# *In silico* identification of off-target pesticidal dsRNA binding in honey bees (*Apis mellifera*)

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## ABSTRACT

**Background.** Pesticidal RNAs that silence critical gene function have great potential in pest management, but the benefits of this technology must be weighed against non-target organism risks.

**Methods.** Published studies that developed pesticidal double stranded RNAs (dsRNAs) were collated into a database. The target gene sequences for these pesticidal RNAs were determined, and the degree of similarity with sequences in the honey bee genome were evaluated statistically.

**Results.** We identified 101 insecticidal RNAs sharing high sequence similarity with genomic regions in honey bees. The likelihood that off-target sequences were similar increased with the number of nucleotides in the dsRNA molecule. The similarities of non-target genes to the pesticidal RNA was unaffected by taxonomic relatedness of the target insect to honey bees, contrary to previous assertions. Gene groups active during honey bee development had disproportionately high sequence similarity with pesticidal RNAs relative to other areas of the genome.

**Discussion.** Although sequence similarity does not itself guarantee a significant phenotypic effect in honey bees by the primary dsRNA, *in silico* screening may help to identify appropriate experimental endpoints within a risk assessment framework for pesticidal RNAi.

**Subjects** Biotechnology, Ecology, Entomology, Toxicology, Ecotoxicology
**Keywords** RNAi, Non-target, Risk assessment, Transgenic crops, Pesticide, Insecticide

## INTRODUCTION

The potential to silence critical gene function in pest species has led to the proposed application of RNA interference (RNAi) as a novel class of agricultural products (*Price & Gatehouse, 2008*; *Gu & Knipple, 2013*) that target several species of economically important pests (*Baum et al., 2007*; *Maori et al., 2009*; *Desai et al., 2012*; *Hajeri et al., 2014*; *Marr et al., 2014*). These RNAi-based pesticides may be delivered to the target pest via a number of methods, including transgenic plants, diet-incorporated suspensions, and topical applications of naked or encapsulated small RNAs, which elicit post-transcriptional gene silencing following ingestion. Once ingested, the insect's cellular machinery cleaves long double stranded RNA (dsRNA) molecules into small-interfering RNAs (siRNAs) that are

19–25 nucleotides in length; these siRNAs serve as the functional unit of RNAi and govern the location of gene suppression through the degradation of complementary messenger RNA molecules (*Fire et al., 1998*; *Martinez et al., 2002*; *Vermeulen et al., 2005*). To date, this process has been investigated in the control of a number of pest groups, including parasites of medical importance, urban pests, pests and pathogens of honey bees, and agricultural pests of economic importance.

While the technology promises to be target specific (*Whyard, Singh & Wong, 2009*; *Bachman et al., 2013*), there is concern that current risk assessment frameworks for genetically modified crops are not adequate to proactively assess the risks to non-target organisms (*Romeis et al., 2008*; *Lundgren & Duan, 2013*; *FIFRA-SAP, 2014*). The risks associated with RNAi to non-target organisms include immune stimulation (*Lu & Liston, 2009*), saturation of an organism's RNAi machinery that could interfere with normal cellular processes (*Grimm, 2011*; *Flenniken & Andino, 2013*), and unintentional gene silencing. Unintentional gene silencing in non-target organisms is the primary risk posed by pesticidal RNAi; within a non-target species, this unintentional gene silencing can be due to silencing the intended gene in an unintended organism (non-target binding) or silencing a different gene with sufficient sequence similarity to the dsRNA (off-target binding) (*Lundgren & Duan, 2013*; *FIFRA-SAP, 2014*). Because pesticidal RNAi poses risks to non-target organisms that are different from other pesticides, a risk assessment framework has been proposed to proactively assess these risks using a series of steps (*FIFRA-SAP, 2014*; *Roberts et al., 2015*). Indeed, the United Nations employs the precautionary principle when conducting risk assessment of genetically modified organisms to ensure that these products do not adversely affect the environment (https://bch.cbd.int/protocol; accessed November 7, 2017).

Bioinformatic analyses that compare pesticidal RNAs to non-target genomes can help focus more extensive risk assessment procedures to predict some risks (*Heinemann, Agapito-Tenfen & Carman, 2013*). The hazard to non-target organisms should be predictable if the functional genome of a non-target organism is known, recognizing that numerous circumstances influence gene silencing even when the sequence is identical between a small RNA and the non-target genome (*Kerschen et al., 2004*). Bioinformatic analyses have thus been advocated as an initial screen of potential risks posed by RNAi (*FIFRA-SAP, 2014*; *Roberts et al., 2015*). In the present study, we used *in silico* searches to determine whether putative pesticidal dsRNAs share sequence similarities with off-target regions of the honey bee (*Apis mellifera* L.), a model non-target organism. We were specifically interested in testing the hypotheses that (1) longer dsRNAs increase the potential for off-target binding, (2) non-target silencing of the target gene is dependent on relatedness of the target and non-target species, and (3) certain gene groups in the honey bee are more prone to off-target sequence similarities with pesticidal dsRNAs.

