UNIVERSIDADE FEDERAL DE PELOTAS

Crop Protection Post-Graduation Program

Thesis

Transcriptome analysis and exploring RNAi and CRISPR in the Neotropical stink bug, *Euschistus heros*

Deise Cagliari

Pelotas, 2021

Deise Cagliari

Transcriptome analysis and exploring RNAi and CRISPR in the Neotropical stink bug, *Euschistus heros*

A dissertation submitted to the Universidade Federal de Pelotas (UFPel) and Ghent University (UGent) as a partial fulfillment of the requirement for the Joint PhD degree in Crop Protection (UFPel) and Bioscience Engineering (UGent).

Promoters: Dr. Moises João Zotti (UFPel)
Dr. Guy Smagghe (UGent)
Co-Advisors: Dr. Flávio Roberto Mello Garcia

Pelotas, 2021

Universidade Federal de Pelotas / Sistema de Bibliotecas
Catalogação na Publicação

C131t Cagliari, Deise

Transcriptome analysis and exploring RNAi and CRISPR in the Neotropical stink bug, *Euschistus heros* / Deise Cagliari ; Moisés João Zotti, Guy Smagghe, orientadores ; Flávio Roberto Mello Garcia, coorientador. — Pelotas, 2021.

237 f.

Tese (Doutorado) — Programa de Pós-Graduação em Fitossanidade, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, 2021.

1. Gene silencing. 2. Gene editing. I. Zotti, Moisés João, orient. II. Smagghe, Guy, orient. III. Garcia, Flávio Roberto Mello, coorient. IV. Título.

CDD : 595.7

Elaborada por Gabriela Machado Lopes CRB: 10/1842

Examination committee:

_____
Vanessa Galli, Dra.
(Federal University of Pelotas)

_____
Godelieve Gheysen, Dr.
(Ghent University)

_____
Diogo Manzano Galdeano, Dr.
(Campinas Agronomic Institute)

_____
Danielle Ribeiro de Barros, Dr.
(Federal University of Pelotas)

_____
Dori Edson Nava, Dr.
(Embrapa Temperate Agriculture)

_____
Guy Smagghe, Dr. ir.
(Ghent University)

_____
Moisés João Zotti, Dr.
(Federal University of Pelotas)

Ao meu querido tio, Paulo Cagliari (in memorium),
minha querida madrinha, Claci Kieling (in memorium) e
meu querido primo, Mauro Cagliari (in memorium)
que vem iluminando nosso caminho lá de cima.

Ofereço

Aos meus amados pais CANISIO e AMÉLIA, por todas as vezes que
abriram mão dos seus sonhos para que nós pudessemos
realizar os nossos e por sempre acreditarem em mim
e me darem todo apoio possível.

Dedico

Acknowledgments

Primeiramente, gostaria de agradeçer à Deus por guiar meus passos, iluminar meu caminho e me dar força nos momentos dificeis.

I would like to thanks the Federal University of Pelotas and the Ghent University for the opportunity to pursue my Joint PhD degree in these prestigious universities.

Gostaria de agradecer ao CNPq pelo auxílio financeiro concedido na realização desse trabalho por meio de uma bolsa de estudos e taxa de bancada, e a CAPES pelo auxílio financeiro concedido para realização do doutorado sanduiche (bolsa de doutorado sanduiche).

Gostaria de agradecer ao meu orientador Moises João Zotti, por me receber em seu laboratório. Isso abriu outras portas importantes na minha carreira e aprendi muito. Sempre serei grata pela oportunidade.

I would also like to express my immense gratitude to my promoter Guy Smagghe, for the opportunity, his guidance, teachings, patience, support and friendship during the PhD. You always believed in me and your continue support was of upmost importance during to finish this work. Thank you!

I must also express my immense gratitude to Clauvis Nji Tizi Taning for his constant and continues help, guidance and support. For always making some time to correct the manuscripts and teaching me important lessons. Without your help and guidance this work would not be complete. I will always be grateful.

I want to thanks also to Clauvis Nji Tizi Taning, Olivier Christiaens and Kristof De Schutter for all the help and support in the lab and reviewing the papers. You helped me a lot, and I am very thankful for that.

Meus agradecimentos aos professores da Pós-Graduação em Fitossanidade e da Pós-Graduação em Biotecnologia da UFPel, por me ensinarem tanto sobre entomologia e biologia molecular.

Um agradecimento especial aos meus co-orientadores Flávio Roberto Mello Garcia e Anderson Dionei Grützmacher, por toda a ajuda durante o meu doutorado. Agradeço também aos professores Dirceu Agostinetto e Luís Antonio de Ávila, por permitirem o uso dos equipamentos do CEHERB (Centro de Herbologia) para realização das minhas pesquisas.

Gostaria de agradecer aos professores Daniel Bernardi, Juliano R. Farias, Leticia R. e Dori E. Nava pelos questionamentos e sugestões durante minha qualificação de doutorado.

Meu muito obrigada ao funcionário do Departamento de Defesa Fitossanitária, Marcelo e aos funcinários da PRPGPI, por sempre me ajudarem a resolver as questões admisnistrativas. Aos demais professores, funcionários e alunos do Departamento de Defesa Fitossanitária por todos os ensinamentos e tempo que passamos juntos.

To the professors of the University Language Centre from UGent for teaching me very important skills to improve my English presentations.

I want also to say thank you to the Leen, Stephanie and Bjorn for always making some time to help me in the lab. You always support me and the other people form the lab. I want also to thank Marleen, for all the help in the administrative issues.

Um agradecimento especial à servidora do Laboratório de Manejo Integrado de Pragas, Mariane Rosenthal, por sua amizade e por toda ajuda e apoio durante o desenvolvimento deste trabalho. A tua ajuda foi imprescindível Mari!

Também quero agradecer a todas os membros do Laboratório de Entomologia Molecular, em especial Kadine, Lizandra, Lethicia, Daniele, Naymã, Ericmar, Maicon e Bruno por toda a ajuda durante meus estudos de doutorado.

Um agradecimento especial a Leticia Rickes pelos ensinamentos iniciais em biologia molecular. Por me ensinar com paciência e me conduzir no início da minha jornada. Ao Frederico, pelo ensinamos em bioinformática e toda a paciência para tirar as inacabáveis dúvidas. A Daiane, pelos ensinamentos sobre qRT-PCR. Ao Diogo por todos os ensimamentos iniciais em RNAi e por sempre tirar as minhas incontáveis dúvidas.

Gostaria de agradeçer ao Laboratório de Virologia da UFPel, a professora Danielle, e as colegas Sílvia e Carol por toda ajuda prestada durante os meus

experiementos. Sem a ajuda de vocês com certeza a minha tese não estaria completa.

Quero expressar minha gratidão aos meus amigos do programa de Pós-Graduação em Fitossindade Aline, Juliano, Joana, Jéssica, Swamy, Paloma, Silvia, Carolina G. N., Alexandra, Júlia, Tiago, Lili, Daia, Bruna, Andrisa, Caroline N., Dalvane, Joanei, Fernanda, Magali e João, e aqueles cujos quais não consegui citar aqui por todo seu apoio e ajuda durante meu doutorado.

Aos colegas do laboratório de Manejo Integrado de Pragas, Francieli, Ronaldo, Mateus, Maíra e Paulo pelos mates e conversas durante o tempo que passei no laboratório.

Aos colegas Cristiano e Joanei por toda ajuda despendida para que fosse possível realizar meus experimentos. Muito obrigada, sem a ajuda de vocês, com certeza meu trabalho estaria incompleto.

Um agradecimento especial aos meus queridos amigos Aline, Juliano, Joana e Jéssica por todo o tempo que sempre tiveram comigo, pelas infinitas conversas sobre o doutorado, por sempre me apoiarem e tornarem meus dias melhores. Sempre serei grato a você.

As minhas amigas de longa data Lia, Andrisa, Suelen, Edicarla, Katherine, Daniele, Daniela e Franciele por sempre estarem ao meu lado. Pelas inumeras conversas, conselhos e amizade ao longo desses anos. Vocês são as melhores amigas que alguém poderia ter. Muito obrigado por tudo.

Às minhas amigas “do trecho”, Cristiane, Juliane e Magali, por me ajudarem tantas vezes. Por todo apoio, amizade e o tempo que passaram juntos nas viagens durante os 3 primeiros anos do meu doutorado (devemos ter uns 30 000 kms na conta). Vocês foram um dos presentes que Pelotas me deu eu sou muito grato por ter tido a oportunidade de conhecê-las e te-lás em minha vida.

I want to say thank you to my friends from Ghent University, Clauvis, Olivier, Nathaly, Zarel, Jaqueline, Claúdia, Louella, Melissa, Sander, Eva Kt, Eva Skoufa, Nomi, Wenxin, Christine, Xueping, Qun, Dong Dong, Pengyu, Xue and Rohit for all the help, support and beers during the time I spend in Ghent. You made my days better. If one day you need something from Brazil, just let me know.

I want to thanks Clauvis, Olivier, Kristofer, Nathaly, Zarel, Rohit, Weidong, Dong Dong, Pengyu and Shunhua for always making some time to explain me something from the lab. A special thanks to Clauvis, Olivier, Nathaly, Rohit and Shunhua for teaching me so much about RNAi and CRISPR. I will always be grateful.

Quero agardeçer em especial a querida Nathaly por toda sua ajuda no laboratório em Ghent e na formatação desse trabalho. Muito obrigada do fundo do meu coração.

Às minhas queridas amigas Jaqueline, Nathaly e Claúdia, por todas as conversas, apoio, ajuda e amizade. Jaqueline, você me recebeu de braços abertos em Ghent e foi de uma importância sem tamanho para mim. Nathaly, você me ajudou e ensinou tanto, no laboratório e fora dele. Claúdia, nem sei agradecer pela parceria, por topar todos os “roles” de bike e de canoa. Vocês fizeram meus dias em Ghent os melhores que eu poderia ter. Sou imensamente grata a vocês!

Ao meu namorado Jonas. Você sempre esteve aqui, me dando seu apoio, ajuda e amor durante todos esses anos. Agradeço a tua imensa paciência para entender minha ausência (as vezes mesmo estando presente fisicamente). As palavras de incentivo e os “puxões de orelha” quando precisei. Fizemos isso juntos, mais de 100 0000 kms ao final desse doutorado, e sem você, não teria conseguido. Não consigo expressar o quanto sou grata por ter você em minha vida e por tudo que construimos ao longo desses quase 13 anos. Eu te amo!

A “ minha segunda familia”, meu sogro Edemar, minha sogra Neli, meu cunhado Joelson, minha cunhada Caroline e minha amada sobrinha Isabela, por todo apoio e suporte ao longo desses anos, e principalemente por sempre me fazerem presente na vida da pequena, mesmo não estando presente fisicamente.

Por último, mas com certreza não menos importante, quero agradecer à minha família. Minha mãe Amélia, meu pai Canísio e minha irmã Dione. Todos os seus ensinamentos, amor, compreensão e apoio foram tão importantes que não posso descrever. Obrigado por compreenderem minha ausência fisica aos longo desses anos. Por me ligarem e me “fazerm companhia” no lab ou ainda, tomarmos mates juntos em tantas videochamdas. Esta tese de doutorado não é

só minha, é NOSSA. Eu amo vocês mais que tudo e sempre estarei aqui para vocês e por vocês.

Muitíssimo obrigada! Thank you so much! Echt heel erg bedankt!

“O melhor dos planos não passa de boas intenções, a não ser que seja deturbado em trabalho.”

– Peter Drucker

Abstract

CAGLIARI, Deise. **Transcriptome analysis and exploring RNAi and CRISPR in the Neotropical stink bug, *Euschistus heros.*** 2021. 219f. Dissertation (PhD) – Post-Graduation Program in Crop Protection (UFPel) and in Bioscience Engineering (UGent)

One of the main factors limiting agricultural production is the attack of insects, pathogens and weeds, which can cause significant losses in the culture. *Euschistus heros* (Heteroptera: Pentatomidae) is one of the most important stink bug species found in soybean, with occurrence distributed throughout the Brazilian production area. In an attempt to reduce the damage caused by this and other insects, producers depend almost exclusively on the use of insecticides. However, the indiscriminate use of these products has been causing serious management problems, such as the selection of resistant populations leading to the inefficiency of products available on the market. Therefore, the development of alternative control tools, such as gene silencing based on RNA interference (RNAi) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is necessary. RNAi is a highly conserved mechanism in eukaryotic organisms and by which the messenger RNA molecule (mRNA) is cleaved by the gene-silencing machinery, leading to inactivation of gene expression (Knockdown). CRISPR, on the other hand, is a gene editing tool with which it is possible to carry out modification at the DNA level, introducing or eliminating specific DNA sequences from the genome (knockout). During the last years, the use of RNAi and CRISPR has attracted the interest of researchers, mainly in the study of the functions of genes during insect's development and reproduction, which will allow the future development of alternative control strategies. Thus, considering the importance of RNAi and the CRISPR / Cas9 system in the study of functional genomics and in the development of new tools for the management of insect pests, the objectives of this study include: i) to identify the main components of the RNAi machinery present in *E. heros*, as well as validating the functionality of this tool in *E. heros*; ii) a review showing the potential use of the RNAi tool in a non-transformative approach; iii) the use of parental RNAi as a tool in the study of gene functions in *E. heros*; iv) the combination of RNAi and CRISPR/Cas9 as functional genomic tools in the Neotropical bug, *E. heros*. This information provides novel and important dataset on RNAi machinery and its efficiency, the future perspective of non-transformative RNAi approaches and the use of RNAi and CRISPR as tools in functional genetic studies, underpinning future strategies to improve RNAi and CRISPR in *E. heros* and other piercing-sucking insects species important in agriculture.

Key words: gene silencing; gene knockdown; gene knockout; pest management; functional genomics.

Resumo

CAGLIARI, Deise. **Análise do transcriptoma e exploração do RNAi e CRISPR no percevejo neotropical, *Euschistus heros*.** 2021. 219f. Tese (doutorado) – Programa de Pós-Graduação em Fitossanidade (UFPel) e Engenharia de Biosciências (UGent).

Um dos principais fatores limitantes a produção agrícola, são o ataque de insetos, patógenos e plantas daninhas, os quais podem causar perdas significativas. *Euschistus heros* (Heteroptera: Pentatomidae) é uma das espécies de maior importância encontradas na cultura da soja, com ocorrência distribuída em toda área produtora brasileira. Na tentativa de reduzir os danos causados por esse e outros insetos, produtores dependem quase que exclusivamente do uso de inseticidas. Entretanto, o uso indiscriminado desses produtos vem causando sérios problemas de manejo, como a seleção de populações resistentes levando a ineficiência dos produtos disponíveis no mercado. Diante disso, é necessário o desenvolvimento de ferramentas alternativas de controle, tais como silenciamento gênico baseado em RNA de interferência (RNAi) e CRISPR (Repetições Palindrômicas Curtas Agrupadas e Regularmente Interespaçadas). O RNAi é um mecanismo altamente conservado em organismos eucariotos e pelo qual a molécula de RNA mensageiro (RNAm) é clivada pela maquinaria de silenciamento, levando a inativação da expressão gênica (Knockdown). Já o CRISPR é uma ferramenta de edição gênica com a qual é possível realizar modificação a nível de DNA, introduzindo ou eliminando sequências específicas do DNA (knockout). Nos últimos anos, a utilização do RNAi e CRISPR tem atraído o interesse de pesquisadores no estudo das funções dos genes no desenvolvimento e reprodução dos insetos, o que permitirá o desenvolvimento de estratégias alternativas de controle. Assim, considerando a importância do RNAi e do sistema CRISPR/Cas9 no estudo da genômica funcional e no desenvolvimento de novas ferramentas para o manejo de insetos-praga, os objetivos deste estudo incluem: i) identificar os principais componentes da maquinaria de RNAi presentes em *E. heros,* assim como validar a funcionalidade dessa ferramenta em *E. heros*; ii) uma revisão mostrando o uso potencial da ferramenta RNAi em uma abordagem não-transformativa; iii) o uso do RNAi parental como ferramenta no estudo das funções gênicas em *E. heros*; iv) a combinação de RNAi e CRISPR/Cas9 como ferramentas genômicas funcionais no percevejo Neotropical, *E. heros*. Essas informações fornecem novos e importantes conjuntos de dados sobre a maquinaria de RNAi e sua eficiência, a perspectiva futura da utilização do RNAi via não-transformativa e do uso de RNAi e CRISPR como ferramentas em estudos genéticos funcionais, apoiando estratégias futuras para melhorar RNAi e CRISPR em *E. heros* e outras espécies de insetos perfuradores e sugadores importantes na agricultura.

Key words: silenciamento gênico; gene knockdown; gene knockout; manejo de insetos-praga; genética funcional.

List of figures

General introduction

List of tables

Content

1.General introduction

1.1 The Neotropical stink bug *Euschistus heros:* an important agricultural insect pest

1.1.1 *Euschistus heros* characteristics

Euschistus heros adults present a dark brown color, with two lateral extensions of the pronotum, that look like thorns (PANIZZI et al. 2000, 2012). Females deposited the eggs mainly in the leaves or pods of the soybean, in small masses of 5-8 eggs, yellowish in color, presenting a pink spot near to hatching (PANIZZI et al. 2012). The eggs take about 7 days to hatch (COSTA et al. 1998). The newly hatched nymphs measure about 1 mm and remain on the eggs shelf, starting to cause damage to the soybean seeds at the 3rd instar, when they reach a size of 5 mm (Hoffmann-campo et al. 2000). The total developmental cycle of the insects (from egg-adult) usually takes around 40 days, and the adults can survive for up to 116 days (PANIZZI et al. 2012) (Table 1). The adults reach sexual maturity at the age of 9-14 days and oviposition starts usually 10 days after mating (Costa et al. 1998). However, several factors can affect the lifespan of the insects, such as temperature, humidity, photoperiod, and food availability, which can significantly affect biological parameters (PANIZZI et al. 2012). After harvesting, insects can take shelter in other host plants, such as weeds (MEDEIROS and AMEGIER 2009; DALAZEN et al. 2016) and during the coldest months remain in diapause until the beginning of the next planting season (CORRÊA-FERREIRA and PANIZZI 1999).

Table 1 – Duration, in days, of *Euschistus heros* different life stages, including eggs, nymphs, and adults.

Stage	Duration of each stage (days)[1]
Eggs	6-8
1st-instar nymph	3-6
2nd-instar nymph	6-8
3rd-instar nymph	4-7
4th-instar nymph	5-9
5th-instar nymph	6-9
Adults	up to 116

[1]Adapted from BORGES et al. 2006; PANIZZI et al. 2012.

Euschistus heros is a polyphagous species that can feed on different parts of the plants of the Fabaceae, Solanaceae, Brassicaceae, Compositae, and Malvaceae families, being one of the main problems, in soybean crop, generating significant losses in the yield and quality of the grains during harvest (LINK and GRAZIA 1987; SORIA et al. 2007; PANIZZI 2015; SMANIOTTO and PANIZZI 2015). In cotton crops, *E. heros* can cause serious damage, mainly in cultivated areas near soybean crops at the end of the cycle, due to insect migration (SORIA et al. 2010; PANIZZI et al. 2012). Also, *E. heros* can cause injuries of varying intensities in seedlings and ears of corn, but the main damages are still registered in the soybean crop (SOSA-GÓMEZ et al. 2010).

1.1.2 *Euschistus heros* distribution

The first report of the occurrence of this species was in South America during the 70's, and at that time, its occurrence was rarely reported (PANIZZI et al. 1977; PANIZZI 2015). However, due to population outbreaks (PANIZZI 2000; SOSA-GÓMEZ et al. 2009), mainly related to the increase in average global temperatures (BUENO et al. 2017), an increase in their occurrence has been noted. Currently, *E. heros* is found in the main soybean production areas in South America: Brazil (PANIZZI 2015), Paraguay (PANIZZI 2015), and Argentina (SALUSO et al. 2011) (Figure 1). In Santa Maria city (Rio Grande do Sul - Brazil), in the 2007/08 harvest season, *E. heros* represented about 12% of the stink bugs present in the soybean crop, representing an increase of about 200% compared to the previous harvest season (2006/07) (KUSS-ROGGIA 2009). In Londrina city (Paraná - Brazil), in the 2007/08 harvest season, *E. heros* represented 84% of the insects sampled (CORRÊA-FERREIRA et al. 2010).

Figure 1 – Occurrence of *Euschistus heros* in South America: Brazil (PANIZZI, 2015), Paraguay (PANIZZI, 2015), and Argentina (SALUSO et al. 2011).

In an attempt to understand the genetic structure of *E. heros* in Brazil, genetic analysis of different populations revealed two divergent Brazilian strains (SOARES et al. 2018). One clade grouped the insects from the North and Northeast regions of Brazil, while the second clade grouped the insects from the South and Southeast regions of Brazil (SOARES et al. 2018). The researchers also found a genetic structure among the subpopulations of *E. heros* according to the biome in which the population was inserted (Amazon, Caatinga, Cerrado, and Atlantic Forest), being the population growth and expansion rates affected by the environmental conditions. Still, the researchers claim that individuals adapted to different environmental conditions, and specific monocultures may be combining themselves into a specific population, which will make the management even more difficult (SOARES et al. 2018).

1.1.3 *Euschistus heros* management

To reduce the damage caused by insect pests, pathogens, and weeds, farmers depend almost exclusively on chemical products. Current recommendations for the management of this insect depend almost exclusively on the use of broad-spectrum insecticides, such as organophosphates (~ 49%), pyrethroids (~ 6%), or the mixture containing any of these chemical groups (~ 28%) (AGROFIT, 2020).

In 2019, Brazil approved the use of *Telenomus podisi* as a biological agent to control *E. heros* in soybean. This natural enemy is an egg parasitoid that can parasitize

the eggs of *E. heros* and other stink bugs (BORTOLOTTO et al. 2016; QUEIROZ et al. 2018). Under natural conditions, *T. podisi* can parasitize up to 80% of the stink bug egg masses (PACHECO and CORRÊA-FERREIRA 2000). So, this represents an option for growers in the field to manage *E. heros* with a biological agent.

Although in Brazil soybean crop presents a consolidated Integrated Pest Management program (IPM), the control of *E. heros*, as well as other stink bugs, is still basically carried out through the use of insecticides, with up to 5 applications carried out during the soybean reproductive period (PANIZZI 2013; BUENO et al. 2015). The increasing use of insecticides can cause serious problems in the management of insects, such as the selection of resistant populations, a fact that has already been reported in populations of *E. heros* (SOSA-GÓMEZ and SILVA 2010; GUEDES 2017; TUELHER et al. 2018). Still, problems related to the resurgence/occurrence of population peaks due to the effect of insecticides on natural enemies and damage to non-target organisms, including humans, are some of the effects caused by the indiscriminate use of insecticides (PANIZZI 2013; GUEDES and CUTLER 2014; MACFADYEN et al. 2014; SANTOS et al. 2016; TUELHER et al. 2018). Therefore, there is a need to develop alternatives for the management of insect pests, which are insect-specific, sustainable, and have a less environmental impact, such as RNAi (RNA interference) and/or CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats).

1.1.4 Establishment of a *Euschistus heros* colony in the laboratory

The maintenance of an insect colony in the laboratory is not just a routine activity. The establishing and maintaining of an insect colony with significant production of individuals requires a thorough knowledge of biology, nutritional ecology, the behavior of the insects, and labor time. To carry out scientific research with the necessary quality, have standardized and healthy insects is extremely important.

In an attempt to reduce the variables that can impair insects behavior, external factors (temperature, lighting, etc.) and internal factors (general physiological condition of the insects, degree of stress, etc.) need to be reduced the maximum as possible (BORGES et al. 2006). To uniformize and obtain healthy insects for the experiments, a colony of insects was kept under laboratory conditions both in Brazil and in Belgium.

To have a healthy and uniform *E. heros* colony, insects need to be kept under standard mass-rearing conditions of 25 ± 2 °C, 60 ± 10% relative humidity, and a Light/Dark photoperiod of 14:10 h. The insects are kept in plastic containers (20 x 21 cm in Brazil and 23 x 8 cm in Belgium) lined with towel paper. Each one of these boxes can support up to 250 adult insects.

Food usually is supplied *ad libitum* with a mixture of fresh green bean pods *Phaseolus vulgaris* (L.), and seeds: raw shelled peanuts *Arachis hypogaea* (L.) and soybean seeds *Glycine max* (L.) (BORGES et al. 2006). The supplies require to be replenished at 3-days-intervals (especially due to fungal growth), taking around 4 hours to the maintenance of the colony (14 plastic boxes with 23 x 8 cm). Every time insects are changed, eggs are collected and placed in Petri dishes for 3-4 days. Then they are transferred to plastic boxes containing food and reared until they reached adulthood.

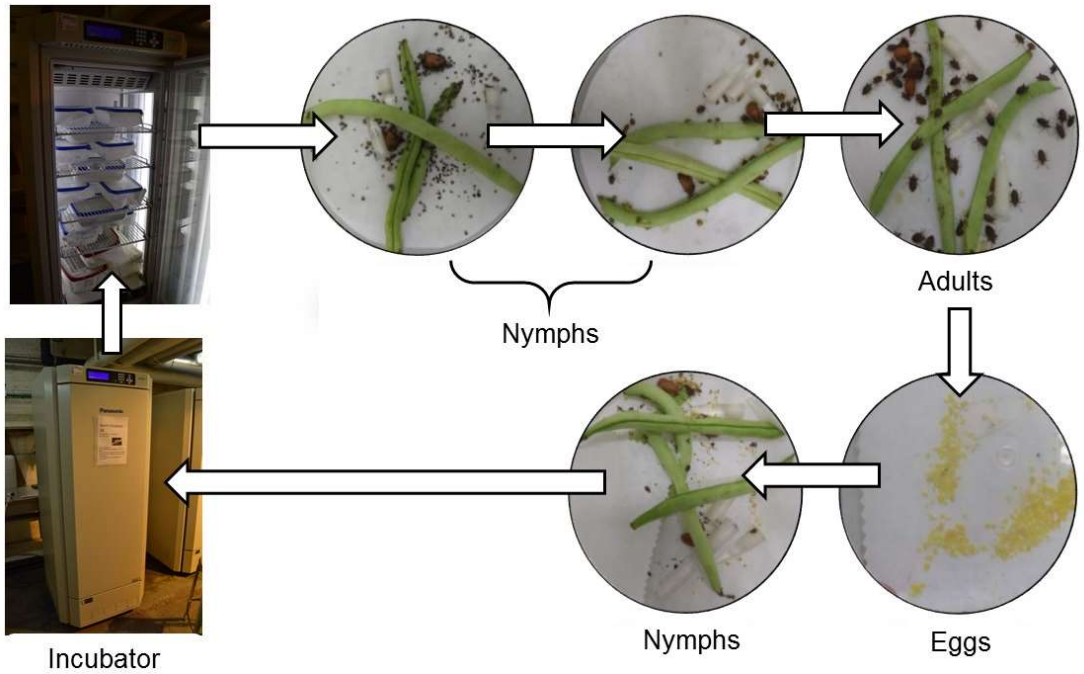


Figure 2 – Schematic representation of the steps involved in *E. heros* rearing. Nymphs are kept in separate plastic boxes, and when the presence of adults is observed, they are collected and placed in another separate plastic box. The plastic boxes are kept in an incubator, under standard mass-rearing conditions of 25 ± 2 °C, 60 ± 10% relative humidity, and a L/D photoperiod of 14:10 h.

This process ensures that the insects used in experiments are healthy and standardized, reducing unintended effects.

1.2 RNA interference (RNAi)

1.2.1 RNAi mechanism

In plants and animals, the RNAi mechanism provides a front line defense mechanism against invading RNA viruses as well as unknown double-stranded RNA (dsRNA) molecules to the cellular machinery (Mello and Conte 2004; Baum and Roberts 2014). In insects, there are currently two main classes of small non-coding RNAs (ncRNAs) active in the RNAi pathway: micro RNAs (miRNAs) and small interference RNAs (siRNAs). MiRNAs are derived from endogenous expression whereas siRNAs are of exogenous origin derived from viruses or transposons (Preall and Sontheimer 2005). Although the biogenesis of miRNA and siRNA is different, these ncRNAs share most of the elements involved in the RNAi pathway (Figure 2) (ZOTTI and SMAGGHE 2015).

Small interfering pathway (siRNA)

Although some RNAi pathways use dsRNAs to generate small RNAs (sRNAs) (*i.e.* microRNA and siRNA) (BERNSTEIN et al. 2001; KETTING 2011), in insects it is known that the siRNA pathway is also activated due to the external supply of dsRNA molecules or direct siRNA (CARTHEW 2009; ZOTTI and SMAGGHE 2015). The dsRNAs are processed by Dicer 2 (DCR-2) enzyme (belonging to the RNase III family) in ~ 21 bp siRNAs, which will guide the silencing machinery (Figure 3) (MEISTER and TUSCHL 2004; TOMARI et al. 2007). To process the siRNAs, DCR-2 requires the auxiliary protein R2D2 (LIU et al. 2003). These siRNA duplexes have ~2-3 nucleotides (nt) overhangs at both 3' ends, which ensures them to be identified by the gene silencing machinery (WYNANT et al. 2014b; MONGELLI and SALEH 2016).

The siRNA pathway in plants, worms, and fungi is much more complex than the one observed in insects and mammals because these organisms present a molecule called RNA-dependent RNA polymerase (RdRPs) (CHAPMAN and CARRINGTON 2007; ZOTTI and SMAGGHE 2015). This enzyme is responsible to increase the silencing signal, through the production of secondary siRNAs (SIOMI and SIOMI 2009), responsible for the so-called systemic RNAi (ZOTTI and SMAGGHE 2015). However, it is very important to point out that RdRps are not necessary to induce systemic RNAi (TOMOYASU et al. 2008).

The complex R2D2/DCR-2 helps to load the siRNA into the RNAi silencing complex (RISC), avoiding the siRNA from diffusing in the cytoplasm (TOMARI and ZAMORE 2005; SIOMI and SIOMI 2009). RISC is a ribonucleoprotein complex that contains several components, including Argonaute 2 (AGO-2), which represents the catalytic domains of the complex (SIOMI and SIOMI 2009). Once the siRNA molecule is loaded into the RISC, one of the strands, called passenger strand (sense), is unloaded from the complex, and eliminated from the system (MATRANGA et al. 2005; MIYOSHI et al. 2005). The remaining strand, called guide strand (antisense), is responsible to guide the enzymatic complex to the complementary sequence (TOMARI and ZAMORE 2005; SIOMI and SIOMI 2009).

Then, the active RISC searches the system for potential target mRNA (SIOMI and SIOMI 2009). Once the RISC identifies the target sequence via the Watson-Crick base pairing of the guide strand and the target mRNA (MONGELLI and SALEH 2016), it cleaves the mRNA approximately in the middle of the formed duplex region (position 10 concerning the 5' end) (AGRAWAL et al. 2003; MEISTER and TUSCHL 2004). After cleaving the mRNA, the resulting products are quickly degraded by the cellular ribonucleases (SCHUSTER et al. 2019).

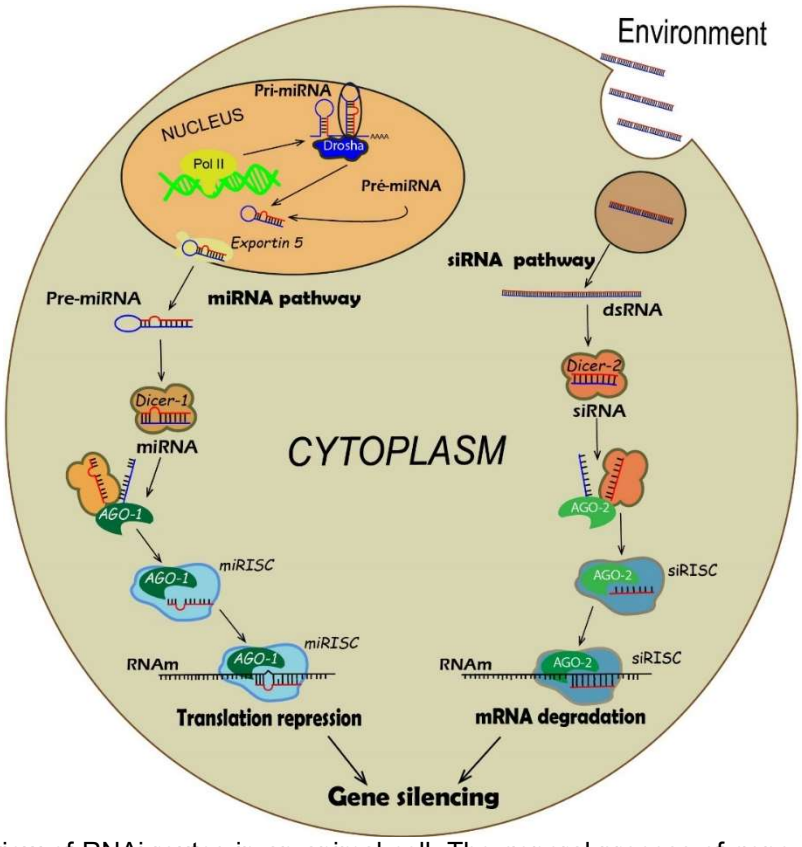


Figure 3 - Overview of RNAi routes in an animal cell. The general process of gene silencing through RNAi is divided into one step in the nucleus (miRNA) and three steps in the cytoplasm (miRNA/siRNA). Nucleus: the RNA Polymerase enzyme transcribes the pri-miRNA. The pri-miRNA is cleaved by the enzyme Drosha, resulting in the pre-miRNA molecule, which is then exported by the protein Exportin 5 to the cytoplasm. Cytoplasm: First, Dicer1/ 2(DCR-1/DCR-2) cleaves the molecule of pre-miRNA/dsRNA in small RNAs (sRNAs); Then, the sRNAs are loaded into the RISC complex which contains the Argonaute enzyme; one of the strands, called guide strand, directs the Argonaute to cleave/block the messenger RNA (mRNA). Adapted from ZOTTI and SMAGGHE, 2015.

1.2.2. Nucleases

Nuclease enzymes (RNases together with other RNA enzymes) participate in the digestion of DNA/RNA in the digestive tract of insects (ARIMATSU et al. 2007), offering an additional defense barrier and gene control. The activity of the nucleases involved in the degradation of dsRNAs (dsRNases) is well defined and has an important role in different groups of insects such as Hemiptera (CHRISTIAENS and SMAGGHE 2014; WANG et al. 2016a), Lepidoptera (LIU et al. 2012; GUAN et al. 2018) and Diptera (SINGH et al. 2017), affecting the efficiency of RNAi.

In *Bombyx mori* (Lepidoptera: Bombycidae), three isoforms of *dsRNases* were found and expressed in different tissues, such as epidermis, fatty body, and intestine, being involved in the innate immune response against invading nucleic acids (LIU et al., 2012). In the aphid *Acyrthosiphon pisum* (Hemiptera: Aphididae), the nucleases present in the hemolymph partially degraded the dsRNAs after 60 min of incubation,

and after 3 hours the dsRNA was completely degraded (CHRISTIAENS and SMAGGHE 2014). Researchers associate this fact with the lack of RNAi responses in this species. In an experiment with *E. heros* saliva, researchers found that the dsRNA was completely degraded after 2 h of incubation, due to the high nuclease activity in the saliva (CASTELLANOS et al. 2019).

In a study with *Cylas puncticollis* (Coleoptera: Brentidae) the authors found that when dsRNA was delivered via oral feeding, gene silencing was lower compared to dsRNA delivered via microinjection (PRENTICE et al. 2016). Thus, the authors notice that the maximum effect of gene silencing in this species is prevented by the action of nucleases present in the intestine. In a study by Lomate e Bonning (2016), in *Nezara viridula* (Hemiptera: Pentatomidae) these authors found that the activity of nucleases (DNase, RNase, and dsRNase) is concentrated in the salivary gland and saliva of the insects and has a small activity in the intestine.

Nucleases activity (dsRNase) can significantly affect the efficiency of gene silencing in the most different species. Understand the activity of nucleases in the target insect is of paramount importance for the successful use of the RNAi.

1.2.3. Cellular uptake of dsRNA molecules

One of the main factors related to the success of RNAi relies on the ability of the insect cells to efficiently uptake the dsRNA from the environment (HUVENNE and SMAGGHE 2010). This process can be mediated by two different pathways: (i) transmembrane channel proteins such as sid-like (systemic interference defective-like) (FEINBERG and HUNTER 2003; ARONSTEIN et al. 2006; KOBAYASHI et al. 2012), or (ii) endocytosis (SALEH et al. 2006; ULVILA et al. 2006; CAPPELLE et al. 2016; PINHEIRO et al. 2018; VÉLEZ and FISHILEVICH 2018), allowing gene silencing in cells/tissues distant from the uptake point (WHANGBO and HUNTER 2008; HUVENNE and SMAGGHE 2010).

In insects, there is evidence to support endocytosis via receptor-mediated clathrin-dependent endocytosis (SALEH et al. 2006; ULVILA et al. 2006; DENECKE et al. 2018). In this system, receptors on the plasma membrane recognize the dsRNA molecule, internalize them through clathrin-coated endocytic vesicles, and dsRNA escapes the endosome, being released in the cytoplasm (SALEH et al. 2006; ULVILA et al. 2006; CAPPELLE et al. 2016; VÉLEZ and FISHILEVICH 2018).

The recognition of the extracellular dsRNA is mediated by two scavenger receptors, SR-CI and Eater, which are responsible for the internalization of the molecules (ULVILA et al., 2006). These receptors together with clathrin-dependent endocytosis are indicated as the ones that play the main roles in dsRNA uptake (CAPPELLE et al. 2016; YOON et al. 2016).

1.2.4 Spreading of the RNAi signal

The spread of the RNAi signal in the organism can be cell-autonomous or non-cell-autonomous (WHANGBO and HUNTER 2008; HUVENNE and SMAGGHE 2010). In cell-autonomous RNAi, gene silencing is observed only in the cells directly exposed to the dsRNA (HUVENNE and SMAGGHE, 2010). On the other hand, in non-cell-autonomous RNAi, gene silencing effects are detected in exposed and non-exposed cells, even in different tissues distant from the initial uptake point (WHANGBO and HUNTER 2008). In this case, dsRNA/siRNA molecules are taken up from the environment by a tissue/cell (environmental RNAi) and spread from one cell to another or from one tissue type to another via systemic RNAi (HUVENNE and SMAGGHE 2010). As discussed previously, in plants, fungi, and the nematode *C. elegans*, RdRp enzyme synthesizes secondary siRNAs producing a systemic spread of the RNAi signaling (ZOTTI et al. 2017).

In insects, the mechanism of systemic RNAi is still unknown. What is known about this process so far is that there is a dsRNA/siRNA spread from cell to cell or tissues (via exosomes), which is highly dependent on the cell's ability to take up the dsRNA or siRNA molecules (VÉLEZ and FISHILEVICH 2018), or mediation from one cell to another through nanotube-like structures (KARLIKOW et al. 2016).

1.2.5. Potential uses of RNAi in agricultural insect pests.

As a control tool

RNAi is a promising tool and the success of this technology in *Diaphorina citri* (Hemiptera: Liviidae) (GALDEANO et al. 2017), *Bemisia tabaci* (Hemiptera: Aleyrodidae) (UPADHYAY et al. 2011), *Nilaparvata lugens* (Hemiptera: Delphacidae) (CHEN et al. 2010), *Halyomorpha hayls* (Hemiptera: Pentatomidae) (GHOSH et al. 2017), among others, is paving the way for the future use of this technology in the field (GULLAN and CRANSTON, et al. 2019). Due to the high specificity of the silencing

mechanism via dsRNA, there is growing interest in the use of this technology in management strategies for a large number of pest insects, either through sprayed insecticides or through modified cultures for expression of silencing genes (CAGLIARI et al. 2018). However, RNAi has different efficiency among different insect groups, developmental stages, or tissues (TERENIUS et al. 2011), and due to its transient characteristic, it is not suitable for some candidate genes.

Currently, the use of RNAi in crop protection is carried out mainly through plant-incorporated protectant (PIP), via plant transformation (transgenics) (ZOTTI et al. 2017). In 2016, the first transgenic cultivar combining *Bt* toxins (*Bacillus thuringiensis*) with RNAi for insect control, was released for cultivation in Canada, and the following year in the United States (US). In general, the delivery of dsRNA in the field is facilitated with the use of transgenic plants (ZHANG et al. 2017a). Currently, 84 events originated from genetically modified (GM) plants using ncRNA approved for the control of pathogens, insect pests, or improvement of specific plant traits (Figure 4) (ISAAA, 2020). Brazil has approved until now, according to ISAAA (2020), two events of GM plants using ncRNA, while the US has 35 and Canada 24 (ISAAA, 2020).

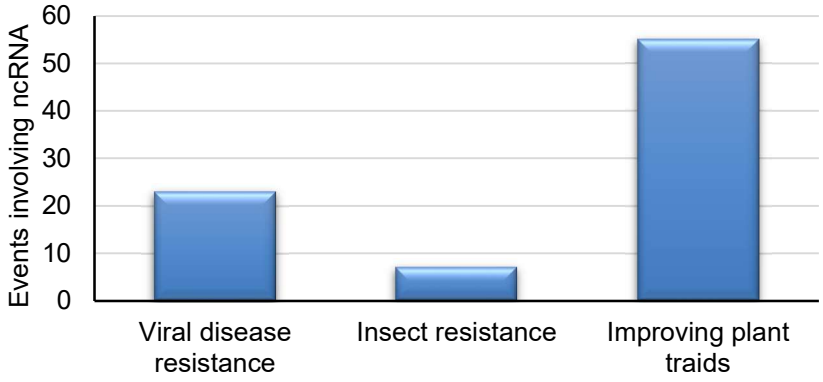


Figure 4 - Genetically modified events based on non-coding RNA (ncRNA) approved worldwide for cultivation. The data was compiled from the database of GM events approved by the International Service for the Acquisition of Agro-Biotechnological Applications (ISAAA, 2020) (http://www.isaaa.org/gmapprovaldatabase/default.asp).

Brazil is far behind regarding the release of genetically engineered (GE) crops involving ncRNAs. Extremely strict legislation and the negative view of society on transgenic plants are factors that affect these numbers and bring up the necessity for alternative strategies. Therefore, the emergence of alternative dsRNA/siRNA delivery strategies, through non-transformative pathways, such as formulations for foliar application (SIGS – Spray-Induced Gene Silencing), providing ways to expand the use of this technology in the field (JOGA et al. 2016; SAN MIGUEL and SCOTT 2016).

Today, the main disadvantage of the non-transformative RNAi strategy is that as the plant grows, new leaves need to be sprayed to ensure protection, while transgenic plants can express dsRNA continuously. This fact implies an increase in cost; however, producers can go for foliar applications of dsRNA targeting specific pest insects, with less aggressive environmental effects than the products currently used, something that consumers are increasingly looking for (Figure 5).

The use of dsRNA carrier particles has been studied and the results are been promising. Researchers from Australia and the United Kingdom used clay nanoparticles as a vehicle for delivering dsRNA to protect plants from attack by viruses (MITTER et al. 2017b). Researchers found that dsRNA remained in plants for up to 30 days after application, protecting plants for up to 20 days after application (Mitter et al. 2017b).

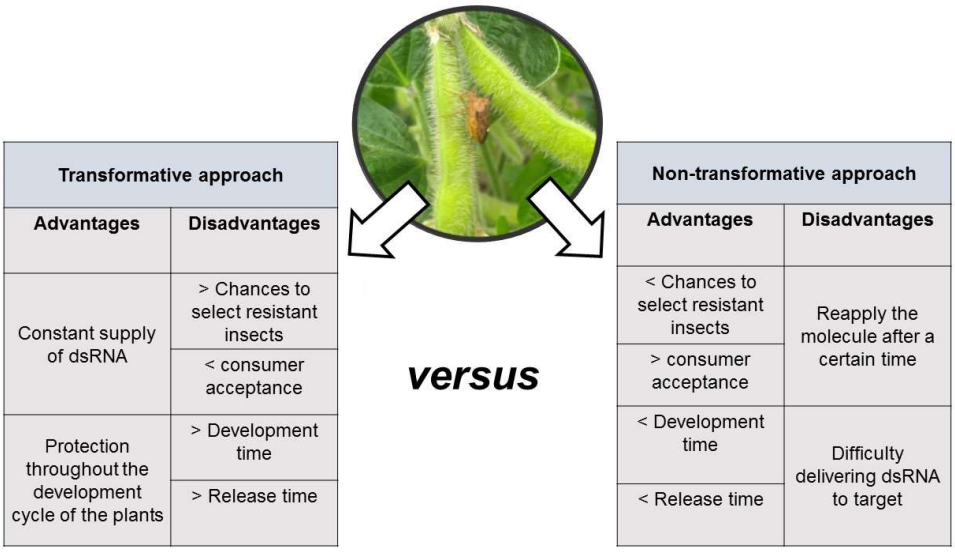


Figure 5 – Transformative versus non-transformative delivery approaches for the control of the stink bug *E. heros*. The principal differences between the two different RNAi delivery approaches are expected to reach the market for growers to control the stink bug *E. heros*. In this case, the non-transformative delivery approach refers to as foliar applications, which is the non-transformative approach expected to be released for the control of *E. heros*.

The application of dsRNA via injection in the trunk also proved to be an efficient dsRNA delivery mechanism for the control of psyllids in citrus plants, with the presence of dsRNA detected for up to 57 days after application (Hunter et al. 2012b). During the last years, there was also a constant reduction in the production price of dsRNA, from about $12,500/gram in 2008, $60/gram in 2019, to approximately $2/gram in 2020 (ZOTTI et al. 2017; DALAKOURAS et al. 2020). The advances in non-transformative dsRNA delivery methods had also brought up the potential uses of this technology.

As a genomic functional tool

Alongside pest control, RNAi and parental RNAi (pRNAi) have been widely used in functional genomic studies, elucidating the role of genes in insect development (HRYCAJ et al. 2010; YATES 2014). Transgenerational or pRNAi is a tool where the gene knockdown effects can be observed in the progeny of the treated parent organism (VÉLEZ et al. 2017). This technique has been explored in a range of different species, including Hemiptera species, such as aphids (COLEMAN et al. 2015), bugs (HUGHES and KAUFMAN 2000; ANGELINI et al. 2005), stink bugs (FISHILEVICH et al. 2016; LU et al. 2017; RIGA et al. 2020), among others. This information can lead to the identification and selection of potential target-genes for the development of new pest management strategies.

The choice of the target gene(s), as well as the relative expression of the gene(s), can significantly affect the efficiency of gene silencing (HONG et al. 2014). The analysis and availability of a database containing the genetic information of different insect species allow the evaluation and identification of potential target genes to be used in pest control through RNAi (FIRMINO et al. 2013; SALVADOR et al. 2014; PERERA et al. 2015).

1.2.6 Environmental risk associate with RNAi technology

Before the release of an RNAi-based GE plant or an RNAi-based product, they need to undergo a risk assessment framework, that will study the potential adverse effects of these products on the non-target organism, including humans, mammals, aquatic and terrestrial organisms as well as the environment itself (ROMEIS et al. 2013; RAMON et al. 2014; CASACUBERTA et al. 2015; ROBERTS et al. 2015; PAPADOPOULOU et al. 2020). The main environmental risk associated with the use of RNAi-based products can be classified into two different classes: (i) sequence-dependent effect, which will affect non-target organisms, being involved in off-target effect; or (ii) sequence-independent, being related to the saturation of the RNAi machinery, activation of the immune system and resistance to RNAi-based molecules.

