellifera* specific dsRNA. Our results are in agreement with those reported by Tan et al. (2015) who observed that dsRNA targeting the *Snf7* gene from *D. v. virgifera* did not affect honey bee adult survival or larval development at concentrations that exceed environmentally relevant amounts. Although our study represents a single acute exposure, the dsRNA concentrations were higher than those tested by Tan et al. (2015). Results of both studies suggest that the risk of *in planta* RNAi is minimal for honey bees.

Previous studies with honey bees have shown that dsRNA concentrations necessary to generate gene knockdown is highly variable. Delivery of dsRNA by feeding has shown that concentrations as low as 7 µg/ml for the honey bee Toll-related receptor 18 W gene (Aronstein et al., 2006) and as high as 450 µg/ml for the phosphatase and tensin (PTEN) homolog fed for four consecutive days (Mutti et al., 2011) are necessary to achieve gene knockdown. Our results and previous studies indicate that each gene performs differently in honey bees and supports the idea that dsRNAs for different genes should be evaluated independently.

Although no effects were observed in honey bee larvae or adults, other potential hazards for non-target arthropods have been identified. Potential hazards include off-target gene silencing (Auer and

Frederick, 2009; Jarosch and Moritz, 2012) and the potential saturation of the RNAi machinery that may affect the insect immune viral response (EFSA, 2014; USEPA, 2014). Previous reports of RNAi in honey bees and cell-based assays with *Drosophila melanogaster* indicate non-specific down regulation of off-target sequences after dsRNA exposure (Kulkarni et al., 2006; Jarosch and Moritz, 2012). The potential silencing of genes other than the target and/or immune responses may have subtle impacts that are not detected in acute bioassays (Auer and Frederick, 2009), especially in social insects such as honey bees. Therefore, other assays that detect behavioral or colony level responses may be important for honey bees (Thompson, 2003; Gill et al., 2012).

The insufficient response of honey bees to *A. mellifera vATPase-A* dsRNA suggests that the activity spectrum of dsRNA does not only depend on the sequence identity to the target gene, but also on the inherent ability of the organism to respond to orally ingested dsRNA (CERA, 2011; EFSA, 2014). A variety of factors including nucleases in the midgut (Liu et al., 2013), salivary secretions (Allen and Walker, 2012; Christiaens et al., 2014) and haemolymph (Garbutt et al., 2013; Christiaens et al., 2014) have shown to degrade dsRNA in different insect orders and may be important in determining specificity. The results from the diet stability experiment suggest that dsRNA enzymatic barriers are most likely occurring after ingestion since no dsRNA degradation was observed from salivary secretions released in both larval and adult diet (Fig. 4). In addition, rootworm specific dsRNA provided to adult rootworms in royal jelly had no effect on adult survival. It is possible that royal jelly somehow compromised the availability of dsRNA and may be another factor in possible selectivity of rootworm specific dsRNAs. Nanoparticles are currently used to stabilize dsRNA delivery (Whyard et al., 2009; Zhang et al., 2010; He et al., 2013). This technology relies on the ability of nanoparticles to bind and condense nucleic acids into stable complexes through electrostatic interactions (He et al., 2013). It is likely that lipids, carbohydrates or peptides present in royal jelly are creating strong bonds with the dsRNA making it unavailable to the insect. Additional studies identifying potential barriers for dsRNA uptake could help to refine the number of species required for testing in tier I assessments (EFSA, 2014; USEPA, 2014).

5. Conclusions

The results obtained in this study support the general risk assessment framework used for Bt crops as a starting point for RNAi based technologies. However, because of the unique mode of action of RNAi, additional information may be gained by measuring gene expression and by testing closely related organisms that may share sequence identity with the target pest species (Bachman et al., 2013). Our results support the consensus among risk assessors that each dsRNA used for *in planta* RNAi may have its own risk and should be independently tested (CERA, 2011; EFSA, 2014). Our study indicated that *D. v. virgifera vATPase-A* dsRNA did not significantly affect larval or adult survival and similarly, no effects were observed by *A. mellifera vATPase-A* dsRNA. In general, these results support the conclusions of Tan et al. (2015) that dsRNA technologies pose little risk to honey bees. However, further studies evaluating other genes and non-target arthropods, the potential for off-target gene silencing and effect on the insect immune viral response will improve our understanding of additional potential hazards of RNAi on non-target arthropods such as honey bees.

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

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Appendix A: Supplementary data

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D.v.v._vATPase-A      GCTCTTTTCCCATGTGTACAGGGTGGTACTACTGCCATTCCCGGAGCTTTCGGTTGTGGA  60
A.m._vATPase-A        TCACTTTTTCCATGCGTTCAAGGTGGTACTACAGCCATTCCTGGTGCTTTTGGTTGTGGC  60
                       * ***** ***** ** ** ********** ******** ** ***** ********

D.v.v._vATPase-A      AAAACTGTAATTTCACAATCTCTTTCCAAATATTCCAACTCTGATGTCATTATCTACGTC  120
A.m._vATPase-A        AAAACTGTAATTTCTCAAGCTTTATCAAAATATTCAAATTCTGATGTTATTATTTATGTT  120
                      ************** *** ** * ** ******** ** ******** ***** ** **

D.v.v._vATPase-A      GGTTGCGGAGAAAGAGGTAACGAAATGTCTGAAGTATTGAGAGATTTCCCTGAATTGACT  180
A.m._vATPase-A        GGTTGTGGGGAACGTGGTAATGAAATGTCTGAAGTATTACGCGATTTTCCTAAATTAACA  180
                      ***** ** *** * ***** *****************  * ***** *** **** **

D.v.v._vATPase-A      GTTGAAATTGACGGGCACACTGAATCTATTATGAAACGTACCGCATTGGTCGCCAACACA  240
A.m._vATPase-A        GTGGAAATTGATGGTATCACTGAATCTATCATGAAGCGTACAGCTTTAGTCGCTAATACT  240
                      ** ******** **   ************ ***** ***** ** ** ***** ** **

D.v.v._vATPase-A      TCTAACATGCCTGTAGCTGCTCGTGAAGCTTCTATCTATACTGGTATTACTCTTTCTGAA  300
A.m._vATPase-A        TCAAATATGCCTGTAGCAGCTCGTGAAGCATCTATTTACACAGGTATTACTCTATCAGAA  300
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D.v.v._vATPase-A      TACTTCCGTGATATGGGTTACAACGTATCTATGATGGCTGACTCGACATCACGTTGGGCC  360
A.m._vATPase-A        TACTTCAGAGATATGGGTTATAATGTATCTATGATGGCCGATTCAACATCTCGTTGGGCA  360
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D.v.v._vATPase-A      GAAGCTTTGAGAGAAATTTCAGGTCGTTTGGCTGAAATGC  400
A.m._vATPase-A        GAAGCACTTCGTGAAATTTCTGGTCGATTAGCTGAAATGC  400
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Fig. S1. Sequence alignment of the 400 nt vATPase-A dsRNA regions tested in *A. mellifera* larvae and adults. D.v.v. corresponds to *D. v. virgifera* vATPase-A sequence and A.m. to *A. mellifera* vATPase-A sequence.