## MATERIALS AND METHODS

### Literature review

In broad terms, our approach was to examine the literature for published pesticidal RNAs against an identified suite of pests, and search the targeted gene sequences in the pests

for similarities with regions of the honey bee genome. Published studies evaluating the effects of *pesticidal* dsRNAs were searched using the ISI Web of Knowledge database, using combinations of the search terms "pesticidal," "insecticidal," "siRNA," "dsRNA," "RNAi," and "RNA interference." See the introduction for a description of these terms. Studies were included if they evaluated the pesticidal effects of a dsRNA or siRNA and provided either the RNA sequence or primer sets that allowed the RNA sequences to be determined from the target species' genome using the NCBI genome database (http://www.ncbi.nlm.nih.gov/genome/). A total of 24 studies were included, with pesticidal qualities being evaluated for 74 dsRNAs and 21 siRNAs targeting 57 genes (Data S1). These included species of medical importance (*Hajdusek et al., 2009*; *Kwon, Park & Lee, 2013*), urban pests (*Zhou et al., 2008*; *Itakura et al., 2009*), parasites and pathogens of honey bees (*Maori et al., 2009*; *Campbell, Budge & Bowman, 2010*; *Desai et al., 2012*), agricultural pests (*Mutti et al., 2006*; *Baum et al., 2007*; *Whyard, Singh & Wong, 2009*; *Tang, Wang & Zhang, 2010*; *Choudhary & Sahi, 2011*; *Wuriyanghan, Rosa & Falk, 2011*; *Gong et al., 2013*; *Ochoa-Campuzano et al., 2013*; *Yao et al., 2013*; *Christiaens, Swevers & Smagghe, 2014*; *Chu et al., 2014*; *Han et al., 2014*; *Meng et al., 2014*; *Miyata et al., 2014*; *Yu et al., 2014*), and others (*Whyard, Singh & Wong, 2009*; *Kelkenberg et al., 2015*; *Petrick et al., 2015*).

### *In silico* sequence similarity identification

Published pesticidal dsRNAs ranged from 19 to 2500+ nucleotides in length. These were queried against the annotated honey bee genome accessed through GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST nucleotide algorithm for somewhat similar sequences (blastn). Similar genetic regions were mostly less than 25 nt long, the length expected for active siRNAs randomly generated from a dsRNA molecule. Sequence similarities of 19/21, 20/21, and 21/21 nt were tallied for each RNA against the honey bee genome, and the off-target gene name was recorded. Each off-target gene was only tallied once per dsRNA, even when that dsRNA targeted multiple locations along that gene. Sequence similarity for the target gene (non-target binding) was also recorded. Low quality proteins (as defined by NCBI) and genes of unknown function were excluded from the analysis, as were any regions with high sequence similarity that did not return any protein or gene information, such that the resultant database represents a conservative estimate of putative binding.

### Statistical analysis

Because data violated parametric assumptions, the number of off-target similarities were $\log(x + 1)$ transformed and dsRNA length were log transformed to uphold assumptions for analysis with linear regression (Systat v.13.1; Systat San Jose, CA, USA). A chi-square test of independence was used to determine whether there was a significant effect of target taxa on the incidence of non-target binding in honey bees, and whether certain functional gene groups were targeted more frequently.

## RESULTS AND DISCUSSION

### dsRNA length-suppression

Each of the 74 pesticidal dsRNAs shared at least one region of perfect or high sequence similarity with the honey bee genome (average $28.6 \pm 3.32$ off-target homologies per dsRNA) (Data S1). However, none of the published pesticidal siRNAs (21 total, 19–23 nt in length) found sequence similarity within the honey bee genome at our specified level (19/21, 20/21, 21/21 nt matches), indicating that these much smaller sequences were more specific when focusing on a single non-target organism. This result was mirrored by *Li et al. (2015)*, though siRNAs are not always this benign: *Qiu, Adema & Lane (2005)* demonstrated that 5–80% of tested siRNAs resulted in off-target binding among diverse organisms.