Sequence-depend effect

Plants that express dsRNA for the control of invertebrate herbivores and formulated products containing dsRNA/siRNA must be highly selective. This can be achieved with the appropriate selection of the target gene(s) and the target

sequence(s) within the target gene(s) (ZOTTI and SMAGGHE 2015; CHRISTIAENS et al. 2018a). Depending on the selected target sequence, a single species can be the target of silencing, or in cases of more conserved sequences, a broader specificity can be achieved (RUNO et al. 2011).

Researchers have shown that dsRNA expressed in GE plants for pest control can achieve a high degree of specificity (DILLIN 2003; WHYARD et al. 2009; PETRICK et al. 2013). The ecological risk assessment of Dv*Snf7* RNA had shown that exposure of invertebrate predators, parasitoids, pollinators, soil biota as well as aquatic and terrestrial vertebrate species to Dv*Snf7* RNA, both directly and indirectly, did not produce any adverse effect (BACHMAN et al. 2016; PETRICK et al. 2016; TAN et al. 2016). The researchers conclude that Dv*Snf7* is safe at the expected field exposure levels. On the other hand, other studies have shown that siRNAs can silence non-target genes (BIRMINGHAM et al. 2006). However, due to the small size of the siRNAs generated, there is the possibility of gene-silencing (sequence-dependent) in non-target organisms (NTOs). Thinking about this, bioinformatics plays an important role in the dsRNA design, in an attempt to predict possible NTO organisms, as well as off-target effects (ZOTTI and SMAGGHE 2015; CHRISTIAENS et al. 2018a). However, there are still considered limitations due to the low amount of genetic data available publicly, limiting the bioinformatic search.

Sequence-independent effect

Saturation of the RNAi machinery

On a sequence-independent base, the saturation of the RNA machinery is also possible (LUNDGREN and DUAN 2013; CHRISTIAENS et al. 2018a). High levels of exogenous dsRNA/siRNAs can saturate the cell's RNAi machinery and thereby reduce its efficiency (LUNDGREN and DUAN 2013). There is a limited number of RISCs present within the organism cells, and if the siRNAs saturate these complexes, the performance of the gene silencing may be compromised (KHAN et al. 2009). Due to this saturation, the RNAi could be temporally inhibited (JACKSON and LINSLEY 2010). However, researchers still don't know if this effect could be reached due to the amounts of dsRNA supplied in the field (CHRISTIAENS et al. 2018a).

Immune stimulation

RNAi is a natural mechanism in antiviral defense in eukaryotic organisms, and the exogenous supply of dsRNA can stimulate the immune system in these organisms (Lundgren and Duan 2013). In mammals, it was found that the injection of small fragments of RNA (< 30 nt) stimulated an immune reaction (ROBBINS et al. 2009). There are some similarities between the innate immune system response of insects and mammals (LUNDGREN and JURAT-FUENTES 2012), but how the immune systems of other organisms will react to a supply of dsRNA/siRNAs is still unknown (LUNDGREN and DUAN 2013).

Resistance to RNAi

Many mechanisms can lead to the development of RNAi-based resistance, especially under high selection pressure. An RNAi-based resistant population of *Diabrotica virgifera virgifera* was first reported in 2018 (KHAJURIA et al. 2018). The insects were continuously exposed to maize plants expressing dsRNA targeting the *Snf7* gene. After several generations, the insects showed impaired dsRNA luminal uptake, which was not only to Dv*Snf7*, showing cross-resistance to all dsRNAs tested.

Another way that insects could develop resistance is through modification in the expression of the main RNAi machinery components. In *D. v. virgifera* when the expression of *DCR-2* and *AGO-2* was artificially reduced, adults were completely protected against an insecticidal dsRNA (VÉLEZ et al. 2016). However, this alteration could impair a long-term fitness cost, affecting the normal development of larvae/adults, but this is still unclear (VÉLEZ et al. 2016; WU et al. 2017).

Environmental RNAi

Insecticidal dsRNA available in the environment is also an important fact that can impair NTO (LUNDGREN and DUAN 2013; ZHANG et al. 2017a; JOAQUIM et al. 2019; BACHMAN et al. 2020). Experiments to evaluate the degradation of naked dsRNA molecule in soil have been conducted, showing that dsRNA is rapidly degraded in soil, within a period time of 15 h to 50 h (DUBELMAN et al. 2014; FISCHER et al. 2016; JOAQUIM et al. 2019; PARKER et al. 2019). In this case, the degradation is independent of the sequence, concentration, structure, or molecular weight (FISCHER et al. 2016). In an aquatic environment, the half-life time of the dsRNA molecules was less than 3h, with no accumulation in the sediments

(ALBRIGHT III et al. 2017; FISCHER et al. 2017). However, when dsRNA architecture is modified to increase the protective effects on plants (time available in the field), there is also an increase in the half-time life of the molecule in the soil (WHITFIELD et al. 2018). Linear and Star Cationic Polymers delayed the degradation of dsRNA by up to 1 week and up to 3 weeks in soil, respectively (WHITFIELD et al. 2018). So, at the same time that this will increase the protective effects of the dsRNA molecules, it will also have more time to impair NTOs. So, this needs to be taken into consideration, when developing a dsRNA molecule.

1.3 Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR

1.3.1 CRISPR/Cas9 mechanism

CRISPR-associated system, also known as CRISPR/Cas system (where Cas stands for CRISPR-associated proteins), is a natural immune system present in bacteria and most archaea (KOONIN and MAKAROVA 2009; KOONIN and MAKAROVA 2013). The CRISPR/Cas9 system was first used as a genome editing tool in 2012, by Jennifer A. Doudna and Emmanuelle Charpentier, generating very specific modifications of the DNA at a genomic level (DOUDNA and CHARPENTIER 2014).

The CRISPR/Cas9 system is formed by three main components: (1) the CRISPR RNA – crRNA, which is a molecule of approximately 21 nucleotides; (2) the trans-activating CRISPR RNA (tracrRNA), and; (3) the Cas 9 enzyme (Figure 6). The dual crRNA:tracrRNA forms the sgRNA (single-guide RNA), which shows two main features: a sequence at the 5’ end that determines the DNA target site and a duplex RNA structure at the 3’ end responsible for Cas9 binding, respectively (DOUDNA and CHARPENTIER 2014). The sgRNA guides the Cas9 endonuclease enzyme to the complementary crRNA sequence in the genome, near the PAM sequence (protospacer adjacent motif). The correct recognition of the DNA target region requires the base pairing of the crRNA sequence and the presence of the PAM sequence near the targeted sequence (GASIUNAS et al. 2012; MARTIN JINEK et al. 2012). If the Cas9 enzyme is derived from *Streptococcus pyogenes,* the PAM sequence is 5′-NGG-3′, and needs to be adjacent to crRNA (HELER et al. 2015).

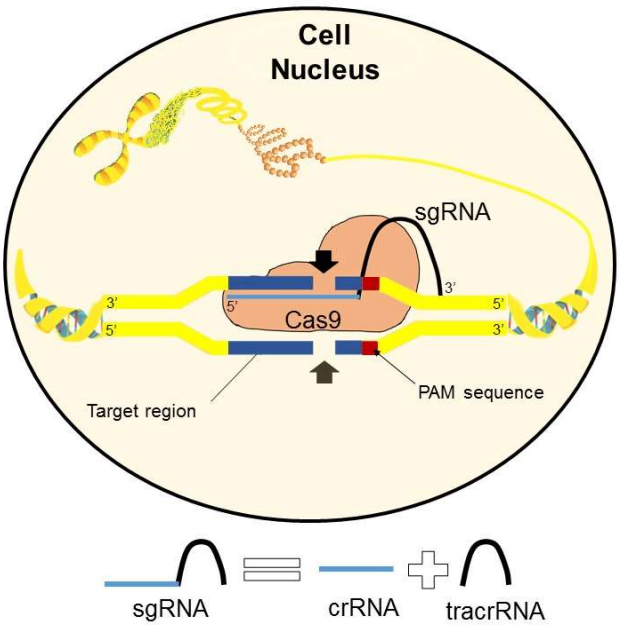


Figure 6 – Gene knockout through the CRISPR/Cas9 system. This system is formed by three main components: (1) a molecule of approximately 21 nucleotides called CRISPR RNA – crRNA; (2) the trans-activating CRISPR RNA (tracrRNA), and; (3) an endonuclease enzyme called Cas9. The combination of crRNA:tracrRNA forms the single-guide RNA (sgRNA), which presents two main features: a sequence at the 5' end that determines the DNA target site and a duplex RNA structure at the 3' end responsible for Cas9 binding, respectively (DOUDNA and CHARPENTIER 2014). The sgRNA guides the Cas9 endonuclease enzyme to the complementary crRNA sequence in the genome, near the protospacer adjacent motif (PAM sequence). The correct recognition of the DNA requires the base pairing of the crRNA sequence and the presence of the PAM sequence near the targeted sequence (GASIUNAS et al. 2012; MARTIN JINEK et al. 2012). Once the systems have found the complementary region, the Cas9 endonuclease cleaves the two DNA strands, generating a double-strand break (DSB) in the target sequence (indicate with the dark arrow) (JINEK et al. 2012; DOUDNA and CHARPENTIER 2014).

Once the systems had found the complementary region, the Cas9 endonuclease cleaves the two DNA strands, generating a double-strand break (DSB) in the target sequence (JINEK et al. 2012; DOUDNA and CHARPENTIER 2014). There are two different approaches that the cell can repair the DSB: (1) error-prone non-homologous end joining (NHEJ) or (2) homology-directed repair (HDR).

The repair by the NHEJ can result in either deletions or insertions known as "indels", or generate nucleotide substitutions, leading to the creation of a mutant version of the target gene (SANDER and JOUNG 2014; SUN et al. 2017; TANING et al. 2017). On the other hand, HDR generates repair based on a donor template, leading to a gene knock-in repair process (SUN et al. 2017; THURTLE-SCHMIDT and LO 2018).

1.3.2 Potential uses of CRISPR in agricultural pests.

Until this moment, mainly RNAi has been used as a tool in the study of gene function. Due to its limitations, this technology may not be suitable for all species and/or target genes selected. On the other hand, with the use of the CRISPR/Cas9 system, it is possible to generate mutant lines by a relatively simple and inexpensive method (TANING et al. 2017). This has been done in a couple of insect species, such as *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) (GUI et al. 2020), *N. lugens* (XUE et al. 2018), *Helicoverpa armigera* (Lepidoptera: Noctuidae) (YE et al. 2017), among others. In these works, researchers were able to study the function of genes, understanding the importance of them in insect development. Nevertheless, both techniques (RNAi and CRISPR) can complement each other in functional gene studies, and the further development of pest management tools.

Also, the CRISPR/Cas9 system can be exploited beyond functional gene studies to generate gene drives, which can result in an insect pest management tool (TANING et al. 2017). Using the gene drive tool in insects' population management, GE individuals containing a DNA cassette are introduced into the wild population, spreading the desired genetic trait into the natural population in a quicker way compared to simple Mendelian genetic inheritance (ESVELT et al. 2014; ALPHEY 2016; COURTIER-ORGOGOZO et al. 2017; RODE et al. 2019; WATSON et al. 2015). Three elements are present in the DNA cassette: (1) a gene coding the Cas9 enzyme; (2) a gene encoding the sgRNA that targets a specific site in the genome; and (3) a flanking sequence which allows the cassette to be inserted in the target site (GANTZ and BIER 2015; GANTZ et al. 2015; HAMMOND et al. 2016).

Briefly, the process of gene drive happens in three different steps: (a) The endonuclease gene cuts the corresponding locus of chromosomes lacking them; (b) the cell repairs the break by copying the DNA cassette onto the damaged chromosome via homologous recombination; (c) and then the process happens again in the second allele of the chromosome (Figure 7) (BURT and KOUFOPANOU 2004). The copying process is referred to as 'homing', while the DNA cassette that is copied is termed as 'gene drive' or just a 'drive' (TANING et al. 2017). Due to the homing process, the fraction of the offspring that inherits the DNA cassette is greater than half, so these genes can spread throughout the population even if there is a reduction in the reproductive fitness in the individuals carrying them (ESVELT et al. 2014). Depending on the characteristic of the gene-drive, it can be classified into three different classes: (i) eradication, (ii) suppression, and (iii) rescue drives (Rode et al.

2019). Briefly, eradication and suppression drives are designed to eliminate or decrease the size of an insect population, relying on the introduction of strong or mild deleterious mutations, respectively (RODE et al. 2019). On the other hand, using rescue drives we could introduce beneficial mutations or remove deleterious ones to save an endangered population (ESVELT et al. 2014). In agriculture, this approach could be used, for example, to make honey bees and other important pollinators less susceptible to insecticides, or even, reintroduce susceptibility in a resistant population of insects (Resistant *Spodoptera frugiperda* for example).

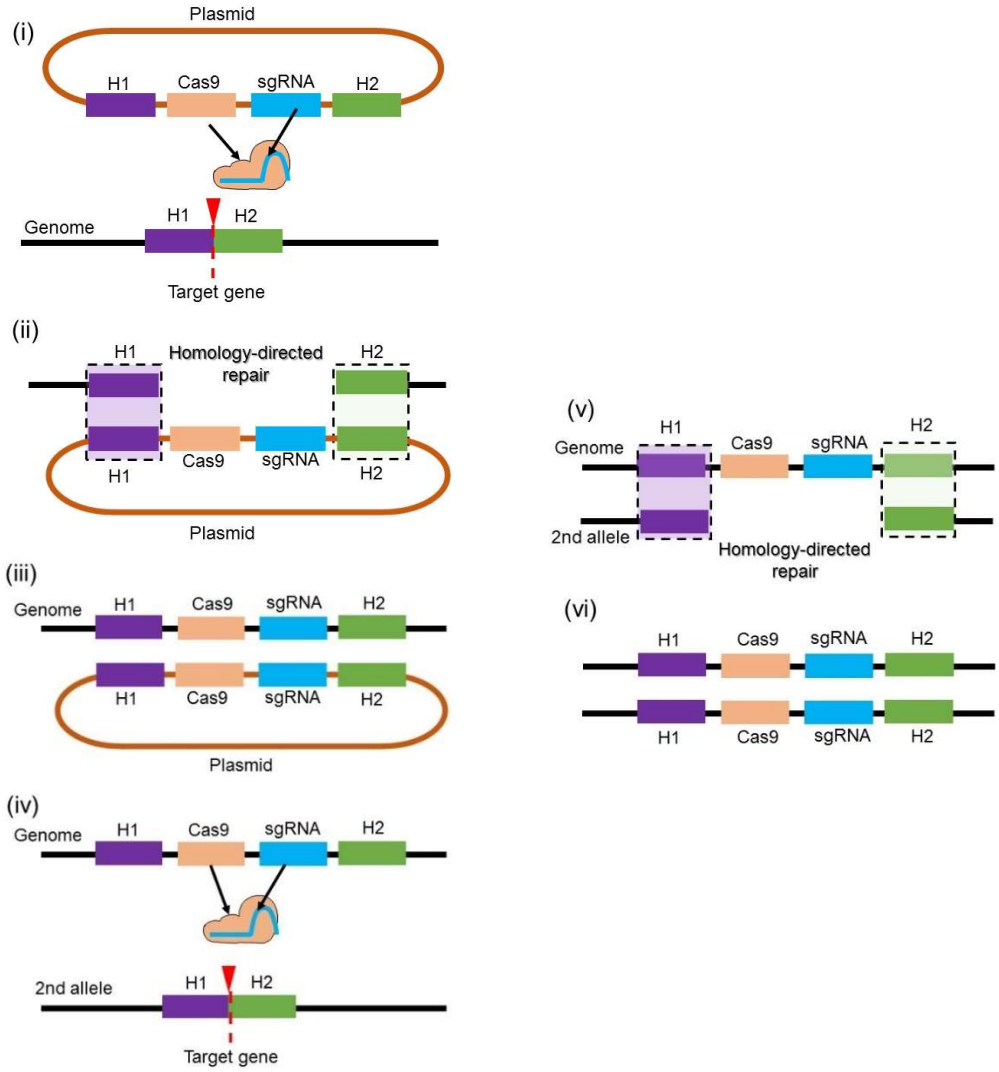


Figure 7 – Schematic representation of the CRISPR/Cas9-based gene drive. (i) A plasmid expressing the DNA cassette (Flanking regions, Cas9, and gRNA), in which the sgRNA directs the Cas9 enzyme to cleave the DNA at the specified target site, generating a double-strand break (DSB). (ii) The DNA cassette is then integrated into the target locus via homologous-directed repair (HDR). This process is first done in one of the chromosome alleles, resulting in (iii) heterozygosity. (iv) The allele expresses the Cas9 and sgRNA, which target the remaining wild-type allele, cleaving the DNA, and (v) via HDR-mediated, the information is copied into the wild-type locus. (vi) Homozygosity is observed for the drive allele, and both alleles show the mutation now. Adapted from DRURY et al. 2017; GANTZ and BIER 2015.

1.3.3 Environmental risk associate with CRISPR/Cas9 technology

Although other tools such as transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs) have been used for a long time to edit insect genomes (MA et al. 2012; WATANABE et al. 2012; SAJWAN et al. 2013), the applications of CRISPR/Cas9 have undergone rapid development over the recent years (COURTIER-ORGOGOZO et al. 2017; TANING et al. 2017). However, even

being considered as one of the most precise and promising gene-editing tools, there are still issues (biosafety and biosecurity) related to the use of this technology.

Biosafety

Off-target effects

These have been a constant problem in CRISPR genetic engineering, being considered one of the most frequently encountered barriers when scientists try to develop a genetically engineered population. One of the main reasons is still the lack of genetic information for many important pest insects, which creates great difficulties in the development of precise sgRNAs molecules. The off-target issues arise from the sgRNA binding to undesired places (FU et al. 2013; SANDER and JOUNG 2014; SCHAEFER et al. 2017), generally in regions with a certain degree of similarity to the target gene of interest (FERREIRA et al. 2017). Furthermore, it is also very important to take into consideration if there is any risk of gene flow between the target species and other species, and so, the adverse trait will also be transferred into the non-target organisms (TANING et al. 2017).

Researchers have been working to optimize the CRISPR/Cas9 tool, such as optimization of the Cas9 enzyme, the gRNA which is complexes itself (and will demand genomic information), and the target DNA sequence for the Cas9 enzyme, among others (BRADDICK and RAMAROHETRA 2020).

Genomic rearrangements and mosaicism

Aside from the direct mutagenesis due to the creation of DSBs, these DSBs may also trigger DNA repair mechanisms that can promote troubling genomic rearrangements (BRUNET et al. 2009). These rearrangements can include deletions, inversions, and translocations, underling biosafety concerns (BRADDICK and RAMAROHETRA 2020). Together with genomic rearrangements, mosaicism plays also an important role in CRISPR/Cas9 bioassays. Although the main reasons for mosaicism are still unknown, it is speculated that the reason may involve the segregation of Cas9 between dividing cells, and/or its continued activity after that could result in a dividing cell passing the active CRISPR/Cas9 to one “daughter cell” but not to all cells resulting from the division (BRADDICK and RAMAROHETRA 2020). This process could hide the effects of gene editing.

Biosecurity

Biosecurity is the field that englobes the potential issues that are posed by the use of biotechnology (BRADDICK and RAMAROHETRA 2020). The main concern related to the use of CRISPR/Cas9 in insects regards the generation and release of CRISPR-edited insects carrying drives that could change entire populations or even the ecosystems (BEUMER et al. 2013; ESVELT et al. 2014; CHAMPER et al. 2016).

In an attempt to avoid unintended ecological consequences, the release of CRISPR-edited insects carrying drives must undergo rigorous pre-release risk assessment of non-target effects (TANING et al. 2017). To avoid this kind of issue, some characteristics must be considered: (1) they must be precise; (2) they need to understand the ecosystem-wide implications; and (3) they also need to be aware and anticipate possible unintended consequences (TANING et al. 2017).

2. Manuscript 1.

First transcriptome of the Neotropical pest *Euschistus heros* (Hemiptera: Pentatomidae) with dissection of its siRNA machinery.*

Deise Cagliari

Naymã Pinto Dias

Ericmar Ávila dos Santos

Leticia Neutzling Rickes

Frederico Schmitt Kremer

Juliano Ricardo Farias

Giuvan Lenz

Diogo Manzano Galdeano

Flávio Roberto Mello Garcia

Guy Smagghe

Moisés João Zotti

*Manuscript published in Scientific Report

First transcriptome of the Neotropical pest *Euschistus heros* (Hemiptera: Pentatomidae) with dissection of its siRNA machinery

Deise Cagliari[1*]; Naymã Pinto Dias[1]; Ericmar Ávila dos Santos[1]; Leticia Neutzling Rickes[1]; Frederico Schmitt Kremer[2]; Juliano Ricardo Farias[3]; Giuvan Lenz[4]; Diogo Manzano Galdeano[5]; Flávio Roberto Mello Garcia[6]; Guy Smagghe[7*]; Moisés João Zotti[1*]

[1] Department of Crop Protection, Molecular Entomology, Federal University of Pelotas, Pelotas, Brazil.

[2] Center for Technological Development, Bioinformatics and Proteomics Laboratory, Federal University of Pelotas, Pelotas, Brazil.

[3] Department of Crop Protection, Universidade Regional Integrada do Alto Uruguai, Santo Ângelo, Brazil.

[4] Agricultural Research and Development Center, UPL, Pereiras, Brazil

[5]Sylvio Moreira Citrus Center, Agronomic Institute of Campinas, Cordeirópolis, São Paulo, Brazil.

[6]Department of Crop Protection, Insect Ecology Laboratory, Federal University of Pelotas, Pelotas, Brazil.

[7]Department of Plants and Crops, Ghent University, Ghent, Belgium.

*Corresponding authors:

Deise Cagliari, Moisés João Zotti, Guy Smagghe

deisycagliari@yahoo.com.br; moises.zotti@ufpel.edu.br; guy.smagghe@ugent.be

+55 55 9 9162-2651; +55 55 9 9671-2207; +32 9 2646150

Abstract

Over the past few years, the use of RNA interference (RNAi) for insect pest management has attracted considerable interest in academia and industry as a pest-specific and environment-friendly strategy for pest control. For the success of this technique, the presence of core RNAi genes and a functional silencing machinery is essential. Therefore, the aim of this study was to test whether the Neotropical brown stinkbug *Euschistus heros* has the main RNAi core genes and whether the supply of dsRNA could generate an efficient gene silencing response. To do this, total mRNA of all developmental stages was sequenced on an Illumina platform, followed by a *de novo* assembly, gene annotation and RNAi-related gene identification. Once RNAi-related genes were identified, nuclease activities in hemolymph were investigated through an *ex vivo* assay. To test the functionality of the siRNA machinery, *E. heros* adults were microinjected with ~28 ng per mg of insect of a dsRNA targeting the *V-ATPase-A* gene. Mortality, relative transcript levels of *V-ATPase-A*, and the expression of the genes involved in the siRNA machinery, *Dicer-2* (*DCR-2*) and *Argonaute 2* (*AGO-2*), were analyzed. Transcriptome sequencing generated more than 126 million sequenced reads, and these were annotated in approximately 80,000 contigs. The search of RNAi-related genes resulted in 47 genes involved in the three major RNAi pathways, with the absence of *sid-like* homologous. Although *ex vivo* incubation of dsRNA in *E. heros* hemolymph showed rapid degradation, there was 35% mortality at 4 days after treatment and a significant reduction in *V-ATPase-A* gene expression. These results indicated that although s*id-like* genes are lacking, the dsRNA uptake mechanism was very efficient. Also, 2-fold and 4-fold overexpression of *DCR-2* and *AGO-2*, respectively, after dsRNA supply indicated the activation of the siRNA machinery. Consequently, *E. heros* has proven to be sensitive to RNAi upon injection of dsRNA into its hemocoel. We believe that this finding together with a publically available transcriptome and the validation of a responsive RNAi machinery provide a starting point for future field applications against one of the most important soybean pests in South America.

Keywords: Brown stink bug, gene silencing, RNA interference, soybean.

Introduction

The Neotropical brown stink bug (BS), *Euschistus heros* (Hemiptera: Pentatomidae), is one of the most important Pentatomidae pests in South America[1], especially in soybean (*Glycine max*) with a reduction in seed quality and yield[2]. Stink bugs use their piercing/sucking mouthparts to inject enzymes into the plant tissues to digest plant components and remove pre-digested fluids[3]. Although rarely reported

before the 70s[2,4], since then population outbreaks[2,5] and rapid population growth have allowed expansion of the range of *E. heros* to all the major South American soybean production regions, including Brazil[2], Paraguay[2], and Argentina[6].

The current recommendations for the management of this insect rely on the use of broad-spectrum insecticides such as organophosphates and pyrethroids (AGROFIT, http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons). However, these are detrimental to the environment and some are harmful to beneficial organisms. Furthermore, the high infestation of *E. heros* has frequently been reported and the lack of a sustainable alternative for pest control has led growers frequently to spray insecticides from the same chemical group, contributing to the selection of resistant strains[7–9]. Moreover, due to favorable weather conditions found in Brazil, Argentina and Paraguay, multiple generations occur during a crop season, making the control even more difficult. Therefore, effective and environmental-friendly multiple control strategies are needed to reduce the use of highly toxic pesticides and to delay resistance development in *E. heros*.

RNA interference (RNAi), also known as Post-Transcriptional Gene Silencing (PTGS), is a natural mechanism of gene regulation and a defense system against viruses in eukaryotic cells [10,11], and since the milestone work done by Baum et al.[12], RNAi towards insect management has significantly attracted interest as an alternative control strategy to synthetic insecticides. In 2017, genetically modified maize using RNAi-based technology against *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae), an important pest in the United States of America (USA), has been approved by the Environmental Protection Agency (EPA) in the USA[13]. Besides the use of RNAi in plants, RNA-based spray insecticides, focusing on non-transformative approaches, are expected to be introduced into the market soon[14], with significant advances in the use of SIGS (Spray-Induced Gene Silencing)[15,16].

RNAi triggers gene silencing through non-coding RNAs (ncRNAs), such as micro RNAs (miRNAs) and small interfering RNA (siRNA), originally generated from double-stranded RNA (dsRNA)[17], and Piwi-interacting RNA (piRNA)[18]. The success of the RNAi relies on the ability of the insect cells to efficiently uptake the dsRNA from the environment[19] and activate the silencing machinery. The process of dsRNA uptake can be mediated by transmembrane channel proteins such as sid-like (systemic interference defective-like)[20–22], or endocytosis[23–27], allowing gene silencing in cells/tissues distant from the uptake point [19,28]. Once inside cells, dsRNAs are processed into siRNA fragments, with ~20 base pairs (bp), by the ribonuclease III enzyme Dicer 2 (DCR-2)[29]. These siRNAs are incorporated into the RISC

(RNA-Induced Silencing Complex), which contains the Argonaute 2 (AGO-2) protein[30] allowing the specific breakdown of messenger RNA (mRNA) and so preventing the protein formation[19].

Transcriptome analysis focusing on RNAi as a control strategy has been reported in insects mainly for Coleoptera[31–33], Lepidoptera[34] and Hemiptera[35]. According to some studies, RNAi is less efficient in Hemiptera[36,37] when compared to Coleoptera because of the presence of double-stranded ribonucleases (*dsRNases*)[38–40]. In the pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphidoidea), the lack of RNAi response was associated with the high nuclease activity in hemolymph[41]. However, the brown marmorated stink bug, *Halyomorpha halys* (Heteroptera: Pentatomidae), has lower nuclease activities and gene silencing can reach up to 70% when compared to *Heliothis virescens* (Lepidoptera: Noctuidae)[42]. Successful use of RNAi through oral delivery has been reported in other hemipteran species such as *Diaphorina citri* (Hemiptera: Lividae)[43,44], *Bemisia tabaci* (Hemiptera: Aleyrodidae)[45], and *Nilaparvata lugens* (Homoptera: Delphacidae)[46], suggesting that RNAi could be further investigated towards a control strategy in *E. heros.*

Transcriptome analysis allows researchers to understand the RNAi mechanism and its main components as well as helping in the selection of target genes, essential genes involved in biological processes and housekeeping genes. Therefore, the main goal of our work was to provide a transcriptome dataset for *E. heros*, characterize the genes involved in the RNAi pathways, and validate the RNAi machinery through a gene silencing assay. In brief, the RNAi core genes were identified, and the efficiency of the siRNA machinery was tested through injection of dsRNA followed by quantitative real-time PCR. Next, considering the importance of dsRNA degradation by nucleases, an *ex vivo* assay was performed with collected hemolymph. Finally, dsRNAs were designed to target *V-ATPase subunit A* gene, resulting in mortality after microinjection. To test the activation of the siRNA machinery, an upregulation of *DCR-2* and *AGO-2* was also investigated. Overall, these data will provide for the first time the dissection of siRNA pathway in *E. heros* and with an efficient dsRNA cellular uptake system, resulting in significant insect mortality. These data could then be further explored to develop a pest control strategy using RNAi.

Results

Analysis of *E. heros* transcriptome

RNA sequencing resulted in a total of 126,455,838 reads of 101 bp long, corresponding to an accumulated length of 12,772,039,638 bp. *De novo* assembling using Trinity software resulted in 147,612

transcripts, assembled into 83,114 contigs with an average length of 1,000 bp and an average GC content of 37.12%.

Based on Diamond analysis, a total of 60,956 hits was produced, representing 41.30% of the total transcripts (Figure 1-A). Out of the sequences, 60,227 hits (98.8%) were from Eukaryotes, with 84.64% of the contigs similar to sequences from Hemiptera species: 20.16% to the *Lygus hesperus* (Hemiptera: Miridae), 17.57% to *Triatoma infestans* (Hemiptera: Reduviidae), 11.69% to *Rhodnius prolixus* (Hemiptera: Reduviidae), 7.03% to *Riptortus pedestris* (Hemiptera: Alydidae), 6.55% to *Panstrongylus megistus* (Hemiptera: Reduviidae), 4.69% to *Triatoma dimidiate* (Hemiptera: Reduviidae), 4.43% to *A. pisum*, 4.26% to *Rhodnius neglectus* (Hemiptera: Reduviidae), 3.15% to *Clastoptera arizonana* (Hemiptera: Clastopteridae), 1.88% to *Graphocephala atropunctata* (Hemiptera: Cicadellidae), 1.73% to *Cuerna arida* (Hemiptera: Cicadellidae), 1.50% to *Homalodisca liturata* (Hemiptera: Cicadellidae). The reminding 15.36% belonged to *Zootermopsis nevadensis* (Isoptera: Archotermopsidae) (1.00%), *Lasius niger* (Hymenoptera: Formicidae) (0.87%), *D. citri* (0.83%), *Tribolium castaneum* (Coleoptera: Tenebrionidae) (0.78%) and *Anoplophora glabripennis* (Coleoptera: Cerambycidae) (0.54%), and other hits (11.34%) (Figure 1-B, Supplementary Table S1). The raw reads have been deposited in the sequence reads archive (SRA) at NCBI, and can be accessed using SRP159293 accession number.

A total of 143,806 predicted GO terms was obtained and grouped into three categories: cellular components, biological processes, and molecular functions. Membrane was the most dominant GO term within the cellular component (28,631; 29.3%), for the biological processes it was RNA-dependent DNA biosynthesis process (46,238; 10.4%), and for the molecular function was nucleic acid binding (68,937; 8.9%) (Figure 2-A-C).

Identification of RNAi-related genes

The result of the *E. heros* transcriptome search for RNAi-related genes revealed the presence of 47 genes associated with dsRNA uptake, RNAi core machinery, auxiliary RISC factors, nucleases, antiviral RNAi, and intracellular transport. Some RNAi-related proteins presented variants, with the presence or absence of conserved domains. Overall, the sequences of *H. halys* showed the highest similarity to sequences from *E. heros*.

dsRNA uptake. The protein sequences involved in dsRNA uptake were searched in the *E. heros* transcriptome, and a total of six proteins related to this process were found, although there was an absence

of *sid-like* genes (Table 1, Supplementary data S1 online). Scavenger protein was found with a CD36 domain region, Ubiquitin-protein transferase (FBX011) with an F-box conserved domain and three beta-helices, and Epsin 2 with an Epsin N-terminal homology (ENTH) domain. The Clathrin heavy chain (Chc) protein and Gap Junction Protein with an Innexin conserved domain were also found in the *E. heros* transcriptome.

Core RNAi machinery. Proteins related to the miRNA, siRNA and piRNA pathways were identified in the *E. heros* transcriptome (Table 2, Supplementary data S2 online).

The DCR-1 protein was found in *E. heros* with the conserved PAZ (Piwi, Argonaute and Zwille) domain, two RNaseIII domains and a Double-stranded RNA-binding domain (DSRBD), with an absence of the helicase domains. DCR-2 was also found in *E. heros* with two isoforms as following: 1 and 2 with 646,601 and 0.618 transcripts per million (TPM), respectively. The DCR-2 isoform 1 contained all the conserved domains: one helicase domain, one PAZ domain, two RNaseIII domains, and a DSRBD, while DCR-2 isoform 2 was found with two RNaseIII domains and a Ribonuclease III C terminal domain (RIBOc). Dicer 3 protein was not found in the *E. heros* transcriptome. Drosha protein was found with two RNaseIII domains and a RIBOc, but with the absence of PAZ, and an amino-terminal DExH-box helicase domain. The dsRNA-binding proteins Pasha, Loquacious and R2D2 were also identified in *E. heros* with conserved domains (DSRBDs). Argonaute superfamily proteins were also searched in the *E. heros* transcriptome and five members of the Argonaute superfamily proteins were identified: AGO-1, AGO-2, AGO-3, Aubergine (Aub) and Piwi (Table 2, Supplementary data S2 online). Four variants of the AGO-1 protein were found: isoforms 1, 3, 4 and 5, presenting 0.585, 0.103, 0.4 and 118,437 TPM, respectively. All AGO-1 isoforms were found with the PAZ and PIWI conserved domains. For the AGO-2 protein, two isoforms were found, isoform 1 and 2, with 146,222 and 0.14 TPM, respectively. AGO-2 isoform 1 was found with PAZ and PIWI conserved domains, while AGO-2 isoform 2 had no PAZ domains. AGO-3, Aub and Piwi proteins presented the PAZ and PIWI conserved domains. Zucchini (Zuc), with a nuclease conserved domain, was also found in the *E. heros* transcriptome.

Phylogenetic analyses revealed distinct groups for DCR and AGO superfamily proteins (Supplementary Fig. S1-S2 online). The protein DCR-1 from *E. heros* was grouped in a clade with the DCR-1 proteins from *Nezara viridula* (Hemiptera: Pentatomidae) and *H. halys,* and the same results were found for *E. heros* DCR-2 (Supplementary Fig. S1). Also, *E. heros* DCR-1 was grouped in a distinct clade compared to *E. heros* DCR-2, but it showed a common ancestor. The phylogenetic analysis of the AGO

superfamily resulted in two main clades, one contained the AGO subfamily proteins, AGO-1 and AGO-2, while the other had the PIWI subfamily proteins, AGO-3, Aub and Piwi (Supplementary Fig. S2). *E. heros* AGO-1 was clustered with AGO-1 from *N. viridula* and *N. lugens*, while *E. heros* AGO-2 was clustered in a second group together with other proteins of this family. *E. heros* AGO-3 was clustered in a distinct group as well as Aub and Piwi proteins.

Auxiliary RISC factors. The *E. heros* transcriptome was searched for RNAi auxiliary factors (Table 3, Supplementary data S3 online). The research resulted in 17 intracellular factors associated with the RISC. The Tudor-SN (TSN) protein sequence, with a Tudor-conserved domain, and the Translin and Translin-associated factor-X (TRAX), conserved subunits of the component 3 promoter of the RISC (C3PO), were identified in *E. heros*. The Armitage (Armi), spindle-E (Spn-E), Maelstrom, Gawky, Staufen (STAU) and CLIP-associating protein (Clp-1) were also present in the *E. heros* transcriptome with all conserved domains. HEN-1 nuclease was also present, but no conserved domain was found (DSRBD, FK506 binding protein-like domain or methyltransferase domain). Other auxiliary RISC factors identified in *E. heros* were the Elongator complex protein 1 (Elp-1), Vasa intronic gene (VIG), DEAD-box RNA helicases, PRP16 with a DExD conserved domain, Belle with the conserved DEAD-box domain, Glucose dehydrogenase (GLD-1) and Cytoplasmic aconitate hydratase (ACO-1).

Nucleases. Exoribonuclease 1 (Eri-1) and DNA/RNA non-specific endonuclease (dsRNase) proteins were found in the *E. heros* transcriptome (Table 4, Supplementary data S4 online). Eri-1 was found with a 5'-3' exonuclease N-terminus domain (XRN_N). The dsRNase protein was found with seven isoforms, 1, 3, 4, 6, 7, 9 and 10 with 0.17, 0.30, 0.46, 702,558, 719,814, 292,033 and 280,771 TMP, respectively. The isoforms presented a DNA/RNA non-specific endonuclease (Endonuclease_NS) conserved domain, except the isoform 3, which did not show any conserved domain. Small RNA degrading nuclease 1 (SDN1-like) and Nibbler were found with the 3'-5' exonuclease conserved domain (Table 4, Supplementary data S4 online). The phylogenetic analyses revealed distinct clades among nuclease proteins, being the Eri-1, dsRNases, SDN1 and Nibbler grouped in clades together with these proteins from other insect species (Supplementary Fig. S3 online)

Antiviral RNAi. The search for proteins related to the antiviral RNAi resulted in four protein sequences: Ars2, ninaC, a seven transmembrane-domain glycosyltransferase, Egghead (egh)[47], and the CG4572 protein (Table 4, Supplementary data S5 online). The phylogenetic analyses revealed distinct clusters for the four antiviral RNAi proteins (Supplementary Fig. S4 online).

200 *Intracellular transport.* Three sequences related to intracellular transport were found: *Vacuolar*
201 *H+ ATPase subunit A* (*vha68*), *Vacuolar H+ ATPase subunit C* (*vha16*) and the *Small Rab GTPases* (Table
202 4, Supplementary data S6 online).
203
204 ***Ex vivo* dsRNA hemolymph degradation**
205 The dsRNA stability in the hemolymph was assessed at 0, 1, 10, 30, 60 and 120 min of incubation.
206 After 10 min of incubation, the dsRNA-*V-ATP-A* was partially degraded, as the gel showed a smear below
207 the band, clearly demonstrating dsRNA degradation (Supplementary Fig. S5 online). At 30 and 60 min of
208 incubation we observed increased degradation, with all dsRNA degraded after 120 min incubation.
209
210 **Mortality of *E. heros* by dsRNA microinjection**
211 Mortality was assessed at 24, 48, 72 and 96 h post-microinjection of dsRNA-*V-ATP-A* (Figure 3).
212 At 24 h, there was 7% mortality and this increased to 19% at 48 h, 28% at 72 h, and at 96 h 35% of the
213 treated insects were killed. Alongside the mortality in dsRNA-*V-ATP-A* treated *E. heros*, reduced mobility
214 was observed compared to the insects microinjected with dsRNA-*GFP*, which were very active. These
215 mobility effects lasted until 72 h, with a recovery in the mobility at 96 h post microinjection.
216
217 **Gene expression of *V-ATPase-A*, *DCR-2* and *AGO-2* in *E. heros***
218 The *V-ATPase-A* transcripts level gradually decreased following dsRNA treatment over time (14%
219 to 74% from 24 h to 48 h, respectively) (Figure 4). At 72 h and 96 h, there was an increase in the relative
220 transcript levels, with ~40% reduction in gene expression, but despite this, these values were still
221 significantly lower than the control (dsRNA-*GFP*-microinjected) insects (p-values <0.001 and 0.014,
222 respectively) (Figure 4).
223 The involvement of the siRNA machinery in the gene silencing mechanism was assessed through
224 a qRT-PCR analysis of *DCR-2* and *AGO-2* genes (Figure 5). The relative transcript levels of *DCR-2* were
225 significantly higher in the insects microinjected with dsRNA-*V-ATP-A* compared to the controls (not
226 exposed to dsRNA), with the highest *DCR-2* expression level observed at 72 h post microinjection and with
227 an increase of ~2.0-fold (Figure 5-A). At 96 h, relative transcript levels of *DCR-2* dropped to ~1.5-fold,
228 still higher than the controls. The expression pattern of *AGO-2* behaved similarly as we saw for *DCR-2*
229 (Figure 5-B). At 48 and 72 h post-microinjection, the relative transcript levels of *AGO-2* were higher with

almost a 4.0-fold increase compared to the control samples.

Discussion

The Neotropical stinkbug *E. heros* is one of the most important soybean pests in Brazil, Argentina, and Paraguay, and the current lack of genetic information is among the factors limiting the prospects of RNAi as an alternative control approach.

The RNAi pathway works primarily through dsRNA uptake, intracellular dsRNA transport, dsRNA processing to sRNA, RISC complex formation and binding, and digestion/repression of the target mRNA[48]. Based on our currently reported *E. heros* transcriptome database, most of the genes involved in these processes above and related to RNAi pathways, are also present in the *E. heros* transcriptome (Table 1-4). However, it is important to note that although these genes are involved in the RNAi process in other organisms, it does not mean that they play the same role in the RNAi mechanism in *E. heros,* and the real involvement of these genes needs to be further confirmed in future functional assays.

To achieve gene silencing through RNAi, dsRNA is taken up by the tissue/cell. In eukaryote organisms, this process occurs through sid-like transmembrane proteins[25,49] or endocytosis-mediated uptake[24,25]. Before Sid-like homologous proteins have been found in Coleoptera, Hymenoptera, Lepidoptera, and Hemiptera, but not in Diptera[49]. Also in the *E. heros* transcriptome, *sid-like* homolog genes were not found. In *Drosophila* (Diptera: Drosophilidae), with the lack of *sid-like* homolog genes, the dsRNA uptake occurs via endocytosis-involving scavenger receptors[24,50]. Indeed previous work demonstrated that the Scavenger protein is involved in endocytic dsRNA uptake in insects[24,50,51] and other organisms, such as mites[19,52,53]. The Chc protein, which is related to an alternative mechanism for endocytic dsRNA uptake in insects[24–26,50], was found in the *E. heros* transcriptome. Consequently, with the absence of *sid-like* genes in the *E. heros* transcriptome, we believe that the Chc protein may be involved in cellular uptake in *E. heros*; however the involvement of this protein in dsRNA uptake needs to be proven in future functional assays. In addition, future experiments need to investigate the importance of endocytosis in *E. heros*.

Core RNAi machinery genes were also searched for in the *E. heros* transcriptome with focus on the miRNA, siRNA and piRNA pathways (Table 2), and most of these were present with the absence of a *RNA-dependent RNA polymerase* (*RdRP*) gene. The lack of *RdRP* was generally expected because, so far, it has been reported only in ticks, plants and in the nematode *Caenorhabditis elegans* (Rhabditida:

Rhabditidae)[54]. The main core domains of Dicer are well known due to their involvement in dsRNA cleavage into small RNA molecules (sRNAs), including miRNAs and siRNAs. In the current work, the DCR-1 protein, which is related to the miRNA pathway, contains a PAZ domain, two RNaseIII domains, and a DSRBD, however no conserved domains for helicase were identified. For DCR-2, two isoforms with distinct structures and abundances were identified. The DCR-2 isoform 1 was the most abundant and showed a helicase domain, a PAZ domain, two RNaseIII domains, and a DSRBD[55,56]. The PAZ domain holds a binding pocket for the 3' overhang of dsRNA substrate and a phosphate-binding pocket that recognizes the phosphorylated 5' end of small RNAs[57,58]. The two RNaseIII domains are the catalytic core components of Dicer and responsible for the cleavage of the dsRNA substrate[59]. The function of the helicase domain remains unclear, but so far it is known that this domain is required to process siRNA but not miRNA[57]. In flies, the loss in the functionality of DEAD/Helicase domain is related to a particular function in the miRNA-based gene regulation[57]. We hypothesize here that the loss of the helicase domain in the DCR-1 protein in *E. heros* may be a functional adaptation, related to the miRNA pathway, but this needs to be further investigated. The canonical conserved domains of DCR-2 isoform were not identified in *E. heros*. Similarly, DCR-2 isoforms with the lack of conserved domains were also identified in mammals[60,61] as well as in *Arabidopsis thaliana*[62]. Due to the lack of important functional domains, it is expected that these DCR-2 variants may not be involved in the siRNA pathway, however the function of these isoforms in insects still remains unclear. To our knowledge, this is the first report of DCR-2 variants in insects. It would be interesting in the future to investigate the role of DCR variants in cellular processes.

In other insects such as *Cylas puncticollis* (Coleoptera: Curculionidae), *N. lugens, D. v. virgifera, L. decemlineata*, *Drosophila* and *Tribolium*, *DCR-1* and *DCR-2* are also present[31,48,63,64]. In *Drosophila* the involvement of *DCR-1* and *DCR-2* is well established in the miRNA and siRNA pathways[63]. In the piRNA pathway, there is no evidence of a dsRNA precursor and the need of DCR endonucleases[65–67]. Drosha protein was identified with two RNaseIII domains plus a RIBOc[55] and with some similar features to Dicer, although it processes miRNA precursors in the nucleus[17]. The dsRNA-binding proteins Pasha, Loquacious and R2D2, which mediate dsRNA binding to the RISC complex, are among the other proteins from the DCR superfamily identified in *E. heros*. These proteins are cofactors required to interact with the RNaseIII genes Drosha, DCR-1, and DCR-2, respectively[31,63] (Table 2).

Five members belonging to the Argonaute superfamily were identified in the *E. heros* transcriptome as follows: AGO-1 and AGO-2 which belong to Argonaute subfamily, and AGO-3,

Aubergine (Aub) and Piwi, which belong to the PIWI subfamily[68,69]. AGO-1 is an essential protein related to the miRNA pathway, and AGO-2 is related to the siRNA pathway[69,70]. More recently, two new functions have been attributed to AGO-1 and AGO-2 in early *Drosophila melanogaster* embryos: the generation of polarity within cells and tissues by modulating an important cell-cell signaling pathway[71]. These proteins are characterized by the presence of PAZ and PIWI domains, which guide sRNA recognition and binding, supporting endonucleolytic cleavage[72]. The PAZ domain forms a pocket for siRNAs binding and, specifically, the characteristic two nucleotides (nt) 3′ overhangs, trimmed by Dicer proteins, while the PIWI domain shares structural similarities with ribonucleases and degrades the corresponding RNAs[73–75]. The lack of a PAZ functional domain in the AGO-2 isoform 2 raises the hypothesis that this isoform may be related in another biological process, as mentioned above for the DCR-2 isoforms. In the shrimp *Marsupenaeus japonicus* (Decapoda: Penaeidae), three AGO-1 isoforms have been identified, and interestingly, two isoforms were more expressed in the lymphoid organ, suggesting a role in immunity[76]. The presence of multiple isoforms of AGO-1 and AGO-2 may indicate a role of AGO in many biological processes, including cell proliferation/differentiation, immune defense, among others[76]. AGO-3, Aub, and Piwi are proteins related to the piRNAs pathway[66,69] and they were also found in *E. heros* (Table 2). Zucchini (Zuc), responsible for piRNA maturation[77] and related to the germline RNAi processes[78], was also identified in the *E. heros* transcriptome.