Off-target sequence similarity increased significantly as the dsRNA increased in length (linear regression: $F_{1,100} = 623$, $P < 0.001$) (Fig. 1A), with every increase of 100 nt in the dsRNA resulting in 6 more predicted hits. This strong relationship between dsRNA length and potential off-target binding can be further demonstrated using only the genes described in *Miyata et al. (2014)*, in which the authors evaluated the effects of dsRNA length on RNAi activity *in vivo* in western corn rootworms. Although the gene targets in this study were not pesticidal specifically, and thus excluded from our overall analysis, the authors evaluated silencing of the same gene targets (*laccase 2* and *ebony*) using different sized dsRNAs to evaluate efficacy. When we examined this suite of genes from a risk assessment perspective using the same methodology as for the pesticidal RNAs, the longer dsRNAs returned significantly more regions of off-target sequence similarity in the honey bee genome (*laccase 2*: $F_{1,5} = 181$, $P < 0.001$; *ebony*: $F_{1,2} = 103$, $P = 0.01$) (Fig. 1B). While intuitive (*Bolognesi et al., 2012*), this is the first demonstration of the possibility for increased length-suppression in a non-target organism. Thus, optimizing RNA length to have maximum gene suppression efficacy in the target pest needs to be balanced against the non-target risks posed by longer molecules.

### Target-species specificity

Taxonomic relatedness of the target organism to honey bees had no effect on potential binding of siRNAs on the original gene target (non-target binding) ($\chi^2 = 9.4$, $df = 7$, $P = 0.23$) (Fig. 2). Contrary to assertions of pesticidal specificity (*Bachman et al., 2013*), this implies that silencing of the target gene in a non-target organism may be more likely to occur from random sequence similarities than based on evolutionary relatedness to the target organism. Although the pool of available literature is limited with regards to targeted applications of RNAi against pest species, with certain species being more frequently researched (e.g., *Diabrotica virgifera*), our results suggest that non-target hazard assessments should focus on species of ecological relevance rather than strictly on phylogenetic relatedness to the target species.

Unfortunately, when conducting bioinformatics analyses for the purposes of a risk assessment, the availability of sequenced genomes from representative species becomes a limiting factor. Further, the potential non-target community will differ depending on the specific pest being targeted, making it difficult to have a standard suite of species
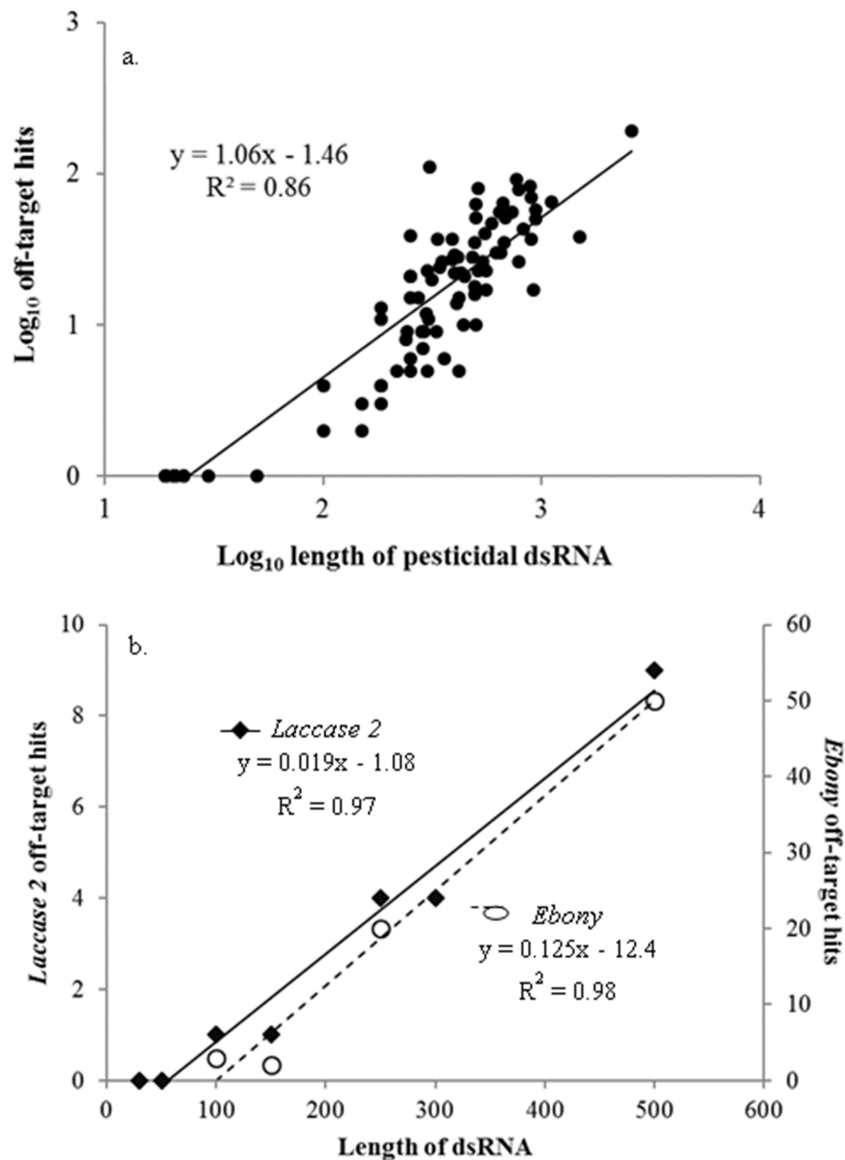
**Figure 1  Pesticidal dsRNA length and potential off-target binding in honey bees.** The relationship between pesticidal dsRNA length and potential off-target binding in honey bees for pesticidal dsRNAs (A) and the non-pesticidal *laccase 2* and *ebony* genes (data from *Miyata et al. (2014)*) (B).