The identification of both DCR-1 and DCR-2 was confirmed through a phylogenetic analysis using sequences from other insects and revealed distinct groups inside DCR proteins (Supplementary Fig. S1 online). The *E. heros* protein DCR-1 was grouped in a clade with DCR-1 proteins from *N. viridula* and *H. halys*, showing a common ancestor. The same results were found for *E. heros* DCR-2. Also, *E. heros* DCR-1 was grouped in a distinct clade compared to *E. heros* DCR-2, but with a common ancestor. The phylogenetic analysis for the AGO superfamily resulted in two main clades; one containing the AGO subfamily proteins AGO-1 and AGO-2, and another with the PIWI subfamily proteins AGO-3, Aub and Piwi (Supplementary Fig. S2 online). *E. heros* AGO-1 clustered with AGO-1 from *N. viridula* and *N. lugens* with the same ancestor. *E. heros* AGO-2 was assembled in a second group together with other proteins of this family. These two clusters showed a common ancestor. The AGO-3 was clustered in a distinct group, as well as Aub and Piwi proteins. *E. heros* AGO-3 was grouped with the AGO-3 proteins from *H. halys* and other insects. Thereby, the phylogenetic analyses were useful to confirm the identification of the core RNAi genes present in the *E. heros* transcriptome.

AGO protein is the core component of the RISC, and guided by the siRNA it promotes mRNA cleavage[73,74]. Next to AGO, other important genes related to RISC were identified in the *E. heros* transcriptome (Table 3). Tudor-SN (TSN) protein is known to interact with Argonaute proteins in the silkworm *Bombyx mori* (Lepidoptera: Bombycidae)[79], while Translin and TRAX, that are conserved subunits of the C3PO, are involved in RISC activation, supporting RNAi activity[80]. The *Armi*, *Spn-E*, *Maelstrom* and *Hen-1* nucleases are involved in piRNA biogenesis[31]. *Maelstrom* mutations in *Drosophila* ovaries resulted in a depletion of Dicer and AGO-2 proteins, the latter two being related to the RNAi pathways[81]. Elp-1, that is also present in *E. heros*, is a component of the polymerase II elongator complex, and although the absence of this protein in *Drosophila* S2 cell lines did not affect the miRNA pathway, it can cause an inhibition of the siRNA pathway[82]. The *Vasa intronic gene* (*VIG*), that encodes a putative RNA-binding protein through association with RISC[83], and related to the production of piRNAs[84], was also identified in the *E. heros* transcriptome. The Gawky protein, a cytoplasmic mRNA component necessary in early embryonic development[85], Staufen (STAU), a DSRBP, and CLIP-associating protein (Clp-1), that is responsible for the phosphorylation of the 5' end of siRNAs[86] and related to the splicing process of transfer RNAs[87], were all also found in the *E. heros* transcriptome of this study. The PRP16 protein plays a role in the pre-mRNA processing[88], while Belle has a function in the endo-siRNA pathway[89]. The proteins GLD-1 and ACO-1, known to inhibit translation of mRNA into protein[82], were also identified in *E. heros*.

Nucleases (RNases together with other RNA enzymes) function in DNA/RNA digestion in the midgut[90] and offer an additional defense and regulatory control layer. The activity of nucleases in dsRNA degradation (dsRNases) is well known, taken an important role in RNAi efficiency across insect groups such as Hemiptera[39,41,91], Lepidoptera[92,93], and Diptera[38]. Four nucleases were identified in the *E. heros* transcriptome: *Eri-1*, *Nibbler*, *SDN1*, and *dsRNase* (Table 4; Supplementary Fig. S3 online). The Eri-1 nuclease is suggested to play a role in the intracellular siRNA and miRNA pathways[94]. In *C. elegans*, Eri-1 forms a complex with Dicer, generating specific classes of siRNAs, while in mouse, Eri-1 negatively regulates the global abundance of miRNA[94]. Nibbler, an exonuclease known to be involved in shaping the 3'end of the miRNAs, and its depletion leading to developmental defects in Drosophila[95], was found with conserved domains in *E. heros*. Another intracellular nuclease found in *E. heros* was SDN1. In *Arabidopsis*, this protein is involved in the degradation of mature miRNA, and the knockdown resulted in developmental defects[95]. However, the involvement of the Eri-1, Nibbler, and SDN1 in RNAi efficiency in insects remains unclear. In *E. heros* we also identified a *dsRNase* gene with six isoforms and with a conserved

Endonuclease_NS domain associated with the degradation of foreign dsRNA molecules[38]. In *B. mori* three dsRNases isoforms were identified and expressed in different tissues, such as epidermis, fat body, and gut; these dsRNases are related to the innate immune response against invasive nucleic acids[93]. The presence of a *dsRNase* nuclease with five isoforms may indicate that *E. heros* has a strong nuclease activity, so this may result in a lower potential to suppress the expression of target genes and so in turn a lower RNAi response.

In the current work, we identified some genes related to antiviral RNAi as follows: *Ars2*, a gene related to RISC regulation, *ninaC*, a gene associated with vesicle transport, and a seven transmembrane-domain glycosyltransferase, *egh*[47] (Table 4; Supplementary Fig. S4 online). These genes are known to be involved in antiviral defense in *Drosophila*[47,96]. The *CG4572* gene was also identified in *E. heros*; it is a carboxypeptidase with unknown function, but related to RNAi in *D. melanogaster*[47]. Three genes involved in intracellular transport were also identified. Two *vacuolar H^+ adenosine triphosphatases* (*V-ATPases*) genes were identified in the *E. heros* transcriptome: *V-ATPase subunit A* (*vha68*) and *V-ATPase subunit C* (*vha16*). These genes are located at different functional V-ATPase domains, the peripheral domain (V1) and the integral domain (V0)[97], respectively, and they are related to dsRNA release by the endocytic vesicles[50]. The Small Rab GTPases and vha68 are essential signaling components linked to the extracellular part with the cytoplasm in *L. decemlineata*[25,48].

The presence of some genes in *E. heros* suggests that it has an active and functional RNAi machinery. However, the lack of *sid-like* gene and the presence of nuclease raise the concern about the RNAi efficiency. So, we first checked the stability of a dsRNA molecule in the hemolymph of adults in which it was rapidly degraded. After 10 min, the dsRNA-*V-ATP-A* was partially degraded, with increasing dsRNA degradation over time up to 120 min (Supplementary Fig. S5-B online). In a similar experiment with the pea aphid *A. pisum*, dsRNA was completely degraded after 3 h incubation and this was associated with the lack of RNAi responses in this species[41]. In *E. heros*, dsRNA was completely degraded after 2 h of incubation with watery saliva[98]. Indeed, high nuclease activity in the hemolymph and saliva of *E. heros* may reduce RNAi efficiency and so some form of dsRNA protection may be needed for future field applications.

To confirm the effectivity of the *E. heros* RNAi machinery, a dsRNA targeting the *V-ATPase-A* gene was microinjected into adults. Previously, targeting the *V-ATPase-A* gene led to mortality in *Pectinophora gossypiella* (Lepidoptera: Gelechiidae)[99], *E. heros* nymphs[98], *A. pisum*[100], *H. halys* nymphs[42],

among others. The main V-ATPase function is the pumping of protons across the membrane[101,102], generating an energy gradient. *E. heros* adults were microinjected with ~28 ng of dsRNA-*V-ATPase-A* per mg of insect fresh weight, and the mortality was evaluated at 24, 48, 72 and 96 h after microinjection. At 24 h post-microinjection, there was 7% mortality and this increased to 35% at 96 h (Figure 3). The same dsRNA concentration previously demonstrated to cause up to 50% mortality in *E. heros* 2nd instar nymphs 7 days post-microinjection[98]. Based on these results, we believe that this species is sensitive to RNAi when we compare to other insects also considered sensitive to RNAi. Fishilevich and collaborators[103] used the same dsRNA concentration through microinjection in *E. heros* adults targeting chromatin remodeling genes and this significantly reduced fecundity and egg viability. Coleoptera insects are considered to be more sensitive to RNAi, presenting a robust RNAi mechanism, while Lepidoptera and Hemiptera appear to be more recalcitrant[104]. Second-instar larvae of the African sweet potato weevil *C. puncticollis* were microinjected with 200 ng/mg of body weight targeting different genes, and mortality reached up to ~50% after six days[105]; this concentration is ~9 times higher than that one used in *E. heros*. One of the main reasons associated with the lack of RNAi response in the *C. puncticollis* weevil was the high nuclease activity[105106]. In the pink bollworm, *P. gossypiella ,* microinjection of 20 ng/mg of body weight of a dsRNA targeting *V-ATPase-A* induced mortality up 26% at 96 h post-microinjection in 3rd-instar larvae[99]. One strategy to increase RNAi efficiency is an adequate formulation of the dsRNA molecules. In *E. heros* nymphs, liposome-encapsulated dsRNA targeting *V-ATPase-A* led to 45% mortality after 14 days as compared to 30% with naked dsRNA[98]. Similar results were found for dsRNA α-tubulin and lipoplexes in the German cockroach, *Blattella germanica* (Blattodea: Blattellidae)[107]. Therefore, the formulation of dsRNA may provide an affordable non-transformative easy-to-use strategy to deliver gene silencing for pest control in the field. However, for successful pest control, it is very important to know the dsRNA concentration, expressed as per mg of insect body weight, to permit a rationalized pest control strategy based on dsRNA concentration and the delivery approach.

Alongside the mortality, other effects were also observed. The treated insects exhibited reduced mobility in contrast to the insects microinjected with dsRNA-*GFP* which were very active. This effect lasted until 72 h post-microinjection. Retardation in larval development was reported in *P. gossypiella*[99] and *Helicoverpa armigera* (Lepidoptera: Noctuidae)[108] treated with dsRNA targeting the *V-ATPase*.

To confirm that the observed mortality in *E. heros* injected with dsRNA-*V-ATPase-A* is a true phenotype of gene silencing, a qRT-PCR assay was performed. Indeed we confirmed the *V-ATPase-A* gene

silencing with a reduction of 74% in the relative level of transcripts. At 72 and 96 h post-microinjection, there is an increase in gene expression but still 40% lower compared to the insects treated with *GFP* (Figure 4). An increase in *DCR-2* and *AGO-2* gene expression was observed with the highest gene expression observed at 48 and 72 h, with a respective increase of ~2.0 and ~4.0-fold, so confirming the activation of the siRNA machinery upon exogenous dsRNA delivery (Figure 5-A-B). This data has shown the activity of the siRNA machinery in *E. heros* through the supply of dsRNA. As expected, due to the high nuclease activity, the RNAi effects were temporary, and at 72 and 96 h, there was a recovery in the relative transcript levels from the target genes. Also, at 96 h post-microinjection, there was a reduction in the expression of the siRNA-related genes. In *Manduca sexta* (Lepidoptera: Sphingidae), an upregulation of *DCR-2* and *AGO-2* expression in response to injection with dsRNA with also only transient effects in the gene upregulation was reported [109]. As discussed above, *DCR-2* and *AGO-2* are core components of the siRNA pathway, and the overexpression of *DCR-2* and *AGO-2* after dsRNA microinjection confirmed the upregulation of the siRNA machinery.

Over the past year, scientists have made enormous progress towards the use of RNAi as a pest control strategy taking advantage of genetic sequences available in public databases, and used this information to understand the RNAi mechanism in insects. To our knowledge, this is the first study of *E. heros* transcriptome, including the identification of RNAi-related genes and dissecting the siRNA pathway. The analyses of the *E. heros* transcriptome have identified the main components of the three RNAi pathways with the surprising lack of *sid-like* genes. Identification of the core RNAi genes, efficient mortality rates and activation of the siRNA machinery, these data provide a novel and important dataset on RNAi machinery and its efficiency, underpinning future strategies to enhance RNAi in *E. heros* and potentially other piercing-sucking insects as models or species important in agriculture.

Material and Methods

Brown stink bug insects

The colony of *E. heros* was originally started with insects collected in Pelotas, Brazil (27°48'1.7352'' S; 52°54'3.834''W) in 2013, and kept for about 73 generations under laboratory conditions before experiments. New insects collected in soybean fields in Rondinha, Brazil (27º48'1.7352'' S; 52º54'3.834'' W) were introduced in the laboratory colony in 2015. All stages were maintained in plastic cages under laboratory conditions with a photoperiod of 14:10 (Light: Dark), temperature of 25±1°C and

75±10% relative humidity. Green beans, peanut and water were supplied *ad libitum* and replaced twice in a week. Eggs were collected twice a week to obtain the insects necessary for microinjection and colony maintenance[110]. Insects were collected every day and insects of four days old were used in the microinjection assays.

cDNA libraries, Illumina sequencing and *de novo* assembly

Eggs, all nymphal stages and adults of *E. heros* were used for total RNA extraction using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer instructions. The RNA pool was prepared with an equally RNA amount from all stages, and the cDNA library preparation and Illumina sequencing were conducted at the Laboratory of Functional Genomics Applied to Agriculture and Agri-Energy, at the University of São Paulo, Brazil. The TruSeq RNA Sample Prep kit (Illumina) protocol was used to construct the cDNA library, following manufacturer instructions. A high-throughput Illumina sequencing platform (HiSeq2000) was used for the final library sequencing, in one lane of a 100 bp paired-end run.

The raw reads originating from the Illumina sequencing were check for quality using the FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). After that, reads were trimmed using Trimmomatic[111], and only high-quality reads, showing a Phred score superior to 30, were used for the *de novo* assembly to generate a set of contigs using Trinity software (http://trinityrnaseq.sourceforge.net)[112]. De Bruijn graph algorithm and a k-mer length of 25 were used as parameters.

Homology search and gene ontology annotation

The generated contigs were analyzed using the UniProt-TrEMBL database[113] via Diamond algorithm[114], with an E-value<10^{-5} as a cut-off parameter. The contigs with insect hits were submitted to a second homology search using QuickGO to identify gene ontology (GO) terms. For this annotation, a similarity search was performed against the UniProt database using Diamond, with an E-value<10^{-5} as a cut-off parameter.

RNAi-related genes

We searched for the genes related to RNAi efficacy and these included genes on dsRNA uptake (Table 1), RNAi core machinery (Table 2), auxiliary factors (Table 3), nucleases, antiviral RNAi and

intracellular transport (Table 4)[31,33,48]. Homologous sequences for these proteins were searched in *UniProt* or Protein database from NCBI, and used as a query to search the *E. heros* transcriptome using the tBLASTn tool from NCBI. Generated contigs with a bitscore >150 and E-value <1e-5 were further used to confirm the identity. To detect the open reading frames (ORFs) in the contigs sequence, the ORF Finder from NCBI was used (https://www.ncbi.nlm.nih.gov/orffinder/), and the protein domains predicted by the NCBI Conserved Domains Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Protein Basic Local Alignment Tool (Protein BLAST) was used for protein homology search against insect non-redundant protein database at NCBI.

To provide additional confirmation on identity and function prediction of the core RNAi proteins, nucleases and antiviral RNAi, members of these groups of proteins were subject to a phylogenetic analysis using the neighbor-joining (MEGA 7.0.26) algorithm with 1,000 bootstrap replicates. A total of 35 Argonaute superfamily protein sequences, 30 endoribonuclease III protein sequences, 28 nuclease protein sequences, and 27 antiviral RNAi sequences were aligned using the MUSCLE program from MEGA 7.0.26 software. ORF Finder from NCBI was used to predict the proteins.

dsRNA synthesis and purification

Specific primers were used to amplify the fragments of the target genes (Table 5). The cDNA was synthesized using the SuperScript First-Strand Synthesis System Kit (Invitrogen) following the manufacturer's instructions. The T7 primer sequence (TAATACGACTCACTATAGGGAGA) was placed in the front of the forward and reverse primers. These primers were used for dsRNA synthesis with cDNA as a template. The PCR reaction was performed with 2 µl of cDNA template, 2 µl of a 10 µM solution of each primer (Integrated DNA Technologies, Coralville, IA, USA), 0.125 µl of Taq DNA polymerase, 2.5 µl of Buffer 10X, 0.5 µl of 10 µM dNTPs, 0.75 µl of $MgCl_2$ (Invitrogen) and 15 µl of nuclease-free water (GE Healthcare, Little Chalfont, UK) in a total volume of 25 µl. The PCR conditions used were 5 min at 94°C for initial denaturation, followed by 30 s at 94°C, 45 s at 59.5°C, 55 s at 72ºC for 30 cycles and final extension for 10 min at 72ºC. The amplified products were purified using a PCR purification kit (Qiagen, Valencia, CA, USA) and analyzed on 1% agarose gels. The PCR product was quantified using a Nanovue spectrophotometer (GE Healthcare) and then samples were stored at -20 °C.

The *V-ATPase-A* dsRNA was synthesized using the MEGAscript T7 RNAi kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. The control group consisted of a dsRNA of the *green*

fluorescent protein (dsRNA-*GFP*) synthesized from a DNA plasmid (pIG1783f) and cloned in *Escherichia coli* (DH5α). Plasmid DNA was extracted and sequenced to confirm the identity of PCR products. The identity of the sequence was confirmed by Sanger sequencing. The dsRNA was analyzed for integrity on 1% agarose gels, its concentration quantified in a Nanovue spectrophotometer (GE Healthcare) and then stored at -20ºC.

***Ex vivo* dsRNA hemolymph degradation assay**

Insects were anesthetized with CO_2 during ~30 s and then taped with the abdomen upwards on a glass plate. Legs and rostrum were cut, and hemolymph collected by a needle, prepared with glass capillary tubes, coupled to an insulin syringe (8 X 0.30 mm) and placed in chilled 1.5 ml tubes containing phenylthiourea (PTU) to prevent melanisation. After that, 30 µl of dsRNAs-V-ATPase-A solution at 200 ng/µl was incubated in 3 µl of RNase-free water or 3 µl of hemolymph at 25°C. Aliquots of 5 µl were collected after 0, 1, 10, 30, 60, and 120 min, and the same volume of EDTA (10 mM) was added to the solution to stop the enzymatic reaction[41]. The integrity of the samples was analyzed on 1% agarose gel.

Adult microinjection

To silence the *V-ATPase-A* gene in *E. heros,* dsRNA-*V-ATP-A* with 623 bp was microinjected in adults (~60 mg) at the concentration of ~28 ng per mg of body weight (0.50 µl of a 3350 ng/µl dsRNA solution)[103]. The control group consisted of insects microinjected with a 560 bp dsRNA molecule targeting *GFP*[31,105]. The dsRNA-*V-ATP-A* was designed to have a length similar to the one used in previous RNAi assays in the hemipterans *D. citri*[43] and *N. lugens*[115].

To perform the microinjection, insects were anesthetized with CO_2 and immobilized in a glass plate with double-sided tape (3M, São Paulo, Brazil). The microinjection was performed using an insulin syringe (8 x 0.30 mm) with a needle (30 g) (Solidor) coupled to a micro-applicator (Burkard, Rickmansworth, UK). In total, 62 adults were injected per treatment, of which 12 individuals were used for qRT-PCR and 50 individuals for mortality assay, at 24, 48, 72 and 96 h post-microinjection. Alongside mortality analysis, visual observations were carried out to analyze other effects related to the dsRNA in the insects. After microinjection, the insects were placed in plastic cages containing green beans, peanut and water *ad libitum*, and kept at 25°C, photoperiod of 14:10 (L:D) and 75±10% RH, as with the colony maintenance. The insect mortality was normalized against the control (dsRNA-*GFP*).

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from whole insect body at 24, 48, 72 and 96 h after microinjection, and each time point had three biological samples containing one insect. RNA extraction was performed using RNAzol RT (MCR, Cincinnati, OH, USA), following the manufacturer's instructions. The samples were quantified using a Nanovue spectrophotometer (GE Healthcare), verified in a 1% agarose gel electrophoresis, and kept at -80°C. First-strand cDNA synthesis proceeded as described in the dsRNA synthesis and purification section.

The qRT-PCR was performed on a Roche LightCycler 480 (LC480) (Roche Diagnostics, Basel, Switzerland) real-time PCR platform. To validate the primers used in the analysis (Table 5), a melting curve analysis with temperatures from 60 to 95ºC and a standard curve based on a serial dilution of cDNA were used to determine the primer annealing efficiency and specificity. The reaction included 6 µl of EvaGreen 2X qPCR MasterMix (ABM, Milton, ON, Canada), 1.25 µl of 10 µM forward primer (Integrated DNA Technologies), 1.25 µl of 10 µM reverse primer (Integrated DNA Technologies), 2.5 µl of nuclease-free water and 2 µl of cDNA, in a total volume of 13 µl. The amplification conditions were 3 min at 95ºC followed by 45 cycles of 30 s at 95ºC, 45 s at 59ºC and 30 s at 77ºC. The reactions were set-up in 96-well microtiter plates (Roche Life Science, Indianapolis, IN, USA), using the cDNA dilution of 1:25 and three technical replicates. The normalization of the data was performed using two endogenous genes, ribosomal protein L32e (rpl32) and 18s ribosomal RNA (18S rRNA) (Table 5); also an appropriate no template *control* (NTC) was included. The equation ratio $2^{-\Delta\Delta Ct}$ was used for normalization of the relative gene expression levels[116]. Data were analyzed using analysis of variance (one-way ANOVA) and unpaired *t*-test (*p*-value ≤ 0.05).

552 **References**

553 1. Medeiros, L. & Amegier, G. A. Ocorrência e Desempenho de *Euschistus heros* (F .) (Heteroptera :
554 Pentatomidae) em Plantas Hospedeiras Alternativas no Rio Grande do Sul. *Neotrop. Entomol.* **38**
555 **(4)**, 459–463 (2009).
556 2. Panizzi, A. R. Stink Bugs Growing Problems with (Hemiptera: Heteroptera: Pentatomidae):
557 Species Invasive to the U.S. and Potential. *Am. Entomol.* **61**, 223–233 (2015).
558 3. Panizzi, A. R., Bueno, A. F. & Silva, F. A. C. Insetos que atacam vagens e grãos. Hoffmann-Campo,
559 C. B., Corrêa-Ferreira, B. S. & Moscardi, F. (ed.) *Soja: manejo integrado de insetos e outros*
560 *artrópodes-praga*. 5, 335–420. (Embrapa, 2012).
561 4. Panizzi, A. R. *et al.* Insetos da Soja no Brasil. *Bol. Técnico nº 1*
562 https://ainfo.cnptia.embrapa.br/digital/bitstream/item/77369/1/CNPSO-BOL.-TEC.-1-77.pdf
563 (1977).
564 5. Sosa-Gómez, D. R. *et al.* Insecticide susceptibility of *Euschistus heros* (Heteroptera: Pentatomidae)
565 in Brazil. *J. Econ. Entomol.* **102**, 1209–1216 (2009).
566 6. Saluso, A., Xavier, L., Silva, F. A. C. & Panizzi, A. R. An invasive pentatomid pest in Argentina:
567 Neotropical brown stink bug, *Euschistus heros (*F.) (Hemiptera: Pentatomidae). *Neotrop. Entomol.*
568 **40**, 704–705 (2011).
569 7. Sosa-Gómez, D. R. & Silva, J. J. da. Neotropical brown stink bug (*Euschistus heros*) resistance to
570 methamidophos in Paraná, Brazil. *Pesqui. Agropecuária Bras.* **45**, 767–769 (2010).
571 8. Guedes, R. N. C. Insecticide resistance, control failure likelihood and the First Law of Geography.
572 *Pest Manag. Sci.* **73**, 479–484 (2017).
573 9. Tuelher, E. S. *et al.* Area-wide spatial survey of the likelihood of insecticide control failure in the
574 neotropical brown stink bug *Euschistus heros*. *J. Pest Sci.* **91**, 849–859 (2018).
575 10. Sagan, S. M. & Sarnow, P. RNAi, antiviral after all. *Science.* **342**, 10–12 (2014).
576 11. Gammon, D. B. & Mello, C. C. RNA interference-mediated antiviral defense in insects. *Curr. Opin.*
577 *Insect Sci.* **8**, 111–120 (2015).
578 12. Baum, J. A. *et al.* Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.*
579 **25**, 1322–1326 (2007).
580 13. Head, G. P. *et al.* Evaluation of SmartStax and SmartStax PRO maize against western corn
581 rootworm and northern corn rootworm: Efficacy and resistance management evaluation. *Pest*

582 *Manag. Sci.* **73**, 1883–1899 (2017).
583 14. Cagliari, D., Santos, E. A. dos, Dias, N., Smagghe, G. & Zotti, M. Nontransformative strategies for
584 RNAi in crop protection. Singh, A. (Ed.). *Modulating Gene Expression - Abridging the RNAi and*
585 *CRISPR-Cas9 Technologies* **2,** 1–17 (2018).
586 15. Palli, S. R. RNA interference in Colorado potato beetle: steps toward development of dsRNA as a
587 commercial insecticide. *Curr. Opin. Insect Sci.* **6,** 1–8 (2014).
588 16. San Miguel, K. & Scott, J. G. The next generation of insecticides: DsRNA is stable as a foliar-
589 applied insecticide. *Pest Manag. Sci.* **72**, 801–809 (2016).
590 17. Carthew, R. W. S. J. E. Origins and Mechanisms of miRNAs and siRNAs. *Natl. Institutes Heal.*
591 **136**, 642–655 (2009).
592 18. Weick, E.-M. & Miska, E. A. piRNAs: from biogenesis to function. *Development* **141**, 3458–3471
593 (2014).
594 19. Huvenne, H. & Smagghe, G. Mechanisms of dsRNA uptake in insects and potential of RNAi for
595 pest control: A review. *J. Insect Physiol.* **56**, 227–235 (2010).
596 20. Feinberg, E. H. & Hunter, C. P. Transport of dsRNA into cells by the transmembrane protein SID-
597 1. *Science* **301**, 1545–1547 (2003).
598 21. Aronstein, K., Pankiw, T. & Saldivar, E. SID-I is implicated in systemic gene silencing in the honey
599 bee. *J. Apic. Res.* **45**, 20–24 (2006).
600 22. Kobayashi, I. *et al.* SID-1 protein of *Caenorhabditis elegans* mediates uptake of dsRNA into
601 *Bombyx* cells. *Insect Biochem. Mol. Biol.* **42**, 148–154 (2012).
602 23. Saleh, M. *et al.* The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing.
603 *Nat. Cell Biol.* **8**, 793–802 (2006).
604 24. Ulvila, J. *et al.* Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis
605 in *Drosophila* S2 cells. *J. Biol. Chem.* **281**, 14370–14375 (2006).
606 25. Cappelle, K., De Oliveira, C. F. R., Van Eynde, B., Christiaens, O. & Smagghe, G. The involvement
607 of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded
608 RNA uptake in the Colorado potato beetle midgut. *Insect Mol. Biol.* **25**, 315–323 (2016).
609 26. Pinheiro, D. H. *et al.* Clathrin-dependent endocytosis is associated with RNAi response in the
610 western corn rootworm, *Diabrotica virgifera virgifera* LeConte. *PLoS One* **13**, e0201849 (2018).
611 27. Vélez, A. M. & Fishilevich, E. The mysteries of insect RNAi: A focus on dsRNA uptake and

612 transport. *Pestic. Biochem. Physiol.* **151**, 25–31 (2018).
613 28. Whangbo, J. S. & Hunter, C. P. Environmental RNA interference. *Trends Genet.* **24**, 297–305
614 (2008).
615 29. Zotti, M. J. & Smagghe, G. RNAi technology for insect management and protection of beneficial
616 insects from diseases: Lessons, challenges and risk assessments. *Neotrop. Entomol.* **44**, 197–213
617 (2015).
618 30. Ketting, R. F. The Many Faces of RNAi. *Developmental Cell* **20**, 148–161 (2011).
619 31. Prentice, K. *et al.* Transcriptome analysis and systemic RNAi response in the African sweetpotato
620 weevil (*Cylas puncticollis*, Coleoptera, Brentidae). *PLoS One* **10**, 1–18 (2015).
621 32. Firmino, A. A. P. *et al.* Transcriptome analysis in cotton boll weevil (*Anthonomus grandis*) and
622 RNA interference in insect pests. *PLoS One* **8**, 1–15 (2013).
623 33. Swevers, L. *et al.* Colorado potato beetle (Coleoptera) gut transcriptome analysis: Expression of
624 RNA interference-related genes. *Insect Mol. Biol.* **22**, 668–684 (2013).
625 34. Camargo, R. de A. *et al. De novo* transcriptome assembly and analysis to identify potential gene
626 targets for RNAi-mediated control of the tomato leafminer (*Tuta absoluta*). *BMC Genomics* **16**, 1–
627 17 (2015).
628 35. Sparks, M. E., Shelby, K. S., Kuhar, D. & Gundersen-Rindal, D. E. Transcriptome of the invasive
629 brown marmorated stink bug, *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae). *PLoS One* **9**,
630 e111646 (2014).
631 36. Terenius, O. *et al.* RNA interference in Lepidoptera: An overview of successful and unsuccessful
632 studies and implications for experimental design. *J. Insect Physiol.* **57**, 231–245 (2011).
633 37. Baum, J. A. & Roberts, J. K. Progress towards RNAi-mediated insect pest management. Dhadialla
634 T. S. & Gill S. S. (ed.) *Advances in Insect Physiology* **47,** 249-295 (2014).
635 38. Singh, I. K., Singh, S., Mogilicherla, K., Shukla, J. N. & Palli, S. R. Comparative analysis of
636 double-stranded RNA degradation and processing in insects. *Sci. Rep.* **7**, 1–12 (2017).
637 39. Wang, K. *et al.* Variation in RNAi efficacy among insect species is attributable to dsRNA
638 degradation *in vivo*. *Insect Biochem. Mol. Biol.* **77**, 1–9 (2016).
639 40. Katoch, R. & Thakur, N. Insect gut nucleases: a challenge for RNA interference mediated insect
640 control strategies. *Int. J. Biochem. Biotechnol.* **1**, 198–203 (2012).
641 41. Christiaens, O., Swevers, L. & Smagghe, G. DsRNA degradation in the pea aphid (*Acyrthosiphon*

642 *pisum*) associated with lack of response in RNAi feeding and injection assay. *Peptides* **53**, 307–
643 314 (2014).
644 42. Mogilicherla, K., Howell, J. L. & Palli, S. R. Improving RNAi in the brown marmorated stink bug:
645 Identification of target genes and reference genes for RT-qPCR. *Sci. Rep.* **8**, 3720 (2018).
646 43. Galdeano, D. M., Breton, M. C., Lopes, J. R. S., Falk, B. W. & Machado, M. A. Oral delivery of
647 double-stranded RNAs induces mortality in nymphs and adults of the Asian citrus psyllid,
648 *Diaphorina citri*. *PLoS One* **12**, e0171847 (2017).
649 44. Taning, C. N. T., Andrade, E. C., Hunter, W. B., Christiaens, O. & Smagghe, G. Asian citrus psyllid
650 RNAi pathway-RNAi evidence. *Sci. Rep.* **6**, 1–10 (2016).
651 45. Upadhyay, S. K. *et al.* RNA interference for the control of whiteflies (*Bemisia tabaci*) by oral route.
652 *J. Biosci.* **36**, 153–161 (2011).
653 46. Chen, J. *et al.* Feeding-based RNA interference of a trehalose phosphate synthase gene in the brown
654 planthopper, *Nilaparvata lugens*. *Insect Mol. Biol.* **19**, 777–786 (2010).
655 47. Saleh, M. C. *et al.* Antiviral immunity in *Drosophila* requires systemic RNA interference spread.
656 *Nature* **458**, 346–350 (2009).
657 48. Yoon, J.-S., Shukla, J. N., Gong, Z. J., Mogilicherla, K. & Palli, S. R. RNA interference in the
658 Colorado potato beetle, *Leptinotarsa decemlineata:* Identification of key contributors. *Insect*
659 *Biochem. Mol. Biol.* **78**, 78–88 (2016).
660 49. Jose, A. M. & Hunter, C. P. Transport of sequence-specific RNA interference information between
661 cells. *Annu. Rev. Genet.* **41**, 305–330 (2007).
662 50. Saleh, M. *et al.* The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing.
663 *Cell* **8**, 793–802 (2006).
664 51. Rocha, J. J. E., Korolchuk, V. I., Robinson, I. M. & O'Kane, C. J. A phagocytic route for uptake of
665 double-stranded RNA in RNAi. *PLoS One* **6**, 2–7 (2011).
666 52. Wynant, N., Santos, D., Van Wielendaele, P. & Van den Broeck, J. Scavenger receptor-mediated
667 endocytosis facilitates RNA interference in the desert locust, *Schistocerca gregaria*. *Insect Mol.*
668 *Biol.* **23**, 320–329 (2014).
669 53. Aung, K. M. *et al.* Scavenger receptor mediates systemic RNA interference in ticks. *PLoS One* **6**,
670 (2011).
671 54. Gordon, K. H. J. & Waterhouse, P. M. RNAi for insect-proof plants. *Nat. Biotechnol.* **25**, 1231–

1232 (2007).

55. Carmell, M. A. & Hannon, G. J. RNase III enzymes and the initiation of gene silencing. *Nat. Struct. Mol. Biol.* **11**, 214–218 (2004).

56. Song, M.-S. & Rossi, J. J. Molecular mechanisms of Dicer: endonuclease and enzymatic activity. *Biochem. J.* **474**, 1603–1618 (2017).

57. Welker, N. C. *et al.* Dicer's helicase domain discriminates dsRNA termini to promote an altered reaction mode. *Mol. Cell* **41**, 589–599 (2011).

58. MacRae, I. J. *et al.* Structural basis for double-stranded RNA processing by Dicer. *Science* **311**, 195–198 (2006).

59. Zhang, H., Kolb, F. A., Jaskiewicz, L., Westhof, E. & Filipowicz, W. Single processing center models for human Dicer and bacterial RNase III. *Cell* **118**, 57–68 (2004).

60. Irvin-Wilson, C. V. & Chaudhuri, G. Alternative initiation and splicing in dicer gene expression in human breast cells. *Breast Cancer Res.* **7**, 563–569 (2005).

61. Potenza, N. *et al.* A novel splice variant of the human dicer gene is expressed in neuroblastoma cells. *FEBS Lett.* **584**, 3452–3457 (2010).

62. Yan, F. *et al.* Identification of novel splice variants of the *Arabidopsis* DCL2 gene. *Plant Cell Rep.* **28**, 241–246 (2009).

63. Xu, H. J. *et al.* Genome-wide screening for components of small interfering RNA (siRNA) and micro-RNA (miRNA) pathways in the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae). *Insect Mol. Biol.* **22**, 635–647 (2013).

64. Miyata, K. *et al.* Establishing an in vivo assay system to identify components involved in environmental RNA interference in the western corn rootworm. *PLoS One* **9**, (2014).

65. Vagin, V. V. *et al.* A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**, 320–324 (2006).

66. Houwing, S. *et al.* A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. *Cell* **129**, 69–82 (2007).

67. Das, P. P. *et al.* Piwi and piRNAs Act Upstream of an endogenous siRNA pathway to suppress Tc3 transposon mobility in the *Caenorhabditis elegans* germline. *Mol. Cell* **31**, 79–90 (2008).

68. Carmell, M. A., Xuan, Z., Zhang, M. Q. & Hannon, G. J. The Argonaute family: tentacles that reach into RNAi. *Genes Dev.* **16,** 2733–2742 (2002).

69. Yigit, E. *et al.* Analysis of the *C. elegans* Argonaute family reveals that distinct Argonautes act sequentially during RNAi. *Cell* **127**, 747–757 (2006).

70. Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R. & Hannon, G. J. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293**, 1146–1150 (2001).

71. Meyer, W. J. *et al.* Overlapping functions of argonaute proteins in patterning and morphogenesis of *Drosophila* embryos. *PLoS Genet.* **2**, 1224–1239 (2006).

72. Parker, J. S., Roe, S. M. & Barford, D. Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. *Nature* **434**, 663–666 (2005).

73. Hutvagner, G. & Simard, M. J. Argonaute proteins: Key players in RNA silencing. *Nat. Rev. Mol. Cell Biol.* **9**, 22–32 (2008).

74. Peters, L. & Meister, G. Argonaute Proteins: Mediators of RNA Silencing. *Mol. Cell* **26**, 611–623 (2007).

75. Ender, M. & Meister, G. Argonaute proteins at a glance. *J. Cell Sci.* **123**, 1819–1823 (2010).

76. Huang, T. & Zhang, X. Contribution of the Argonaute-1 Isoforms to Invertebrate Antiviral Defense. *PLoS One* **7**, e50581 (2012).

77. Hartig, J. V. & Tomari, Y. piRNAs—the ancient hunters of genome invaders. *Genes Dev.* **21,** 1707–1713 (2007).

78. Pane, A., Wehr, K. & Schüpbach, T. Zucchini and squash encode two putative nucleases required for rasiRNA production in the *Drosophila* germline. *Dev. Cell* **12**, 851–862 (2007).

79. Zhu, L. *et al.* Molecular cloning of BmTUDOR-SN and analysis of its role in the RNAi pathway in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Entomol. Zool.* **47**, 207–215 (2012).

80. Liu, Y., Ye, X., Jiang, F., Liang, C. & Chen, D. C3PO, an endoribonuclease that promotes RNAi by facilitating RISC activation. *Science.* **325**, 750–753 (2009).

81. Findley, S. & Maelstrom, D. a *Drosophila* spindle-class gene, encodes a protein that colocalizes with Vasa and RDE1/AGO1 homolog, Aubergine, in nuage. *Development* **130**, 859–871 (2003).

82. Lipardi, C. & Paterson, B. M. Identification of an RNA-dependent RNA polymerase in *Drosophila* establishes a common theme in RNA silencing. *Fly (Austin)* **4**, 30–35 (2010).

83. Caudy, A. A., Myers, M., Hannon, G. J. & Hammond, S. M. Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev.* **16**, 2491–2496 (2002).

84. Lasko, P. The DEAD-box helicase Vasa: Evidence for a multiplicity of functions in 3 RNA processes and developmental biology. *Biochim. Biophys. Acta J.* **1829**, 810–816 (2013).

85. Schneider, M. D. *et al.* Gawky is a component of cytoplasmic mRNA processing bodies required for early *Drosophila* development. *J. Cell Biol.* **174**, 349–358 (2006).

86. Weitzer, S. & Martinez, J. The human RNA kinase hClp1 is active on 3′ transfer RNA exons and short interfering RNAs. *Nature* **447**, 222–226 (2007).

87. Siomi, H. & Siomi, M. C. On the road to reading the RNA-interference code. *Nature* **457**, 396–404 (2009).

88. Liu, Y. & Cheng, S. Functional roles of DExD/H-box RNA helicases in pre-mRNA splicing. *J. Biomed. Sci.* **22**, 1–9 (2015).

89. Wang, Y. A. N. & Guthrie, C. PRP16 , a DEAH-box RNA helicase , is recruited to the spliceosome primarily via its nonconserved N-terminal domain. *RNA* **2**, 1216–1229 (1998).

90. Arimatsu, Y., Kotani, E., Sugimura, Y. & Furusawa, T. Molecular characterization of a cDNA encoding extracellular dsRNase and its expression in the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.* **37**, 176–183 (2007).

91. Christiaens, O. & Smagghe, G. The challenge of RNAi-mediated control of hemipterans. *Curr. Opin. Insect Sci.* **6**, 15–21 (2014).

92. Guan, R.-B. *et al.* A nuclease specific to lepidopteran insects suppresses RNAi. *J. Biol. Chem.* **293,** 6011-6021 (2018).

93. Liu, J., Swevers, L., Iatrou, K., Huvenne, H. & Smagghe, G. *Bombyx mori* DNA/RNA non-specific nuclease: Expression of isoforms in insect culture cells, subcellular localization and functional assays. *J. Insect Physiol.* **58**, 1166–1176 (2012).

94. Thomas, M. F., L'Etoile, N. D. & Ansel, K. M. Eri1: A conserved enzyme at the crossroads of multiple RNA processing pathways. *Trends Genet.* **7**, 298–307 (2014).

95. Han, B. W., Hung, J. H., Weng, Z., Zamore, P. D. & Ameres, S. L. The 3′-to-5′ exoribonuclease Nibbler shapes the 3′ ends of microRNAs bound to *Drosophila* Argonaute1. *Curr. Biol.* **21**, 1878–1887 (2011).

96. Sabin, L. R. *et al.* Ars2 regulates both miRNA- and siRNA-dependent silencing and suppresses RNA virus infection in *Drosophila*. *Cell* **138**, 340–351 (2009).

97. Beyenbach, K. W. & Wieczorek, H. The V-type H+ ATPase: molecular structure and function,

physiological roles and regulation. *J. Exp. Biol.* **209**, 577–589 (2006).

98. Castellanos, N. L., Smagghe, G., Sharma, R., Oliveira, E. E. & Christiaens, O. Liposome encapsulation and EDTA formulation of dsRNA targeting essential genes increase oral RNAi-caused mortality in the Neotropical stink bug *Euschistus heros*. *Pest Manag. Sci.* **75**, 537–548 (2018).

99. Mohammed, A. M. A. RNAi-based silencing of genes encoding the vacuolar-ATPase subunits a and c in pink bollworm (*Pectinophora gossypiella*). *Afr. J. Biotechnol.* **15**, 2547–2557 (2016).

100. Whyard, S., Singh, A. D. & Wong, S. Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem. Mol. Biol.* **39**, 824–832 (2009).

101. Finbow, M. E. & Harrison, M. A. The vacuolar H^+-ATPase: a universal proton pump of eukaryotes. *Biochem. J.* **324,** 697–712 (1997).

102. Nelson, N. *et al.* The cellular biology of proton-motive force generation by V-ATPases. *J. Exp. Biol.* **203**, 89–95 (2000).

103. Fishilevich, E. *et al.* 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*). *Insect Biochem. Mol. Biol.* **71**, 58–71 (2016).

104. Zotti, M. *et al.* RNAi technology in crop protection against arthropod pests, pathogens and nematodes. *Pest Manag. Sci.* **74,** 1239-1250 (2017).

105. Prentice, K. *et al.* RNAi-based gene silencing through dsRNA injection or ingestion against the African sweet potato weevil *Cylas puncticollis* (Coleoptera: Brentidae). *Pest Manag. Sci.* **73**, 44–52 (2016).

106. Prentice, K., Smagghe, G., Gheysen, G. & Christiaens, O. Nuclease activity decreases the RNAi response in the sweetpotato weevil *Cylas puncticollis*. *Insect Biochem. Mol. Biol.* **110**, 80–89 (2019).

107. Lin, Y. H., Huang, J. H., Liu, Y., Belles, X. & Lee, H. J. Oral delivery of dsRNA lipoplexes to German cockroach protects dsRNA from degradation and induces RNAi response. *Pest Manag. Sci.* **73**, 960–966 (2017).

108. Jin, S., Singh, Li, L., N. D., Zhang, X. & Daniell, H. Engineered chloroplast dsRNA silences *cytochrome p450 monooxygenase*, *v-ATPase* and *chitin synthase* genes in the insect gut and disrupts *Helicoverpa armigera* larval development and pupation . *Plant Biotechnol. J.* **13**, 435–446 (2015).

109. Garbutt, J. S. & Reynolds, S. E. Induction of RNA interference genes by double-stranded RNA; implications for susceptibility to RNA interference. *Insect Biochem. Mol. Biol.* **42**, 621–628 (2012).

110. Borges, M. *et al.* Metodologias de criação e manejo de colônias de percevejos da soja (Hemíptera - Pentatomidae) para estudos de comportamento e ecologia química. https://ainfo.cnptia.embrapa.br/digital/bitstream/CENARGEN/27988/1/doc182.pdf (Embrapa, 2006).

111. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).

112. Haas, B. J. *et al. De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* **8**, 1494–1512 (2013).

113. Apweiler R, Bairoch A, Wu CH, Barker WC, Boeckmann B, Ferro S, et al. UniProt: the universal protein knowledgebase. *Nucleic Acids Res.* **46**, 115–119 (2004).

114. Buchfink, B., Xie, C. & Huson, D. H. Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* **12**, 59–60 (2014).

115. Liu, S., Ding, Z., Zhang, C., Yang, B. & Liu, Z. Gene knockdown by intro-thoracic injection of double-stranded RNA in the brown planthopper, *Nilaparvata lugens*. *Insect Biochem. Mol. Biol.* **40**, 666–671 (2010).

116. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* **25**, 402–408 (2001).

Acknowledgements

The authors acknowledge support over the years for their RNAi-related research from the National Council for Scientific and Technological Development (CNPq) and Coordination for the Improvement of Higher Education Personnel (CAPES) in Brazil, and the Research Foundation-Flanders (FWO-Vlaanderen) in Belgium. We also thank support by COST (European Cooperation in Science and Technology) under grant agreement No. CA15223, and the European Union's Horizon 2020 Programme "EUCLID" under grant agreement No. 633999. The authors also thank Prof. Dirceu Agostinetto for permission to use his laboratory equipment, Dr. Daiane de Pinho Benemann for her support in the qPCR analysis, Dr. Mariane D'Avila Rosenthal for her assistance in the *E. heros* rearing, and Prof. Paul Dyson (U.Swansea, UK) for English text editing.

Author Contributions Statement

D.C., N.D., G.S. and M.Z. contributed conception and design the study; D.C. and E.A.S. provide insects; D.C. and F.S.K. organized the database; D.C. performed the assays, statistical analysis and wrote the first draft of the manuscript; D.C., L.N.R., D.M.G., F.R.M.G., G.S. and M.Z. wrote sections of the manuscript; J.R.F. and G.L. provide support getting resources; All authors contributed to manuscript revision, read and approved the submitted version.

Additional Information

Competing interests

The author(s) declare no competing interests

This paper reports the results of research only.

Ethical approval

This article does not contain any studies with human participants or vertebrate performed by any of the authors

Figures

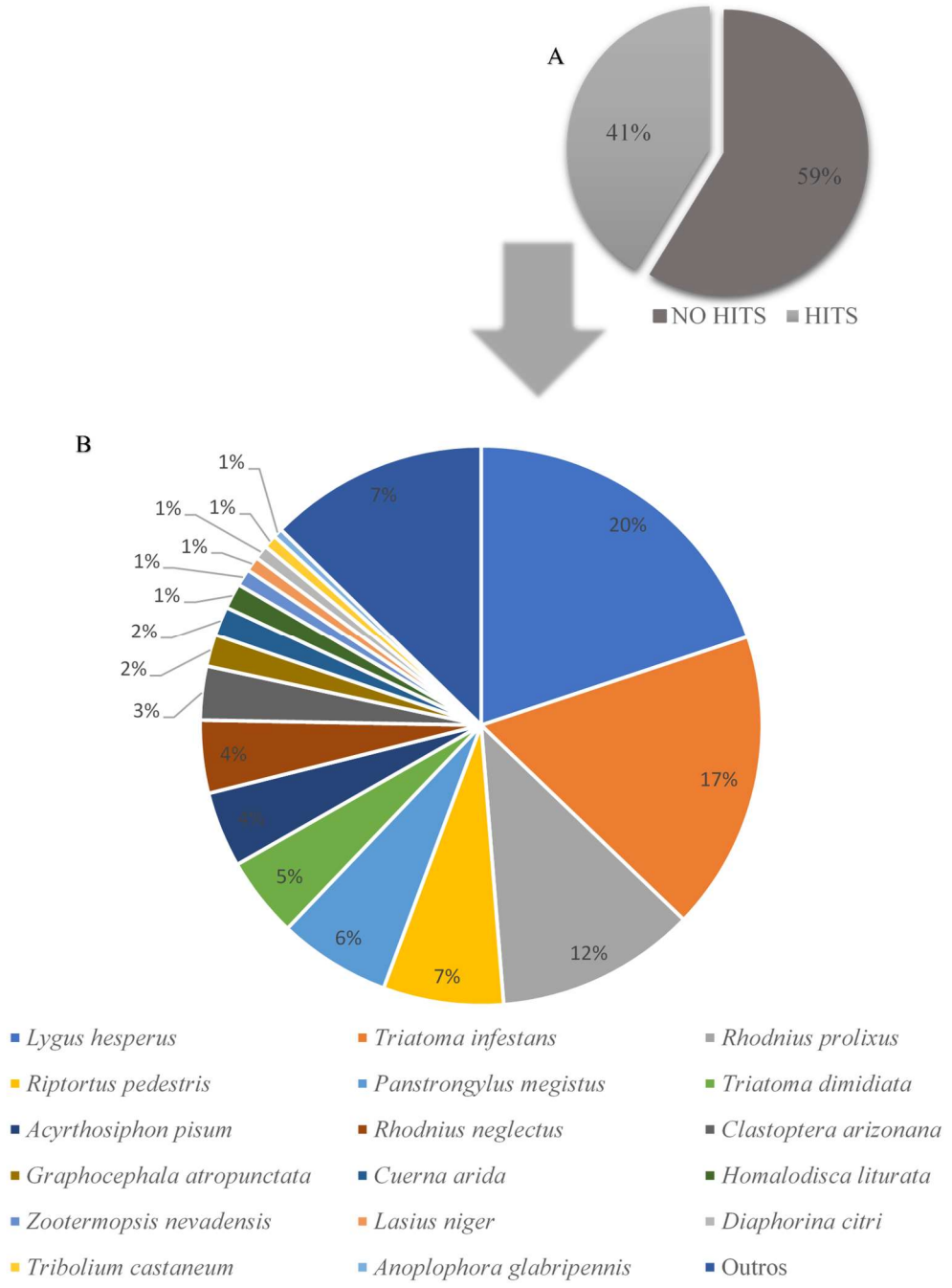


Figure 1. *Euschistus heros* sequence comparison to other insect species. (A) Total transcripts (%) with known and unknown protein sequences in *E. heros* using BLASTx search. (B) BLASTx comparison of *E. heros* known sequences to other insect genera (bitscore>50) against the nr protein database of the NCBI.

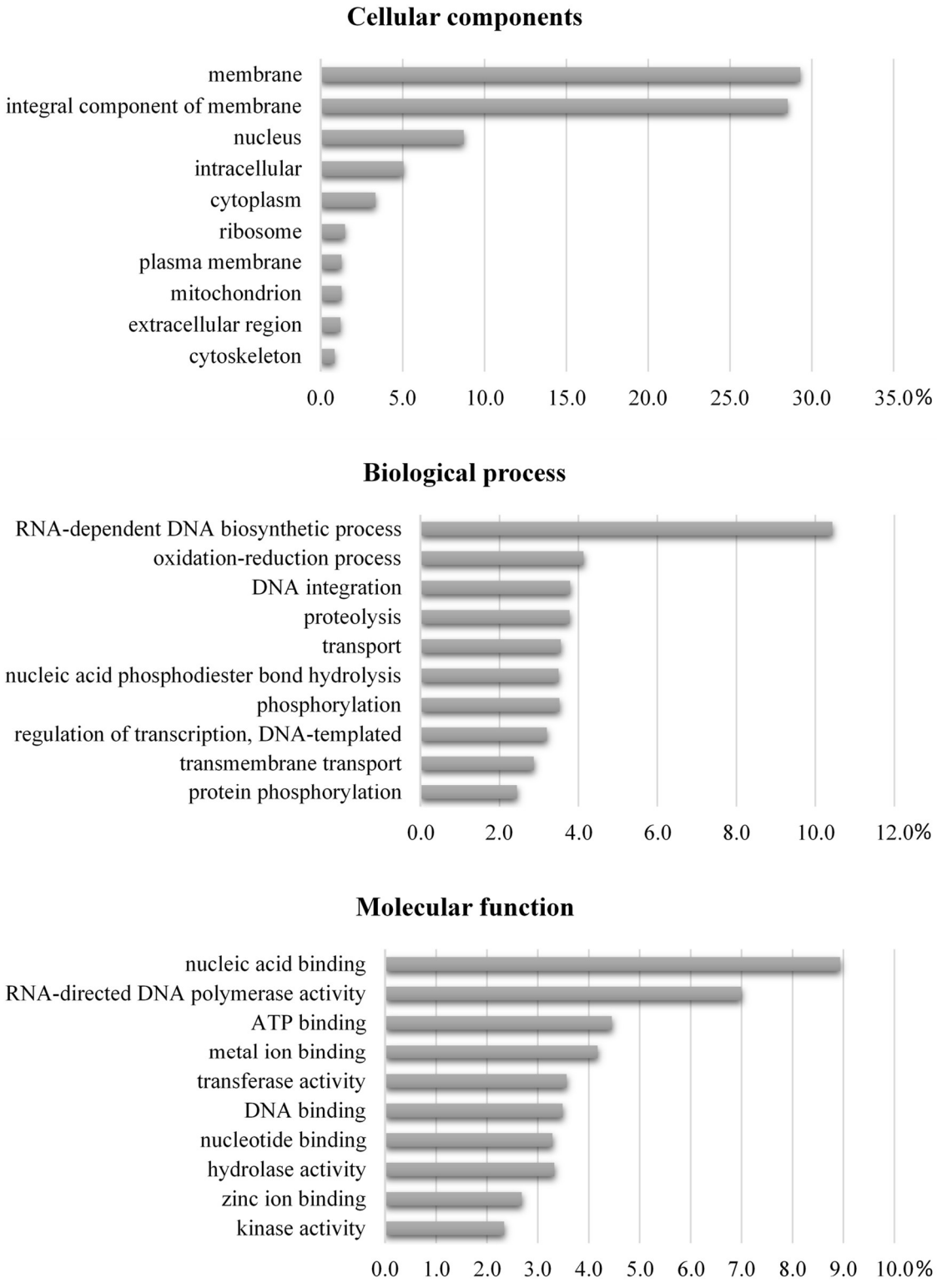


Figure 2. Percentage of *E. heros* contigs assigned to a gene ontology term as predicted by QuickGO from EBI. (A) Cellular components. (B) Biological process. (C) Molecular function.

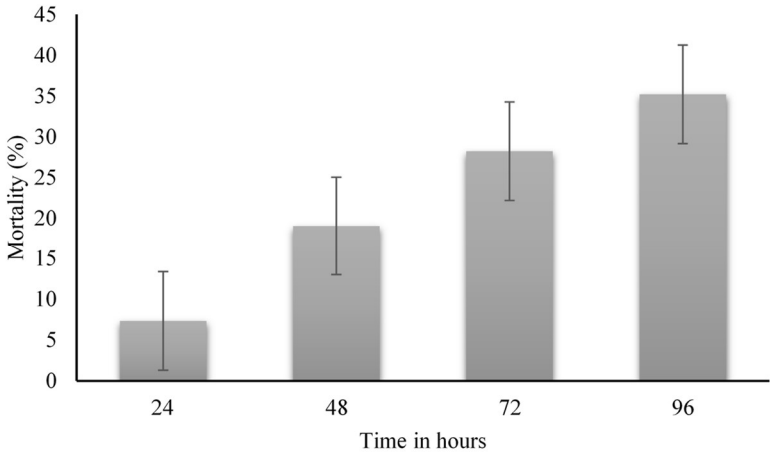


Figure 3. *V-ATPase subunit A* gene silencing mortality effects on *Euschistus heros*. Mortality after microinjection with dsRNA targeting *V-ATPase-A* (dsRNA-*V-ATP-A*) (24-96 h) expressed in percentage. Mortality in adults microinjected with dsRNA-*V-ATP-A* was normalized against the insects microinjected with dsRNA-*GFP*. The columns represent the mean ± SE. (N=50).

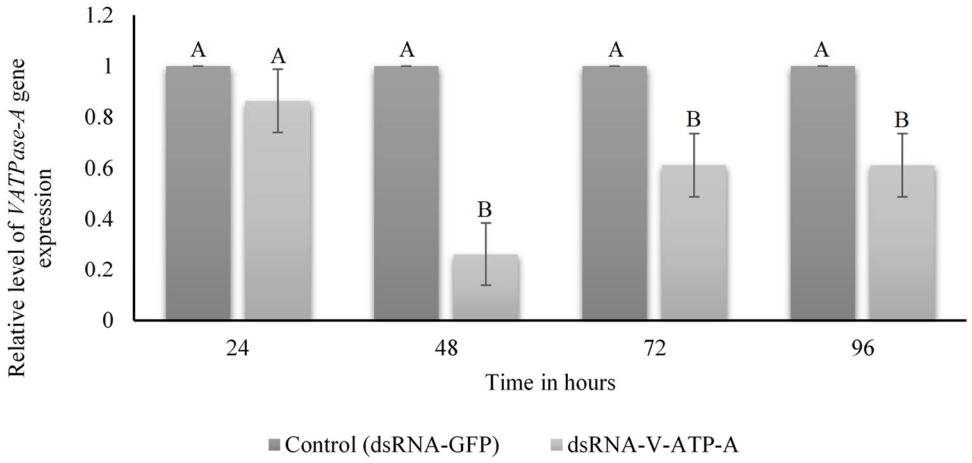


Figure 4. Effects of dsRNA targeting *V-ATPase subunit A* (dsRNA-*V-ATP-A*) on the relative levels of gene expression in *E. heros*. Four days old adults of *E. heros* microinjected with ~28 ng/µL per mg body weight. The adults were sampled at 24, 48, 72 and 96 h post-microinjection at both treatments. Gene expression was normalized against positive controls that were exposed to *gfp* dsRNA (dsRNA-*GFP*) (control). The bars represent the mean ± SE based on 3 biological repeats. The *p*-values were calculated by an unpaired t-test. Bars with different letters indicate that the treatments differed significantly at that time point with $p \leq 0.05$ (N=50).

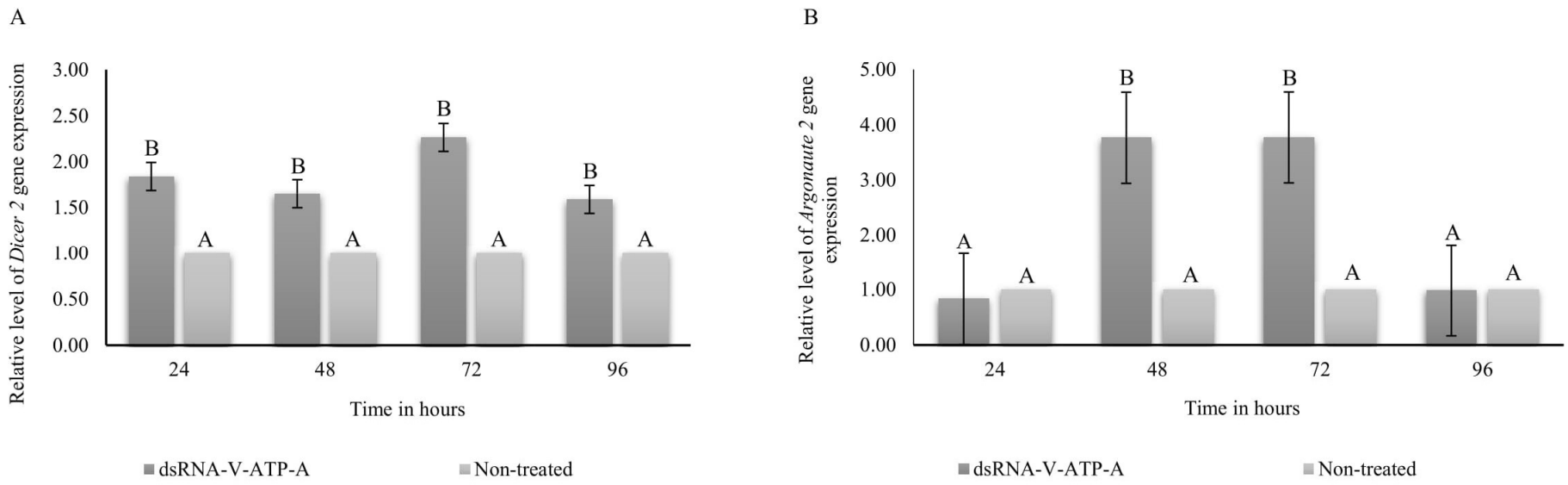


Figure 5. Effects of dsRNA targeting *V-ATPase subunit A* (dsRNA-*V-ATP-A*) on the relative levels of (A) *Dicer 2* (*DCR-2*) and (B) *Argonaute 2* (*AGO-2*) gene expression in *E. heros*. Four days old adults of *E. heros* were microinjected with ~28 ng/µL per mg body weight. The adults (12 in total) were sampled at 24, 48, 72 and 96 h post-microinjection. Gene expression was normalized against negative control that was not exposed to dsRNA. The bars represent the mean ± SE based on 3 biological repeats. The *p*-values were calculated by an unpaired t-test. Bars with different letters indicate that the treatments differed significantly at that time point with $p \leq 0.05$ (N=50).

Table 1. Overview of identified genes related to the dsRNA uptake in *E. heros.*

Gene ID	Transcripts Per Million (TPM)	First hit BLASTp	Homologue ID	Comparison	Identity (%)
Scavenger	818,716	Scavenger receptor class B member 1, partial	XP_024218066.1 (*Halyomorpha halys*)	E= 0.0; bits= 1039	96
CG4966 = orthologous to the Hermansky-Pudlak Syndrome4	310,363	Uncharacterized protein LOC106688690	XP_014288755.1 (*Halyomorpha halys*)	E= 0.0; bits= 1271	90
F-box protein 11 (FBX011)	102,285	F-box only protein 11	XP_014287303.1 (*Halyomorpha halys*)	E= 0.0; bits= 1794	99
Clathrin heavy chain (Chc)	960,642	Clathrin heavy chain	XP_014287090.1 (*Halyomorpha halys*)	E= 0.0; bits= 3477	99
Epsin 2 (Epn2)	141,317	Epsin-2 isoform X5	XP_014270392.1 (*Halyomorpha halys*)	E= 0.0; bits= 900	92
Gap Junction protein (Innexin2)	324,716	Innexin inx2	XP_014292574.1 (*Halyomorpha halys*)	E= 0.0; bits= 736	60

Table 2. Overview of the core RNAi-related genes in *E. heros.*

Gene ID	Transcripts Per Million (TPM)	First hit BLASTp	Homologue ID	Comparison	Identity (%)
miRNA					
DCR-1	0.775	Dcr-1	AVK59457.1 (*Nezara viridula*)	E= 0.0; bits= 2109	91
AGO-1 isoform 1	0.585	Argonaute-1-PC	AVK59466.1 (*Nezara viridula*)	E=0.0; bits= 1924;	99.89
AGO-1 isoform 3	0.103	Argonaute-1-PC	AVK59466.1 (*Nezara viridula*)	E=0.0; bits= 1923;	99.89
AGO-1 isoform 4	0.407	Argonaute-1-PC	AVK59466.1 (*Nezara viridula*)	E=0.0; bits= 1924;	99.89
AGO-1 isoform 5	118,437	Protein argonaute-2 isoform X3	XP_014287705.1 (*Halyomorpha halys*)	E= 0.0; bits= 1877	99
Loquacious	379,404	RISC-loading complex subunit tarbp2-like isoform X1	XP_014274312.1 (*Halyomorpha halys*)	E= 0.0; bits= 521	96
Drosha	112,718	Ribonuclease 3	XP_014278529.1 (*Halyomorpha halys*)	E= 0.0; bits= 2366	91
Pasha	692,682	Microprocessor complex subunit DGCR8	XP_014282581.1 (*Halyomorpha halys*)	E= 0.0; bits= 1078	89
Exportin-5	60,305	Exportin-5	XP_014280932.1 (*Halyomorpha halys*)	E= 0.0; bits= 2420	98
siRNA					
DCR-2 isoform 1	646,601	Endoribonuclease Dicer isoform X1	XP_014275310.1 (*Halyomorpha halys*)	E= 0.0; bits= 2795	83
DCR-2 isoform 2	0.618	Endoribonuclease Dicer isoform X2	XP_014275311.1 (*Halyomorpha halys*)	E= 0.0; bits= 852	88
AGO-2 isoform 1	146,222	Argonaute 2	AVK59468.1 (*Nezara viridula*)	E= 0.0; bits= 565	80
AGO-2 isoform 2	0.137	Argonaute 2	AVK59468.1 (*Nezara viridula*)	E= 0.0; bits= 1516	75
R2D2	350,347	Interferon-inducible double-stranded RNA-dependent protein kinase activator A-like isoform X1	XP_014288218.1 (*Halyomorpha halys*)	E= 0.0; bits= 559	82
piRNA					
AGO-3	227,644	Protein argonaute-3	XP_014276831.1 (*Halyomorpha halys*)	E= 0.0; bits= 1595	85

Aubergine (AUB)	0.750	Protein Aubergine-like	XP_014270559.1 (*Halyomorpha halys*)	E= 0.0; bits= 1676	96
Piwi	579,184	Protein Aubergine-like isoform X3	XP_014275927.1 (*Halyomorpha halys*)	E= 0.0; bits= 1172;	63
Zucchini (Zuc)	0.13543	Mitochondrial cardiolipin hydrolase	XP_014288409.1 (*Halyomorpha halys*)	E= 1e-152; bits= 432	86

Table 3. Overview of identified genes associated to RISC complex in *E. heros*.

Gene ID	Transcripts Per Million (TPM)	First hit BLASTp	Homologue ID	Comparison	Identity (%)
Tudor-SN	0.144	Tudor domain-containing protein 1-like isoform X2	XP_014284230.1 (*Halyomorpha halys*)	E= 0.0; bits= 2031	76
Translin	569,424	Translin	XP_014290495.1 (*Halyomorpha halys*)	E= 1e-154; bits= 434	85
Similar to translin associated factor-X (TRAX)	257,569	Translin-associated protein X isoform X1	XP_014289754.1 (*Halyomorpha halys*)	E= 3e-162; bits= 456	85
Armitage	0.284	Probable RNA helicase armi	XP_014289817.1 (*Halyomorpha halys*)	E= 0.0; bits= 1110	96
Homeless (spindle-E)	0.963535	Probable ATP-dependent RNA helicase spindle-E	XP_014286769.1 (*Halyomorpha halys*)	E= 0.0; bits= 2707	89
Maelstrom	126,192	Protein maelstrom homolog	XP_014290039.1 (*Halyomorpha halys*)	E= 0.0; bits= 694	79
HEN1	0.292	Uncharacterized protein LOC106685926	XP_014284423.1 (*Halyomorpha halys*)	E= 0.0; bits= 656	70
PRP16, mut6 homolog	351,652	Pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16	XP_014279344.1 (*Halyomorpha halys*)	E= 0.0; bits= 2423	96
Clp1 homolog (kinase)	0.999	CLIP-associating protein	XP_014275582.1 (*Halyomorpha halys*)	E: 0.0; bits= 2731	94
Elp-1	221,567	Elongator complex protein 1	XP_014290480.1 (*Halyomorpha halys*)	E= 0.0; bits= 2045	82
GLD-1 homolog	0.03	Glucose dehydrogenase [FAD, quinone]-like isoform X1	XP_014290348.1 (*Halyomorpha halys*)	E= 0.0; bits= 1073	87
ACO-1 homolog	281,389	Cytoplasmic aconitate hydratase-like	XP_014275296.1 (*Halyomorpha halys*)	E: 0.0; bits= 1660	92
Vasa intronic gene (VIG)	658,838	Plasminogen activator inhibitor 1 RNA-binding protein-like isoform X2	XP_014292052.1 (*Halyomorpha halys*)	E= 0.0; bits= 644	96
Staufen	0.147	Double-stranded RNA-binding protein Staufen homolog 2 isoform X5	XP_014282526.1 (*Halyomorpha halys*)	E= 0.0; bits= 956	96
RNA helicase Belle	763,119	ATP-dependent RNA helicase bel isoform X2	XP_014279436.1 (*Halyomorpha halys*)	E= 0.0; bits= 1377	97
Protein arginine methyltransferase 7 (PRMT)	244,103	Protein arginine methyltransferase NDUFAF7, mitochondrial	XP_014292128.1 (*Halyomorpha halys*)	E= 0.0; bits= 726	84
Gawky	135,069	Protein Gawky isoform X1	XP_014288686.1 (*Halyomorpha halys*)	E= 0.0; bits= 2803	97

Table 4. Overview of identified genes associated with RNAi in *E. heros*.

Gene ID	Transcripts Per Million (TPM)	First hit BLASTp	Homologue ID	Comparison	Identity (%)
Nucleases					
Exoribonuclease 1 (Eri1)	388,358	5'-3' exoribonuclease 1	XP_014290344.1 (*Halyomorpha halys*)	E= 0.0; bits= 2701	83
DNA/RNA non-specific endonuclease isoform 1	0.171	Uncharacterized protein LOC106684787	XP_024218583.1 (*Halyomorpha halys*)	E: 6e-172; bits= 490	83.4
DNA/RNA non-specific endonuclease isoform 3	0.294	Uncharacterized protein LOC106691872	XP_014293261.1 (*Halyomorpha halys*)	E= 2e-18; bits= 83.6	56
DNA/RNA non-specific endonuclease isoform 4	0.456	Uncharacterized protein LOC106684787	XP_024218583.1 (*Halyomorpha halys*)	E= 8e-173; bits= 486	85
DNA/RNA non-specific endonuclease isoform 6	702,558	Uncharacterized protein LOC106684787	XP_024218583.1 (*Halyomorpha halys*)	E= 4e-170; bits= 486	83.4
DNA/RNA non-specific endonuclease isoform 7	719,814	Uncharacterized protein LOC106684787	XP_024218583.1 (*Halyomorpha halys*)	E= 4e-170; bits= 486	83
DNA/RNA non-specific endonuclease isoform 9	292,033	Uncharacterized protein LOC106684787	XP_024218583.1 (*Halyomorpha halys*)	E= 5e-170; bits= 486	83
DNA/RNA non-specific endonuclease isoform 10	280,771	Uncharacterized protein LOC106684787	XP_024218583.1 (*Halyomorpha halys*)	E= 7e-172; bits= 490	83
Small RNA degrading nuclease 1 (SDN1-like)	66,023	Uncharacterized exonuclease C637.09 isoform X1	XP_014279339.1 (*Halyomorpha halys*)	E= 0.0; bits= 895	75
Nibbler	743,764	Exonuclease mut-7 homolog	XP_024216394.1 (*Halyomorpha halys*)	E= 0.0; bits= 1402	84
Antiviral					
Ars2	149,588	Serrate RNA effector molecule homolog isoform X1	XP_014277995.1 (*Halyomorpha halys*)	E= 0.0; bits= 1523	98
NinaC	0.352	Neither inactivation nor after potential protein C	XP_014281724.1 (*Halyomorpha halys*)	E= 0.0; bits= 525	95
Beta 1,4-mannosyltransferase (egh)	262,137	Beta-1,4-mannosyltransferase egh	XP_014283435.1 (*Halyomorpha halys*)	E= 0.0; bits= 914	97
CG4572	420,293	Venom serine carboxypeptidase-like	XP_014280828.1 (*Halyomorpha halys*)	E= 0.0; bits=857	89
Intracellular transport					
Vacuolar H+ ATPase sub unit A (vha68)	0.437	V-type proton ATPase catalytic subunit A	XP_014272529.1 (*Halyomorpha halys*)	E= 0.0; bits=1250	99

Vacuolar H+ ATPase sub unit C (vha16)	63,065	V-type proton ATPase 16 kDa proteolipid subunit	XP_014275063.1 (*Halyomorpha halys*)	E= 4e-100; bits= 289	99
Small Rab GTPases	206,763	Ras-related protein Rab-7a	XP_014286452.1 (*Halyomorpha halys*)	E= 3e-152; bits= 425	99

Table 5. Primers used in qRT-PCR and dsRNA synthesis.

	Gene name	Primer	Sequence (5'-3')	Product size (bp)	Amplification factor	R^2
qRT-PCR	*18s ribosomal RNA*	rp*18Sr*RNA-F rp*18Sr*RNA-R	TACAACAAGACAACGCTCGC TTGCGCTCAGTGACATCTCT	150	2.07	0.997
	Ribosomal protein L32e	rp*rpl32*-F rp*rpl32*-R	TCAGTTCTGAGGCGTGCAT TCCGCAAAGTCCTCGTTCA	175	2.15	0.992
	V-ATPase subunit A	rpdsRNA-*V-ATP-A*-F rpdsRNA-*V-ATP-A*-F	GATTATGGTCGTGCGATTTC GAACACCAGCTCTCACTAA	102	1.93	0.998
	Dicer 2	rpDCR2-F rp*DCR2*-R	GAAGCAGGATAACCTCCTAA GGATGCAATTGTTCTACTGGA	156	1.94	1
	Argonaute 2	rp*AGO2*-F rp*AGO2*-R	GACCATCTCCACAACAAATG GTCAGAGGATTGAGGTCTAATA	113	1.97	0.994
dsRNA synthesis	*V-ATPase subunit A*	dsRNA-*V-ATP-A*-F dsRNA-*V-ATP-A*-R	TAATACGACTCACTATAGGGAGACAGGTTTCGACCAATGCCAA TAATACGACTCACTATAGGGAGAACCTCAGAACACCAGCTCTC	623	-	-
	Green Fluorescent Protein	dsRNA-*GFP*-F dsRNA-*GFP*-R	TAATACGACTCACTATAGGGAGATCGTGACCACCCTGACCTAC TAATACGACTCACTATAGGGAGATCGTCCATGCCGAGAGTGAT	560	-	-

Supplementary material

First transcriptome of the Neotropical pest *Euschistus heros* (Hemiptera: Pentatomidae) with dissection of its siRNA machinery

Deise Cagliari[1*]; Naymã Pinto Dias[1]; Ericmar Ávila dos Santos[1]; Leticia Neutzling Rickes[1]; Frederico Schmitt Kremer[2]; Juliano Ricardo Farias[3]; Giuvan Lenz[4]; Diogo Manzano Galdeano[5]; Flávio Roberto Mello Garcia[6]; Guy Smagghe[7*]; Moisés João Zotti[1*]

[1] Department of Crop Protection, Molecular Entomology, Federal University of Pelotas, Pelotas, Brazil.

[2] Center for Technological Development, Bioinformatics and Proteomics Laboratory, Federal University of Pelotas, Pelotas, Brazil.

[3] Department of Crop Protection, Universidade Regional Integrada do Alto Uruguai, Santo Ângelo, Brazil.

[4] Agricultural Research and Development Center, UPL, Pereiras, Brazil

[5] Sylvio Moreira Citrus Center, Agronomic Institute of Campinas, Cordeirópolis, São Paulo, Brazil.

[6] Department of Crop Protection, Insect Ecology Laboratory, Federal University of Pelotas, Pelotas, Brazil.

[7] Department of Plants and Crops, Ghent University, Ghent, Belgium.

*Corresponding authors:

Deise Cagliari, Moisés João Zotti, Guy Smagghe

deisycagliari@yahoo.com.br; moises.zotti@ufpel.edu.br; guy.smagghe@ugent.be

+55 55 9 9162-2651; +55 55 9 9671-2207; +32 9 2646150

Data S1: Sequences of *E. heros* dsRNA uptake.

https://www.nature.com/articles/s41598-020-60078-3#Sec25

Data S2: Sequences of *E. heros* core machinery proteins.

https://www.nature.com/articles/s41598-020-60078-3#Sec25

Data S3: Sequences of *E. heros* RISC-associated auxiliary factors.

https://www.nature.com/articles/s41598-020-60078-3#Sec25

Data S4: Sequences of *E. heros* Nucleases.

https://www.nature.com/articles/s41598-020-60078-3#Sec25

Data S5: Sequences of *E. heros* Antiviral

https://www.nature.com/articles/s41598-020-60078-3#Sec25

Data S6: Sequences of *E. heros* Intracellular transport.

https://www.nature.com/articles/s41598-020-60078-3#Sec25

Table S1: *Euschistus heros* sequence comparison to other insect species. BLASTx comparison of *E. heros* known sequences to other insect genera (bitscore>50) against the nr protein database of the NCBI with hits <0.54%, grouped in other hits.

https://www.nature.com/articles/s41598-020-60078-3#Sec25

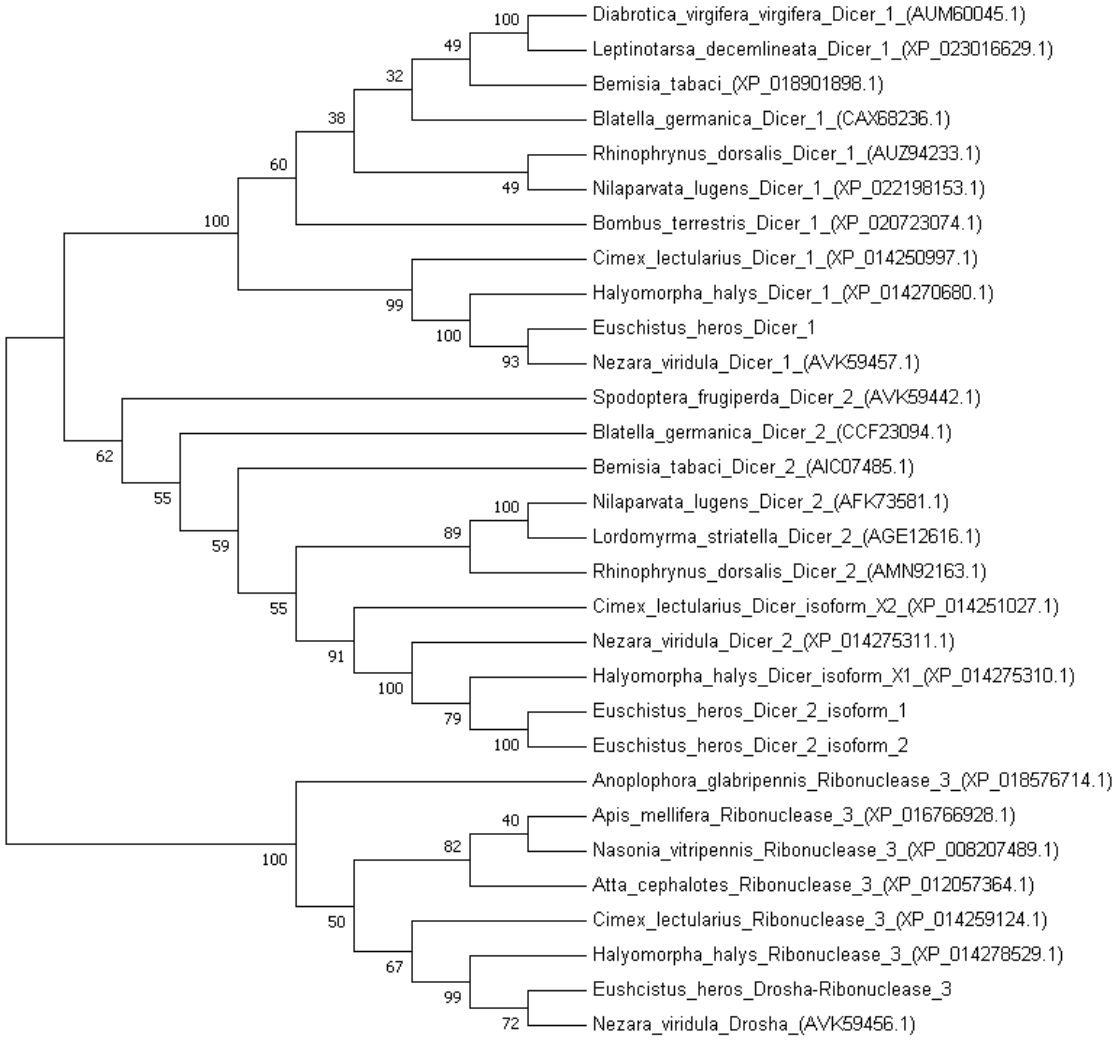


Fig. S1 - Phylogenetic tree of *Euschistus heros* Dicer 1 (DCR-1), Dicer 2 (DCR-2) and Drosha with the DCRs of other insect species. Proteins sequences were aligned using the MUSCLE and tested using the Neighbor-Joining Three. Numbers at each branch node represent the values calculated by bootstrap analysis (1,000 replications).

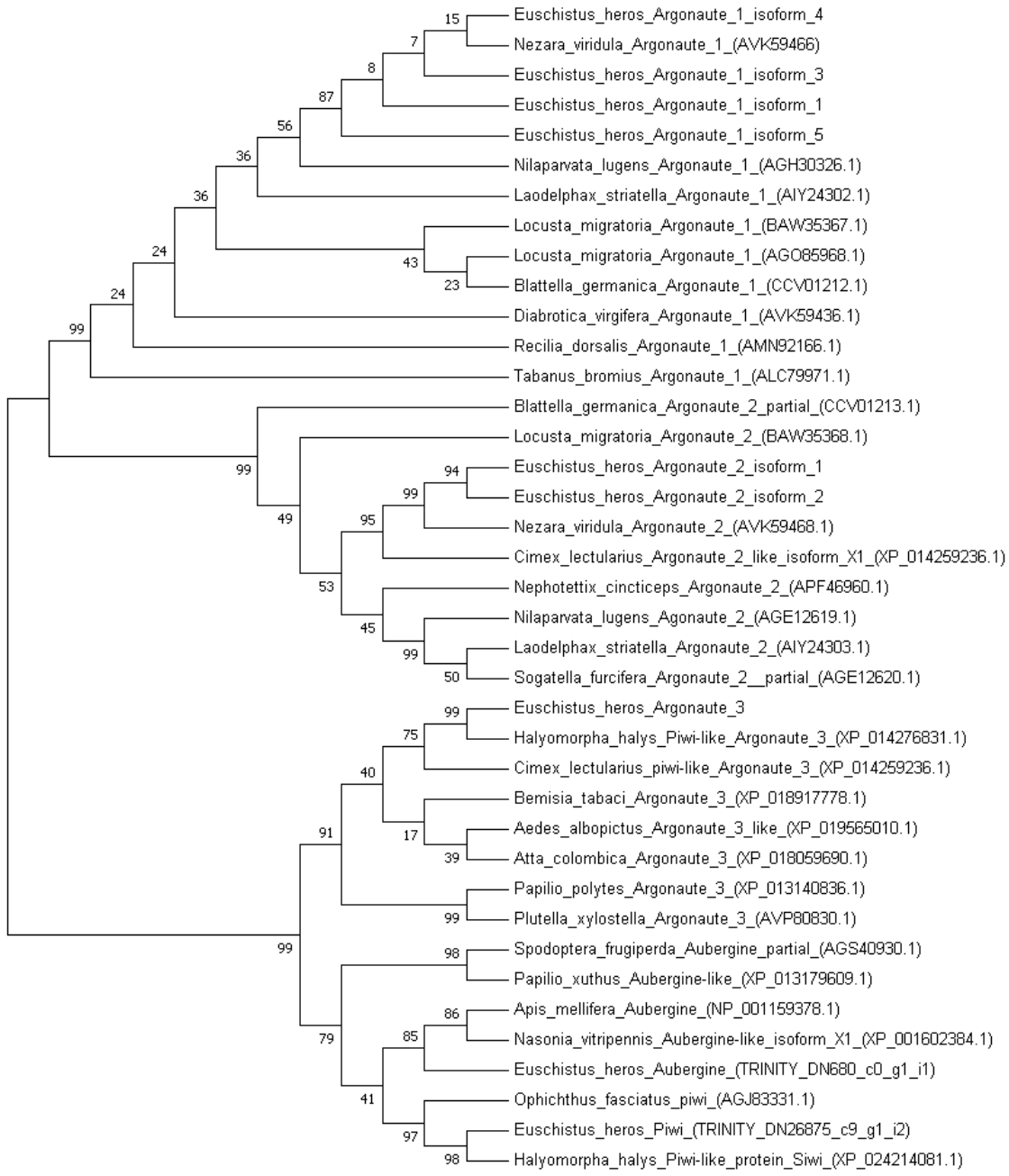


Fig. S2 - Phylogenetic tree of *Euschistus heros* Argonaute 1 (AGO-1), Argonaute 2 (AGO-2), Argonaute 3 (AGO-3), Aubergine (AUB) and Piwi with the AGOs of other insect species. Proteins sequences were aligned using the MUSCLE and tested using the Neighbor-Joining Three. Numbers at each branch node represent the values calculated by bootstrap analysis (1,000 replications).

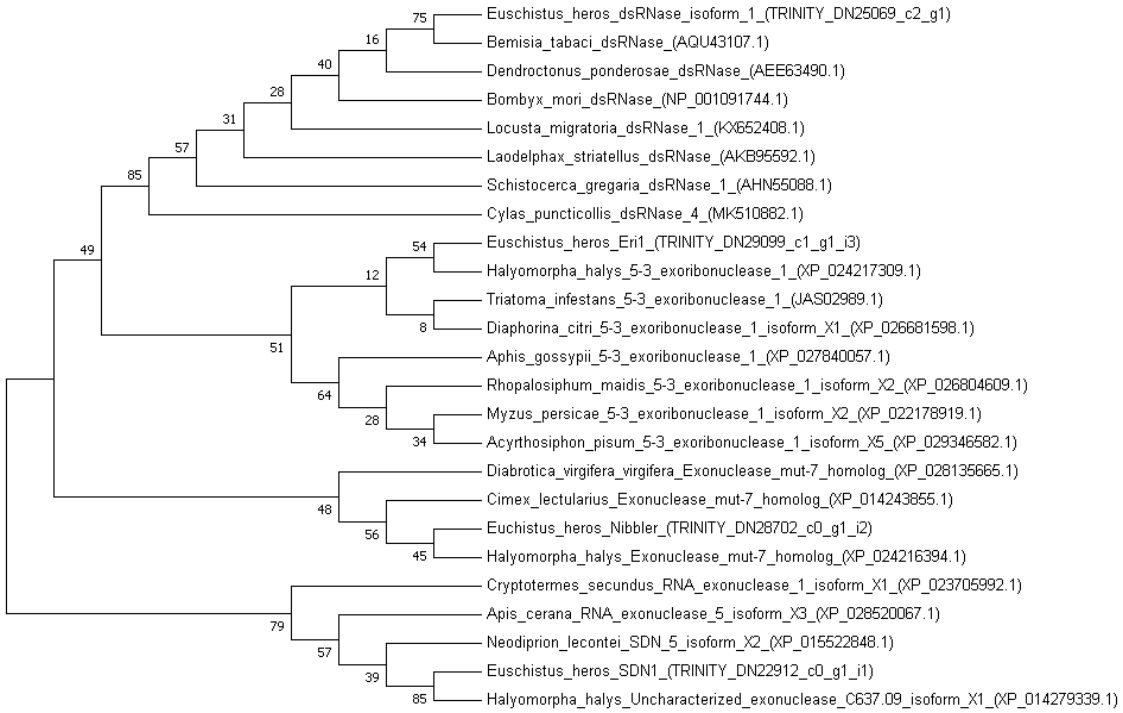


Fig. S3 - Phylogenetic tree of *Euschistus heros* nucleases, Eri-1, Nibbler, SDN1, and dsRNase with the nucleases of other insect species. Proteins sequences were aligned using the MUSCLE and tested using the Neighbor-Joining Three. Numbers at each branch node represent the values calculated by bootstrap analysis (1,000 replications).

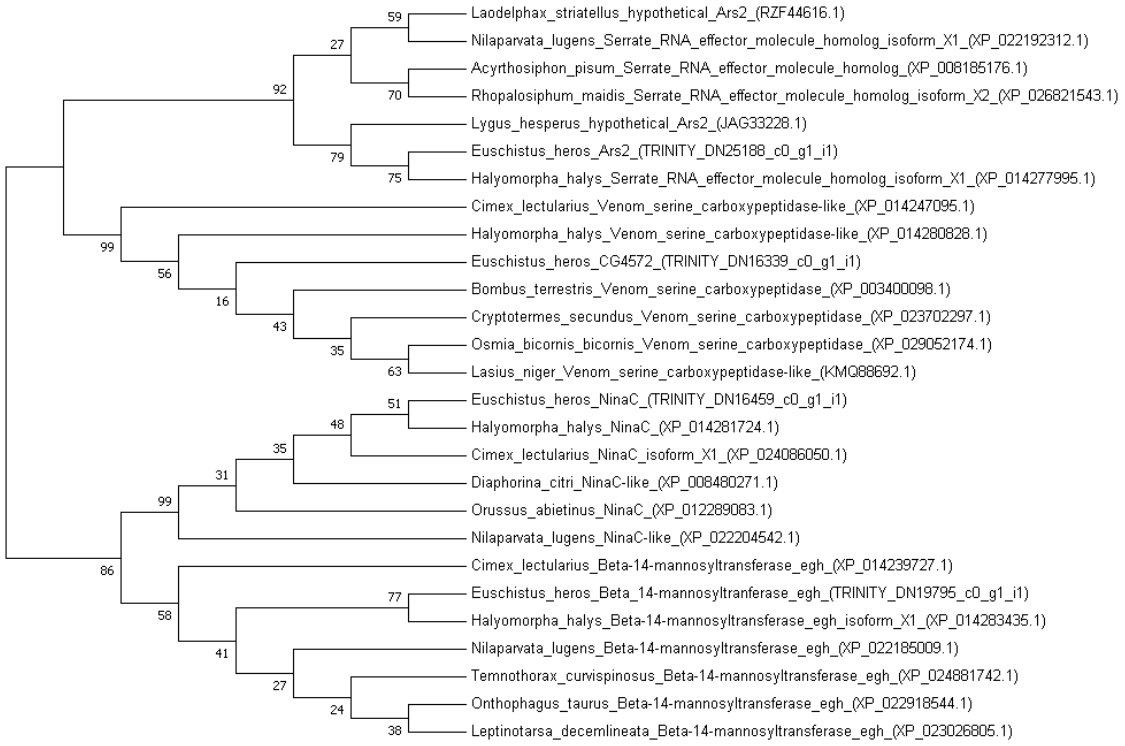


Fig. S4 - Phylogenetic tree of *Euschistus heros* antiviral RNAi proteins, Ars2, ninaC, egh, and CG4572 with the antiviral RNAi proteins of other insect species. Proteins sequences were aligned using the MUSCLE and tested using the Neighbor-Joining Three. Numbers at each branch node represent the values calculated by bootstrap analysis (1,000 replications).

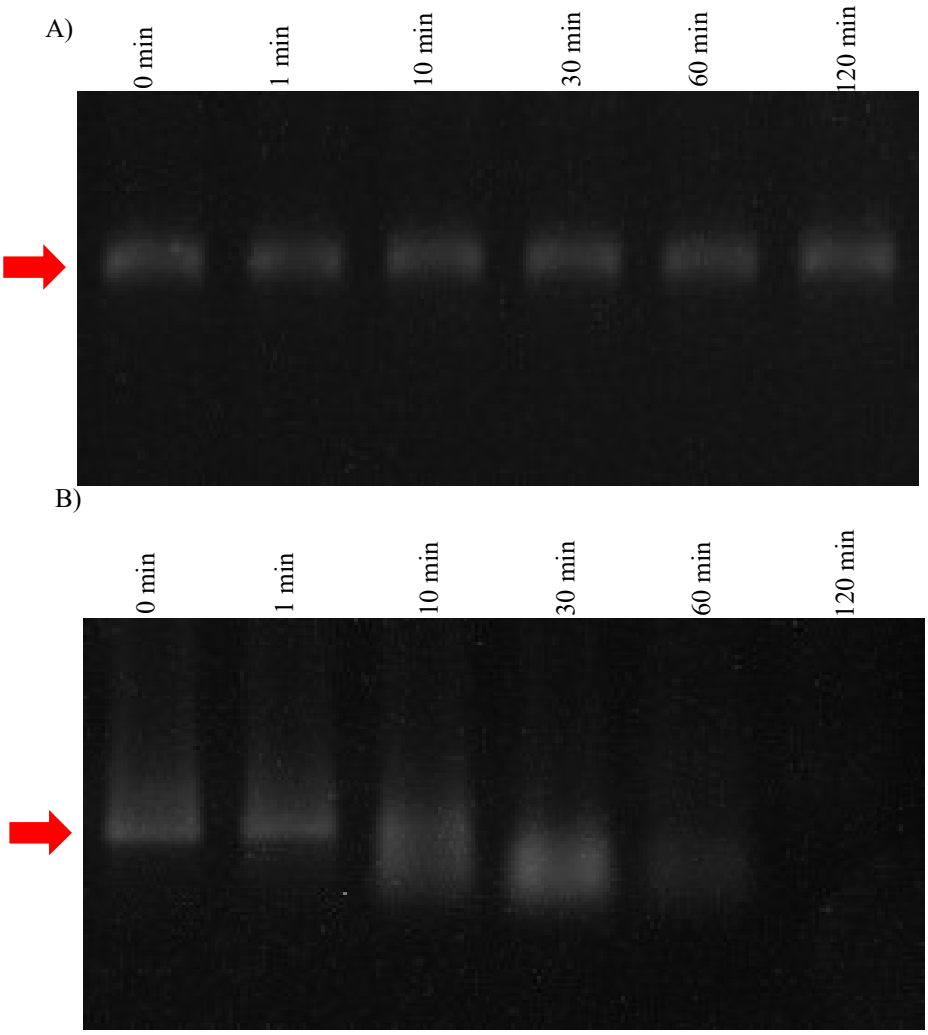


Fig. S5 - *Ex vivo* dsRNA degradation assay of different dsRNA formulations: (A) dsRNA-*V-ATPase-A* with water, (B) dsRNA-*V-ATPase-A* with hemolymph. The hemolymph of *E. heros* was extracted and incubated with 200 ng/µl of dsRNA-*V-ATPase-A* for different periods and run in 1 % agarose gel. The red arrow indicates the size of ~600 base pair.

3. Manuscript 2.

Management of pest insects and plant diseases by non-transformative RNAi.*

Deise Cagliari
Naymã P. Dias
Diogo Manzano Galdeano
Ericmar Ávila dos Santos
Guy Smagghe
Moisés João Zotti

* Manuscript published in Frontiers in Physiology

Management of Pest Insects and Plant Diseases by Non-transformative RNAi

Deise Cagliari[1*†], Naymã P. Dias[1†], Diogo Manzano Galdeano[2], Ericmar Ávila dos Santos[1], Guy Smagghe[3*]; Moisés João Zotti[1*]

[1]Laboratory of Molecular Entomology, Department of Crop Protection, Federal University of Pelotas, Pelotas, Brazil.

[2]Sylvio Moreira Citrus Center, Campinas Agronomic Institute (IAC), Cordeirópolis, São Paulo, Brazil

[3]Department of Plants and Crops, Ghent University, Ghent, Belgium.