to evaluate for non-target effects. Bioinventories are crucial for identifying appropriate non-target species for each target pest. Supporting initiatives such as i5K (*i5K Consortium, 2013*), which strives to sequence the genomes of 5,000 representative invertebrates, and making these genomes freely available, will bolster the applicability of future *in silico* analyses aimed at identifying potential risks of gene-oriented pest control.

## Targeted gene groups

The homeobox genes and other genes involved in embryonic and developmental pathways in honey bees frequently shared sequence similarity with the pesticidal dsRNAs, particularly
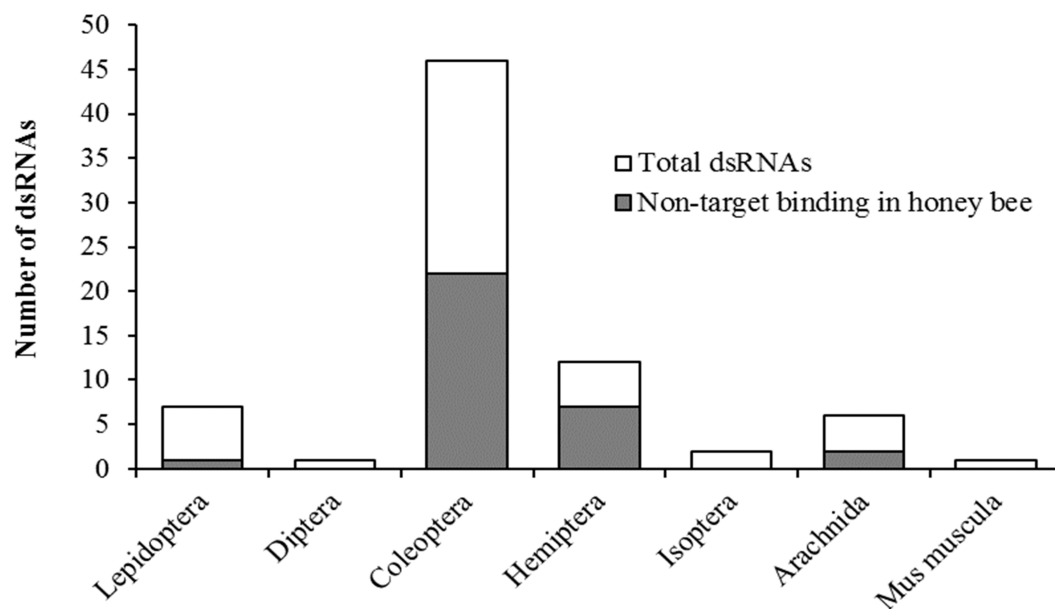
**Figure 2** **Pesticidal dsRNA target organisms and the likelihood of off-target binding in the honey bee genome.** Potential non-target binding of pesticidal dsRNAs in honey bees ($y$-axis, shaded area) versus the original target taxa ($x$-axis), in relation to the total number of examined pesticidal dsRNAs. Taxa are ordered by increasing relative divergence time from honey bees.

Full-size ⬙ DOI: 10.7717/peerj.4131/fig-2

when vATPase subunits were the pesticidal targets ($\chi^2 = 10$, $df = 4$, $P = 0.03$). 67% of all tested dsRNAs had the high potential to bind to honey bee developmental genes that were not the target, and 33% of these shared high similarity with homeobox genes specifically (Data S1). Although we have an incomplete picture of which genes are expressed in most genomes at any given time, many of these genes, while important during embryogenesis and development, support additional critical functions such as cell proliferation and apoptosis, and are highly conserved across metazoans. In this instance, *in silico* analysis identified some of the potential gene targets that could present a hazard requiring unique assessments across life stages to properly identify a phenotypic effect. If validated in future *in vivo* assessments, this screening method may prove useful in identifying appropriate experimental endpoints in non-target risk assessments.