***Correspondence:**
Deise Cagliari
deisycagliari@yahoo.com.br
Guy Smagghe
guy.smagghe@ugent.be
Moisés João Zotti
moises.zotti@ufpel.edu.br;

[†]These authors contributed equally to the work.

Keywords: RNAi, non-transgenic RNAi, RNA-based products, gene silencing, pest insects, plant diseases.

Abstract

Since the discovery of RNA interference (RNAi), scientists have made significant progress towards the development of this unique technology for crop protection. The RNAi mechanism works at the mRNA level by exploiting a sequence-dependent mode of action with high target specificity due the design of complementary dsRNA molecules, allowing growers to target pests more precisely compared to conventional agrochemicals. The delivery of RNAi through transgenic plants is now a reality with some products currently in the market. Conversely, it is also expected that more RNA-based products reach to the market as non-transformative alternatives. For instance, topically applied dsRNA/siRNA (SIGS – Spray Induced Gene Silencing) has attracted attention due to its feasibility and low-cost compared to transgenic plants. Once on the leaf surface, dsRNAs can move directly to target pest cells (*e.g.*, insects or pathogens) or indirectly being taken up by plant cells and then transferred into the pest cells. Water-soluble formulations containing pesticidal dsRNA provide alternatives, especially in some cases where plant transformation is not possible or takes years and cost millions to be developed (e.g., perennial crops). The ever-growing understanding of the RNAi mechanism and its limitations has allowed scientist to develop non-transgenic approaches such as trunk injection, soaking, and irrigation. While the technology has been considered promising for pest management, some issues such as RNAi efficiency, dsRNA degradation, environmental risk assessments, and resistance evolution still needs to be addressed. Here, our main goal was to review some possible strategies for non-transgenic delivery systems, addressing important issues related to the use of this technology.

Introduction

From the earliest days of agriculture, humankind cultivated the land to feed their descendants, allowing increase in population growth over the years. Now, thousands of years later, modern agriculture is facing one of its biggest challenge: How we are going to produce food in a profitable, efficient and sustainable way to feed about 10 billion people by 2050? Agricultural productivity has been facing several issues that limit crop production below maximum potential, namely damage by insects, diseases and competition with weeds. For instance, insects are responsible for 20 to 40% of yield loss (Oerke, 2006). Moreover, researchers expect 10 to 25% increase on insect damage per global temperature degree increment in the next years, with the main problems being in the temperate regions (Deutsch et al., 2018).

In an attempt to reduce the damage caused by pests, growers heavily rely on synthetic chemicals, which have been developed and applied since the 1930s. Pesticides allowed growers to increase production, improve product quality, and yield better profits. In 2012, growers around the world spent nearly $56 billion on pesticides, amounting to nearly 6 billion pounds of chemicals used in 2011 and 2012 (Atwood and Paisley-Jones, 2017). The high amount of chemicals used every year is leading to an increase in pesticide resistance, with a significant increase in resistance cases in insects (APRD 2019, https://www.pesticideresistance.org/search.php).

Modern agriculture is now entering the third green revolution, based on the significant progress in the use of reverse genetics to elucidate gene function and applying this knowledge in pest management. Major progress was made by Fire and Mello in 1998 by elucidating the gene silencing mechanism in eukaryotic organisms named as RNA interference (RNAi) (Fire et al. 1998). RNAi, also known as Post Transcriptional Gene Silencing (PTGS), is a natural mechanism of gene regulation and defense system against virus in eukaryotic cells (Baum and Roberts, 2014; Hannon, 2002) by degradation of the messenger RNA (mRNA), and reduction or complete elimination of the expression of a target gene (Fire et al., 1998).

Since the elucidation of the gene silencing mechanism in eukaryotic organisms, significant advances have been made related to the use of this technique in the management of insect pest (de Andrade and Hunter, 2016; Gordon and Waterhouse, 2007; Huvenne and Smagghe, 2010; Joga et al., 2016; Price and Gatehouse, 2008; San Miguel and Scott, 2016; Zhang et al., 2013; Zotti et al., 2017) and plant diseases (Fu et al., 2005; Jahan et al., 2015; Koch et al., 2013, 2016; Tiwari et al., 2017; Wang et al., 2016b, 2017). Recently, the development by Bayer and approval of SmartStax PRO maize carrying event MON87411 in Canada (2016) and the United States of America (USA) (2017) to control *Diabrotica virgifera virgifera* is considered a milestone in the use of RNAi technology in agriculture (Head et al., 2017). This technology is now available to growers as a tool for pest management. Delivery of double-stranded RNA (dsRNA) through this RNAi transformative approach (*i.e.,* transgenic plants) is a promising way to induce gene silencing in a specific pest (Baum and Roberts, 2014; Ghag, 2017), however it is not practical to every target organism or crop. Also, one of the key disadvantages of transgenic plants and seeds relies on regulatory approval, which takes years, and it is costly.

We are witnessing a constant decrease in the cost of dsRNA production together with an increased attraction from companies towards the development of improved dsRNA production techniques. Therefore it is believed that non-transformative RNAi soon will reach the market (Cagliari et al., 2018; Dubrovina and Kiselev, 2019; Mat Jalaluddin et al., 2018; San Miguel and Scott, 2016). However, some issues are still hindering the development of non-

transformative RNA-based products. In this paper, we aim to present the successful studies using non-transformative delivery systems, and discuss limitations and possible solutions.

RNAi mechanism: from RNA delivery to gene silencing

RNAi-based gene silencing can be triggered in the target organism by the supply of RNAs in two forms: (1) delivery of dsRNA molecules or (2) direct delivery of small RNAs (sRNAs). Currently, there are two major classes of sRNAs acting on the RNAi pathway: microRNAs (miRNAs) and small-interfering RNAs (siRNAs). MiRNAs are endogenously derived and involved in the regulation of gene expression, while siRNAs can be of exogenous origin from viruses or artificial supply (Preall; Sontheimer, 2005; Matranga; Zamore, 2007), or endogenous derived from transposons (Lippman; Martienssen, 2004; Golden; Gerbase; Sontheimer, 2008). It is known that in most cases, insects take up dsRNAs longer than 50 bp but not sRNAs (Feinberg and Hunter, 2003; Ivashuta et al., 2015; Saleh et al., 2006), although some studies have shown that sRNA could trigger gene silencing (Borgio, 2010; Gong et al., 2013). Differently, fungi and plants take up both dsRNAs and sRNAs (Koch et al., 2016; Wang et al., 2016b), suggesting that these organisms have a different uptake mechanism (Wang et al., 2017)(Wang et al., 2017)(Wang et al., 2017)(Wang et al., 2017)(Wang et al., 2017)(Wang et al., 2017).

Once RNA molecules are delivered in the field (*i.e.,* via transgenic plant, foliar spray, trunk injection), they need to enter the cell of target organism to trigger gene silencing. This process can occur through (a) direct or (b) indirect uptake (Figure 1). Direct uptake occurs when the RNA molecules are taken up through topical contact or feeding on plant tissues. In contrast, indirect uptake of RNA molecules involves first entering into the plant vascular system and then uptake by the insect/pathogen (Cagliari et al., 2018). The uptake process in the target pest is closely related to the delivery strategy, as demonstrated in several studies (Table 1).

Successful direct uptake via topical application has already been reported in different organisms (El-Shesheny et al., 2013; Killiny et al., 2014; Pridgeon et al., 2008). Zheng et al. (2019) reported that a dsRNA formulated in nanocarrier plus a detergent was able to cross the cuticle in *Aphis glycines*, leading to a reduction of 95.4% in gene expression. Also, indirect uptake of dsRNA has been reported in some insects (Ghosh et al., 2017) and pathogens (Koch et al., 2016). However, there are some limitations related to the indirect uptake process, such as efficiency of translocation of the RNA molecules inside the plant vascular system and dsRNA processing by the plant RNAi machinery. Although it is known that RNAs can move through the plant vascular systems and plant cells (Gogoi et al., 2017; Melnyk et al., 2011; Molnar et al., 2011), some results have shown inefficient translocation of these molecules inside the plant vascular system. For example, in *Malus domestica* and *Vitis vinifera* treated with dsRNA and siRNA, the RNA molecules spread from treated to non-treated tissues but were restricted to the xylem vessels (Dalakouras et al., 2018). This study also found that in *Nicotiana benthamiana*, siRNA molecules were not efficiently translocated. In pathogens, studies on gene silencing found evidence of external dsRNA processing into siRNAs (Koch et al., 2016; Konakalla et al., 2016; Mitter et al., 2017a). In *Hordeum vulgare*, dsRNA locally applied on detached leaves was taken up by plant cells, translocated through the vascular system and were processed into siRNAs by the plant Dicer enzyme, resulting in inhibition of *Fusarium graminearum* growth at local and distal unsprayed leaves (Koch et al., 2016). In this study, the dsRNA molecules were found in xylem and phloem parenchymal cells, companion cells, mesophyll cells, and in trichomes and stomata showing that the plant cells took up the dsRNAs. In citrus and grapevine

plants treated with dsRNA, siRNAs were found in plants up to three months after treatment, indicating that the dsRNA was processed by the plant RNAi machinery (Hunter et al., 2012).

In some organisms, the process of dsRNA uptake by the cells can be mediated by transmembrane channel proteins such as sid-1 (Aronstein et al., 2006; Feinberg and Hunter, 2003; Kobayashi et al., 2012) or endocytosis (Cappelle et al., 2016; Pinheiro et al., 2018; Saleh et al., 2006; Ulvila et al., 2006; Vélez and Fishilevich, 2018). Recently, in *Drosophila*, scientists elucidated the involvement of nanotube-like structures, which mediate cell-to-cell trafficking of sRNA and RNAi machinery components, allowing gene silencing in cells and tissues distant from the uptake point (Karlikow et al., 2016). However, the RNAs uptake system varies among insects, even within the same order (Vélez and Fishilevich, 2018), resulting in variations in the efficiency of gene silencing.

Although a number of RNAi pathways use dsRNAs to generate sRNAs (*i.e.* microRNA and siRNA) (Bernstein et al., 2001; Ketting, 2011), in insects and fungi the siRNA pathway is known to be activated due to dsRNA molecules or direct siRNA supply (Carthew, 2009; Zotti and Smagghe, 2015). Once inside the cell, dsRNAs are processed into siRNA fragments of ~20 base pairs (bp) in length by a ribonuclease III enzyme called Dicer 2 (DCR-2) (Meister and Tuschl, 2004; Tomari et al., 2007). The siRNA fragments are then incorporated into the RISC complex (RNA-induced Silencing Complex), which contains the Argonaute 2 (AGO-2) protein (Ketting, 2011; Matranga et al., 2005; Miyoshi et al., 2005). After unloading the non-incorporated passenger strand, the complex binds in a sequence-specific manner to the complementary messenger RNA (mRNA), cleaving it and preventing translation to protein (Agrawal et al., 2003; Huvenne and Smagghe, 2010).

Spread of the RNAi signal in the organism can be cell-autonomous or non-cell-autonomous (Huvenne and Smagghe, 2010; Whangbo and Hunter, 2008). In cell-autonomous RNAi, silencing effects are observed only in the cells directly exposed to the dsRNA (Huvenne and Smagghe, 2010). In contrast, in non-cell-autonomous RNAi the silencing effects are detected in exposed and non-exposed cells, even in different tissues (Whangbo and Hunter, 2008). Non-cell-autonomous RNAi is classified as environmental RNAi, a concept describing all processes in which dsRNA/siRNA are taken up from the environment by a tissue/cell, and spread from one cell to another or from one tissue type to another through systemic RNAi (Huvenne and Smagghe, 2010). In plants, fungi and the nematode *Caenorhabditis elegans*, the RNA-dependent RNA polymerase (RdRp) enzyme synthesizes secondary siRNAs by targeting single-stranded RNA molecules (ssRNA) and synthesizing a second strand, consequently generating dsRNA molecules and producing a systemic spread of the RNAi signaling (Zotti et al., 2017). The systemic nature of RNAi has already been observed in insects (Tomoyasu et al., 2008; Whyard et al., 2009; Wynant et al., 2012), however, the mechanism of systemic RNAi is still unknown in this group. What is known about this process so far is that the dsRNA/siRNA spread from cell to cell or tissues is highly dependent on the cell's ability to take up the dsRNA or siRNA molecules (Vélez and Fishilevich, 2018), or mediation through nanotube-like structures (Karlikow et al., 2016).

Why use non-transformative delivery strategies for pest management?

RNAi in crop protection can be achieved by plant-incorporated protectants (PIPs) through plant transformation (*i.e.,* transgenic plants) or by non-transformative strategies through a spray-induced gene silencing (SIGS) process (Table 2). Regardless of the delivery strategy, the use

of RNA-based products to confer plant protection against insects and pathogens is a potential alternative to conventional pesticides (Koch et al., 2016).

Currently, approved RNAi-based GM plants are based on ncRNA (non-coding RNA) to control insects (8%) and diseases (27%) or to improve specific plant traits (65%), with an increase of the approved events over the last years (Figure 2). In 2016, the first transgenic RNAi crop (SmartStax PRO maize) combining *Bt* (*Bacillus thuringiensis*) toxin with RNAi for insect control was released for cultivation in Canada, and a year later in the USA (Head et al., 2017). In general, the delivery of dsRNA in the field is facilitated by the use of GM plants, however, this strategy still cannot be adopted in all plants/crop, due to the high cost of production and the time for development. For instance, the commercial availability of "HoneySweet", a cultivar resistant to the *Plum pox virus* (PPV), took 20 years to reach the market (Scorza et al., 2013). Also, there are no established transformation protocols for most of the cultivated plants, which may cause a substantial delay in the development of RNAi-based GM plants (Mitter et al., 2017b). Therefore, alternative strategies for delivery of RNA biopesticides are necessary, providing alternative ways to use this technology in the field. Given that non-transgenic RNAi-based products would silence genes without introducing hereditary changes in the genome, it is expected that they will not be regulated as GM products, thereby reducing the time and processes for the release of use as well as potentially improving public acceptance (Cagliari et al., 2018).

Studies are being carried out prospecting non-transformative approaches to control insects, diseases, nematodes, and weeds, and it is expected that RNAi-based products will reach the market in the form of sprayable products for foliar application, trunk injection, root dipping or seed treatment as direct control agents (Berger and Laurent, 2019; Cagliari et al., 2018; Dubrovina and Kiselev, 2019; San Miguel and Scott, 2016; Zotti et al., 2017; Zotti and Smagghe, 2015). The RNA-based new generation of biopesticides could circumvent the technical limitation of plant transformation and the public's concerns about GM plants, providing an easy-to-use tool for crop production and storage as well as an environmentally friendly pest management strategy (Wang et al., 2017; Zotti et al., 2017). Furthermore, RNA-based biopesticides could be efficiently designed to target multiple insects or pathogen species.

The development of resistance is an important point regarding the use of non-transformative delivery strategies. Although dsRNAs longer than 200 nucleotides result in many siRNAs post-cleavage, maximizing the RNAi response and reducing resistance issues (de Andrade and Hunter, 2016), in transgenic plants there is a continuous supply of dsRNA, which increases the selection pressure and favors resistance development in the population. The development of RNAi resistance may be related to a reduction in cellular uptake (Khajuria et al., 2018), mutations in mRNA, production of RNAi suppressors (Zheng et al., 2005), upregulation of the target gene or downregulation of the silencing machinery genes (Garbutt and Reynolds, 2012), increased nuclease activity (dsRNases) (Spit et al., 2017) or even behavioral changes. However, when non-transformative delivery techniques are adopted, insects and pathogens have limited exposure to the dsRNA molecules due to the transient feature of such molecules, preventing the development of resistance in the target organisms.

Non-transformative delivery methods can be developed for use on several crops, targeting pests in different regions. Although GM event approval is more complicated, RNA-based non-transformative products will also undergo regulation procedures, though probably less complicated and time-consuming than GM plants. Also, an important aspect related to the legislation of non-transformative products is that RNA-based biopesticides probably will need

to be approved only in the producing country, unlike GM plants, which needs approval in both import and export countries.

Successful non-transformative delivery cases

Based on the advances made in the last decades regarding the use of RNAi in crop protection, it is believed that this technology will soon reach growers as dsRNA/siRNA-based products (Cagliari et al., 2018; Mat Jalaluddin et al., 2018). The application of RNAs targeting essential insect or fungi genes can significantly impair growth, increase mortality rate, and in some cases suppress insecticide/fungicide resistance (Killiny et al., 2014; Pridgeon et al., 2008). Although RNAi is not currently functional in every delivery method and every insect, life stage or target gene (San Miguel and Scott, 2016), this technology has great potential especially for insects and disease with high insecticide and fungicide resistance problems.

On the development of non-transformative delivery technologies, in 2011 Monsanto company published the patent WO 2011/112570 in which the company uses sprayable polynucleotide molecules to regulate gene expression in plants (Sammons et al., 2011). According to the patent, dsRNAs, siRNAs, and even single-stranded DNA oligonucleotides triggered efficient local and systemic silencing of *N. benthamiana* endogenous genes. However, in another experiment, researchers were unsuccessful in inducing gene silencing in plants through siRNA application, including spraying, syringe injection or siRNAs infiltration, yet they achieved success through high-pressure spraying of siRNAs (Dalakouras et al., 2016).

The delivery system varies according to the target organism and crop (Table 1). The selection of the delivery strategies (*i.e.,* foliar sprays, irrigation, trunk injection, baits, among others) is the first step to achieve good control results, determining the success of the technology usage. The correct choice of the delivery system will expedite the entire process and save years of development and commercialization (de Andrade and Hunter, 2016). Hence, the main non-transformative delivery methods and their applications in insect and disease management, shown in table 3, will be further discussed in the following sections.

Foliar application

For pests feeding/growing on stems, foliage or fruit/seeds foliar spraying may be an alternative for delivery of RNA molecules. Thus, the RNA-based formulations are evaluated similarly to topical insecticides where the RNA solution is sprayed on leaves, fed to the target insects, and the effects are observed (de Andrade and Hunter, 2016). Due to chemical properties of RNAs, a short half-life is expected compared to chemical pesticides, hence, sprayable RNAs would be an environmentally friendly alternative to synthetic pesticides (Fire and Won, 2013; Wang and Jin, 2017).

One of the first studies exploring applications of sprayable RNA molecules to control insect pest was conducted using siRNA molecules against the diamondback moth, *Plutella xylostella.* Mortality rates of ~60% were observed when larvae were fed with *Brassica* spp. leaves sprayed with chemically synthesized siRNAs targeting the *acetylcholine esterase* genes *AChE2* (Gong et al., 2013). In an attempt to control the Colorado potato beetle, *Leptinotarsa decemlineata,* foliar application of naked dsRNA targeting the *actin* gene was sufficiently stable for at least 28 days under greenhouse conditions, resulting in significant insect control (San Miguel and Scott, 2016). The same strategy was tested with the aim to control the xylem-feeding leafhopper (*Homalodisca vitripennis*), the phloem-feeding Asian citrus psyllid (*Diaphorina citri*) (Hunter et al., 2012) and the Diaprepes root weevil (*Diaprepes abbreviates)* on citrus leaves, showing

a promising alternative to control these insects (de Andrade and Hunter, 2016). In tomato leaves gently rubbed with dsRNA solution, the molecules were rapidly absorbed by tomato plants and were taken up by aphids (*Myzus persicae*), mites (*Tetranychus urticae*) and in fewer levels whiteflies (*Trialeurodes vaporariorum*) (Gogoi et al., 2017). Hence, siRNA molecules were only detected in tomato plants, aphids and mites being absent in the whiteflies, in which the dsRNA amounts did not reach the threshold necessary to induce RNAi machinery.

The use of RNAs in foliar application to manage pathogen infections and resistance in crops was also explored. In 2013, scientist discovered that Dicer-like protein 1 and 2 from *Botrytis* (Bc-DCL1; Bc-DCL2) fungus produces small RNAs (Bc-sRNAs), which are delivered into plant cells, silencing host immunity genes (Weiberg et al., 2013). Years later, researches applied siRNAs and dsRNAs targeting *Botrytis cinerea* DCL1 and DCL 2 (Bc-DCL1/2) on the surface of fruits (tomato, strawberry, and grape), vegetables (lettuce and onion) and flowers (roses), which resulted in a significant inhibition in grey mold disease development. In both cases, naked dsRNA/siRNA treatment was able to protect plants from the microbial pathogen for up to ten days after spraying. Moreover, these researchers showed that plants infected with another pathogen, *Verticillium dahlia,* displayed severe wilt disease symptoms, indicating that Bc-DCL1/2 RNAs were specific to *B. cinerea* DCL genes, not causing non-target effects (Wang et al., 2016b). In the same year, a breakthrough work showed the foliar application of dsRNA targeting the *cytochrome P450* (*CYP3*) gene in *F. graminearum,* resulting in successful inhibition of fungal growth in the local directly sprayed leaves as well as the non-sprayed distal leaves in barley plants (Koch et al., 2016). DsRNA foliar applications also conferred protection against *Sclerotinia sclerotiorum* and *B. cinerea* in *Brassica napus* (McLoughlin et al., 2018). Due to the relative ease of design, high specificity and applicability to a wide range of pathogens, the use of “RNA fungicides” as anti-fungal agents offers unprecedented potential as a new plant protection strategy and less harmful to the environment.

Furthermore, the use of RNA to target pathogen resistance to regular fungicides is also under development. Wheat plants sprayed with a dsRNA targeting the *Fusarium asiaticum myosin 5* gene, resulted in increased pathogen sensitivity to phenamacril with a reduction in infection (Song et al., 2018). Although dsRNA has a high specificity, it is also possible for dsRNA molecules to target a specific group. DsRNA molecules of a *β2-tubulin* gene derived from *F. asiaticum* suppressed the fungal activity of *F. asiaticum*, *B. cinerea*, *Magnaporthe oryzae* and *Colletotrichum truncatum* in wheat, cucumber, barley, and soybean, respectively (Gu et al., 2019). Alongside, the dsRNA molecule also functioned reducing the dosage of carbendazim (MBC) fungicide to control the pathogens. Thus, the combination of dsRNA and site-specific fungicide can be a control strategy against resistant pathogen infection in the field, rather than the individual use of dsRNA or fungicides.

Co-inoculation of synthesized dsRNA to protect plants against virus/viroid is effective at preventing virus infection in a range of plants through mechanical inoculation, increasing the prospect for foliar dsRNA application in virus management in plants (Carbonell et al., 2008; Konakalla et al., 2016; Šafářová et al., 2014; Tenllado and Díaz-Ruíz, 2001). Recently, Niehl *et al.* (2018) suggested the term “plants vaccines” citing the use of sprayable dsRNA to control the *Tobacco mosaic virus* (TMV) in tobacco, similarly to vaccines for animals that use dead or living (but weakened) microorganisms. These researchers used fragments of the virus genetic material to produce the "vaccines" (dsRNA) together with the plant's immune system as a defense mechanism. This system opens a range of opportunities for the use of RNAi in a non-transformative approach in the control of viruses in crops.

The potential applications of SIGS for plant protection have had significant improvement due to the recent advances in nanoparticle technology. To overcome problems related to dsRNA stability, a double-layered hydroxide (LDH) nanoparticle was developed and combined with dsRNA molecules to yield "BioClay" (Mitter et al., 2017b). The clay nanoparticles are positively charged and thus bind and protect the negatively charged dsRNAs; delivery occurs when atmospheric carbon dioxide and moisture reacts with the clay nanoparticles breaking the LDH and gradually releasing the dsRNAs. Using the dsRNA-LDH complex, researchers were able to achieve long-term gene silencing results by protecting tobacco plants from a virus for up to 20 days with a single spray, extending the period from five to seven days using naked dsRNA (Mitter et al., 2017b, 2017a). In another experiment, researchers sprayed tobacco and cowpea plants with BioClay nanosheets of dsRNA from the coat protein from the *Bean common mosaic virus* (BCMV) five days before exposure to viruliferous aphids (Worrall et al., 2019). The researchers found that BioClay molecules protected plants from BCMV infection due to aphid-mediated virus transmission, considered this an important step toward the development of a practical application of dsRNA in crop protection. These results using sprayable dsRNA are encouraging, and although more progress is needed on several fronts, RNA-based biopesticides are expected to reach the market soon. Monsanto is developing the use of RNAi through a technology called "BioDirect", in which dsRNA formulation is applied exogenously to protected plants against insect and pathogen attack (https://monsanto.com/innovations/agricultural-biologicals/). Syngenta scientists also are developing lines of biocontrol products based on RNAi to protect potato plants from the attack of Colorado potato beetle (https://www.youtube.com/embed/BiVZbAy4NHw?ecver=1). These technologies will help growers to improve pest control in crops resulting in increased yields and improved quality.

Trunk-injection

The use of trunk injection to deliver dsRNA to control insects has been tested and showed great progress, especially in perennial plants such as citrus. Developed citrus plants (2.5 meters tall) and grapevines were treated with 2 g of dsRNA in 15 L of water solution applied by root drench and injection into the trunk, and dsRNA was taken up into whole plant systems over three months. In citrus plants, the dsRNA was detected in the psyllid and the spittlebug from 5 to 8 days after entering the plants, allowing the development of pest suppression (Hunter et al., 2012).

Recently, researchers showed that hairpin RNAs (hpRNAs) and siRNAs delivered through petiole absorption or trunk injection to *M. domestica* and *V. vinifera* plants, were restricted to the xylem vessels and apoplast, being efficiently translocated (Dalakouras et al., 2018). Due to this characteristic, the plant Dicer-like (DCL) endonucleases were unable to process the hpRNA, hence, injected RNA molecules were detected in plants for at least ten days post-application. However, when siRNA was delivered to *N. benthamiana* through petiole absorption, the molecules were not efficiently translocated. These innovative methods may have significant impact in pest management against chewing or xylem sap-feeding insects and eukaryotic pathogens that reside in the xylem, allowing an essay reposition of the RNA-based solution and efficient plant protection for a longer period.

Irrigation

Hunter and collaborators showed that the dsRNA applied through root drench in adult citrus plants (2.5 m tall) could effectively control psyllids and leafhoppers for up to 57 days (Hunter

et al., 2012). They were able to detect the RNA molecules in the citrus plants for over three months. Rice plant roots soaked in a solution containing dsRNA targeting *carboxylesterase* (*Ces*) and *CYP18A1* genes from brown planthopper (BPH), *Nilaparvata lugens*, significantly knocked-down these genes, resulting in high mortality when BPH nymphs were fed on treated plants (Li et al., 2015). This study also showed maize seedlings irrigated with dsRNA of the Kunitz-type trypsin inhibitors (dsKTI) from Asian corn borer (ACB), *Ostrinia furnacalis,* resulted in high larval mortality rates. Recently, Ghosh and collaborators showed that *Halyomorpha hayls* nymphs fed on green beans soaked in dsRNA solution targeting *JHAMT* (*Juvenile hormone acid O-methyltransferas*) and *Vg* (*Vitellogenin*) genes, resulted in a significant reduction in gene expression, indicating that RNAi can be efficiently employed through vegetable delivery in plant-sap feeding insects. The delivery of gene silencing molecules through irrigation can be an alternative for crops that use irrigation in the normal growing system, allowing the continuous supply of RNA molecules. However, Dubelman et al. (2014) reported short persistence of dsRNA molecules in soil, with a rapid breakdown within 2–3 days. Therefore, the dsRNA stability in the soil is still an issue affecting RNAi efficiency (Joga et al., 2016) and the feasibility of this delivery strategy relies on the advances of formulations to protect RNA molecules from degradation.

Microbes-induced gene silencing

Many microbes such as virus, bacteria, yeast and fungi can be engineered to generate a vector for RNAi induction through the continuous production of dsRNA into the host, and this is being considered a promising dsRNA delivery method for insect and disease management (Cagliari et al., 2018; Dubrovina and Kiselev, 2019; Fjose et al., 2001; Goulin et al., 2019; Whitten et al., 2016).

Virus-induced gene silencing (VIGS) is a naturally occurring and very effective defense system consistent with the normal dynamics of host–pathogen interactions, which is widely harnessed as a powerful tool for the study of gene function in plants (Baulcombe, 2015; Lu et al., 2003; Ratcliff et al., 1997; Robertson, 2004; Waterhouse et al., 2001). VIGS is transiently transformative and does not cause alterations on the plant's genetic composition, unlike stable RNAi and mutant plants. Furthermore, VIGS can be transmitted to plant progeny and actively co-opts the plant for expression of dsRNA (Senthil-Kumar and Mysore, 2011). Moreover, VIGS enables high throughput screening of potential targets genes to control insect pest (Gu and Knipple, 2013; Kolliopoulou et al., 2017; Nandety et al., 2015). In Lepidoptera, three midgut-expressed *CYP* genes in *Manduca sexta* were targeted through the engineering of *Tobacco Rattle Virus* (TRV) for dsRNA delivery in *Nicotiana attenuata* (Kumar et al., 2012). Also, plant-virus based dsRNA delivery vectors are promising tools for targeting phloem-feeding insects because almost all plant-infecting viruses infect and move systemically via the phloem (Nandety et al., 2015). To demonstrate this, researchers used a recombinant TMV to express RNAi effectors in *N. benthamiana* plants against the citrus mealybug, *Planococcus citri*, and observed lower fecundity and pronounced death of crawlers after feeding on recombinant TMV-infected plants (Khan et al., 2013). Similarly, tomatillo (*Physalis philadelphica*) plants infected with recombinant TMV expressing RNAi effectors also resulted in a decrease in *Bactericera cockerelli* progeny production after feeding (Wuriyanghan and Falk, 2013). In another study, researchers engineered *Citrus tristeza virus* (CTV), a common virus of citrus, with *D. citri* truncated *abnormal wing disc* (*awd*) RNA sequence triggering *awd* gene silencing after *D. citri* nymphs fed on infected plants, causing wing malformation and mortality of adult insects (Hajeri et al., 2014). The *Potato virus X* (PVX) engineered with

Bursicon and *V-ATPase* genes sequences significantly reduced the population of cotton mealybug, *Phenacoccus solenopsis,* after insects fed on *Nicotiana tabacum* plants inoculated with the recombinant PVX (Khan et al., 2018). Furthermore, insect-specific viruses can be exploited as VIGS vectors to control insect pests (Kolliopoulou et al., 2017; Nouri et al., 2018). For instance, researchers investigated the ability of engineered *Flock house virus* (FHV) to induce gene suppression through RNAi in S2 cells derived from *D. melanogaster* embryos and insects at the adult stage. The recombinant FHV carrying the target gene sequences caused significantly higher mortality (60–73% and 100%) than the wild type virus (24 and 71%), in both S2 cells and adult insects, respectively (Taning et al., 2018).

To date, the sources of RNA-based molecules (dsRNA or siRNA) commonly utilized in insect and disease management studies are costly synthetic molecules or are produced through time-consuming, laborious procedures. To overcome the shortages of these methods, the potential of delivering dsRNA expressed in bacteria has been investigated, providing an alternative method for large-scale target genes screening (de Andrade and Hunter, 2016; Zotti et al., 2017). In Lepidoptera, the cotton bollworm (*Helicoverpa armigera*) larvae exposed to artificial diet coated with engineered bacteria for five days showed high mortality and inhibition in the expression levels of target genes, causing drastic reductions in body weight, body length and pupation rate (Ai et al., 2018). Oral toxicity of *E. coli* expressing dsRNA targeting the *integrin β1 subunit*, was observed in *Spodoptera exigua* larvae, resulting in insect mortality, damage in the midgut epithelium tissue, exhibiting a marked loss of cell-cell contact and underwent remarkable cell death, resulting in increased susceptibility to a Cry insecticidal protein from *B. thuringiensis* (Kim et al., 2015). Also, the growth and development of *S. exigua* larvae fed with *E. coli* expressing dsRNA targeting *chitin synthase A* was disturbed, resulting in mortality (Tian et al., 2009). Moreover, in the gypsy moth, *Lymantria dispar*, a serious insect pest of the North American forests, bacterial expression of dsRNA resulted in target-gene knockdown and subsequent reduction in body mass and egg masses (Ghosh and Gundersen-Rindal, 2017). In the oriental armyworm, *Mythimna separata*, a study showed that oral delivery of bacterially expressed dsRNA led to RNAi effects, with knockdown of target genes, reduction of body weight and increased mortality (Ganbaatar et al., 2017). In Diptera, *Bactrocera dorsalis* adults fed an artificial diet coated with *E. coli* expressing dsRNA, exhibited reduction in target genes mRNA levels and reduction in egg-laying (Li et al., 2011). In Coleoptera, the potential of feeding dsRNA expressed in bacteria to manage populations of Colorado potato beetle, *L. decemlineata*, was observed by the knockdown of five target genes tested, causing significant mortality and reduced body weight gain in treated beetles (Zhu et al., 2011).

Besides the use of bacteria as dsRNA delivery method to pests, these microorganisms have being used to produce large amounts of dsRNAs and it can be sprayed on crops at any time with lower costs (Joga et al., 2016). For example, the *Escherichia coli* HT115 (DE3) strain has been used to produce large amounts of dsRNA since it lacks the enzyme which degrades dsRNAs (Ahn et al., 2019; Papic et al., 2018). Also, studies have shown the efficiency of dsRNA produced in bacteria to control plant viruses (Mitter et al., 2017b; Robinson et al., 2014). Crude extracts of *E. coli* HT115 containing dsRNA targeting the *Sugarcane mosaic virus* (SCMV) *coat protein* gene were applied in maize plants as a preventive spray and inhibited the SCMV infection (Gan et al., 2010). Other works reported the use of bacteria to produce dsRNAs from *Pepper mild mottle virus* (PMMoV), PPV and TMV to protect plants against these pathogens. The application of bacterial crude preparation via spray onto tobacco plants surfaces provided protection against infection by this virus (Tenllado et al., 2003; Yin et al., 2009). Moreover, this system of dsRNA production in bacteria can deliver multiple virus dsRNAs to disrupt

several virus species at once and may achieve multiple virus resistances at one time (Tenllado and Díaz-Ruíz, 2001; Yin et al., 2009).

Recently, the advances in sequencing technology and the characterization of insect gut microbiota are leading to the identification of novel symbiotic microorganisms suitable to be genetically modified and used as dsRNA delivery vectors to control insects (Krishnan et al., 2014). Symbiont-mediated RNAi is an intriguing strategy in which the relationship between culturable symbiotic gut bacteria or yeast and the hosts can be exploited in order to constitutively produce dsRNA to induce RNAi in the host and the use of symbiotic bacteria has been shown to be a promising delivery strategy to control insects (Abrieux and Chiu, 2016; Joga et al., 2016; Whitten and Dyson, 2017). Also, dsRNA can be delivered into target pests through infection of entomopathogenic fungus and may result in the development of a new RNAi methodology for pest control. For instance, the application of *Isaria fumosorosea*, a common fungal pathogen of the B-biotype *Bemisia tabaci*, expressing dsRNA of whitefly immunity-related gene, resulted in knockdown of the target gene and increased whitefly mortality (Chen et al., 2015).

Although viruses and bacteria after genetic modification to express dsRNA and induce gene silencing, are promising strategies to deliver dsRNA in the field, they will be considered as GM products and will suffer the same regulatory and public acceptance downsides as GM crops.

Other applications

In relation to the natural role of RNAi to protect cells from virus infections, this technology could be used to protect beneficial insects, such as bees, from viral diseases. In 2010, large-scale field trials tested the efficiency of Rembee™ (Beeologics, LLC, Miami, FL, USA), a dsRNA product designed to protect honeybees (*Apis mellifera*) from *Israeli acute paralysis virus* (IAPV) infection (Hunter et al., 2010). The product successfully protected the hives from the virus infection, resulting in several bees twice as large in the dsRNA-treated hives compared to untreated. As a result, dsRNA-treated hives produced 3-fold more honey compared to untreated ones. In another study, a similar result was observed in bumblebees (*Bombus terrestris*), which upon feeding of IAPV virus-specific dsRNAs showed decreased mortality (Piot et al., 2015). In other studies carried in *A. mellifera*, RNAi was also efficient against the internal microsporidian parasites *Nosema* (Paldi et al., 2010; Rodríguez-García et al., 2018) and the obligatory ectoparasite *Varroa destructor* (Garbian et al., 2012). The control of these organisms associated with colony decline improved the health of hives and shines a light on the development of effective treatment alternatives for diseases of bees and other beneficial insects in the future.

Issues involving non-transformative delivery approaches

In the near future, the exogenous application of RNA molecules to induce RNAi-mediated gene silencing will influence the traditional way we protect crops from insects and pathogens. Due to uptake restrictions, it is believed that the development of RNA-based products will focus on the use of dsRNA as the molecule to induce gene silencing (Sammons et al., 2011). The minimal required length of a dsRNA to achieve an RNAi effect will vary depending on target genes and species (Bolognesi et al., 2012). Consequently, the formulations can contain only one dsRNA molecule or be a combination of short and long dsRNAs targeting one or more genes, or yet be a combination of dsRNA and insecticide or fungicide, managing resistant population and reaching better results.

Under field conditions, RNA-based biopesticides would need periodical applications following plant growth to ensure plant protection. Also, while the RNA-based products are a new highly specific mode of action, the timing issues of "when should I spray?", a question that growers already have with current chemical control approaches, is also something that needs to be studied and understood. Although the vascular system of plants translocate RNAs (Melnyk et al., 2011) allowing RNA molecules to travel through long distances inside the plant protecting untreated areas, the necessity of reapplication implies an increase in cost. Thus, it is expected that with the use of non-transformative strategies to control insects and pathogens, the dsRNA molecule will remain active long enough to effectively control the target pest. Moreover, although selection of the most effective target gene is desirable, even partial suppression can cause severe damage and irreversible lethal effects (Huvenne and Smagghe, 2010). Transient effects of this technique should not be an overwhelming drawback to the use of non-transformative approaches. Alongside, the development of more efficient dsRNA mass production systems will reduce the costs and together with the release of new formulation strategies will allow foliar spray, trunk injection, irrigation among other approaches to be exploited as potential control strategies (de Andrade and Hunter, 2016; Hunter et al., 2012).

DsRNA production costs have been dropping significantly over the last years from ~ $12,500 USD per gram in 2008 to less than $60 USD per gram in 2018 (Cagliari et al., 2018), with an expectation of a further significant reduction in prices in the next years. Mass dsRNA production systems such as *in vitro* or *in vivo* production systems allow high dsRNA production with the reduction in costs. These are strategies based on the hybridization of two single-stranded RNAs (ssRNAs) enzymatically synthesized, which can be performed *in vitro* (Koch et al., 2016; Konakalla et al., 2016; Tenllado and Díaz-Ruíz, 2001; Wang et al., 2016b) or *in vivo* (bacterial cells deficient of enzyme RNase III that degrades dsRNAs) (Gan et al., 2010; Tenllado et al., 2003). Although an *in vivo* system allows the production of bulk amounts of dsRNA compared to *in vitro* synthesis, it still runs under a high cost, hard purification and high labor demand (AgroRNA, http://www.agrorna.com/sub_02.html), and after all, is still naked dsRNA, that under field condition presents a shorter half-life. Thus, dsRNA formulation is a promising alternative to increase stability and boost the efficiency of gene-silencing in recalcitrant species in Lepidoptera and Hemiptera, allowing plants to be protected for longer time.

The technology called "BioClay", a layered double hydroxide (LDH) clay nanosheet, provided high dsRNA stability under field conditions, increasing the residual period of dsRNA on plants and protecting them from virus infection for up to 30 days compared to naked dsRNA (Mitter et al., 2017a). Guanylate Polymers increased RNAi efficiency in *S. exigua* (Christiaens et al., 2018b) and *Spodoptera frugiperda* (Parsons et al., 2018), and pave the way for future applications of RNA-based pest control strategies in lepidopteran insects. This technology is based on the use of formulations to enhance stability of the dsRNA in insects. Encapsulation of dsRNA molecules in liposome complexes also increased the dsRNA stability and enhanced cellular uptake in Dipteran insects (Taning et al., 2016; Whyard et al., 2009) and Blattodea (Lin et al., 2017). In *Euschistus heros*, liposome complexes increased nymph mortality compared to naked dsRNA (Castellanos et al., 2018). However, in some cases, even with the use of formulation the dsRNA molecules were unable to initiate the RNAi process. This was the case in the migratory locust, *Locusta migratoria*, where liposome encapsulation was not efficient to protect the dsRNA, leading to inefficient RNAi in this species (Luo et al., 2013).

Considering the hostile environmental conditions to which dsRNA molecules are exposed in the field, a biotechnology company called RNAagri (former APSE) developed a system where

APSE RNA Containers (ARCs) are produced by *E. coli* bacteria, allowing the mass production of encapsulated ready-to-spray dsRNA (APSE technology; www.apsellc.com). This technology is based on bacteria engineered with a plasmid to produce naturally occurring proteins such as capsids, which are then co-transformed with another plasmid coding for the target dsRNA or siRNA together with a sequence called the "packing site". The double-transformed *E. coli* are then purified, resulting in self-assemble particles which have encapsulated the desired RNAs. These particles protect the RNAs and enhance resistance to adverse environmental conditions, and once sprayed, they are expected to be rapidly taken up by the insect (Kolliopoulou et al., 2017). The development of formulations to carry dsRNA efficiently up to the target organism is of paramount importance for the success in developing non-transformative strategies for pest control, and advances in this area in the future will boost the use of these strategies.

Successful cases using foliar spray, irrigation, and trunk injection have already been reported (Table 3), but the application range may be much broader. The selection of the dsRNA delivery strategy is of great importance in the development of non-transformative delivery methods, and it will vary according to the target pest and crop. RNAi efficiency naturally varies among the target species, life stage and delivery strategy, and the choice of a correct combination of these factors will save years of research and resources. Regardless of the delivery strategy or target species, for a successful non-transformative RNAi strategy it is also of paramount importance to identify unique regions in essential target genes, so that little changes in expression level will provoke severe consequences. For example, foliar application of dsRNA was unable to induce the RNAi machinery in *T. vaporariorum* due to the low dsRNA uptake by the insects (Gogoi et al., 2017). In order to achieve success using RNAi-based gene silencing as a control strategy, low amounts of RNA molecules need to be enough to trigger the machinery, leading to insect or pathogen mortality. In insects, screening for target genes through artificial diet containing dsRNA is an easy procedure to screen large numbers of dsRNA molecules, resembling field conditions (Araujo et al., 2007; Aronstein et al., 2011; Whyard et al., 2009) and addressing important issues such as better target genes, effective dsRNA and effective lethal concentration (LC50) (Araujo et al., 2007; Bachman et al., 2013; Baum et al., 2007). However, under field conditions it is difficult to establish the dsRNA amount uptaken by the target pest, hindering determination of the LC50.

Coleopteran insects are considered very susceptible to RNAi (Baum et al., 2007; Baum and Roberts, 2014), while insects in the order Lepidoptera are considered recalcitrant and high dsRNA concentrations are required to achieve successful gene silencing results (Terenius et al., 2011). Limiting factors, such as dsRNA degradation (Guan et al., 2018; Wang et al., 2016a) and entrapment of internalized dsRNA in endosomes (Yoon et al., 2017), have recently been associated with unsuccessful RNAi (Niu et al., 2018). In some hemipteran insects, such as *Acyrthosiphon pisum*, the lack of response under dsRNA supply is also associated with high nuclease activity (Christiaens et al., 2014). Thus, we believe significant advances in dsRNA formulation will occur in the next years, and so the development of RNA-based non-transformative products will be focused on non-recalcitrant groups.

Another important point in the use of non-transformative strategies for RNA delivery, mainly via foliar application, is that during the application, not only the target pest will receive the RNA molecules, but also non-target insects. In GM plants, researchers have shown that expressed dsRNA has a high degree of specificity to control insects (Dillin, 2003; Petrick et al., 2013; Whyard et al., 2009) or pathogens (Koch et al., 2013). However, other studies have shown that siRNAs can knockdown non-target genes (Birmingham et al., 2006). In mammals, studies

have shown that even with differences between the nucleotides sequences from siRNA and the target mRNA, gene silencing still occurs (Huang et al., 2009; Jackson et al., 2003; Schwarz et al., 2006). However, there is no consensus among scientists on the number of nucleotides from the siRNA that must match the target sequence identically, and more research is needed to determine if the same issues found in mammalian cells apply to other organisms such as insects or pathogens (Christiaens et al., 2018a). Therefore, the target region and the dsRNA molecule design are very important. Baum et al. (2007) tested the specificity of dsRNA molecules based on the identity of the nucleotide sequence of the *V-ATPase* gene *subunits A* and *E* between *D. v. virgifera* and *L. decemlineata*. The target sequences of the *V-ATPase subunit A* shared 83% identity, while the target sequences of the V-ATPase E subunit of these insects shared 79% identity. Feeding both *D. v. virgifera* and *L. decemlineata* with the non-specific dsRNAs caused mortality in both species (Baum et al., 2007). However, researchers already expected this response, since most of the ~ 21 nt siRNAs obtained had similarity to both species, causing non-specific silencing. GM tobacco plants expressing a dsRNA targeting the *EcR* gene in *H. armigera*, were also effective against another lepidopteran pest, *S. exigua* (Zhu et al., 2012). The target sequence of both species had high similarity in the nucleotides sequences (89%), and when both species fed on the GM tobacco plants, this resulted in mortality levels between 40-50%. However, when the necessary care at the time of dsRNA design is taken, it is possible to obtain extremely specific or broad range molecules. To show the specificity of dsRNA-based gene silencing, the molecules were designed to target the *V-ATPase* gene in four different species, *D. melanogaster* (Diptera), *Tribolium castaneum* (Coleoptera), *A. pisum* (Hemiptera) and *M. sexta* (Lepidoptera), resulting in target gene silencing with no effects over non-target species (Whyard et al., 2009). They also demonstrated the feasibility of designing specific dsRNA molecules even within species from the same genus. Hence, the design of the dsRNA will determine the action spectrum of the molecules, and not necessarily molecules with a larger action spectrum are harmful. If carefully designed, broad-spectrum RNA-based molecules can be used to protect plants against diverse insects and pathogens.

Perspectives in a global view

During the last decade, significant advances have been made in an attempt to find better ways to control insects and pathogens in crops, reducing environmental impacts, and improving profits. Scientists have harnessed technologies such as RNAi-based gene silencing to turn off essential genes in target organisms, leading to mortality. Studies using foliar applications, trunk injection, and irrigation have demonstrated the feasibility and efficacy of RNAi-based gene silencing through non-transformative delivery strategies (Table 3). Other delivery methods still need to be investigated, such as seed coats or baits. To our knowledge, no studies for development of RNA-based products as seed coat or powder/granules formulations are available. While the main objective of the seed coat is to protect plants from the attack of insects and pathogens during the initial growth phase, powder/granules formulations could be applied on the soil or substrate surface. Similarly, the use of baits (spray or station) containing RNA is a promising non-transformative delivery strategy that could be developed for pest control, especially in orchards. The bait spray can consist of an attractant mixed with a specific RNA, while bait stations can be containers with sRNA molecules and attractants which will attract the pest to the bait. These are techniques that can be further explored in the use of RNAi in crop protection.

RNA biopesticides are compounds naturally occurring in the environment and inside organisms, thus are potentially less harmful than synthetic pesticides. These molecules are

naturally internalized by eukaryotic organisms, being subject to RNAi pathways and are degraded by natural cellular processes. Also, dsRNAs are rapidly degraded when present in water or soil (Albright III et al., 2017; Dubelman et al., 2014; Fischer et al., 2017; Parker et al., 2019), reducing the chances to leave residues in the environment or food products. As with any control method, targeted insects, pathogens and viruses can develop resistance.

The use of genomic tools will allow the development of technologies such as RNA-based products to increase crop resistance against insects, pathogens, and viruses. Also, the development of RNA formulations will improve RNAi efficiency and field stability. So, these could even replace chemical pesticides in some applications or when in combination, reducing the use of chemical pesticides at least.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

DC, NPD, GS, and MJZ contributed to the conception of the manuscript. DC and NPD wrote the first draft. DC, NPD, DMG, EAS, GS, and MJZ wrote sections of the manuscript. GS and MJZ revised and edited the manuscript. All authors read, contributed critically to the drafts, and approved the final version.

Funding

DC is a recipient of a scholarship (140733/2017-5) form the National Council for Scientific and Technological Development (CNPq) in Brazil. The Foundation Research-Flanders (FWO-Vlaanderen) in Belgium, and the EUCLID project (No. 633999) and the COST (European Cooperation in Science and Technology) under grant agreement No. CA15223 also supported this work.

Acknowledgments

The authors thank Dr. Juan Luis Jurat-Fuentes (The University of Tennessee, USA) for English text editing.

Reference

Abrieux, A., and Chiu, J. C. (2016). Oral delivery of dsRNA by microbes: Beyond pest control. *Commun. Integr. Biol.* 9, 1–4. doi:10.1080/19420889.2016.1236163.

Agrawal, N., Dasaradhi, P. V. N., Mohammed, A., Malhotra, P., Bhatnagar, R. K., and Mukherjee, S. K. (2003). RNA Interference: Biology, Mechanism, and Applications. *Microbiol. Mol. Biol. Rev.* 67, 657–685. doi:10.1128/MMBR.67.4.657.

Ahn, S., Donahue, K., Koh, Y., Martin, R. R., and Choi, M. (2019). Microbial-Based Double-Stranded RNA Production to Develop Cost-Effective RNA Interference Application for Insect Pest Management. *Int. J. Insect Sci.* 11, 1–8. doi:10.1177/1179543319840323.

669 Ai, X., Wei, Y., Huang, L., Zhao, J., Wang, Y., and Liu, X. (2018). Developmental control of
670 *Helicoverpa armigera* by ingestion of bacteria expressing dsRNA targeting an arginine
671 kinase gene. *Biocontrol Sci. Technol.* 28, 253–267.
672 doi:10.1080/09583157.2018.1441368.

673 Albright III, V. C., Wong, C. R., Hellmich, R. L., and Coats, J. R. (2017). Dissipation of
674 double-stranded RNA in aquatic microcosms. *Environ. Toxicol. Chem.* 36, 1249–1253.
675 doi:10.1002/etc.3648.

676 Araujo, R. N., Santos, A., Pinto, F. S., Gontijo, N. F., Lehane, M. J., and Pereira, M. H.
677 (2007). RNA Interference of the Salivary Gland Nitrophorin 2 in the Triatomine Bug
678 *Rhodnius Prolixus* (Hemiptera : Reduviidae) by dsRNA Ingestion or Injection. *Insect*
679 *Biochem. Mol. Biol.* 36, 683–693. doi:10.1016/j.ibmb.2006.05.012.RNA.

680 Aronstein, K., Oppert, B., and Lorenzen, M. D. "RNAi in Agriculturally-Important
681 Arthropods," In: Grabowski P. editor. RNA Processing. IntechOpen (2011). p. 157–180.

682 Aronstein, K., Pankiw, T., and Saldivar, E. (2006). SID-I is implicated in systemic gene
683 silencing in the honey bee. *J. Apic. Res.* 45, 20–24.
684 doi:10.1080/00218839.2006.11101307.

685 Atwood, D., and Paisley-Jones, C. (2017). Pesticides Industry Sales and Usage 2008-2012
686 Estimates.

687 Bachman, P. M., Bolognesi, R., Moar, W. J., Mueller, G. M., Paradise, M. S., Ramaseshadri,
688 P., et al. (2013). Characterization of the spectrum of insecticidal activity of a double-
689 stranded RNA with targeted activity against Western Corn Rootworm (*Diabrotica*
690 *virgifera virgifera* LeConte). *Transgenic Res.* 22, 1207–1222. doi:10.1007/s11248-013-
691 9716-5.

692 Baulcombe, D. C. (2015). VIGS, HIGS and FIGS: Small RNA silencing in the interactions of
693 viruses or filamentous organisms with their plant hosts. *Curr. Opin. Plant Biol.* 26, 141–
694 146. doi:10.1016/j.pbi.2015.06.007.

695 Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., et al. (2007).
696 Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 25,
697 1322–1326.

698 Baum, J. A., and Roberts, J. K. "Progress Towards RNAi-Mediated Insect Pest
699 Management,"In: Dhadialla T. S. and Gill S. S. editors. Advances in Insect Physiology
700 (2014). p. 249-295 doi: 10.1016/B978-0-12-800197-4.00005-1.

701 Berger, C., and Laurent, F. (2019). Trunk injection of plant protection products to protect
702 trees from pests and diseases. *Crop Prot.* 124, 104831.
703 doi:10.1016/j.cropro.2019.05.025.

704 Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate
705 ribonuclease in the initiation step of RNA interference.pdf. *Nature* 409, 363–366.
706 doi:10.1038/35053110.

Birmingham, A., Anderson, E. M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., et al. (2006). 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat. Methods* 3, 199–204. doi:10.1038/nmeth854.

Bolognesi, R., Ramaseshadri, P., Anderson, J., Bachman, P., Clinton, W., Flannagan, R., et al. (2012). Characterizing the Mechanism of Action of Double-Stranded RNA Activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). *PLoS One* 7, e47534. doi:10.1371/journal.pone.0047534.

Borgio, J. F. (2010). RNAi mediated gene knockdown in sucking and chewing insect pests. *J. Biopestic.* 3, 386–393.

Cagliari, D., Santos, E. A. dos, Dias, N., Smagghe, G., and Zotti, M. "Nontransformative Strategies for RNAi in Crop Protection," In: Singh A.and Khan M. W. editors. Modulating Gene Expression - Abridging the RNAi and CRISPR-Cas9 Technologies, IntechOpen (2018).p. 1–18. doi:10.5772/32009.

Cappelle, K., De Oliveira, C. F. R., Van Eynde, B., Christiaens, O., and Smagghe, G. (2016). The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. *Insect Mol. Biol.* 25, 315–323. doi:10.1111/imb.12222.

Carbonell, A., Martínez de Alba, Á. E., Flores, R., and Gago, S. (2008). Double-stranded RNA interferes in a sequence-specific manner with the infection of representative members of the two viroid families. *Virology* 371, 44–53. doi:10.1016/j.virol.2007.09.031.

Carthew, R. W. S. J. E. (2009). Origins and Mechanisms of miRNAs and siRNAs. *Natl. Institutes Heal.* 136, 642–655. doi:10.1016/j.cell.2009.01.035.Origins.

Castellanos, N. L., Smagghe, G., Sharma, R., Oliveira, E. E., and Christiaens, O. (2018). Liposome encapsulation and EDTA formulation of dsRNA targeting essential genes increase oral RNAi-caused mortality in the Neotropical stink bug *Euschistus heros*. *Pest Manag. Sci.* 75, 537–548. doi:10.1002/ps.5167.

Chen, X., Li, L., Hu, Q., Zhang, B., Wu, W., Jin, F., et al. (2015). Expression of dsRNA in recombinant Isaria fumosorosea strain targets the TLR7 gene in Bemisia tabaci. *BMC Biotechnol.* 15, 64. doi:10.1186/s12896-015-0170-8.

Christiaens, O., Dzhambazova, T., Kostov, K., Arpaia, S., Joga, M. R., Urru, I., et al. (2018a). Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi-based GM plants. *EFSA Support. Publ.* 15. doi:10.2903/sp.efsa.2018.EN-1424.

Christiaens, O., Swevers, L., and 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.

Christiaens, O., Tardajos, M. G., Reyna, Z. L. M., Dash, M., Dubruel, P., and Smagghe, G. (2018b). Increased RNAi efficacy in *Spodoptera exigua* via the formulation of dsRNA

with guanylated polymers. *Front. Physiol.* 9, 1–13. doi:10.3389/fphys.2018.00316.

Dalakouras, A., Jarausch, W., Buchholz, G., Bassler, A., Braun, M., Manthey, T., et al. (2018). Delivery of Hairpin RNAs and Small RNAs Into Woody and Herbaceous Plants by Trunk Injection and Petiole Absorption. *Front. Plant Sci.* 9, 1–11. doi:10.3389/fpls.2018.01253.

Dalakouras, A., Wassenegger, M., McMillan, J. N., Cardoza, V., Maegele, I., Dadami, E., et al. (2016). Induction of Silencing in Plants by High-Pressure Spraying of In vitro-Synthesized Small RNAs. *Front. Plant Sci.* 7, 1–5. doi:10.3389/fpls.2016.01327.

de Andrade, E. C., and Hunter, W. B. "RNA Interference – Natural Gene-Based Technology for Highly Specific Pest Control (HiSPeC)," In: Abdurakhmonov, I. Y. editor. RNA Interference, IntechOpen (2016). p. 391–409. doi:10.5772/61612.

Deutsch, C. A., Tewksbury, J. J., Tigchelaar, M., Battisti, D. S., Merrill, S. C., Huey, R. B., et al. (2018). Increase in crop losses to insect pests in a warming climate. *Science* 919, 916–919. doi:10.1126/science.aat3466.

Dillin, A. (2003). The specifics of small interfering RNA specificity. *Proc. Natl. Acad. Sci.* 100, 6289–6291. doi:10.1073/pnas.1232238100.

Dubelman, S., Fischer, J., Zapata, F., Huizinga, K., Jiang, C., Uffman, J., et al. (2014). Environmental fate of double-stranded RNA in agricultural soils. *PLoS One* 9, e93155. doi:10.1371/journal.pone.0093155.

Dubrovina, A. S., and Kiselev, K. V. (2019). Exogenous RNAs for Gene Regulation and Plant Resistance. *Int. J. Mol. Sci.* 20, 2282. doi:10.3390/ijms20092282.

El-Shesheny, I., Hajeri, S., El-Hawary, I., Gowda, S., and Killiny, N. (2013). Silencing Abnormal Wing Disc Gene of the Asian Citrus Psyllid, *Diaphorina citri* Disrupts Adult Wing Development and Increases Nymph Mortality. *PLoS One* 8, 2–9. doi:10.1371/journal.pone.0065392.

Feinberg, E. H., and Hunter, C. P. (2003). Transport of dsRNA into cells by the transmembrane protein SID-1. *Science* 301, 1545–1547. doi:10.1126/science.1087117.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811. doi:10.1038/35888.

Fire, W. A., and Won, C. M. (2013). A lethal dose of RNA. *Science* 341, 732–733.

Fischer, J. R., Zapata, F., Dubelman, S., Mueller, G. M., Uffman, J. P., Jiang, C., et al. (2017). Aquatic fate of a double-stranded RNA in a sediment water system following an over-water application. *Environ. Toxicol. Chem.* 36, 727–734. doi:10.1002/etc.3585.

Fjose, A., Ellingsen, S., Wargelius, A., and Seo, H. C. (2001). RNA interference: Mechanisms and applications. *Biotechnol. Annu. Rev.* 7, 31–57. doi:10.1016/S1387-2656(01)07032-6.

782 Fu, D. Q., Zhu, B. Z., Zhu, H. L., Jiang, W. B., and Luo, Y. B. (2005). Virus-induced gene
783 silencing in tomato fruit. *Plant J.* 43, 299–308. doi:10.1111/j.1365-313X.2005.02441.x.

784 Gan, D., Zhang, J., Jiang, H., Jiang, T., Zhu, S., and Cheng, B. (2010). Bacterially expressed
785 dsRNA protects maize against SCMV infection. *Plant Cell Rep.* 29, 1261–1268.
786 doi:10.1007/s00299-010-0911-z.

787 Ganbaatar, O., Cao, B., Zhang, Y., Bao, D., Bao, W., and Wuriyanghan, H. (2017).
788 Knockdown of *Mythimna separata chitinase* genes *via* bacterial expression and oral
789 delivery of RNAi effectors. *BMC Biotechnol* 17, 1–11. doi:10.1186/s12896-017-0328-7.

790 Garbian, Y., Maori, E., Kalev, H., Shafir, S., and Sela, I. (2012). Bidirectional Transfer of
791 RNAi between Honey Bee and Varroa destructor: Varroa Gene Silencing Reduces
792 Varroa Population. *PLoS Pathog.* 8, e1003035. doi:10.1371/journal.ppat.1003035.

793 Garbutt, J. S., and Reynolds, S. E. (2012). Induction of RNA interference genes by double-
794 stranded RNA; implications for susceptibility to RNA interference. *Insect Biochem. Mol.*
795 *Biol.* 42, 621–628. doi:10.1016/j.ibmb.2012.05.001.

796 Ghag, S. B. (2017). Host induced gene silencing, an emerging science to engineer crop
797 resistance against harmful plant pathogens. *Physiol. Mol. Plant Pathol.* 100, 242–254.
798 doi:10.1016/j.pmpp.2017.10.003.

799 Ghosh, S. K. B., and Gundersen-Rindal, D. E. (2017). Double strand RNA-mediated RNA
800 interference through feeding in larval gypsy moth, *Lymantria dispar* (Lepidoptera:
801 Erebidae). *Eur. J. Entomol.* 114, 170–178. doi:10.14411/eje.2017.022.

802 Ghosh, S. K. B., Hunter, W. B., Park, A. L., and Gundersen-Rindal, D. E. (2017). Double
803 strand RNA delivery system for plant-sap-feeding insects. *PLoS One* 12, e0171861.
804 doi:10.1371/journal.pone.0171861.

805 Gogoi, A., Sarmah, N., Kaldis, A., Perdikis, D., and Voloudakis, A. (2017). Plant insects and
806 mites uptake double-stranded RNA upon its exogenous application on tomato leaves.
807 *Planta* 246, 1233–1241. doi:10.1007/s00425-017-2776-7.

808 Golden, D. E., Gerbase, V. R., and Sontheimer, E. J. (2008). An Inside Job for siRNAs. *Mol.*
809 *Cell* 31, 309–312. doi:10.1016/j.molcel.2008.04.008.Comprehensive.

810 Gong, L., Chen, Y., Hu, Z., and Hu, M. (2013). Testing Insecticidal Activity of Novel
811 Chemically Synthesized siRNA against *Plutella xylostella* under Laboratory and Field
812 Conditions. *PLoS One* 8, 1–8. doi:10.1371/journal.pone.0062990.

813 Gordon, K. H. J., and Waterhouse, P. M. (2007). RNAi for insect-proof plants. *Nat.*
814 *Biotechnol.* 25, 1231–1232. doi:10.1038/nbt1107-1231.

815 Goulin, E. H., Galdeano, D. M., Granato, L. M., Matsumura, E. E., Dalio, R. J. D., Dalio, D.,
816 et al. (2019). RNA interference and CRISPR: Promising approaches to better understand
817 and control citrus pathogens. *Microbiol. Res.* 226, 1–9.
818 doi:10.1016/j.micres.2019.03.006.

819 Gu, K. X., Song, X. S., Xiao, X. M., Duan, X. X., Wang, J. X., Duan, Y. B., et al. (2019). A β
820 2 -tubulin dsRNA derived from *Fusarium asiaticum* confers plant resistance to multiple
821 phytopathogens and reduces fungicide resistance. *Pestic. Biochem. Physiol.* 153, 36–46.
822 doi:10.1016/j.pestbp.2018.10.005.

823 Gu, L., and Knipple, D. C. (2013). Recent advances in RNA interference research in insects:
824 Implications for future insect pest management strategies. *Crop Prot.* 45, 36–40.
825 doi:10.1016/j.cropro.2012.10.004.

826 Guan, R.-B., Li, H.-C., Fan, Y.-J., Hu, S.-R., Christiaens, O., Smagghe, G., et al. (2018). A
827 nuclease specific to lepidopteran insects suppresses RNAi. *J. Biol. Chem.* 293, 6011–
828 6021. doi:10.1074/jbc.RA117.001553.

829 Hajeri, S., Killiny, N., El-Mohtar, C., Dawson, W. O., and Gowda, S. (2014). Citrus tristeza
830 virus-based RNAi in citrus plants induces gene silencing in *Diaphorina citri*, a phloem-
831 sap sucking insect vector of citrus greening disease (Huanglongbing). *J. Biotechnol.* 176,
832 42–49. doi:10.1016/j.jbiotec.2014.02.010.

833 Hannon, G. J. (2002). RNA interference. *Nature* 418, 244–251. doi:10.1038/418244a.

834 Head, G. P., Carroll, M. W., Evans, S. P., Rule, D. M., Willse, A. R., Clark, T. L., et al.
835 (2017). Evaluation of SmartStax and SmartStax PRO maize against western corn
836 rootworm and northern corn rootworm: efficacy and resistance management. *Pest*
837 *Manag. Sci.* 73, 1883–1899. doi:10.1002/ps.4554.

838 Huang, H., Qiao, R., Zhao, D., Zhang, T., Li, Y., Yi, F., et al. (2009). Profiling of mismatch
839 discrimination in RNAi enabled rational design of allele-specific siRNAs. *Nucleic Acids*
840 *Res.* 37, 7560–7569. doi:10.1093/nar/gkp835.

841 Hunter, W. B., Glick, E., Paldi, N., and Bextine, B. R. (2012). Advances in RNA interference:
842 dsRNA treatment in trees and grapevines for insect pest suppression. *Southwest.*
843 *Entomol.* 37, 85–87. doi:dx.doi.org/10.3958/059.037.0110.

844 Hunter, W., Ellis, J., Vanengelsdorp, D., Hayes, J., Westervelt, D., Glick, E., et al. (2010).
845 Large-scale field application of RNAi technology reducing Israeli acute paralysis virus
846 disease in honey bees (*Apis mellifera*, Hymenoptera: Apidae). *PLoS Pathog.* 6, 1–10.
847 doi:10.1371/journal.ppat.1001160.

848 Huvenne, H., and Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential
849 of RNAi for pest control: A review. *J. Insect Physiol.* 56, 227–235.
850 doi:10.1016/j.jinsphys.2009.10.004.

851 Ivashuta, S., Zhang, Y., Wiggins, B. E., Ramaseshadri, P., Segers, G. C., Johnson, S., et al.
852 (2015). Environmental RNAi in herbivorous insects. *Rna* 5, 1–11.
853 doi:10.1261/rna.048116.114.2.

854 Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V, Burchard, J., Mao, M., et al.
855 (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nat.*
856 *Biotechnol.* 21, 635–637. doi:10.1038/nbt831.

Jahan, S. N., Åsman, A. K. M., Corcoran, P., Fogelqvist, J., Vetukuri, R. R., and Dixelius, C. (2015). Plant-mediated gene silencing restricts growth of the potato late blight pathogen Phytophthora infestans. *J. Exp. Bot.* 66, 2785–2794. doi:10.1093/jxb/erv094.

Joga, M. R., Zotti, M. J., Smagghe, G., and Christiaens, O. (2016). RNAi Efficiency, Systemic Properties, and Novel Delivery Methods for Pest Insect Control: What We Know So Far. *Front. Physiol.* 7, 1–14. doi:10.3389/fphys.2016.00553.

Karlikow, M., Goic, B., Mongelli, V., Salles, A., Schmitt, C., Bonne, I., et al. (2016). *Drosophila* cells use nanotube-like structures to transfer dsRNA and RNAi machinery between cells. *Sci. Rep.* 6, 1–9. doi:10.1038/srep27085.

Ketting, R. F. (2011). The Many Faces of RNAi. *Dev. Cell* 20, 148–161. doi:10.1016/j.devcel.2011.01.012.

Khajuria, C., Ivashuta, S., Wiggins, E., Flagel, L., Moar, W., Pleau, M., et al. (2018). Development and characterization of the first dsRNA-resistant insect population from western corn rootworm, *Diabrotica virgifera virgifera* LeConte. *PLoS One* 13, 1–19. doi:10.1371/journal.pone.0197059.

Khan, A. M., Ashfaq, M., Khan, A. A., Naseem, M. T., Faisalabad, G. E., and Layyah, B. C. (2018). Evaluation of potential RNA-interference-target genes to control cotton mealybug, *Phenacoccus solenopsis* (Hemiptera: Pseudococcuidae). *Insect Sci.* 25, 778–786. doi:10.1111/1744-7917.12455.

Khan, A. M., Ashfaq, M., Kiss, Z., Khan, A. A., Mansoor, S., and Falk, B. W. (2013). Use of Recombinant Tobacco Mosaic Virus To Achieve RNA Interference in Plants against the Citrus Mealybug, *Planococcus citri* (Hemiptera: Pseudococcidae). *PLoS One* 8, e73657. doi:10.1371/journal.pone.0073657.

Killiny, N., Tiwari, S., Hajeri, S., Gowda, S., and Stelinski, L. L. (2014). Double-Stranded RNA Uptake through Topical Application, Mediates Silencing of Five CYP4 Genes and Suppresses Insecticide Resistance in *Diaphorina citri*. *PLoS One* 9, e110536. doi:10.1371/journal.pone.0110536.

Kim, E., Park, Y., and Kim, Y. (2015). A Transformed Bacterium Expressing Double-Stranded RNA Specific to Integrin β1 Enhances Bt Toxin Efficacy against a Polyphagous Insect Pest, *Spodoptera exigua*. *PLoS One* 10, e0132631. doi:10.1371/journal.pone.0132631.

Kobayashi, I., Tsukioka, H., Kômoto, N., Uchino, K., Sezutsu, H., Tamura, T., et al. (2012). SID-1 protein of *Caenorhabditis elegans* mediates uptake of dsRNA into *Bombyx* cells. *Insect Biochem. Mol. Biol.* 42, 148–154. doi:10.1016/j.ibmb.2011.11.007.

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., et al. (2016). An RNAi-Based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs Involves a Plant Passage and is Controlled by the Fungal Silencing Machinery. *PLOS Pathog.* 12, e1005901. doi:10.1371/journal.ppat.1005901.

Koch, A., Kumar, N., Weber, L., Keller, H., Imani, J., and Kogel, K.-H. (2013). Host-induced

gene silencing of cytochrome P450 lanosterol C14 -demethylase-encoding genes confers strong resistance to *Fusarium* species. *Proc. Natl. Acad. Sci.* 110, 19324–19329. doi:10.1073/pnas.1306373110.

Kolliopoulou, A., Taning, C. N. T., Smagghe, G., and Swevers, L. (2017). Viral Delivery of dsRNA for Control of Insect Agricultural Pests and Vectors of Human Disease: Prospects and Challenges. *Front. Physiol.* 8, 1–24. doi:10.3389/fphys.2017.00399.

Konakalla, N. C., Kaldis, A., Berbati, M., Masarapu, H., and Voloudakis, A. E. (2016). Exogenous application of double-stranded RNA molecules from TMV p126 and CP genes confers resistance against TMV in tobacco. *Planta* 244, 961–969. doi:10.1007/s00425-016-2567-6.

Krishnan, M., Bharathiraja, C., Pandiarajan, J., Prasanna, V. A., Rajendhran, J., and Gunasekaran, P. (2014). Insect gut microbiome - An unexploited reserve for biotechnological application. *Asian Pac. J. Trop. Biomed.* 4, 16–21. doi:10.12980/APJTB.4.2014C95.

Kumar, P., Pandit, S. S., and Baldwin, I. T. (2012). Tobacco rattle virus vector: A rapid and transient means of silencing *Manduca sexta* genes by plant mediated RNA interference. *PLoS One* 7, e31347. doi:10.1371/journal.pone.0031347.

Li, H., Guan, R., Guo, H., and Miao, X. (2015). New insights into an RNAi approach for plant defence against piercing-sucking and stem-borer insect pests. *Plant Cell Environ.* 38, 2277–2285. doi:10.1111/pce.12546.

Li, X., Zhang, M., and Zhang, H. (2011). RNA interference of four genes in adult *Bactrocera dorsalis* by feeding their dsRNAs. *PLoS One* 6, e17788. doi:10.1371/journal.pone.0017788.

Lin, Y. H., Huang, J. H., Liu, Y., Belles, X., and Lee, H. J. (2017). Oral delivery of dsRNA lipoplexes to German cockroach protects dsRNA from degradation and induces RNAi response. *Pest Manag. Sci.* 73, 960–966. doi:10.1002/ps.4407.

Lippman, Z., and Martienssen, R. (2004). The role of RNA interference in heterochromatic silencing. *Nature* 431, 364–370.

Lu, R., Martin-Hernandez, A. M., Peart, J. R., Malcuit, I., and Baulcombe, D. C. (2003). Virus-induced gene silencing in plants. *Methods* 30, 296–303. doi:10.1016/S1046-2023(03)00037-9.

Luo, Y., Wang, X., Wang, X., Yu, D., Chen, B., and Kang, L. (2013). Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding. *Insect Mol. Biol.* 22, 574–583. doi:10.1111/imb.12046.

Mat Jalaluddin, N. S., Othman, R. Y., and Harikrishna, J. A. (2018). Global trends in research and commercialization of exogenous and endogenous RNAi technologies for crops. *Crit. Rev. Biotechnol.* 39, 67–78. doi:10.1080/07388551.2018.1496064.

Matranga, C., Tomari, Y., Shin, C., Bartel, D. P., and Zamore, P. D. (2005). Passenger-strand

cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* 123, 607–620. doi:10.1016/j.cell.2005.08.044.

Matranga, C., and Zamore, P. D. (2007). Primer. Small silencing RNAs. *Curr Biol* 17, R789–R793. doi:10.1016/j.cub.2007.07.014.

McLoughlin, A. G., Wytinck, N., Walker, P. L., Girard, I. J., Rashid, K. Y., De Kievit, T., et al. (2018). Identification and application of exogenous dsRNA confers plant protection against *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *Sci. Rep.* 8, 1–14. doi:10.1038/s41598-018-25434-4.

Meister, G., and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature* 431, 343–349. doi:10.1038/nature02873.

Melnyk, C. W., Molnar, A., and Baulcombe, D. C. (2011). Intercellular and systemic movement of RNA silencing signals. *EMBO J.* 30, 3553–3563. doi:10.1038/emboj.2011.274.

Mitter, N., Worrall, E. A., Robinson, K. E., Li, P., Jain, R. G., Taochy, C., et al. (2017a). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants* 3, 16207. doi:10.1038/nplants.2016.207.

Mitter, N., Worrall, E. A., Robinson, K. E., Xu, Z. P., and Carroll, B. J. (2017b). Induction of virus resistance by exogenous application of double-stranded RNA. *Curr. Opin. Virol.* 26, 49–55. doi:10.1016/j.coviro.2017.07.009.

Miyoshi, K., Miyoshi, K., Tsukumo, H., Tsukumo, H., Nagami, T., Nagami, T., et al. (2005). Slicer function of *Drosophila* Argonautes and its involvement in RISC formation. *Genes Dev.* 2, 2837–2848. doi:10.1101/gad.1370605.specific.

Molnar, A., Melnyk, C., and Baulcombe, D. C. (2011). Silencing signals in plants: A long journey for small RNAs. *Genome Biol.* 12, 1–8. doi:10.1186/gb-2010-11-12-219.

Mutti, N. S., Park, Y., Reese, J. C., Reeck, G. R., Amdam, G., Simoes, Z., et al. (2006). RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. *J. Insect Sci.* 6, 1–7. doi:10.1673/031.006.3801.

Nandety, R. S., Kuo, Y. W., Nouri, S., and Falk, B. W. (2015). Emerging strategies for RNA interference (RNAi) applications in insects. *Bioengineered* 6, 8–19. doi:10.4161/21655979.2014.979701.

Niehl, A., Soininen, M., Poranen, M. M., and Heinlein, M. (2018). Synthetic biology approach for plant protection using dsRNA. *Plant Biotechnol. J.* 16, 1679–1687. doi:10.1111/pbi.12904.

Niu, J., Taning, C. N. T., Christiaens, O., Smagghe, G., and Wang, J. J. (2018). *Rethink RNAi in Insect Pest Control: Challenges and Perspectives*. In: Smagghe G. editor. Advances in Insect Physiology. 1st ed. Elsevier Ltd. p. 1-17. doi:10.1016/bs.aiip.2018.07.003.

Nouri, S., Matsumura, E. E., Kuo, Y., and Falk, B. W. (2018). Insect-specific viruses: from

discovery to potential translational applications. *Curr. Opin. Virol.* 33, 33–41. doi:10.1016/j.coviro.2018.07.006.

Oerke, E. C. (2006). Crop losses to pests. *J. ofAgricultural Sci.*, 31–43. doi:10.1017/S0021859605005708.

Paldi, N., Glick, E., Oliva, M., Zilberberg, Y., Aubin, L., Pettis, J., et al. (2010). Effective gene silencing in a microsporidian parasite associated with honeybee (*Apis mellifera*) colony declines. *Appl. Environ. Microbiol.* 76, 5960–5964. doi:10.1128/AEM.01067-10.

Papic, L., Rivas, J., Toledo, S., and Romero, J. (2018). Double-stranded RNA production and the kinetics of recombinant *Escherichia coli* HT115 in fed-batch culture. *Biotechnol. Reports* 20, 10–13. doi:10.1016/j.btre.2018.e00292.

Parker, K. M., Barragán Borrero, V., Van Leeuwen, D. M., Lever, M. A., Mateescu, B., and Sander, M. (2019). Environmental Fate of RNA Interference Pesticides: Adsorption and Degradation of Double-Stranded RNA Molecules in Agricultural Soils. *Environ. Sci. Technol.* 53, 3027–3036. doi:10.1021/acs.est.8b05576.

Parsons, K. H., Mondal, M. H., McCormick, C. L., and Flynt, A. S. (2018). Guanidinium-Functionalized Interpolyelectrolyte Complexes Enabling RNAi in Resistant Insect Pests. *Biomacromolecules* 19, 1111–1117. doi:10.1021/acs.biomac.7b01717.

Petrick, J. S., Brower-Toland, B., Jackson, A. L., and Kier, L. D. (2013). Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: A scientific review. *Regul. Toxicol. Pharmacol.* 66, 167–176. doi:10.1016/j.yrtph.2013.03.008.

Pinheiro, D. H., Vélez, A. M., Fishilevich, E., Wang, H., Carneiro, N. P., Valencia-Jiménez, A., et al. (2018). Clathrin-dependent endocytosis is associated with RNAi response in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. *PLoS One* 13, e0201849. doi:10.1371/journal.pone.0201849.

Piot, N., Snoeck, S., Vanlede, M., Smagghe, G., and Meeus, I. (2015). The effect of oral administration of dsRNA on viral replication and mortality in *Bombus terrestris*. *Viruses* 7, 3172–3185. doi:10.3390/v7062765.

Preall, J. B., and Sontheimer, J. E. (2005). RNAi: RISC Gets Loaded. *Cell* 123, 543–545. doi:10.1016/j.cell.2005.11.006.

Price, D. R. G., and Gatehouse, J. A. (2008). RNAi-mediated crop protection against insects. *Trends Biotechnol.* 26, 393–400. doi:10.1016/j.tibtech.2008.04.004.

Pridgeon, J. W., Zhao, L., Becnel, J. J., Strickman, D. A., Clark, G. G., and Linthicum, K. J. (2008). Topically applied AaeIAP1 double-stranded RNA kills female adults of *Aedes aegypti*. *J. Med. Entomol.* 45, 414–420. doi:10.1603/0022-2585(2008)45[414:taadrk]2.0.co;2.

Ratcliff, F., Harrison, B. D., and Baulcombe, D. C. (1997). A Similarity Between Viral Defense and Gene Silencing in Plants. *Science* 276, 1558–1560.

doi:10.1126/science.276.5318.1558.

Robertson, D. (2004). VIGS Vectors for Gene Silencing: Many Targets, Many Tools. *Annu. Rev. Plant Biol.* 55, 495–519. doi:10.1146/annurev.arplant.55.031903.141803.

Robinson, K. E., Worrall, E. A., and Mitter, N. (2014). Double stranded RNA expression and its topical application for non-transgenic resistance to plant viruses. *J. Plant Biochem. Biotechnol.* 23, 231–237. doi:10.1007/s13562-014-0260-z.

Rodríguez-García, C., Evans, J. D., Li, W., Branchiccela, B., Li, J. H., Heerman, M. C., et al. (2018). Nosemosis control in European honey bees, *Apis mellifera* , by silencing the gene encoding *Nosema ceranae* polar tube protein 3. *J. Exp. Biol.* 221, jeb184606. doi:10.1242/jeb.184606.

Šafářová, D., Brázda, P., and Navrátil, M. (2014). Effect of artificial dsRNA on infection of pea plants by pea seed-borne mosaic virus. *Czech J. Genet. Plant Breed.* 50, 105–108. doi:10.17221/120/2013-CJGPB.

Saleh, M., Rij, R. P. Van, Hekele, A., Gillis, A., Foley, E., Farrell, P. H. O., et al. (2006). The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nat. Cell Biol.* 8, 793–802. doi:10.1038/ncbl439.The.

Sammons, R. D., Ivashuta, S., Liu, H., Wang, D., Feng, P. C. C., Kouranov, A. Y., et al. (2011). Polynucleotide molecules for gene regulation in plants. *U.S. Pat. 2011/0296556 A1* 1.

San Miguel, K., and Scott, J. G. (2016). The next generation of insecticides: DsRNA is stable as a foliar-applied insecticide. *Pest Manag. Sci.* 72, 801–809. doi:10.1002/ps.4056.

Schwarz, D. S., Ding, H., Kennington, L., Moore, J. T., Schelter, J., Burchard, J., et al. (2006). Designing siRNA that distinguish between genes that differ by a single nucleotide. *PLoS Genet.* 2, e140. doi:10.1371/journal.pgen.0020140.

Scorza, R., Callahan, A., Dardick, C., Ravelonandro, M., Polak, J., Malinowski, T., et al. (2013). Genetic engineering of *Plum pox virus* resistance: “HoneySweet” plum-from concept to product. *Plant Cell. Tissue Organ Cult.* 115, 1–12. doi:10.1007/s11240-013-0339-6.

Senthil-Kumar, M., and Mysore, K. S. (2011). New dimensions for VIGS in plant functional genomics. *Trends Plant Sci.* 16, 656–665. doi:10.1016/j.tplants.2011.08.006.

Song, X. S., Gu, K. X., Duan, X. X., Xiao, X. M., Hou, Y. P., Duan, Y. B., et al. (2018). A *myosin5* dsRNA that reduces the fungicide resistance and pathogenicity of *Fusarium asiaticum*. *Pestic. Biochem. Physiol.* 150, 1–9. doi:10.1016/j.pestbp.2018.07.004.

Spit, J., Philips, A., Wynant, N., Santos, D., Plaetinck, G., and Vanden Broeck, J. (2017). Knockdown of nuclease activity in the gut enhances RNAi efficiency in the Colorado potato beetle, *Leptinotarsa decemlineata*, but not in the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* 81, 103–116. doi:10.1016/j.ibmb.2017.01.004.

1046 Taning, C. N. T., Christiaens, O., Berkvens, N., Casteels, H., Maes, M., and Smagghe, G.
1047 (2016). Oral RNAi to control *Drosophila suzukii*: laboratory testing against larval and
1048 adult stages. *J. Pest Sci. (2004).* 89, 803–814. doi:10.1007/s10340-016-0736-9.

1049 Taning, C. N. T., Christiaens, O., Li, X., and Swevers, L. (2018). Engineered Flock House
1050 Virus for Targeted Gene Suppression Through RNAi in Fruit Flies (*Drosophila*
1051 *melanogaster*) in Vitro and in Vivo. *Front. Physiol.* 9, 805.
1052 doi:10.3389/fphys.2018.00805.

1053 Tenllado, F., and Díaz-Ruíz, J. R. (2001). Double-stranded RNA-mediated interference with
1054 plant virus infection. *J. Virol.* 75, 12288–12297. doi:10.1128/JVI.75.24.12288.

1055 Tenllado, F., Martínez-García, B., Vargas, M., and Díaz-Ruíz, J. R. (2003). Crude extracts of
1056 bacterially expressed dsRNA can be used to protect plants against virus infections. *BMC*
1057 *Biotechnol.* 3, 1–11. doi:10.1186/1472-6750-3-3.

1058 Terenius, O., Papanicolaou, A., Garbutt, J. S., Eleftherianos, I., Huvenne, H., Kanginakudru,
1059 S., et al. (2011). RNA interference in Lepidoptera: An overview of successful and
1060 unsuccessful studies and implications for experimental design. *J. Insect Physiol.* 57,
1061 231–245. doi:10.1016/j.jinsphys.2010.11.006.

1062 Tian, H., Peng, H., Yao, Q., Chen, H., Xie, Q., Tang, B., et al. (2009). Developmental Control
1063 of a Lepidopteran Pest *Spodoptera exigua* by Ingestion of Bacteria Expressing dsRNA of
1064 a Non-Midgut Gene. *PLoS One* 4, e6225. doi:10.1371/journal.pone.0006225.

1065 Tiwari, I. M., Jesuraj, A., Kamboj, R., Devanna, B. N., Botella, J. R., and Sharma, T. R.
1066 (2017). Host Delivered RNAi, an efficient approach to increase rice resistance to sheath
1067 blight pathogen (*Rhizoctonia solani*). *Sci. Rep.* 7, 1–14. doi:10.1038/s41598-017-07749-
1068 w.

1069 Tomari, Y., Du, T., and Zamore, P. D. (2007). Sorting of *Drosophila* small silencing RNAs.
1070 *Cell* 130, 299–308. doi:10.1016/j.cell.2007.05.057.

1071 Tomoyasu, Y., Miller, S. C., Tomita, S., Schoppmeier, M., Grossmann, D., and Bucher, G.
1072 (2008). Exploring systemic RNA interference in insects: A genome-wide survey for
1073 RNAi genes in Tribolium. *Genome Biol.* 9, 1–22. doi:10.1186/gb-2008-9-1-r10.

1074 Ulvila, J., Parikka, M., Kleino, A., Sormunen, R., Ezekowitz, R. A., Kocks, C., et al. (2006).
1075 Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in
1076 *Drosophila* S2 cells. *J. Biol. Chem.* 281, 14370–14375. doi:10.1074/jbc.M513868200.

1077 Vélez, A. M., and Fishilevich, E. (2018). The mysteries of insect RNAi: A focus on dsRNA
1078 uptake and transport. *Pestic. Biochem. Physiol.* 151, 25–31. doi:.1037//0033-
1079 2909.I26.1.78.

1080 Wang, K., Peng, Y., Pu, J., Fu, W., Wang, J., and Han, Z. (2016a). Variation in RNAi
1081 efficacy among insect species is attributable to dsRNA degradation *in vivo*. *Insect*
1082 *Biochem. Mol. Biol.* 77, 1–9. doi:10.1016/j.ibmb.2016.07.007.

1083 Wang, M., and Jin, H. (2017). Spray-Induced Gene Silencing: a Powerful Innovative Strategy

for Crop Protection. 25, 4–6. doi:10.1109/EMBC.2016.7590696.Upper.

Wang, M., Thomas, N., and Jin, H. (2017). Cross-kingdom RNA trafficking and environmental RNAi for powerful innovative pre- and post-harvest plant protection. *Curr. Opin. Plant Biol.* 38, 133–141. doi:10.1016/j.pbi.2017.05.003.

Wang, M., Weiberg, A., Lin, F.-M., Thomma, B. P. H. J., Huang, H.-D., and Jin, H. (2016b). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* 2, 16151. doi:10.1038/nplants.2016.151.

Waterhouse, P. M., Wang, M. B., and Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature* 411, 834–842. doi:10.1038/35081168.

Weiberg, A., Wang, M., Lin, F.-M., Zhao, H., Zhang, Z., Kaloshian, I., et al. (2013). Fungal Small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* 342, 118–123. doi:10.1126/science.1239705.

Whangbo, J. S., and Hunter, C. P. (2008). Environmental RNA interference. *Trends Genet.* 24, 297–305. doi:10.1016/j.tig.2008.03.007.

Whitten, M., and Dyson, P. (2017). Gene silencing in non-model insects: Overcoming hurdles using symbiotic bacteria for trauma-free sustainable delivery of RNA interference. *Prospect. Overviews* 39, 1–12. doi:10.1002/bies.201600247.

Whitten, M. M. A., Facey, P. D., Sol, R. Del, Evans, M. C., Mitchell, J. J., Bodger, O. G., et al. (2016). Symbiont-mediated RNA interference in insects. *Proc. R. Soc. B* 283, 20160042. doi:10.1098/rspb.2016.0042.

Whyard, S., Singh, A. D., and Wong, S. (2009). Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem. Mol. Biol.* 39, 824–832. doi:10.1016/j.ibmb.2009.09.007.

Worrall, E. A., Bravo-Cazar, A., Nilon, A. T., Fletcher, S. J., Robinson, K. E., Carr, J. P., et al. (2019). Exogenous Application of RNAi-Inducing Double-Stranded RNA Inhibits Aphid-Mediated Transmission of a Plant Virus. *Front. Plant Sci.* 10, 265. doi:10.3389/fpls.2019.00265.

Wuriyanghan, H., and Falk, B. W. (2013). RNA Interference towards the Potato Psyllid, *Bactericera cockerelli*, Is Induced in Plants Infected with Recombinant *Tobacco mosaic virus* (*TMV*). *PLoS One* 8, e66050. doi:10.1371/journal.pone.0066050.

Wynant, N., Verlinden, H., Breugelmans, B., Simonet, G., and Vanden Broeck, J. (2012). Tissue-dependence and sensitivity of the systemic RNA interference response in the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* 42, 911–917. doi:10.1016/j.ibmb.2012.09.004.

Yin, G., Sun, Z., Liu, N., and Zhang, L. (2009). Production of double-stranded RNA for interference with TMV infection utilizing a bacterial prokaryotic expression system. *Appl. Microbiol. Biotechnol.* 84, 323–333. doi:10.1007/s00253-009-1967-y.

Yoon, J. S., Gurusamy, D., and Palli, S. R. (2017). Accumulation of dsRNA in endosomes contributes to inefficient RNA interference in the fall armyworm, *Spodoptera frugiperda*. *Insect Biochem. Mol. Biol.* 90, 53–60. doi:10.1016/j.ibmb.2017.09.011.

Zhang, H., Li, H. C., and Miao, X. X. (2013). Feasibility, limitation and possible solutions of RNAi-based technology for insect pest control. *Insect Sci.* 20, 15–30. doi:10.1111/j.1744-7917.2012.01513.x.

Zheng, Z. M., Tang, S., and Tao, M. (2005). Development of resistance to RNAi in mammalian cells. *Ann. N. Y. Acad. Sci.* 1058, 105–118. doi:10.1196/annals.1359.019.

Zhu, F., Xu, J., Palli, R., Ferguson, J., and Palli, S. R. (2011). Ingested RNA interference for managing the populations of the Colorado potato beetle, *Leptinotarsa decemlineata*. *Pest Manag. Sci.* 67, 175–182. doi:10.1002/ps.2048.

Zhu, J. Q., Liu, S., Ma, Y., Zhang, J. Q., Qi, H. S., Wei, Z. J., et al. (2012). Improvement of pest resistance in transgenic tobacco plants expressing dsRNA of an insect-associated gene EcR. *PLoS One* 7, e38572. doi:10.1371/journal.pone.0038572.

Zotti, M., dos Santos, E. A., Cagliari, D., Christiaens, O., Taning, C. N. T., and Smagghe, G. (2017). RNAi technology in crop protection against arthropod pests, pathogens and nematodes. *Pest Manag. Sci.* 74, 1239–1250. doi:10.1002/ps.4813.

Zotti, M. J., and Smagghe, G. (2015). RNAi Technology for Insect Management and Protection of Beneficial Insects from Diseases: Lessons, Challenges and Risk Assessments. *Neotrop. Entomol.* 44, 197–213. doi:10.1007/s13744-015-0291-8.

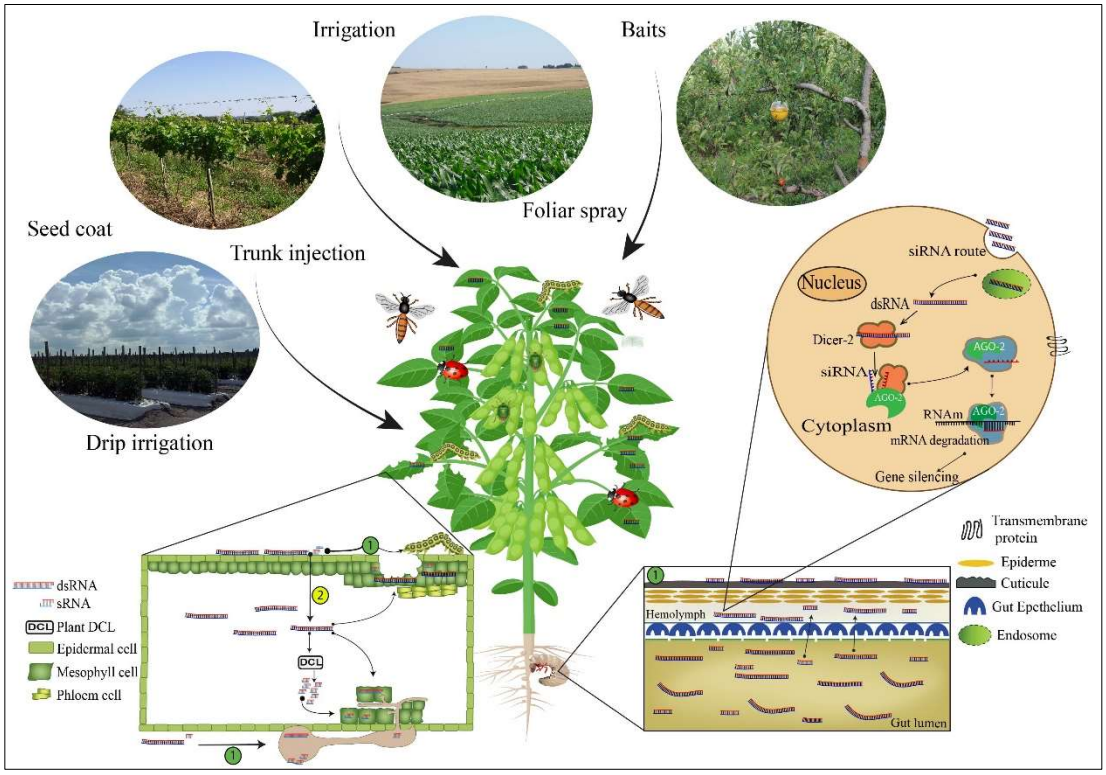


Figure 1- Non-transformative delivery strategies routes for RNAi-based gene silencing induction. The first step to achieve successful RNAi-based gene silencing results via non-transformative approaches is the selection of the RNAs (dsRNA or siRNA) delivery strategy: Foliar spray, trunk injection, irrigation, drip irrigation, seed coat, baits, and powder or granules for soil applications. Once the RNAs are delivered the insects and pathogens need to internalize the RNAs molecules, and this process can occur (1) directly or (2) indirectly. The direct uptake occurs when the organisms get in contact with the RNAs molecules during application or feed on tissues containing the RNA molecules on the surface. However, when the RNA molecules are absorbed, translocated in the plant vascular system then taken up by the organism (Koch et al., 2016), the process is classified as indirect uptake (Cagliari et al., 2018). Inside the organism system, the cell uptake of dsRNA can be mediate by transmembrane channel proteins such as sid-1 (Aronstein et al., 2006; Feinberg and Hunter, 2003; Kobayashi et al., 2012) or endocytosis (Cappelle et al., 2016; Pinheiro et al., 2018; Saleh et al., 2006; Ulvila et al., 2006; Vélez and Fishilevich, 2018). The RNAi-based gene silencing depends on the release at cellular levels of dsRNA or siRNA molecules (Carthew, 2009; Zotti and Smagghe, 2015). When dsRNAs are unloaded in the cytoplasm, these molecules are processed into siRNA fragments by an enzyme called Dicer 2 (DCR-2) (Meister and Tuschl, 2004; Tomari et al., 2007). The siRNA fragments are then incorporated into the RISC complex (RNA-induced Silencing Complex), which contains the Argonaute 2 (AGO-2) protein (Ketting, 2011; Matranga et al., 2005; Miyoshi et al., 2005), and in a sequence-specific manner binds to a complementary messenger RNA (mRNA), cleaving it and preventing the protein formation (Agrawal et al., 2003; Huvenne and Smagghe, 2010), affecting the target organism survival.