## CONCLUSIONS

Our bioinformatics-based *in silico* analysis provides a conservative assessment of potential off-target binding of pesticidal dsRNAs in the honey bee genome; the actual binding affinity of RISC is more nuanced than 100% or similar sequence similarity for subsequent mRNA degradation. While some have documented suppression of off-target gene expression with 20/21 nt similarity (*Jarosch & Moritz, 2012*), others have found silencing with even less sequence similarity in certain study systems, particularly in the 2–8 nt seed region of the siRNA. For example, in experiments with cultured human cells, *Saxena, Jonsson & Dutta (2003)* found gene silencing with as many as 3–4 bp mismatches in addition to G.U wobbles

(guanine and uracil have a slight affinity for each other), while *Jackson et al. (2003)* found mRNA degradation with only 11/21 contiguous nt. The locations of the mismatches along the siRNA are also important; perfect sequence similarity of the seed region is particularly crucial for mRNA recognition (*Jackson et al., 2006*; *Chu et al., 2014*). Additionally, the off-target effects may not be easily predicted, as downstream effects may result in gene suppression unrelated to the sequence of the original pesticidal molecule (*Hanning et al., 2013*). Another area of research that merits attention from a risk assessment perspective is that of RNA replication of the primary pesticidal molecule within a non-target organism. It has been repeatedly shown that environmental RNAi is replicated within a cell, and that the secondary RNAs produced do not always perfectly match the original sequence of the insecticidal RNA (*Pak & Fire, 2007*; *Sijen et al., 2007*). Focusing *in silico* analyses on only the primary insecticidal molecule may overlook these potential non-target effects.

However, *in silico* identification of sequence similarity between a pesticidal dsRNA and non-target organism's genome does not imply that RNAi will occur in the non-target organism. A fundamental difference between RNAi and chemical pesticides resides in their spectrum and mode of activity. Arguably, biochemical pesticides work on a limited number of physiological targets within an organism, and the list of potential non-target species is restricted to those sharing these targets with the pest. The absence of a relationship of taxonomic relatedness of target and off-target species and the likelihood of gene similarity between them indicates that the list of species potentially at risk from RNAi initially includes all of those species that use mRNA for gene expression and have the cellular machinery to process small RNAs. This spectrum of activity, and broad set of potential unintended phenotypic effects of the pesticidal RNAi may make predicting the risk of this technology more challenging than other pesticides. Unintended gene silencing will depend on a number of factors. The organism would need to possess behavioral characteristics that would put it into contact with contaminated materials, e.g., leaf tissue *versus* pollen *versus* nectar feeding at a contaminated location. Other factors include the length of the dsRNA and whether the organism is exposed to siRNA or dsRNA, the identity of the target or off-target mRNA, the size of a non-target organism's genome (more off-target binding would be expected when there are more potential gene targets), the necessary binding affinity of a particular siRNA, exposure concentration of the dsRNA, and the physiological state of the insect (*Qiu, Adema & Lane, 2005*; *Baum et al., 2007*; *Huvenne & Smagghe, 2010*; *Gu et al., 2014*).

Ecological risk assessment is a complex and multi-stepped process, and no single piece of work is sufficient to fully quantify the risk of a toxicological event. We have demonstrated that an *in silico* analysis may be used as a first step in establishing whether off-target binding could pose a significant threat for a particular pesticidal dsRNA in a non-target organism such as the honey bee. Future experiments to evaluate the usefulness of this tool are planned that would quantify up/down gene regulation of honey bees exposed to pesticidal dsRNA. Taken together, these data may provide a basis for designing biologically appropriate experiments to optimize hazard assessments for applications of this novel pesticidal technology in field settings where honey bees and other non-target organisms may be exposed.

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## ADDITIONAL INFORMATION AND DECLARATIONS

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### Grant Disclosures

### Competing Interests

The research was funded through public funding, and neither author has patents on RNAi-based pesticides nor affiliations with entities that have interest in RNAi-based pesticide technology. Jonathan Lundgren is the director of Ecdysis Foundation.

### Author Contributions

- Christina L. Mogren conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Jonathan Gary Lundgren conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

### Data Availability

The following information was supplied regarding data availability:
The raw data has been uploaded as a Supplemental File.

### Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.4131#supplemental-information.

## REFERENCES

**Bachman PM, Bolognesi R, Moar WJ, Mueller GM, Paradise MS, Ramaseshadri P, Tan J, Uffman JP, Warren J, Wiggins BE, Levine SL. 2013.** Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity

against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). *Transgenic Research* **22(6)**:1207–1222 DOI 10.1007/s11248-013-9716-5.

Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, Johnson SD, Plaetinck G, Munyikwa T, Pleau M, Vaughn T, Roberts J. 2007. Control of coleopteran insect pests through RNA interference. *Nature Biotechnology* **25(11)**:1322–1326 DOI 10.1038/nbt1359.

Bolognesi R, Ramaseshadri P, Anderson J, Bachman P, Clinton W, Flannagan R, Ilagan O, Lawrence C, Levine S, Moar W, Mueller G, Tan J, Uffman J, Wiggins E, Heck G, Segers G. 2012. Characterizing the mechanism of action of double-stranded RNA activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). *PLOS ONE* **7(10)**:e47534 DOI 10.1371/journal.pone.0047534.

Campbell EM, Budge GE, Bowman AS. 2010. Gene-knockdown in the honey bee mite Varroa destructor by a non-invasive approach: studies on a glutathione S-transferase. *Parasite Vector* **3**:73 DOI 10.1186/1756-3305-3-73.

Choudhary M, Sahi S. 2011. *In silico* designing of insecticidal small interfering RNA (siRNA) for *Helicoverpa armigera* control. *Indian Journal of Experimental Biology* **49(6)**:469–474.

Christiaens O, Swevers L, Smagghe G. 2014. dsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. *Peptides* **53**:307–314 DOI 10.1016/j.peptides.2013.12.014.

Chu C-C, Sun W, Spencer JL, Pittendrigh BR, Seufferheld MJ. 2014. Differential effects of RNAi treatments on field populations of the western corn rootworm. *Pesticide Biochemistry and Physiology* **110**:1–6 DOI 10.1016/j.pestbp.2014.02.003.

i5K Consortium. 2013. The i5K Initiative: advancing arthropod genomics for knowledge, human health, agriculture, and the environment. *Journal of Heredity* **104(5)**:595–600 DOI 10.1093/jhered/est050.

Desai SD, Eu YJ, Whyard S, Currie RW. 2012. Reduction in deformed wing virus infection in larval and adult honey bees (*Apis mellifera* L.) by double-stranded RNA ingestion. *Insect Molecular Biology* **21(4)**:446–455 DOI 10.1111/j.1365-2583.2012.01150.x.

FIFRA-SAP. 2014. RNAi technology: program formulation for human health and ecological risk assessment. FIFRA Scientific Advisory Panel Minutes No. 2014-2.

Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391(6669)**:806–811 DOI 10.1038/35888.

Flenniken ML, Andino R. 2013. Non-specific dsRNA-mediated antiviral response in the honey bee. *PLOS ONE* **8(10)**:e77263 DOI 10.1371/journal.pone.0077263.

Gong L, Chen Y, Hu Z, Hu M. 2013. Testing insecticidal activity of novel chemically synthesized siRNA against *Plutella xylostella* under laboratory and field conditions. *PLOS ONE* **8(5)**:e62990 DOI 10.1371/journal.pone.0062990.

Grimm D. 2011. The dose can make the poison: lessons learned from adverse *in vivo* toxicities caused by RNAi overexpression. *Science* **2**:8–8 DOI 10.1186/1758-907X-2-8.

Gu L, Knipple DC. 2013. Recent advances in RNA interference research in insects: Implications for future insect pest management strategies. *Crop Protection* **45**:36–40 DOI 10.1016/j.cropro.2012.10.004.

Gu S, Zhang Y, Jin L, Huang Y, Zhang F, Bassik MC, Kampmann M, Kay MA. 2014. Weak base pairing in both seed and 3′ regions reduces RNAi off-targets and enhances si/shRNA designs. *Nucleic Acids Research* **42(19)**:12169–12176 DOI 10.1093/nar/gku854.

Hajdusek O, Sojka D, Kopacek P, Buresova V, Franta Z, Sauman I, Winzerling J, Grubhoffer L. 2009. Knockdown of proteins involved in iron metabolism limits tick reproduction and development. *Proceedings of the National Academy of Sciences of the United States of America* **106(4)**:1033–1038 DOI 10.1073/pnas.0807961106.

Hajeri S, Killiny N, El-Mohtar C, Dawson WO, Gowda S. 2014. *Citrus tristeza* virus-based RNAi in citrus plants induces gene silencing in *Diaphorina citri*, a phloem-sap sucking insect vector of citrus greening disease (Huanglongbing). *Journal of Biotechnology* **176**:42–49 DOI 10.1016/j.jbiotec.2014.02.010.