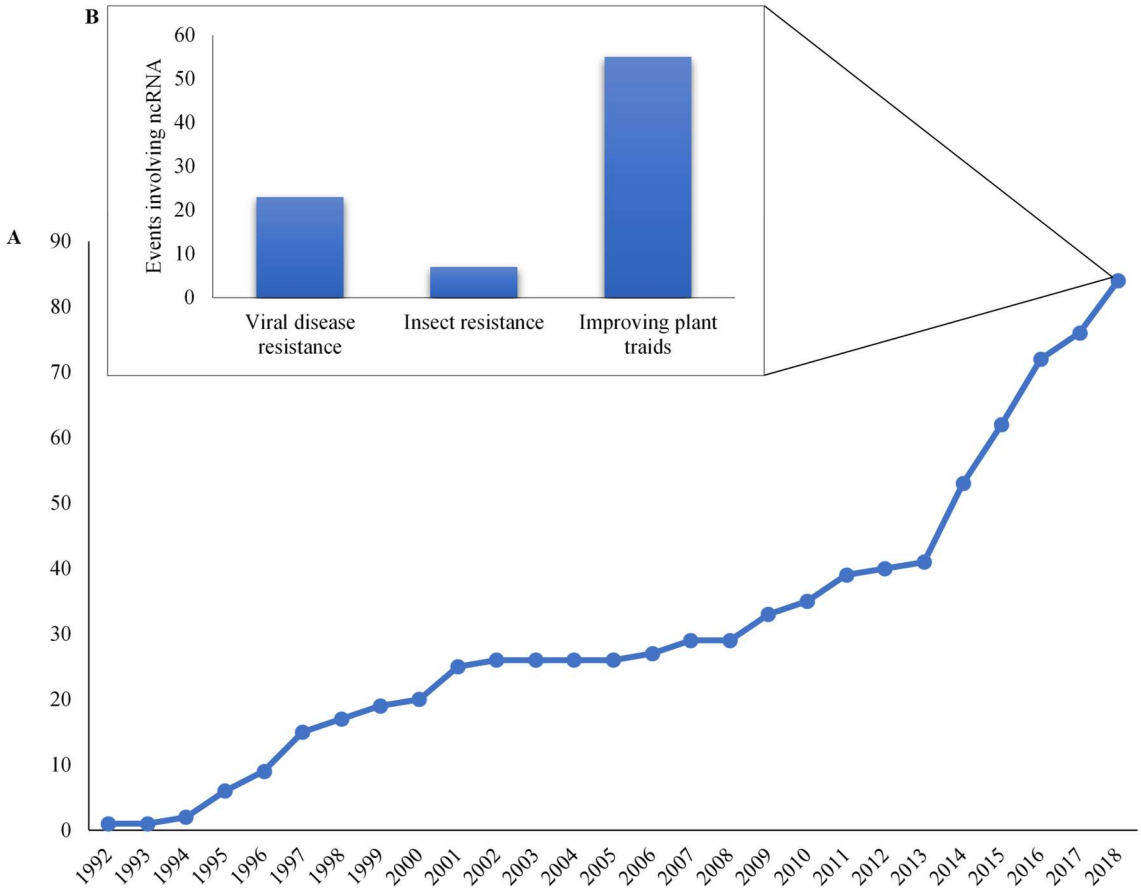


Figure 2 – Accumulated approved genetically modified events based on non-coding RNA (ncRNA) worldwide for cultivation since 1992. **A –** Total approved ncRNA GM events worldwide since the first ncRNA approved event in 1992; **B -** Number of ncRNA GM events according to the desired features. The data used to make the graphics were compiled from the GM Approval Database at the International Service for the Acquisition of Agri-Biotech Applications (ISAAA) (http://www.isaaa.org/gmapprovaldatabase/default.asp).

Table 1 – Non-transformative delivery approaches and the relation with the organism location at the plant and initial RNA uptake process.

Non-transformative delivery system	**Insect/Pathogen location**	**RNA uptake process by the target organism**	**Reference**
Soil drench; Drip irrigation; Irrigation	Roots; Stem; Leaves	Direct/Indirect	(Ghosh et al., 2017; Hunter et al., 2012; Li et al., 2015)
Seed coat or powder/granules	Roots; Stem	Direct/Indirect	-
Sprayable products	Stem; Leaves; Fruits/seeds	Direct/Indirect	(de Andrade and Hunter, 2016; Gogoi et al., 2017; Gu et al., 2019; Hunter et al., 2012; Koch et al., 2016; McLoughlin et al., 2018; Mitter et al., 2017b; Niehl et al., 2018; San Miguel and

			Scott, 2016; Song et al., 2018; Wang et al., 2016b; Weiberg et al., 2013; Worrall et al., 2019)
Trunk injection	Roots; Stem; Leaves; Fruits/seeds	Indirect	(Berger and Laurent, 2019; Dalakouras et al., 2018; Hunter et al., 2012)
Baits	Fruits	Direct	-

Table 2 –Different features affecting the development of RNAi-based products: Transformative vs. Non-transformative methods.

Feature	**Strategy**	
	Transformative	**Non-transformative[1]**
Development time	High	Low[2]
Development costs	High	Low
Feasibility according to culture	Unviable for some plant species	Viable for all cultures[1]
Delivery of sRNA	Continuous	Transient
Feasibility according to the pest	Most pests can be targeted due to continuous dsRNA supply feature	Not all pests can be targeted due to recalcitrant features
Development of resistance	High	Low
Regulatory process	Extensive	Simple
Acceptance by consumers	Low	High

[1]Non-transformative delivery approaches: foliar application, trunk injection, irrigation water, among others; [2]Non-transforative strategy compared to transformative strategy.

Table 3 – Non-transformative delivery strategies for insects, pathogens, and virus management.

Target pest	Crop	Delivery strategy	Target gene	Molecule	Size	Molecule concentration	Results	Reference
	Insects							
Plutella xylostella	Kale	Foliar spray	AChE2	siRNA	18–27 bp	200 µg/ml	Approximately 60% mortality.	(Gong et al., 2013)
Leptinotarsa decemlineata	Potato	Foliar spray	Actin	dsRNA	50 – 297 bp	5 µg leaf $^{-1}$	Significant mortality in dsRNA length-depend pattern.	(San Miguel and Scott, 2016)
Diaprepes abbreviates	Citrus	Foliar spray	Not informed	dsRNA	Not informed	Not informed	Control started 4-5 days after dsRNA application.	(de Andrade and Hunter, 2016)
Diaphorina citri; *Bactericera cockerelli;* *Homalodisca vitripennis*	Citrus approximately 2.5 m tall and Grapevines	Trunk injection; root drench	Arginine kinase	dsRNA	Not informed	2 g in 15 liters of water	Insects successfully uptake dsRNA from the treated plants; dsRNA was detected in plants for at least 57 days.	(Hunter et al., 2012)
Nilaparvata lugens	Rice	Roots soaking	Ces	dsRNA	Not informed	1 mL (1.0 mg mL^{-1} of water)	Gene knocked down; nymph mortality.	(Li et al., 2015)
			CYP18A1					
Ostrinia furnacalis	Maize	Irrigation	KTI	dsRNA		10 mL (0.5 mg mL^{-1} water)	Gene knocked down; larval mortality.	
Myzus persicae	Tomato	Foliar application	ZYMV HC-Pro	dsRNA	588 bp	10.5 µg dsRNA in 10 µL water	Insect successfully uptake dsRNA; the dsRNA was processed into siRNA by the insect RNAi machinery.	(Gogoi et al., 2017)
Tetranychus urticae								
Trialeurodes vaporariorum							Low dsRNA uptake; No siRNA in insects.	
Halyomorpha halys	Green beans	Soaking	JHAMT	dsRNA	200-500 bp	300 µl (0.017 µg µL^{-1} of water)	Significant reduction in gene expression.	(Ghosh et al., 2017)
			Vg			300 µl (0.067 µg µL^{-1} of water)		

Planococcus citri	Tobacco	VIGS using recombinant TMV	Actin CHS1 V-ATPase	siRNA	Not informed	-	Crawlers feed on recombinant TMV-infected plants showed lower fecundity and pronounced death.	(Khan et al., 2013)
Bactericera cockerelli	Tomato Tomatillo Tobacco	VIGS using recombinant TMV	Actin	siRNA	21 nt	-	Gene knocked down in insects feed on these plants; Insects fed on infected tomatillo plants showed a decreased progeny production.	(Wuriyanghan and Falk, 2013)
Diaphorina citri	Citrus	VIGS using recombinant CTV	Awd	siRNA	20-22 nt	-	Adults showed malformed-wing phenotype and increased mortality.	(Hajeri et al., 2014)
Phenacoccus solenopsis	Tobacco	VIGS using recombinant PVX	Bur V-ATPase	siRNA	-	-	Insects fed on treated plants showed physical deformities or died.	(Khan et al., 2018)
Drosophila melanogaster	-	VIGS using recombinant FHV; microinjection	RPS13 Vha26 Alpha COP	siRNA	-	-	Significantly higher mortality in insects.	(Taning et al., 2018)
Helicoverpa armigera	-	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115; artificial diet coated with engineered bacteria	AK	dsRNA	379-426 bp	30 µL (10^9 cells)	Knocked down the target gene caused drastic reductions in body weight, body length, and pupation rate, resulting in high mortality.	(Ai et al., 2018)
Spodoptera exigua	Chinese cabbage	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115	INT	dsRNA	410 bp	10^7 cells per larva	Significant reduction of the SeINT expression resulting in insect mortality; Pretreatment with an ultra-sonication increased the	(Kim et al., 2015)

							insecticidal activity of the recombinant bacteria, and treated larvae became s susceptible to Cry toxin.	
	-	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115; artificial diet containing engineered bacteria	CHSA	dsRNA	635 bp	High dose (250X), medium dose (50X), and low dose (10X) based on the dilution factors.	Significant reduction in survival rates. Levels of target gene expression, tissue structure, and survival rates were dose-dependent.	(Tian et al., 2009)
Lymantria dispar	-	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115; diet with engineered bacteria	Locus 365 Locus 28365	dsRNA	-	300 µl of bacteria culture	Target-gene knocked down, reduction in body mass and egg masses.	(Ghosh and Gundersen-Rindal, 2017)
Mythimna separata	-	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115; artificial diet containing engineered bacteria	Chi	dsRNA	700 bp	-	Target gene knocked down after oral delivery of engineered bacteria, resulting in resulted in increased mortality and reduction in body weight of the feeding larvae.	(Ganbaatar et al., 2017)
Bactrocera dorsalis	-	dsRNA expressed in bacteria, using recombinant *E.*	Rpl19 V-ATPase	dsRNA	-	200 ml 250X of bacteria culture expressing dsRNA.	Successful gene silencing of the target genes after insects were fed on a diet containing engineered	(Li et al., 2011)

		coli strain HT115; artificial diet containing engineered bacteria	Rab11					bacteria. An over-expression of the target genes after continuously supply of engineered bacteria was also observed.	
			Noa						
Bemisia tabaci	Hibiscus	dsRNA expressed in fungus, using engineered *Isaria fumosorosea*	TLR7		dsRNA	548 bp	$2x10^7$,$1x10^7$,$5×10^6$, $2.5x10^6$ spores mL^{-1}	The engineered IfB01-TRL7 strain increased the mortality of whitefly nymphs compared to the IfB01 strain. The IfB01-TRL7 strain also show higher virulence, with decreased and shortened values of LC50 and LT50.	(Chen et al., 2015)
Manduca sexta	Tobacco	VIGS using recombinant TRV	DCL1	In tobacco plants	dsRNA	≥ 300 bp	-	Knocked down of the DCL target genes in engineered tobacco plants to express a 312 bp fragment of *Ms*CYP6B46 gene increased the gene silencing results.	(Kumar et al., 2012)
			DCL2						
			DCL3						
			DCL4						
			CYP6	In tobacco hornworm					
Diseases									
Fusarium graminearum	Barley	Foliar spray	CYP3		dsRNA	791 bp	500 µL (20 ng $µL^{-1}$ of water)	Inhibition of fungal growth.	(Koch et al., 2016)
SCMV	Maize	Bacterial crude extract foliar spraying (*E. coli* strain HT115)	CP		dsRNA	147-247 bp	One-half diluted extraction crude	Inhibition of SCMV infection.	(Gan et al., 2010)
Botrytis cinerea	Tomato, Strawberry, Grape, Lettuce, Onion, Rose	Foliar application	DCL1		sRNA	21-24 nt	400 µl (20 ng $µL^{-1}$)	Both sRNA and dsRNA were uptake by the fungus resulting in fungal growth inhibition.	(Wang et al., 2016b)
					dsRNA	252 bp			
			DCL2		sRNA	21-14 nt			
					dsRNA	238 bp			

Sclerotinia sclerotiorum *Botrytis cinerea*	Canola	Foliar spray	59 target genes	dsRNA	200-450 bp	10–25 µL of 200–500 ng dsRNA plus 0.02–0.03% Silwet L-77.	From the 59 dsRNAs tested, 20 showed antifungal activity with a reduction in lesion size ranging from 26-85%.	(McLoughlin et al., 2018)
BCMV	Tobacco; cowpea	Foliar spray	Nib CP	dsRNA naked or loaded onto LDH	480 bp 461 bp	100 µg of in a 1 mL or 250 ng of dsRNA.	Plants were protected from aphid-mediated virus transmission.	(Worrall et al., 2019)
Fusarium asiaticum	Wheat	Foliar spray	Myosin 5	dsRNA	496 bp	0.1 pM	Reduced pathogen sensitivity to phenamacril with a reduction in infection.	(Song et al., 2018)
PPV PMMoV	Tobacco	Bacterial crude extract foliar spraying (*E. coli* strain HT115)	IR 54 HC; CP	hpRNA dsRNA	977 bp 1492 bp; 1081 bp	Dilution series (1/2 to 1/20) using 3 µg of total nucleic acid/µl. One-half diluted French Press preparations derived from engineered bacteria.	Dilutions of 1/10 or less did not display disease symptoms upon completion of their life cycles Plants treated with dsRNA-expressing preparations showed no virus symptoms (HC: 82% or CP: 73%).	(Tenllado et al., 2003)
TMV	Tobacco	Bacterial crude extract foliar spraying (Different *E. coli* strain tested)	CP	dsRNA	480 bp	One-half diluted French Press preparations derived from engineered bacteria.	M-JM109 or M-JM109lacY strains and the pGEM-CP480 vector exhibited the best results producing great quantities of dsRNA. Tobacco plants sprayed with dsRNA crude bacterial extract showed inhibition in TMV infection.	(Yin et al., 2009)
PMMoV CMV	Tobacco Cowpea	Foliar spray	RP 2b supressor	dsRNA naked or loaded onto LDH	977 bp 330 bp	125 µL per cm^2 (1.25 µg of dsRNA and/or 3.75 µg of LDH).	Virus protection for at least 20 days.	(Mitter et al., 2017a)
Fusarium asiaticum.	Wheat	Foliar spray after leaves were	β2-tubulinX	dsRNA	480 bp	40 ng μL^{-1} of water	Antifungal activity against these fungi with a reduction in the	(Gu et al., 2019)

Botrytis cinerea	Cucumber	wounded using quartz sand					dosage of carbendazim fungicides necessary to control the pathogens.	
Magnaporthe oryzae	Barley							
Colletotrichum truncatum	Soybean							

AChE2: *acetylcholine esterase*; CP: *Coat Protein*; Ces: *carboxylesterase*; ZYMV: *Zucchini yellow mosaic virus*; JHAMT: *Juvenile hormone acid O-methyltransferase*; Vg: *Vitellogenin*; CYP: *cytochrome P450*; KTI: *Kunitz-type trypsin inhibitor*; DCL: *Dicer-like*; BCMV: *Bean common mosaic virus*; PMMoV: *Pepper mild mottle virus*; CMV: *Cucumber mosaic virus*; LDH: double-layered hydroxide; RP: *Replicase*; CTV: *Citrus tristeza virus*; Awd: *abnormal wing disc*; BUR: *Bursicon*; FHV: *Flock house virus;* RPS13: *Ribosomal protein S13;* Vha26: *Vacuolar H[+]-ATPase 26kD E subunit;* Alpha COP: *Alpha-coatomer protein;* AK: *Arginine kinase;* INT: *β1 integrin gene;* CHSA: *Chitin synthase gene A;* Chi: *chitinase;* Rpl19: *ribosomal protein Rpl19;* Sec23: *Protein transport protein sec23;* vATPaseE: *Vacuolar ATP synthase subunit E;* vATPaseB: *Vacuolar ATP synthase subunit B;* COPβ: *Coatomer subunit beta;* SCMV: *Sugarcane Mosaic Virus;* HC: *Helper component;* IR: *replicase;* TLR7: *Toll-like receptor 7;* LC50: Lethal Concentration 50; LT50: Lethal Time 50; VIGS: Virus-induced gene silencing.

4. Manuscript 3.

Parental RNA interference as a tool to study genes involved in rostrum development in the Neotropical brown stink bug, *Euschistus heros.* ***

Deise Cagliari

Clauvis Nji Tizi Taning

Olivier Christiaens

Kristof De Schutter

Benny Lewille

Koen Dewettinck

Moises Zotti

Guy Smagghe

Manuscript published in Journal of Insect Physiology

Parental RNA interference as a tool to study genes involved in rostrum development in the Neotropical brown stink bug, *Euschistus heros*

Deise Cagliari[1,2*]; Clauvis Nji Tizi Taning[1*]; Olivier Christiaens[1]; Kristof De Schutter[1]; Benny Lewille[3]; Koen Dewettinck[3]; Moises Zotti[2]; Guy Smagghe[1*]

[1] Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium.

[2] Department of Crop Protection, Molecular Entomology Laboratory, Federal University of Pelotas, Pelotas, Brazil.

[3] Food Structure & Function Research Group, Department of Food Technology, Safety and Health, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium.

[*]Corresponding authors: deise.cagliari@ugent.be (D. Cagliari), tiziclauvis.taningnji@ugent.be (C.N.T. Taning), guy.smagghe@ugent.be (G. Smagghe)

Abstract

In insects, the identity of body segments is controlled by homeotic genes and the knockdown of these genes during embryogenesis can lead to an abnormal development and/or atypical phenotypes. The main goal of this study was to investigate the involvement of *labial (lab)*, *deformed (dfd)*, *sex comb reduced (scr)*, *extradenticle (exd)* and *proboscipedia (pb)* in rostrum development in the Neotropical brown stink bug *Euschistus heros*, using parental RNAi (pRNAi). To achieve this objective, 10-day-old adult females were first microinjected with double-stranded RNAs (dsRNA) targeting these five genes. Then, the number of eggs laid per female, the percentage of hatched nymphs with normal or abnormal phenotype and target gene

25 silencing were evaluated. Except for the ds*Dfd*-treatment, the number of eggs laid per female
26 per day was not affected by the different dsRNA-treatments compared to the control (ds*GFP*).
27 However, treatment with either ds*Lab, dsDfd*, ds*Scr* or ds*Exd* caused a strong reduction in egg
28 hatching. The ds*Exd*-treatment caused no apparent change in phenotype in the nymphs while
29 hatched nymphs from the *dsDfd*, ds*Scr* and ds*Pb*-treatment showed abnormalities in the
30 rostrum. Particularly for the ds*Pb*-treatment, 91% of the offspring displayed a bifurcated
31 rostrum with a leg-like structure. Overall, these results indicate that these five genes are
32 involved in *E. heros* embryonic development and that the knockdown of *dfd*, *scr* and *pb* leads
33 to an abnormal development of the rostrum. Additionally, this study demonstrates the efficiency
34 of pRNAi in studying genes involved in embryogenesis in *E. heros,* with clear phenotypes and
35 a strong target gene silencing in the next generation, after treatment of the parent female adult
36 with gene-specific dsRNA.
37 ***Keywords***: Pentatomidae, parental RNAi, embryonic development, homeotic genes.
38

39 **1. Introduction**

40 The Neotropical brown stink bug *Euschistus heros* (Fabr. 1798) (Hemiptera:
41 Pentatomidae) is an important Pentatomidae pest present in southern America, in Brazil
42 (Panizzi, 2015), Paraguay (Panizzi, 2015) and Argentina (Saluso et al., 2011). This
43 polyphagous stink bug feeds on different parts of Fabaceae, Solanaceae, Brassicaceae,
44 Compositae and Malvaceae plants (Panizzi et al., 2012; Smaniotto and Panizzi, 2015; Soares et
45 al., 2018; Soria et al., 2007). The majority of the crop damage occurs during their reproductive
46 period, when the insect population rapidly increases, resulting in significant losses in yield and
47 quality (Panizzi, 2015). Stink bugs use their piercing/sucking mouthparts to inject enzymes that
48 pre-digest the plant tissues, after which they suck up the fluid (Panizzi et al., 2012). The

piercing/sucking mouthparts are generated by a labium which holds the stylet, formed by the juxtaposition of a pair of mandibula and maxillae, creating the channel for liquid flow (Depieri and Panizzi, 2010; Hughes and Kaufman, 2000).

The differentiation of body segments in insects is controlled by a family of conserved genes known as homeotic genes (Dhawan and Gopinathan, 2005; Hughes and Kaufman, 2002; Lewis, 2007). In the large milkweed bug, *Oncopeltus fasciatus* (Hemiptera: Lygaeidae), *labial* (*lab*), *deformed* (*dfd*), *sex comb reduced* (*scr*) and *proboscipedia* (*pb*) have been reported as the *Hox* genes responsible for giving identity to the piercing/sucking segments of the mouth (Angelini et al., 2005; Hughes and Kaufman, 2000). *Extradenticle* (*exd*) is known for its role in body segmentation in *Drosophila* (Peifer and Wieschaus, 1990), however, its function is not yet explored in hemipteran insects. The temporal and spatial regulation of homeotic genes is essential for the correct development of the insects and incorrect expression of these genes (in place or time) and/or the repression of these genes can lead to the development of a completely different appendage (Hughes and Kaufman, 2002).

As a tool in functional genomics, RNA interference (RNAi) has been exploited to elucidate the role of genes in insect development (Hrycaj et al., 2010; Yates, 2014) and to identify potential targets for pest management. In the context of pest control, it has proven its efficiency in stink bugs such as *E. heros* (Cagliari et al., 2020; Castellanos et al., 2018), *Nezara viridula* (Gurusamy et al., 2020; Riga et al., 2019; Sharma et al., 2020), *Murgantia histrionica* (Howell et al., 2020), among others. Transgenerational or parental RNAi (pRNAi) is the phenomenon where a target gene knockdown phenotype is observed in the progeny of the treated parent organism (Vélez et al., 2017). Parental RNAi as a tool for studying gene functions and in the context of pest management has been explored in a range of insect species belonging to different insect orders, including Coleoptera (Bucher et al., 2002; Khajuria et al., 2015;

73 Prentice et al., 2015; Vélez et al., 2017) and Hemiptera (Angelini et al., 2005; Hughes and
74 Kaufman, 2000; Coleman et al., 2015; Fishilevich et al., 2016; Lu et al., 2017; Riga et al., 2019).
75 The ability to affect gene expression in the next generation makes pRNAi an interesting
76 research tool to study the role of genes involved in embryogenesis, without having to create
77 mutants for genes of interest.

78 The main goal of this study was to exploit pRNAi in *E. heros* to investigate the functional
79 role of *lab*, *dfd*, *scr*, *exd* and *pb* in rostrum development. Female insects were microinjected
80 with gene-specific dsRNA targeting these five genes, subsequently, the numbers of eggs laid
81 per female and target gene silencing were evaluated. Next, we evaluated hatching of the eggs
82 and studied the resulting effects from target gene silencing in the unhatched eggs and neonate
83 nymphs. Using light- and scanning electron microscopy (SEM), the rostrum structure was
84 investigated in detail for abnormalities. Altogether, this study highlights the usefulness of
85 pRNAi as a tool to study developmental genes in *E. heros* and possibly other essential genes
86 involved in embryogenesis and rostrum development, both in this species or other
87 Pentatomidae. In addition, pRNAi could eventually be developed as a strategy to control such
88 important pest insects in agriculture.

89 **2. Material and methods**

90 **2.1. Insects**

91 A colony of *E. heros* was kept under standard mass-rearing conditions of 25 ± 2 °C, 60 ±
92 10% relative humidity and an L/D photoperiod of 14:10 h. The insects were kept in plastic
93 boxes and fed *ad libitum* with a mixture of fresh green bean pods *Phaseolus vulgaris* (L.), raw
94 shelled peanuts *Arachis hypogaea* (L.) and soybean seeds *Glycine max* (L.) (Borges et al.,
95 2006). The supplies were replenished at 3-days-intervals. Eggs were removed and placed in
96 Petri dishes for five days, then transferred to plastic boxes and reared until they reached

adulthood. For the experiments, newly emerged adults (females and males) were collected daily to ensure that the insects used for microinjection were of the same age.

2.2. Target gene identification and phylogenetic analysis

Lab, *Dfd*, *Scr* and *Pb* were selected based on previously published RNAi research (Angelini et al., 2005; Hughes and Kaufman, 2000). Using these genes as inputs, a protein-protein interaction network (https://string-db.org/) was generated in an attempt to identify other genes with a potential role in rostrum development in *E. heros*. Based on these interactions, *extradenticle* (*exd*) was also selected to be further evaluated.

Hemipteran protein sequences of the selected genes were collected from the NCBI protein database (https://www.ncbi.nlm.nih.gov/). These sequences were used in tBLASTn searches against the published *E. heros* transcriptome database (Cagliari et al., 2020) to identify candidate gene orthologs in *E. heros*. For the detection of the open reading frames (ORFs) in the contig sequences, the ORF Finder tool from NCBI was used (https://www.ncbi.nlm.nih.gov/orffinder/). The Protein Basic Local Alignment Tool (Protein BLAST) was used for protein homology searches against the insect non-redundant protein database at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To confirm the identity of the selected genes, the nucleotide sequences were aligned in MEGA7 using the MUSCLE algorithm with default settings. Subsequently, the alignments were trimmed using trimAL with automated settings (Capella-Gutiérrez et al., 2009) and a phylogenetic tree was built using the maximum likelihood algorithm in MEGA7 with default settings (Kumar et al., 2016).

2.3. cDNA preparation and dsRNA synthesis

Total RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany), then residual genomic DNA was removed from the RNA samples using the Turbo DNA-free kit (Invitrogen), following the manufacturer's instructions. RNA was then quantified using a NanoDrop ND-

1000 (Nanodrop Technologies) and verified by 1.5% agarose gel electrophoresis. The cDNA was reverse transcribed, starting from 500 ng of total RNA template, with oligo (dT) primers using the SuperScript III First-Strand synthesis (Invitrogen, Merelbeke, Belgium). Primers were designed using the PrimerQuest Tool from Integrated DNA Technologies (IDT) (https://www.idtdna.com/pages) and T7 promoter sequences were placed at the 5′ -ends of both the forward and reverse primers (Supplementary Table 1). The designed primers together with Taq DNA polymerase (Invitrogen) and 500 ng of cDNA (as a template) were used to amplify the target gene DNA template for subsequent dsRNA synthesis. For the negative control, a green fluorescent protein (GPF) fragment was amplified from a plasmid containing a *GFP* insert (Genbank ID: NC_011521.1). The amplified target gene DNA templates were purified using the Wizard clean-up system (Promega, Madison, WI, USA) and used for dsRNA synthesis with the MEGAscript RNAi kit (Ambion, Austin, TX, USA), according to the instructions of the manufacturer. The synthesized dsRNA was quantified on a NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) at 260 nm and analyzed by gel electrophoresis to determine integrity.

2.4. DsRNA microinjection

Virgin females (10 days after emergence) were microinjected using a nanoinjector (FemtoJet, Eppendorf, Hamburg, Germany), equipped with an injection needle prepared with capillary glass tubes. Each female (approximate fresh weight of 60 mg) was injected with 2.5 µL of a 4 µg/µL dsRNA solution (i.e. 10 µg dsRNA per insect, which corresponds to approximately 167 ng per mg of insect body weight), based on an established protocol (Cagliari et al., 2020; Castellanos et al., 2019). The females were anesthetized on ice for 10 min and then injected in the ventral metathoracic region, near the hind coxa. DsRNA targeting *GFP* was used as a negative control. Ten females were injected per treatment. After microinjection, females

were individually placed into different Petri dishes containing green bean slices and were allowed to rest for 6 h. After that, two males were added into every Petri dish. The number of fertilized eggs per female and the percentage of hatched nymphs with normal or abnormal rostrum development were analyzed every day, starting from the third day after microinjection and continuing until 21 days after microinjection.

2.5. Phenotype analyses

Photographs of the rostrum were taken under a dissection stereomicroscope (Leica M420, Wetzlar, Germany). For Cryo-scanning electron microscopy (SEM), samples of the insects were placed in slots of a stub, plunge-frozen in liquid nitrogen and then transferred into the cryo-preparation chamber (PP3010T cryo-SEM preparation system, Quorum Technologies, Lewes, UK). Samples were then freeze-fractured, sublimated and subsequently sputter-coated with Pt, and examined in a JEOL JSM 7100F SEM (JEOL, Tokyo, Japan).

2.6. Real-time quantitative PCR

All qPCR measures were performed with three biological repeats and each consisted of three technical repeats. Specifically, we collected 7-days-old eggs from ds*Lab*- (N=29, 17, and 16), ds*Dfd*- (N=7, 7, and 11), ds*Scr*- (N=13, 5 and 9) and ds*GFP*- (N=9, 11 and 8) treated groups that were deposited at 8, 9 and 11 days after microinjection, respectively. For ds*Exd* (N=20, 15, and 13), we collected the eggs immediately upon deposition at 4, 5 and 6 days after microinjection. For ds*Pb*, we collected 1st-instar nymphs (N=9, 19 and 14) after hatching from eggs deposited at 9, 10 and 11 days after microinjection, and the target gene expression was compared to a respective control, i.e. ds*GFP* (N=19, 14 and 22). Samples collected from the bioassay were stored at -80 °C until further analyses.

For all samples, total RNA was isolated using the RNeasy kit (Qiagen) following the suppliers' recommendations as described above. qRT-PCR specific primers were designed

using PrimerQuest Tool from IDT (Supplementary Table 2). The qRT-PCR reactions were performed in the CFX 96TM real-time system (Bio-Rad, Hercules, CA, USA) with SYBR green dye as the fluorescence reporter. The primers used in the analysis were validated with a standard curve based on a serial dilution of cDNA to determine the primer annealing efficiency and a melting curve analysis with a temperature range from 60 to 95 °C. The reaction included 10 µL of GoTaq qPCR Master Mix for Dye-Based Detection (Promega), 1 µL of 10 µM forward primer (Invitrogen), 1 µL of 10 µM of reverse primer (Invitrogen), and 8 µL of cDNA (dilution 1:100), in a total volume of 20 µL. The amplification conditions were 3 min at 95 °C followed by 39 cycles of 10 s at 95 °C and 30 s at 60 °C. The reactions were set-up in 96-well format Microseal PCR plates (Bio-Rad). The endogenous controls, *ribosomal protein 18S* and *RPL32*, were used for normalization of the data. A no-template control was also included in the assay. Relative expression values of genes in biological samples were calculated using the equation ratio $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

2.7. Data analysis

The data were checked for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests, respectively. Given that the data were overall not normally distributed, the nonparametric Kruskal-Wallis test followed by a Dunn's multiple comparison test was used for analysis in the SigmaPlot 12.0 software (Systat Software, San Jose, CA, USA).

3. Results

3.1. Phylogeny of the genes

While *lab*, *dfd*, *scr* and *pd* were previously shown to be involved in the development of the rostrum, the potential role of *exd* was suggested through a protein-protein interaction network (Supplementary figure S1). The *lab*, *dfd*, *scr, pb* and *exd* genes were identified in the *E. heros* transcriptome (Cagliari et al., 2020) based on homology with sequences obtained from

other hemipterans. The presence of the conserved homeotic domain was confirmed in *lab*, *dfd*, *scr* and *exd*, while *pb* presented only a partial fragment of the domain. The phylogenetic analysis confirmed the correct identification of the proteins, generating five clusters, each one representing one of the genes (Supplementary figure S2).

3.2. Parental RNAi effects in oviposition and egg hatching rates

Fertilized eggs change color from yellow to reddish during embryonic development, while unfertilized eggs stay yellow and show no change in color (Supplementary figure S3). This characteristic enables the identification of fertilized and unfertilized eggs. The number of fertilized eggs laid per female was evaluated daily from the 3rd to the 21st day after microinjection (Supplementary file S1 and S2). As shown in Figure 1a, the treatment of the females with either of the tested gene-specific dsRNAs did not affect the number of fertilized eggs laid/female/day when compared to the ds*GFP* control, with the exception of the ds*Dfd*-treatment. Each female injected with ds*GFP* produced an average±SE of 3.5±1.7 eggs per day and this was similar ($p>0.05$) for ds*Lab,* ds*Scr*, ds*Exd* and ds*Pb* with 4.5±2.1, 2.2±1.3, 2.9±1.2, and 3.8±2.2 eggs per day, respectively, while the ds*Dfd*-treated females only produced 1.4±1.4 egg per day ($p<0.001$).

In addition, we scored the viability of the eggs produced by the treated females as the number of nymphs hatching from the eggs. In the control (ds*GFP*), there were 519 eggs and 370 of them developed into nymphs, which is representing a hatching percentage of 71%. For the treatments with ds*Pb* the hatching was similar ($p>0.05$) with 66% (196 nymphs out of 295 eggs) (Figure 1b). In contrast, the egg hatching percentage in the four other dsRNA treatments was significantly lower ($p<0.001$). Specifically, for ds*Lab*-, ds*Dfd*-, ds*Scr*- and ds*Exd*-treatments, only 4% (25 nymphs out of 650 eggs), 12% (13 nymphs out of 107 eggs), 6% (19 nymphs out of 300 eggs) and 1% (3 nymphs out of 204 eggs) of the eggs hatched, respectively.

This represented a high decrease of over 90% in the total number of eggs that hatched to nymphs for these treatments.

3.3. Parental RNAi phenotypes in the embryo inside the egg and the hatched nymph

Typically, the eggs of the control (ds*GFP*) are yellow when laid by the female and they change color to reddish/orange prior to hatching (6-7 days after being laid). At this moment, the embryo presents all developed structures, such as legs, antennae and rostrum (Figure 2a). The embryos from the eggs deposited by females treated with ds*Lab,* presented all developed appendage structures, such as legs, antennae and rostrum. However, they were dead before hatching, suggesting lethal effects during the embryogenesis as a result of target gene knockdown (Figure 2c, d). With ds*Exd*, it was clear that the embryos did not develop. Specifically, these embryos did not complete their development and did not show any appendage structures such as legs, antennae or rostrum (Figure 2e, f).

After egg hatching, the nymphs of the control (ds*GFP*) had a needle-like shaped rostrum with a sharp tip (Figure 3a, b, c). In these insects, the labium and the stylet (maxilla and mandible) form the rostrum. In contrast, with ds*Dfd*, we observed two abnormal structures, each on one side of the head, between the antennae and the labium (Figure 3d), and this phenotype was observed in one of the 13 nymphs. In contrast, the nymphs that developed from the ds*Scr*- (Figure 3e) and ds*Pb*-treated (Figure 3f, g, h) females showed a clear malformed rostrum, specifically a bifurcated rostrum with a leg-like structure. The proximal first two sections of the labium were normal as observed in the control, while the third section was split into two leg-like structures with claws. In the ds*Scr*- and ds*Pb*-treatment, this leg-like rostrum phenotype was observed in 74% (14 nymphs out of the total 19 nymphs hatched) and 91% (178 nymphs out of the total 196 nymphs hatched) of the hatched nymphs, respectively. Interestingly, these nymphs with the malformed rostrum phenotype were unable to feed and died shortly after

241 hatching, resulting in a total loss of the next generation after treatment of the female parent.

242 **3.4. Parental RNAi effects at gene expression level**

243 For eggs collected from ds*Dfd*-, ds*Scr*-, and ds*Exd*-treated females, there was a significant
244 ($p \leq 0.01$) decrease in the transcript level of the target genes by 40±9%, 60±20% and 62±4%,
245 respectively. In contrast, an unexpected increase ($p < 0.01$) in the target gene transcript level was
246 recorded for eggs collected from ds*Lab*-treated females compared to the control (ds*GFP*). In
247 the ds*Pb*-treatment, a 63±8% reduction in the target gene transcript level was recorded for
248 nymphs with a strong malformed rostrum phenotype, when compared to the respective control
249 (ds*GFP*).

250 **4. Discussion**

251 Parental RNAi to elucidate gene function has been used in insect species, such as
252 *Diabrotica virgifera* (Coleoptera: Chrysomelidae) (Khajuria et al., 2015), *O. fasciatus*
253 (Hemiptera: Lygaeidae) (Hughes and Kaufman, 2000) and *Periplaneta americana* (Blattodea:
254 Blattidae) (Hrycaj et al., 2010). In this study, parental gene silencing in *E. heros* targeting *lab*,
255 *dfd*, *scr* and *exd* resulted in embryonic lethality in the offspring for up to 21 days after treatment.
256 Moreover, upon silencing of *Dfd*, *Scr* and *Pb*, nymphs from successfully hatched eggs had a
257 malformed rostrum. These results indicate the involvement of these genes in *E. heros*
258 embryonic and/or rostrum development, and also confirm the potential of pRNAi as a valuable
259 tool for studying developmental genes in this species. Furthermore, a high loss of progeny
260 following exposure of adult *E. heros* females to target gene-specific dsRNA suggests that these
261 genes could be potential targets for RNAi-based pest control. However, further research will be
262 required to investigate their relevance for pest control in comparison to or in combination with
263 targets that can cause direct mortality in adults. In addition, the designed dsRNAs against these
264 target genes should be safe for non-target organisms as beneficials insects with pollinators and

natural enemies (Bachman et al., 2013, Roberts et al., 2015, Christiaens et al., 2018, Mezzetti et al., 2020, Taning et al., 2020).

Homeotic genes are crucial for proper embryonic development and the loss of homeotic function can result in abnormal phenotypes and/or embryonic death (Robertson and Mahaffey, 2005). In this study, we found that targeting *lab*, *dfd, scr,* and *exd* reduced the egg hatching rates significantly. *Lab, scr* and *dfd* are genes mainly expressed in the head of *Drosophila* (Diederich et al., 1989), *O. fasciatus* (Angelini et al., 2005) and *Apis mellifera* (Hymenoptera: Apidae) (Fleig et al., 1992) during embryonic development. Disruption in the expression of these genes can result in defective head formation (Merrill et al., 1989), leading to embryonic death. While *lab* was not required for normal embryo development in the hemipteran milkweed bug *O. fasciatus* (Angelini et al., 2005), targeting this gene in *Drosophila* resulted in embryo lethality before hatching (Merrill et al., 1989). In *E. heros, lab* knockdown resulted in high embryo mortality, leading to a severe reduction in egg hatching rates. Also, targeting *scr* in *E. heros* resulted in embryo mortality. A similar phenotype was observed when *scr* was targeted in *H. halys*, where the researchers reported a decrease in egg hatching percentage (Lu et al. 2017). In *Drosophila*, during embryonic development, the expression of *dfd* is also essential and required during the first hours of embryogenesis. Mutation of *dfd* leads to a lethal phenotype during embryonic development (Merrill et al., 1987). In *E. heros*, the knockdown of this gene also caused embryonic lethality. In eggs from ds*Exd*-treated females, the embryos do not complete their development, showing no structures such as legs, antennae or rostrum. In *Drosophila*, *exd* acts through its selective homeodomain proteins, altering the regulation of other homeoproteins (Kurant et al., 1998; Rieckhof et al., 1997). This gene is required for proper segmentation and appropriate segmental identity (Peifer and Wieschaus, 1990). Loss of *exd* expression disrupts embryonic development (Robertson and Mahaffey, 2005), wherein

289 embryos lacking *exd* die during late embryogenesis (Peifer and Wieschaus, 1990). Due to its
290 regulatory function, the knockdown of this gene in *E. heros* probably leads to a cascade process,
291 which affects proper embryonic development and ultimately leads to the death of the embryo.
292 Also, the knockdown of either *exd, dfd* or *scr* expression in the female parent can also lead to
293 disruptions in embryo development. Nevertheless, there still exists knowledge gaps on the
294 detailed mechanism(s) through which these genes regulate certain processes during embryonic
295 development, warranting further investigation.

296 Besides the effects in embryo development, inappropriate expression of homeotic genes
297 can also result in the transformation of one body part to another (Heffer and Pick, 2013). While
298 the rostrum of control 1st-instar nymphs has a needle-like shape with a sharp tip, consisting of
299 the labium (Lb) and stylet (St), pRNAi of *dfd* resulted in an abnormal rostrum in the offspring.
300 Although only one nymph showed this phenotype in our study with *E. heros*, it is very similar
301 to the phenotype observed in *O. fasciatus*, where silencing of *dfd* caused the appearance of two
302 short and curled structures at the posterior of the rostrum (Hughes and Kaufman, 2000). The
303 offspring from ds*Scr*- and ds*Pb*-treated females showed a bifurcated rostrum with a leg-like
304 structure. These pRNAi phenotypes observed in *E. heros* are largely consistent with the
305 phenotypes described in *O. fasciatus* for these target genes (Hughes and Kaufman, 2000).
306 Hughes and Kaufman (2000) found that the depletion of *scr* and *pb* led to abnormal
307 development of the labium to a leg-like structure in *O. fasciatus*. Also, silencing of *scr* in
308 *Nezara viridula* (Hemiptera: Pentatomidae) led to a similar phenotype, further confirming the
309 involvement of this gene in rostrum development (Riga et al., 2019). However, this observation
310 could vary between species considering that *scr* has been reported to have no function in the
311 head of the cockroach *P. americana* during post-embryogenesis (Hrycaj et al., 2010).
312 Altogether, pRNAi was a useful tool to study genes involved in rostrum development due to its

long-lasting effects, resulting in offspring with clear phenotypes. However, further studies will be required to uncover the underlying mechanisms that lead to the observed phenotypes when these target genes are knocked down, providing a better understanding of their role in embryo development.

5. Conclusion

Here, we demonstrated the use of pRNAi to study the function of homeotic genes in rostrum development in the Neotropical brown stink bug *E. heros*. Knockdown of *lab*, *dfd*, *scr,* and *exd* led to a reduction in the egg hatching rate, indicating an involvement of these genes in embryonic development. The nymphs that hatched from ds*Scr*- and ds*Pb*-treated females showed a malformed rostrum with a bifurcated leg-like structure instead of the labium, while for ds*Dfd*, the rostrum showed two short and curled structures. These results support pRNAi effects in this species and demonstrate the involvement of these genes in embryonic and/or rostrum development. This agrees with our opinion for the need of a wider phylogenetic investigation into the function and interactions of *Hox* genes, as well as other essential developmental and regulatory genes, for fundamental insect research and/or RNAi-mediated pest control.

Author contribution

Deise Cagliari: Experimental design, performed the assays, data organization and analysis, writing - original draft. **Clauvis Nji Tizi Taning:** Experimental design, data organization and analysis, writing – review and editing. **Olivier Christiaens:** Data analysis, writing – review and editing. **Kristof De Schutter:** Data analysis, writing – review and editing. **Benny Lewille:** scanning electron microscopy, writing, and editing. **Koen Dewettinck:** scanning electron microscopy, writing and editing. **Moises Zotti:** Writing – review and editing. **Guy Smagghe:** Conceptualization, supervision, funding acquisition, writing – review and editing.

Declaration of Competing Interest

The author(s) declare no competing interests.

Acknowledgments

This research was support by the coordination for the Improvement of Higher Education Personnel (CAPES) in Brazil, the Special Research Fund of Ghent University (BOF) and the Research Foundation-Flanders (FWO-Vlaanderen) in Belgium. Hercules foundation is acknowledged for its financial support in the acquisition of the scanning electron microscope JEOL JSM-7100F equipped with cryo-transfer system Quorum PP3000T and Oxford Instruments Aztec EDS (grant number AUGE-09-029). Olivier Christiaens is a recipient of a postdoctoral fellowship from the Research Foundation – Flanders (FWO).

References

Angelini, D.R., Liu, P.Z., Hughes, C.L., Kaufman, T.C., 2005. Hox gene function and interaction in the milkweed bug *Oncopeltus fasciatus* (Hemiptera). Dev. Biol. 287, 440–455. https://doi.org/10.1016/j.ydbio.2005.08.010

Bachman P.M, Bolognesi R., Moar W.J. Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). Transgenic Res., 22 (2013), pp. 1207-1222, https://doi.org/10.1007/s1124 8-013-9716-5

Borges, M., Laumann, R.A., Da Silva, C.C. a, Moraes, M.C.B., Dos Santos, H.M., Ribeiro, D.T., 2006. Metodologias de criação e manejo de colônias de percevejos da soja (Hemíptera - Pentatomidae) para estudos de comportamento e ecologia química. Embrapa Recur. Genéticos e Biotecnol. Documentos, 18.