Han P, Fan J, Liu Y, Cuthbertson AGS, Yan S, Qiu B-L, Ren S. 2014. RNAi-mediated knockdown of serine protease inhibitor genes increases the mortality of *Plutella xylostella* challenged by destruxin A. *PLOS ONE* **9(5)**:e97863.

Hanning JE, Saini HK, Murray MJ, Van Dongen S, Davis MPA, Barker EM, Ward DM, Scarpini CG, Enright AJ, Pett MR, Coleman N. 2013. Lack of correlation between predicted and actual off-target effects of short-interfering RNAs targeting the human papillomavirus type 16 E7 oncogene. *British Journal of Cancer* **108**:450–460 DOI 10.1038/bjc.2012.564.

Heinemann JA, Agapito-Tenfen SZ, Carman JA. 2013. A comparative evaluation of the regulation of GM crops or products containing dsRNA and suggested improvements to risk assessments. *Environment International* **55**:43–55 DOI 10.1016/j.envint.2013.02.010.

Huvenne H, Smagghe G. 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *Journal of Insect Physiology* **56(3)**:227–235 DOI 10.1016/j.jinsphys.2009.10.004.

Itakura S, Murayama S, Kamata Y, Tanaka H, Enoki A. 2009. RNA interference in symbiotic protists of the termite *Coptotermes formosanus* (Isoptera: Rhinotermitidae) through ingestion of siRNA by the host termite. *Sociobiology* **54(1)**:77–87.

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao MK, Li B, Cavet G, Linsley PS. 2003. Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnology* **21(6)**:635–637 DOI 10.1038/nbt831.

Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, Chau BN, Cleary M, Lim L, Linsley PS. 2006. Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. *RNA* **12(7)**:1197–1205 DOI 10.1261/rna.30706.

Jarosch A, Moritz RFA. 2012. RNA interference in honeybees: off-target effects caused by dsRNA. *Apidologie* **43(2)**:128–138 DOI 10.1007/s13592-011-0092-y.

Kelkenberg M, Odman-Naresh J, Muthukrishnan S, Merzendorfer H. 2015. Chitin is a necessary component to maintain the barrier function of the peritrophic

matrix in the insect midgut. *Insect Biochemistry and Molecular Biology* **56**:21–28 DOI 10.1016/j.ibmb.2014.11.005.

**Kerschen A, Napoli CA, Jorgensen RA, Müller AE. 2004.** Effectiveness of RNA interference in transgenic plants. *FEBS Letters* **566**:223–228 DOI 10.1016/j.febslet.2004.04.043.

**Kwon DH, Park JH, Lee SH. 2013.** Screening of lethal genes for feeding RNAi by leaf disc-mediated systematic delivery of dsRNA in *Tetranychus urticae*. *Pesticide Biochemistry and Physiology* **105(1)**:69–75 DOI 10.1016/j.pestbp.2012.12.001.

**Li H, Khajuria C, Rangasamy M, Gandra P, Fitter M, Geng C, Woosely A, Hasler J, Schulenberg G, Worden S, McEwan R, Evans C, Siegfried B, Narva KE. 2015.** Long dsRNA but not siRNA initiates RNAi in western corn rootworm larvae and adults. *Journal of Applied Entomology* **139(6)**:432–445 DOI 10.1111/jen.12224.

**Lu L-F, Liston A. 2009.** MicroRNA in the immune system, microRNA as an immune system. *Immunology* **127(3)**:291–298 DOI 10.1111/j.1365-2567.2009.03092.x.

**Lundgren JG, Duan JJ. 2013.** RNAi-based insecticidal crops: potential effects on nontarget species. *BioScience* **63(8)**:657–665 DOI 10.1525/bio.2013.63.8.8.

**Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, Sela I. 2009.** IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. *Insect Molecular Biology* **18(1)**:55–60 DOI 10.1111/j.1365-2583.2009.00847.x.

**Marr EJ, Sargison ND, Nisbet AJ, Burgess STG. 2014.** RNA interference for the identification of ectoparasite vaccine candidates. *Parasite Immunology* **36(11)**:614–624 DOI 10.1111/pim.12132.

**Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. 2002.** Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110(5)**:563–574 DOI 10.1016/S0092-8674(02)00908-X.

**Meng X, Li LM, Gao G, Jin FL, Ren SX. 2014.** The gene expression of the protein SLAWD, mediating the toxic effect of destruxin A on *Spodoptera litura* larvae, in prokaryotic cells: purification and characterization. *Molekuliarnaia Biologiia* **48(6)**:908–914 DOI 10.1134/S0026893314060120.

**Miyata K, Ramaseshadri P, Zhang Y, Segers G, Bolognesi R, Tomoyasu Y. 2014.** Establishing an *in vivo* assay system to identify components involved in environmental RNA interference in the western corn rootworm. *PLOS ONE* **9(7)**:e101661 DOI 10.1371/journal.pone.0101661.

**Mutti NS, Park Y, Reese JC, Reeck GR. 2006.** RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. *Journal of Insect Science* **6**:1–7 DOI 10.1673/031.006.3801.

**Ochoa-Campuzano C, Martinez-Ramirez AC, Contreras E, Rausell C, Real MD. 2013.** Prohibitin, an essential protein for Colorado potato beetle larval viability, is relevant to *Bacillus thuringiensis* Cry3Aa toxicity. *Pesticide Biochemistry and Physiology* **107(3)**:299–308 DOI 10.1016/j.pestbp.2013.09.001.

**Pak J, Fire A. 2007.** Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. *Science* **315**:241–244 DOI 10.1126/science.1132839.

**Petrick JS, Moore WM, Heydens WF, Koch MS, Sherman JH, Lemke SL. 2015.** A 28-day oral toxicity evaluation of small interfering RNAs and a long double-stranded

RNA targeting vacuolar ATPase in mice. *Regulatory Toxicology and Pharmacology* **71(1)**:8–23 DOI 10.1016/j.yrtph.2014.10.016.

**Price DRG, Gatehouse JA. 2008.** RNAi-mediated crop protection against insects. *Trends in Biotechnology* **26(7)**:393–400 DOI 10.1016/j.tibtech.2008.04.004.

**Qiu S, Adema CM, Lane T. 2005.** A computational study of off-target effects of RNA interference. *Nucleic Acids Research* **33(6)**:1834–1847 DOI 10.1093/nar/gki324.

**Roberts AF, Devos Y, Lemgo GNY, Zhou X. 2015.** Biosafety research for non-target organism risk assessment of RNAi-based GE plants. *Frontiers in Plant Science* **6**:Article 958.

**Romeis J, Bartsch D, Bigler F, Candolfi MP, Gielkens MMC, Hartley SE, Hellmich RL, Huesing JE, Jepson PC, Layton R, Quemada H, Raybold A, Rose R, Schiemann J, Sears MK, Shelton AM, Sweet J, Vaituzis Z, Wolt JD. 2008.** Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. *Nature Biotechnology* **2008(26)**:203–208 DOI 10.1038/nbt1381.

**Saxena S, Jonsson ZO, Dutta A. 2003.** Small RNAs with imperfect match to endogenous mRNA repress translation—implications for off-target activity of small inhibitory RNA in mammalian cells. *Journal of Biological Chemistry* **278(45)**:44312–44319 DOI 10.1074/jbc.M307089200.

**Sijen T, Steiner FA, Thijssen KL, Plasterk RH. 2007.** Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. *Science* **315**:244–247 DOI 10.1126/science.1136699.

**Tang B, Wang S, Zhang F. 2010.** Two storage hexamerins from the beet armyworm *Spodoptera exigua*: cloning, characterization and the effect of gene silencing on survival. *BMC Molecular Biology* **11**:65 DOI 10.1186/1471-2199-11-65.

**Vermeulen A, Behlen L, Reynolds A, Wolfson A, Marshall WS, Karpilow J, Khvorova A. 2005.** The contributions of dsRNA structure to Dicer specificity and efficiency. *RNA* **11(5)**:674–682 DOI 10.1261/rna.7272305.

**Whyard S, Singh AD, Wong S. 2009.** Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochemistry and Molecular Biology* **39(11)**:824–832 DOI 10.1016/j.ibmb.2009.09.007.

**Wuriyanghan H, Rosa C, Falk BW. 2011.** Oral delivery of double-stranded RNAs and siRNAs induces RNAi effects in the potato/tomato psyllid, *Bactericerca cockerelli*. *PLOS ONE* **6(11)**:e27736 DOI 10.1371/journal.pone.0027736.

**Yao J, Rotenberg D, Afsharifar A, Barandoc-Alviar K, Whitfield AE. 2013.** Development of RNAi methods for *Peregrinus maidis*, the corn planthopper. *PLOS ONE* **8(8)**:e70243 DOI 10.1371/journal.pone.0070243.

**Yu R, Xu X, Liang Y, Tian H, Pan Z, Jin S, Wang N, Zhang W. 2014.** The insect ecdysone receptor is a good potential target for RNAi-based pest control. *International Journal of Biological Sciences* **10(10)**:1171–1180 DOI 10.7150/ijbs.9598.

**Zhou X, Wheeler MM, Oi FM, Scharf ME. 2008.** RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA. *Insect Biochemistry and Molecular Biology* **38(8)**:805–815 DOI 10.1016/j.ibmb.2008.05.005.