Bucher, G., Scholten, J., Klingler, M., 2002. Parental RNAi in *Tribolium* (coleoptera). Curr. Biol. 12, 85–86. https://doi.org/10.1016/S0960-9822(02)00666-8

Cagliari, D., Dias, N.P., dos Santos, E.Á., Rickes, L.N., Kremer, F.S., Farias, J.R., Lenz, G., Galdeano, D.M., Garcia, F.R.M., Smagghe, G., Zotti, M.J., 2020. First transcriptome of the Neotropical pest *Euschistus heros* (Hemiptera: Pentatomidae) with dissection of its siRNA machinery. Sci. Rep. 10, 1–16. https://doi.org/10.1038/s41598-020-60078-3

Capella-Gutiérrez, S., Silla-Martínez, J.M., Gabaldón, T., 2009. TrimAl: A tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics 25, 1972–1973. https://doi.org/10.1093/bioinformatics/btp348

Castellanos, N.L., Smagghe, G., Sharma, R., Oliveira, E.E., Christiaens, O., 2019. Liposome encapsulation and EDTA formulation of dsRNA targeting essential genes increase oral RNAi-caused mortality in the Neotropical stink bug *Euschistus heros*. Pest Manag. Sci. 75, 537–548. https://doi.org/10.1002/ps.5167

Coleman, A.D., Wouters, R.H.M., Mugford, S.T., Hogenhout, S.A., 2015. Persistence and transgenerational effect of plant-mediated RNAi in aphids. J. Exp. Bot. 66, 541–548. https://doi.org/10.1093/jxb/eru450

Depieri, R.A., Panizzi, A.R., 2010. Rostrum length, mandible serration, and food and salivary canals areas of selected species of stink bugs (Heteroptera, Pentatomidae). Rev. Bras. Entomol. 54, 584–587. https://doi.org/10.1590/S0085-56262010000400008

Dhawan, S., Gopinathan, K.P., 2005. Phylogeny of the insect homeobox gene (Hox) cluster. Genomics, Proteomics Bioinforma. 3, 42–46. https://doi.org/10.1016/S1672-0229(05)03006-8

Diederich, R.J., Merrill, V.K., Pultz, M.A., Kaufman, T.C., 1989. Isolation, structure, and expression of labial, a homeotic gene of the Antennapedia Complex involved in *Drosophila* head development. Genes Dev. 3, 399–414. https://doi.org/10.1101/gad.3.3.399

385 Fishilevich, E., Velez, A.M., Khajuria, C., Frey, M.L.F., Hamm, R.L., Wang, H.,
386 Schulenberg, G.A., Bowling, A.J., Pence, H.E., Gandra, P., Arora, K., Storer, N.P.,
387 Narva, K.E., Siegfried, B.D., 2016. Use of chromatin remodeling ATPases as RNAi
388 targets for parental control of western corn rootworm (*Diabrotica virgifera virgifera*) and
389 Neotropical brown stink bug (*Euschistus heros*). Insect Biochem. Mol. Biol. 71, 58–71.
390 https://doi.org/10.1016/j.ibmb.2016.02.004
391 Fleig, R., Walldorf, U., Gehring, W.J., Sander, K., 1992. Development of the Deformed
392 protein pattern in the embryo of the honeybee *Apis mellifera* L. (Hymenoptera). Roux's
393 Arch. Dev. Biol. 201, 235–242. https://doi.org/10.1007/BF00188754
394 Gurusamy, D., Howell, J.L., Chereddy, S.C.R.R., Koo, J., Palli, S.R., 2020. Transport of
395 orally delivered dsRNA in southern green stink bug, *Nezara viridula*. Arch. Insect
396 Biochem. Physiol. 104, 1–18. https://doi.org/10.1002/arch.21692
397 Heffer, A., Pick, L., 2013. Conservation and variation in Hox genes: How insect models
398 pioneered the evo-devo field . Annu. Rev. Entomol. 58, 161–179.
399 https://doi.org/10.1146/annurev-ento-120811-153601
400 Howell, J.L., Mogilicherla, K., Gurusamy, D., Palli, S.R., 2020. Development of RNAi
401 methods to control the harlequin bug, *Murgantia histrionica*. Arch. Insect Biochem.
402 Physiol. 104, 1–14. https://doi.org/10.1002/arch.21690
403 Hrycaj, S., Chesebro, J., PopadiC, A., 2010. Functional analysis of Scr during embryonic and
404 post-embryonic development in the cockroach, *Periplaneta americana*. Dev. Biol. 341,
405 324–334. https://doi.org/10.1016/j.ydbio.2010.02.018
406 Hughes, C.L., Kaufman, T.C., 2002. Hox genes and the evolution of the arthropod body plan.
407 Evol. Dev. 4, 459–499. https://doi.org/10.1046/j.1525-142X.2002.02034.x
408 Hughes, C.L., Kaufman, T.C., 2000. RNAi analysis of deformed, proboscipedia and sex

combs reduced in the milkweed bug *Oncopeltus fasciatus*: Novel roles for Hox genes in the Hemipteran head. Development 127, 3683–3694.

Khajuria, C., Vélez, A.M., Rangasamy, M., Wang, H., Fishilevich, E., Frey, M.L.F., Carneiro, N.P., Gandra, P., Narva, K.E., Siegfried, B.D., 2015. Parental RNA interference of genes involved in embryonic development of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Insect Biochem. Mol. Biol. 63, 54–62. https://doi.org/10.1016/j.ibmb.2015.05.011

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054

Kurant, E., Pai, C.Y., Sharf, R., Halachmi, N., Sun, Y.H., Salzberg, A., 1998. Dorsotonals/homothorax, the *Drosophila* homologue of meis1 interacts with extradenticle in patterning of the embryonic PNS. Development 125, 1037–1048.

Lewis, E.B., 2007. A gene complex controlling segmentation in *Drosophila*. Genes, Dev. Cancer Life Work Edward B. Lewis 229–242. https://doi.org/10.1007/978-1-4020-6345-9_10

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Lu, Y., Chen, M., Reding, K., Pick, L., 2017. Establishment of molecular genetic approaches to study gene expression and function in an invasive hemipteran, *Halyomorpha halys*. Evodevo 8, 1–11. https://doi.org/10.1186/s13227-017-0078-6

Merrill, V.K.L., Diederich, R.J., Turner, F.R., Kaufman, T.C., 1989. A genetic and developmental analysis of mutations in labial, a gene necessary for proper head

433 formation in *Drosophila melanogaster*. Dev. Biol. 135, 376–391.
434 https://doi.org/10.1016/0012-1606(89)90187-5
435 Merrill, V.K.L., Turner, F.R., Kaufman, T.C., 1987. A genetic and developmental analysis of
436 mutations in the Deformed locus in *Drosophila melanogaster*. Dev. Biol. 122, 379–395.
437 https://doi.org/10.1016/0012-1606(87)90303-4
438 Mezzetti B., Smagghe G., Arpaia S., Christiaens O., Dietz-Pfeilstetter A., Jones H.D., Ventura
439 V. RNAi: What is its position in agriculture? J. Pest Sci., 93 (2020), pp. 1123-1130,
440 https://doi.org/10.1007/s10340-020-01238-2
441 Panizzi, A.R., 2015. Stink bugs growing problems with (Hemiptera: Heteroptera:
442 Pentatomidae): Species invasive to the U.S. and potential. Am. Entomol. 61, 223–233.
443 Panizzi, A.R., Bueno, A.F., Silva, F.A.C., 2012. Insetos que atacam vagens e grãos, in: Soja:
444 Manejo Integrado de Insetos e Outros Artrópodes-Praga. pp. 335–420.
445 Peifer, M., Wieschaus, E., 1990. Mutations in the *Drosophila* gene extradenticle affect the
446 way specific homeo domain proteins regulate segmental identity. Genes Dev. 4, 1209–
447 1223. https://doi.org/10.1101/gad.4.7.1209
448 Rieckhof, G.E., Casares, F., Ryoo, H.D., Abu-Shaar, M., Mann, R.S., 1997. Nuclear
449 translocation of extradenticle requires homothorax, which encodes an extradenticle-
450 related homeodomain protein. Cell 91, 171–183. https://doi.org/10.1016/S0092-
451 8674(00)80400-6
452 Riga, M., Denecke, S., Livadaras, I., Geibel, S., Nauen, R., Vontas, J., 2019. Development of
453 efficient RNAi in *Nezara viridula* for use in insecticide target discovery. Arch. Insect
454 Biochem. Physiol. 1–9. https://doi.org/10.1002/arch.21650
455 Roberts A.F., Devos Y., Lemgo G.N.Y, Zhou X. Biosafety research for non-target organism
456 risk assessment of RNAi-based GE plants. Front. Plant Sci. 6:958 (2015).

https://doi.org/10.3389/fpls.2015.00958

Robertson, L.K., Mahaffey, J.W., 2005. Insect homeotic complex genes and development, lessons from *Drosophila* and beyond. Compr. Mol. Insect Sci. 1–6, 247–303. https://doi.org/10.1016/B0-44-451924-6/00004-1

Saluso, A., Xavier, L., Silva, F.A.C., Panizzi, A.R., 2011. An invasive pentatomid pest in Argentina: Neotropical brown stink bug, *Euschistus heros* (F.) (Hemiptera: Pentatomidae). Neotrop. Entomol. 40, 704–705.

Sharma, R., Christiaens, O., Taning, C.N.T., Smagghe, G., 2020. RNAi-mediated mortality in southern green stinkbug *Nezara viridula* by oral delivery of dsRNA. Pest Manag. Sci. https://doi.org/10.1002/ps.6017

Smaniotto, L.F., Panizzi, A.R., 2015. Interactions of selected species of stink bugs (Hemiptera: Heteroptera: Pentatomidae) from leguminous crops with plants in the Neotropics. Florida Entomol. 98, 7–17. https://doi.org/10.1653/024.098.0103

Soares, P.L., Cordeiro, E.M.G., Santos, F.N.S., Omoto, C., Correa, A.S., 2018. The reunion of two lineages of the Neotropical brown stink bug on soybean lands in the heart of Brazil. Sci. Rep. 8, 1–12. https://doi.org/10.1038/s41598-018-20187-6

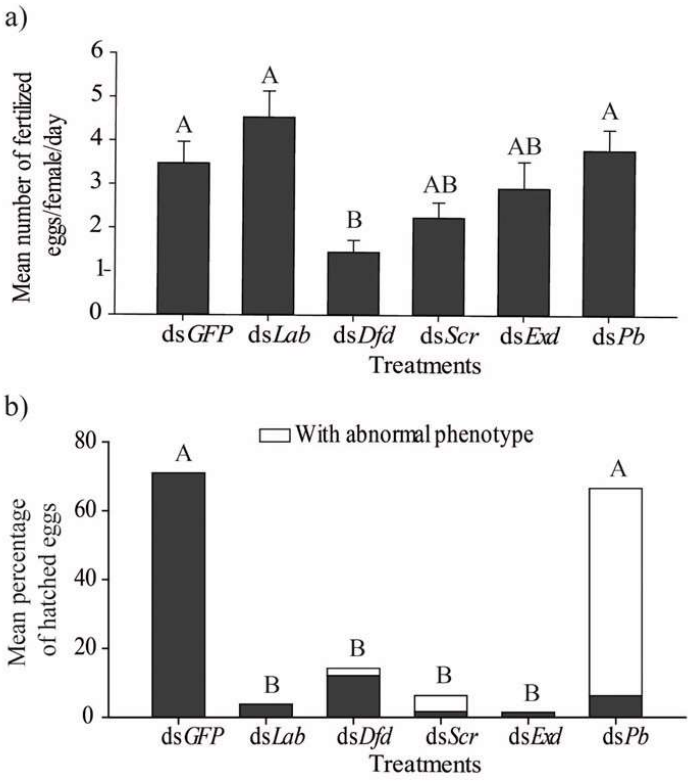
Soria, M.F., Degrade, P.E., Panizzi, A.R., 2007. Algodão invadido. Rev. Cultiv. 11, 274.

Taning C.N.T., Gui S,De Schutter K., Jahani M., Castellanos N.L., Christiaens O., Smagghe G. A sequence complementarity-based approach for evaluating off-target transcript knockdown in *Bombus terrestris*, following ingestion of pest-specific dsRNA. J. Pest Sci. (2020), https://doi.org/10.1007/s10340-020-01273-z

Vélez, A.M., Fishilevich, E., Matz, N., Storer, N.P., Narva, K.E., Siegfried, B.D., 2017. Parameters for successful parental RNAi as an insect pest management tool in western corn rootworm, *Diabrotica virgifera virgifera*. Genes (Basel). 8, 1–18.

https://doi.org/10.3390/genes8010007

Yates, A.D., 2014. RNA interference as a tool for the functional analysis of genes in the Colorado potato beetle , *Leptinotarsa decemlineata* (Say). Diss. Student Res. Entomol. http://digitalcommons.unl.edu/entomologydiss/33.



487

488 **Figure 1.** The brown stink bug *Euschistus heros* oviposition (fertilized eggs) and percent egg
489 hatch rates following knockdown of *labial*, *deformed*, *sex comb reduced*, *extradenticle* and
490 *proboscipedia*. Females were microinjected with 10 µg/µl of gene-specific dsRNA and the eggs
491 collected daily from the 3rd to the 21st day after microinjection **a)** Mean number of eggs per
492 female per day. **b)** Mean percent of egg hatching with normal (dark bars) and abnormal
493 phenotype (white bars). Bars represent the mean observed in every treatment. The bars with
494 different letters denote significant differences ($p<0.05$) according to a Dunn's test. Confidence
495 bars are shown for ± SE.

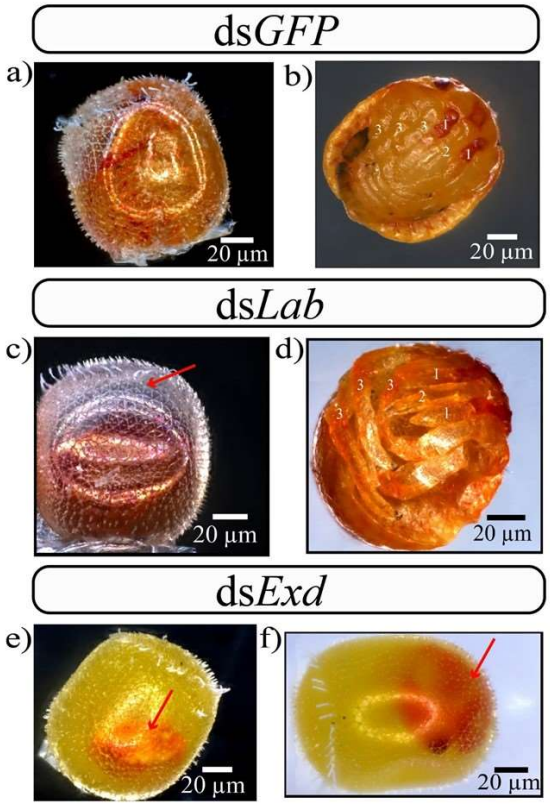
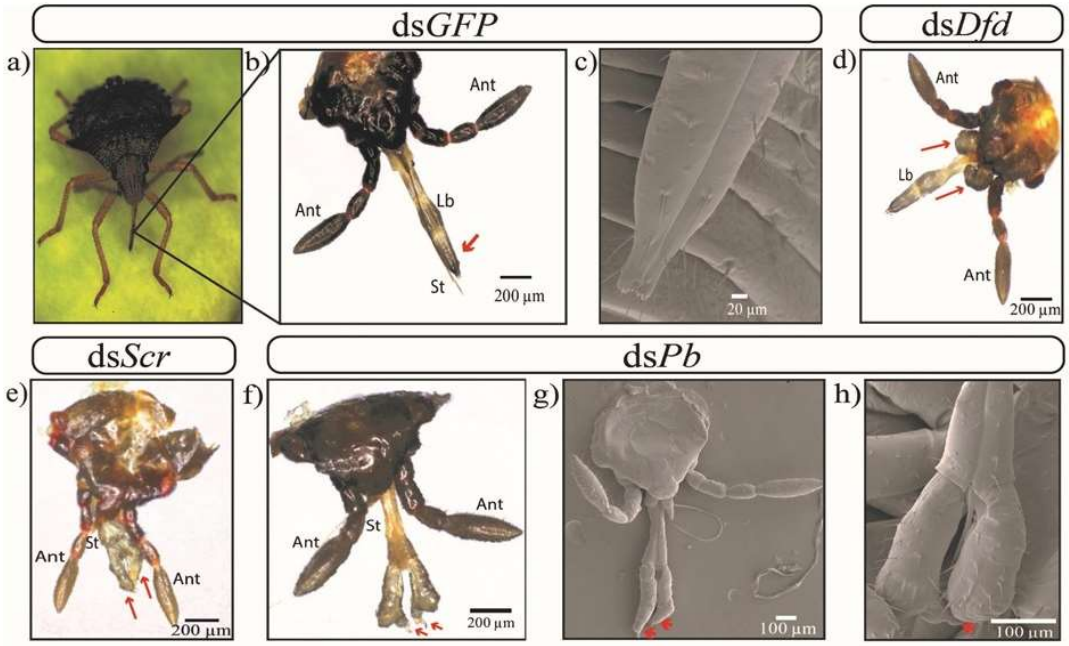
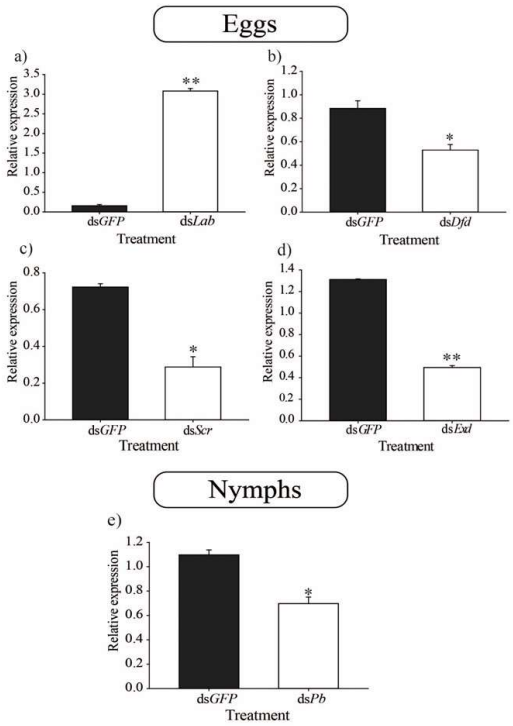


Figure 2. The RNAi phenotype in eggs laid by *GFP*, *labial,* and *extradenticle* dsRNA-treated females of the brown stink bug *Euschistus heros*. a) lateral view of a 7-days-old egg laid by a ds*GFP*-treated female; b) ventral view of a 7-days-old dissected embryo from ds*GFP*-treated female; c) lateral view of a 7-days-old egg laid by a ds*Lab*-treated female; the red arrow shows a bigger blank space in comparison with *GFP* eggs (a), indicating the death of the embryo prior to hatching; d) ventral view of a 7-days-old dissected embryo from ds*Lab*-treated female; asterisk indicates a hollow part right behind the legs; e-f) lateral view of 7-days-old eggs laid by a ds*Exd*-treated female; red arrow shows the embryos that did not develop. 1-antenna; 2-rostrum; 3-legs.



506

507 **Figure 3.** The RNAi phenotype in nymphs of the brown stink bug *Euschistus heros* from
508 females treated with dsRNA targeting *GFP*, *deformed, sex comb reduced,* and *proboscipedia*.
509 **a,b,c)** Control of the brown stink bug *Euschistus heros* nymph treated with ds*GFP*. **a)** nymphs
510 feeding on green beans, where the insect inserts the piercing/sucking structure into the plant
511 tissue, injecting enzymes that pre-digest the tissue content and then followed by sucking of the
512 pre-digested fluids (Panizzi et al., 2012). **b)** Details of the piercing/sucking mouthparts. The red
513 arrow shows the tip of the labium. **c)** Detail of the labium tip part under the SEM. Ant, antenna;
514 Lb, labium; St, stylet. **d)** ds*Dfd* phenotype: the insect presented two curl structures one in each
515 side of the head, between the labium and antenna. This insect presented a normal labium
516 appendage. **e)** ds*Scr* phenotype: the Lb appendage is transformed into a bifurcated rostrum with
517 a leg-like structure, while the St structure is normal; Ant, antenna; Lb, labium; St, stylet. **f,g,h)**
518 ds*Pb* phenotype showing the Lb appendage is transformed into a leg-like structure. On the
519 transformed labium we can see claws (red arrows), and the St structure is normal as in the
520 control. **h)** Details of the distal part under the SEM, showing the splitted appendage with two
521 leg-like structures with claws (red arrows).



522

523 **Figure 4.** Percent knockdown of *labial*, *deformed*, *sex comb reduced* and *extradenticle* in eggs
524 and *pb* in nymphs of the brown stink bug *Euschistus heros*. Relative expression was normalized
525 to *ribosomal protein 18S* and *RPL32*. Females were microinjected with 10 µg of dsRNA. For
526 ds*Lab* (N=29, 17 and 16), ds*Dfd* (N=7, 7 and 11), ds*Scr* (N=13, 5 and 9), and ds*GFP* (N=9, 11
527 and 8), eggs were collected 8, 9, and 11 days after microinjection, tracked for seven days and
528 then collected for RNA extraction. For ds*Exd* (N=20, 15, and 13), eggs were collected at 4, 5,
529 and 6 days after microinjection. The nymphs of ds*Pb* (N=9, 19 and 14) and ds*GFP* (N=19, 14
530 and 22) were collected from eggs laid at 9, 10, and 11 days after microinjection, tracked for
531 seven days and then RNA extracted. **a)** Relative *lab* transcript expression. **b)** Relative *dfd*
532 transcript expression. **c)** Relative *scr* transcript expression. **d)** Relative *exd* transcript
533 expression. **e)** Relative *pb* transcript expression. Comparison of the means was performed with
534 *GFP* as control, using the Kruskal-Wallis test and Dunn's multiple comparison test in
535 SigmaPlot 12.0 (Systat Software, San Jose, CA, USA), ** $p<0.001$, * $p<0.05$. N=number of
536 nymphs.

Supplementary material

Parental RNA interference as a tool to study genes involved in rostrum development in the Neotropical brown stink bug, *Euschistus heros*

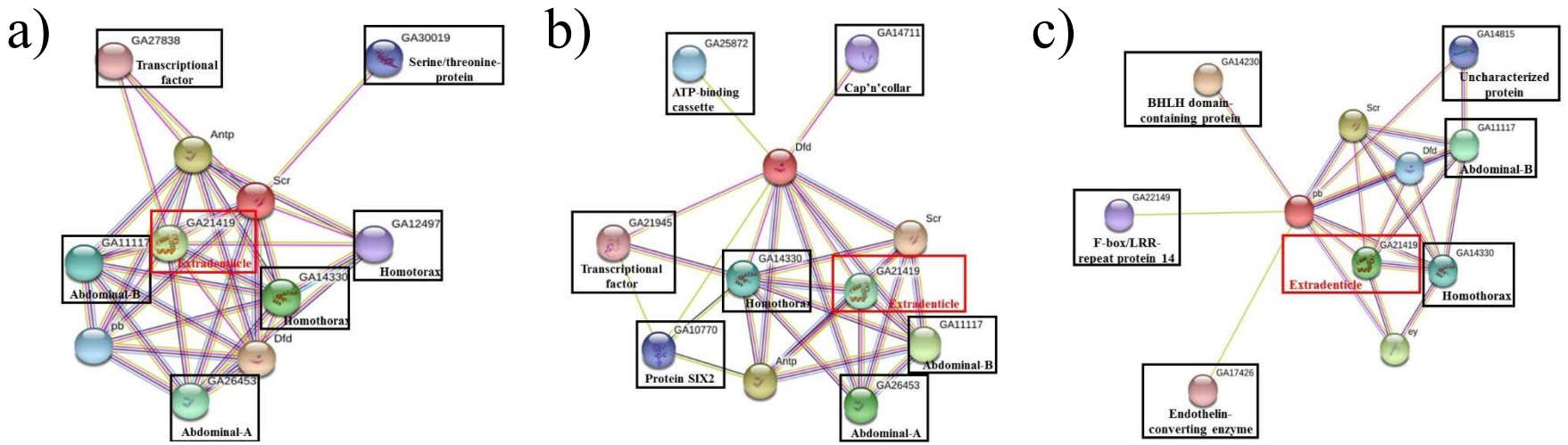
Deise Cagliari[1,2*]; Clauvis Nji Tizi Taning[1*]; Olivier Christiaens[1]; Kristof De Schutter[1]; Benny Lewille[3]; Koen Dewettinck[3]; Moises Zotti[2]; Guy Smagghe[1*]

[1] Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium.

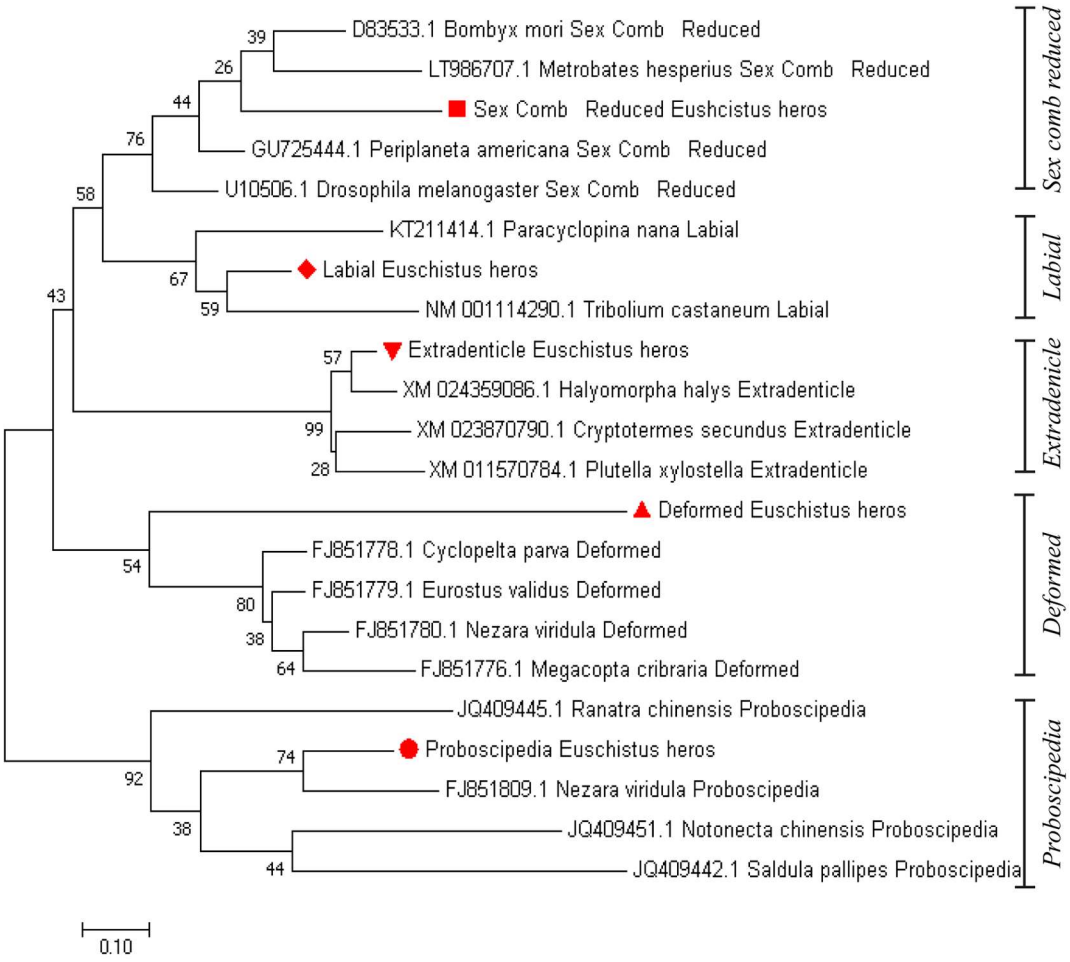
[2] Department of Crop Protection, Molecular Entomology Laboratory, Federal University of Pelotas, Pelotas, Brazil.

[3] Food Structure & Function Research Group, Department of Food Technology, Safety and Health, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium.

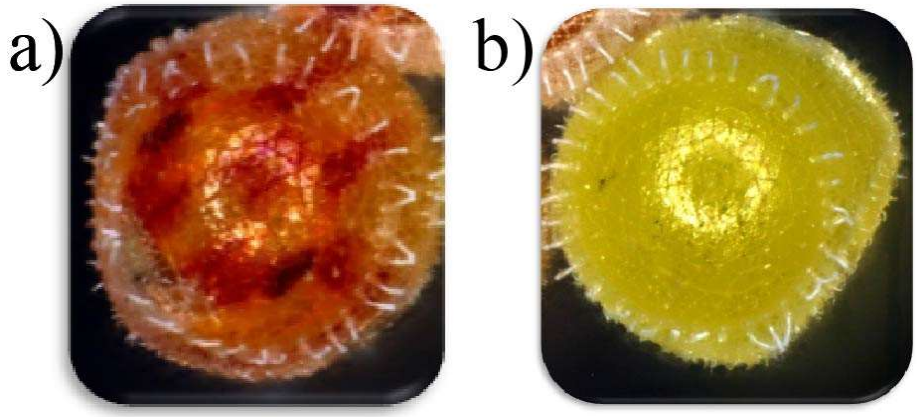
[*]Corresponding authors: deise.cagliari@ugent.be (D. Cagliari), tiziclauvis.taningnji@ugent.be (C.N.T. Taning), guy.smagghe@ugent.be (G. Smagghe)



Supplementary figure S1 – Protein interaction network. a) Sex comb reduced (scr) as input; b) Deformed (dfd) as input; c) Proboscipedia (pb) as input. Antp: antennapedia; ey: eyeless.



Supplementary figure S2. Phylogenetic tree of *labial*, *deformed*, *sex comb reduced*, *extradenticle,* and *proboscipedia* and their homologs from selected species. The full-length amino acid sequences of the genes from the brown stink bug *Euschistus heros* were aligned using MUCSLE with those of selected sequences from other species.



579

580 **Supplementary figure S3 –** Seven-days-old fertilized and unfertilized eggs of *Euschistus*
581 *heros*. a) fertilized egg: changes color from yellow to orange/reddish prior to hatching. b)
582 unfertilized egg: does not change color and stays yellow.

583

584 **Supplementary table S1**. Primers used for dsRNA synthesis, preceded by the T7 adaptor
585 sequence TAATACGACTCACTATAGGG. Product size without T7 sequence. F: Forward; R:
586 Reverse.

Gene name	Gene symbol	Sequence	Amplicon length
Labial	ds*Lab*	F CATGAACCTCGGGATGTA R CTGTGTTGTTGAGGAGTTG	473
Deformed	ds*Dfd*	F CACTTGAACAAGCGGTAAG R CACGATCTGCACGAGTAT	644
Sex comb reduced	ds*Scr*	F TTCCGGGATGATGGACTA R CCCGTTAGCATTGACTGTA	466
Extradenticle	ds*Exd*	F GAATTGAGCGGTTCGTATTT R CCCTATGTCCTGCTTTCTT	401
Proboscipedia	ds*Pb*	F GGCTACTATGAGAACCAGATG R TCAGTTGACTAACGTATCTCAG	309
GFP	ds*GFP*	F TACGGCGTGCAGTGCT R TGATCGCGCTTCTCG	455

587
588 **Supplementary table S2.** qRT-PCR primers and efficacy results.

Target gene	Primer sequence	Amplicon size	Efficiency (%)	R^2
Eggs				
Lab	F[1] CCCTTATTTGTGAGCTCTAGG R[2] GATGGATCAGCCCTCTTTG	90	93.5	0.995
Dfd	F TCGTGGAGGTGTGGTAT R ACTAAGAACGTCAGGAGAAAG	134	103.2	0.996
Scr	F GTGAGTGGAACCTCTGATAC R TAGTAGTCTGGGCTCTGG	122	92.1	0.994
Exd	F TTTGAATCGGCTCGGTAG R GTGTCATCCGGTAGTAATGT	124	99.5	0.998
18S	F TACAACAAGACAACGCTCGC R TTGCGCTCAGTGACATCTCT	150	95.7	0.998
RPL32	F TCAGTTCTGAGGCGTGCAT R TCCGCAAAGTCCTCGTTCA	175	90.9	0.999
Nymphs				
Pb	F CTCCTCCGACTTCAACTTC R TCAGTTGACTAACGTATCTCAG	88	107.0	0.992
18S	F TACAACAAGACAACGCTCGC R TTGCGCTCAGTGACATCTCT	150	94.5	0.999
RPL32	F TCAGTTCTGAGGCGTGCAT R TCCGCAAAGTCCTCGTTCA	175	97.8	1.0

589 [1]F: forward; [2]R: reverse.

Supplementary file S1

Control ds*GFP*

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of eggs	**1**	13	23	4	4	10	0	12														
	2	8	11	6	0	8	6	0	8	0	8	0	12									
	3	0	1	0	3	5	3	1	1	7	0	1	0									
	4	4	18	1	0	6	4	7	10	13	0	22	8	0	16	16	0	8	0	9	7	0
	5	0	16	0	10	0	0	15	11	0	0	0	0	0	8	0	0	0	0	0	0	0
	6	0	0	7	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	7	7	9	10	16	14	13	0	0	0	0	18	0
	8	0	0	0	0	2	0	7	2	8	0	11	0	14	18	0	9	0	12	4		
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	2	7	0	8	0	5	0	0
	10	2	0	0	0	0	0	10	0	6	0	17	10	0	0							

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Fertilized eggs	**1**	13	23	4	4	10	0	12														
	2	8	11	6	0	8	6	0	8	0	7	0	12									
	3	0	1	0	3	5	3	1	1	7	0	1	0									
	4	0	17	1	0	6	4	7	10	12	0	22	8	0	16	16	0	8	0	9	7	0
	5	0	16	0	10	0	0	15	11	0	0	0	0	0	8	0	0	0	0	0	0	0
	6	0	0	7	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	7	7	9	10	16	14	12	0	0	0	0	18	0
	8	0	0	0	0	2	0	7	2	7	0	11	0	14	18	0	9	0	10	4		
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	2	7	0	8	0	5	0	0
	10	0	0	0	0	0	0	10	0	5	0	17	10	0	0							

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Unfertilized eggs	**1**	0	0	0	0	0	0	0														
	2	0	0	0	0	0	0	0	0	0	1	0	0									
	3	0	0	0	0	0	0	0	0	0	0	0	0									
	4	4	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2	0		
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10	2	0	0	0	0	0	0	0	1	0	0	0	0	0							

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Total number of nymphs	**1**	13	18	1	4	6	0	12														
	2	8	9	6	0	6	6	0	6	0	7	0	0									
	3	0	1	0	2	5	1	1	0	5	0	0	0									
	4	0	17	1	0	5	3	7	8	11	0	22	7	0	6	14	0	6	0	9	0	0
	5	0	10	0	4	0	0	14	7	0	0	0	0	0	7	0	0	0	0	0	0	0
	6	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	1	7	8	6	9	5	8	0	0	0	0	6	0
	8	0	0	0	0	1	0	7	2	7	0	10	0	13	16	0	9	0	6	4		
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	0	3	0	1	0	0
	10	0	0	0	0	0	0	6	0	3	0	12	9	0	0							

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Number of normal nymphs	1	13	18	1	4	6	0	12														
	2	8	9	6	0	6	6	0	6	0	7	0	0									
	3	0	1	0	2	5	1	1	0	5	0	0	0									
	4	0	17	1	0	5	3	7	8	11	0	22	7	0	6	14	0	6	0	9	0	0
	5	0	10	0	4	0	0	14	7	0	0	0	0	0	7	0	0	0	0	0	0	0
	6	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	1	7	8	6	9	5	8	0	0	0	0	6	0
	8	0	0	0	0	1	0	7	2	7	0	10	0	13	16	0	9	0	6	4		
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	0	3	0	1	0	0
	10	0	0	0	0	0	0	6	0	3	0	12	9	0	0							

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Number of nymphs with phenotype	1	0	0	0	0	0	0	0														
	2	0	0	0	0	0	0	0	0	0	0	0	0									
	3	0	0	0	0	0	0	0	0	0	0	0	0									
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0							

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Remining eggs	1	0	5	3	0	4	0	0														
	2	0	2	0	0	2	0	0	2	0	0	0	12									
	3	0	0	0	1	0	2	0	1	2	0	1	0									
	4	0	0	0	0	1	1	0	2	1	0	0	1	0	10	2	0	2	0	0	7	0
	5	0	6	0	6	0	0	1	4	0	0	0	0	0	1	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	6	0	1	4	7	9	4	0	0	0	0	12	0
	8	0	0	0	0	1	0	0	0	0	0	1	0	1	2	0	0	0	4	0		
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	5	0	4	0	0
	10	0	0	0	0	0	0	4	0	2	0	5	1	0	0							

ds*Lab*

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Number of eggs	1	0	9	7	0	7																
	2	13	0	1	0	0	3	0	11	0	3	7	0	0	19	9						
	3	0	5	18	0	7	0	6	6	0	8	0	0	0	24	0	10	0	7	2	0	2
	4	8	2	4	0	0																
	5	40	0	8	12	13	9	11	17	11	13	12	8	0	15	0	18	10	0	3	4	0
	6	0	1	13	0	0	0	0	9	0	0	0	0	10	0	5	0	2	0	0	0	0
	7	0	17	0	15	0	18	11	0	18	16	0	15	0	4	19	0	11	0	0	0	0
	8	12	1	0	1	11	8	1	4	0	7	4	0	5	1	0	0	0	3	0	0	0
	9	14	16	4	23	0	8															
	10	14	13	0	23	0	10	0	18	0	19	0	0	15	0	0	14	0	0	5	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Fertilized eggs	**1**	0	9	7	0	7																
	2	13	0	1	0	0	3	0	11	0	2	6	0	0	19	9						
	3	0	5	14	0	7	0	6	6	0	7	0	0	0	24	0	10	0	7	2	0	2
	4	2	2	4	0	0																
	5	26	0	8	11	13	9	11	17	11	13	12	8	0	14	0	17	10	0	3	4	0
	6	0	1	13	0	0	0	0	7	0	0	0	0	10	0	5	0	2	0	0	0	0
	7	0	14	0	13	0	18	11	0	17	15	0	15	0	4	19	0	11	0	0	0	0
	8	10	1	0	1	9	8	1	4	0	7	4	0	5	1	0	0	0	3	0	0	0
	9	0	16	4	16	0	8															
	10	9	13	0	23	0	10	0	18	0	19	0	0	15	0	0	14	0	0	5	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Unfertilized eggs	**1**	0	0	0	0	0																
	2	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0						
	3	0	0	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	4	6	0	0	0	0																
	5	14	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
	6	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	3	0	2	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
	8	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	9	14	0	0	7	0	0															
	10	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Total number of nymphs	**1**	0	0	0	0	0																
	2	8	0	1	0	0	0	0	0	0	0	0	0	0	0	0						
	3	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	1	2	1	0	0																
	5	23	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	1	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	9	0	10	4	0	0	0															
	10	9	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of normal nymphs	**1**	0	0	0	0	0																
	2	8	0	1	0	0	0	0	0	0	0	0	0	0	0	0						
	3	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	1	2	1	0	0																
	5	23	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	1	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	9	0	10	4	0	0	0															
	10	9	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Number of nymphs with phenotype	**1**	0	0	0	0	0																
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0																
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	0															
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Remining eggs	**1**	0	9	7	0	7																
	2	5	0	0	0	0	3	0	11	0	2	6	0	0	19	9						
	3	0	5	7	0	7	0	6	6	0	7	0	0	0	24	0	10	0	7	2	0	2
	4	1	0	3	0	0																
	5	3	0	7	11	13	9	11	17	11	13	12	8	0	14	0	17	10	0	3	4	0
	6	0	0	2	0	0	0	0	7	0	0	0	0	10	0	5	0	2	0	0	0	0
	7	0	4	0	13	0	18	11	0	17	15	0	15	0	4	19	0	11	0	0	0	0
	8	5	0	0	1	9	8	1	4	0	7	4	0	5	1	0	0	0	3	0	0	0
	9	0	6	0	16	0	8															
	10	0	0	0	23	0	10	0	18	0	19	0	0	15	0	0	14	0	0	5	0	0

599 **ds*Dfd***

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of eggs	**1**	5	8																			
	2	0	0																			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	4	4	7	3	11	0	1														
	5	7	23	9	2	0																
	6	2	19																			
	7	0	0	0	0	0	0	0														
	8	16	0	0	0																	
	9	3	5	0	0	5	6	3	7	9	0	11	0	5	5	0	2	2	3	0	6	0
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Fertilized eggs	**1**	0	0																			
	2	0	0																			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	4	4	7	3	10	0	1														
	5	7	13	9	2	0																
	6	0	19																			
	7	0	0	0	0	0	0	0														
	8	11	0	0	0																	
	9	3	5	0	0	5	6	3	7	9	0	11	0	5	5	0	2	2	3	0	6	0
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Unfertilized eggs	**1**	5	8																			
	2	0	0																			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	1	0	0														
	5	0	10	0	0	0																
	6	2	0																			
	7	0	0	0	0	0	0	0														
	8	5	0	0	0																	
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Total number of nymphs	**1**	0	0																			
	2	0	0																			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	4	1	2	0	0	0	0														
	5	0	9	9	0	0																
	6	0	0																			
	7	0	0	0	0	0	0	0														
	8	6	0	0	0																	
	9	0	4	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of normal nymphs	**1**	0	0																			
	2	0	0																			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	4	1	2	0	0	0	0														
	5	0	9	9	0	0																
	6	0	0																			
	7	0	0	0	0	0	0	0														
	8	6	0	0	0																	
	9	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of nymphs with phenotype	**1**	0	0																			
	2	0	0																			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0														
	5	0	0	0	0	0																
	6	0	0																			
	7	0	0	0	0	0	0	0														
	8	0	0	0	0																	
	9	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Remining eggs	1	0	0																			
	2	0	0																			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	3	5	3	10	0	1														
	5	7	4	0	2	0																
	6	0	19																			
	7	0	0	0	0	0	0	0														
	8	5	0	0	0																	
	9	3	1	0	0	5	6	3	7	7	0	11	0	5	5	0	2	2	3	0	6	0
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

ds*Scr*

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Number of eggs	1	0	10	0	0	0	6	7	6	0	5	4	0	0	0	0	0	0	9	0	4	0
	2	22	0	0	0	0	7	5	6	10												
	3	3	13	1	0	7	0	5	13	0	2	11	0	0								
	4	0	0	0	0	0	7	24	0	0	0	12	0	0	0	0	4	0	1	0	0	0
	5	0	0	0	1	8	0	4	4	1												
	6	0	0	0	0	9	0	0	0	0	1	0	5	5	0	5	0	3	0	0	0	0
	7	11	14	1	10	16	0	1	0	0	7	9	20	0	0	5	9	0	0	7	0	1
	8	0	0	0	0	0	0	0	0													
	9	0	0	0	0	0	0	0	12													
	10	0	0	0	0	0	0	3	2	5	3	0	6	4	7	7	0	6	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Fertilized eggs	**1**	0	7	0	0	0	6	6	5	0	4	4	0	0	0	0	0	0	9	0	2	0
	2	22	0	0	0	0	7	5	6	10												
	3	0	13	0	0	7	0	4	12	0	1	8	0	0								
	4	0	0	0	0	0	3	24	0	0	0	11	0	0	0	0	3	0	1	0	0	0
	5	0	0	0	0	0	0	0	0	0												
	6	0	0	0	0	7	0	0	0	0	1	0	5	5	0	5	0	3	0	0	0	0
	7	11	14	1	9	16	0	1	0	0	5	9	19	0	0	5	9	0	0	7	0	1
	8	0	0	0	0	0	0	0	0													
	9	0	0	0	0	0	0	0	11													
	10	0	0	0	0	0	0	3	2	5	3	0	6	4	7	7	0	6	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Unfertilized eggs	**1**	0	3	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	2	0
	2	0	0	0	0	0	0	0	0	0												
	3	3	0	1	0	0	0	1	1	0	1	3	0	0								
	4	0	0	0	0	0	4	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
	5	0	0	0	1	8	0	4	4	1												
	6	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	1	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0													
	9	0	0	0	0	0	0	0	1													
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Total number of nymphs	**1**	0	7	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	19	0	0	0	0	3	0	0	2												
	3	0	13	0	0	0	0	0	3	0	0	0	0	0								
	4	0	0	0	0	0	1	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0												
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	11	0	0	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0													
	9	0	0	0	0	0	0	0	0													
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of normal nymphs	**1**	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	19	0	0	0	0	0	0	0	0												
	3	0	13	0	0	0	0	0	0	0	0	0	0	0								
	4	0	0	0	0	0	1	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0												
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0													
	9	0	0	0	0	0	0	0	0													
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Number of nymphs with phenotype	**1**	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	3	0	0	2												
	3	0	0	0	0	0	0	0	3	0	0	0	0	0								
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0												
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0													
	9	0	0	0	0	0	0	0	0													
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Remining eggs	**1**	0	0	0	0	0	3	6	5	0	4	4	0	0	0	0	0	0	9	0	2	0
	2	3	0	0	0	0	4	5	6	8												
	3	0	0	0	0	7	0	4	9	0	1	8	0	0								
	4	0	0	0	0	0	2	21	0	0	0	10	0	0	0	0	3	0	1	0	0	0
	5	0	0	0	0	0	0	0	0	0												
	6	0	0	0	0	7	0	0	0	0	1	0	5	5	0	5	0	3	0	0	0	0
	7	0	14	1	7	16	0	1	0	0	5	9	18	0	0	5	9	0	0	7	0	1
	8	0	0	0	0	0	0	0	0													
	9	0	0	0	0	0	0	0	11													
	10	0	0	0	0	0	0	3	2	5	3	0	6	4	7	7	0	6	0	0	0	0

606
607

608 **ds*Exd***

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of eggs	**1**	11	0	0	0	3	0	8	0	0	0	0	0	0								
	2	3	10																			
	3	17	12	2																		
	4	0	0	0	0	3	12	0	7	0	0	10	0									
	5	0	0	0	4	6	0	2	8	0	0	12	0	0								
	6	0	0	0	0	0	0	0	0	0	0	0										
	7	11	19																			
	8	10	16																			
	9	22	1	2	12	9	0	8	0	14	4	0	8	0	6	3						
	10	0	3	1	0	0	6	0	0	0	0	8	6	13	11	4	0	10	4	0	0	1

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Fertilized eggs	**1**	11	0	0	0	3	0	8	0	0	0	0	0	0								
	2	0	9																			
	3	16	11	1																		
	4	0	0	0	0	3	12	0	6	0	0	10	0									
	5	0	0	0	4	6	0	2	8	0	0	12	0	0								
	6	0	0	0	0	0	0	0	0	0	0	0										
	7	2	19																			
	8	0	16																			
	9	19	1	2	12	8	0	8	0	14	4	0	8	0	6	3						
	10	0	0	1	0	0	6	0	0	0	0	8	6	13	11	4	0	10	4	0	0	1

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Unfertilized eggs	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0								
	2	3	1																			
	3	1	1	1																		
	4	0	0	0	0	0	0	0	1	0	0	0	0									
	5	0	0	0	0	0	0	0	0	0	0	0	0	0								
	6	0	0	0	0	0	0	0	0	0	0	0										
	7	9	0																			
	8	10	0																			
	9	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0						
	10	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Total number of nymphs	**1**	8	0	0	0	0	0	0	0	0	0	0	0	0								
	2	0	9																			
	3	12	9	0																		
	4	0	0	0	0	2	0	0	0	0	0	0	0									
	5	0	0	0	0	0	0	0	0	0	0	0	0	0								
	6	0	0	0	0	0	0	0	0	0	0	0										
	7	1	16																			
	8	0	15																			
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
	10	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Number of normal nymphs	**1**	8	0	0	0	0	0	0	0	0	0	0	0	0								
	2	0	9																			
	3	12	9	0																		
	4	0	0	0	0	2	0	0	0	0	0	0	0									
	5	0	0	0	0	0	0	0	0	0	0	0	0	0								
	6	0	0	0	0	0	0	0	0	0	0	0										
	7	1	16																			
	8	0	15																			
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
	10	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Number of nymphs with phenotype	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0								
	2	0	0																			
	3	0	0	0																		
	4	0	0	0	0	0	0	0	0	0	0	0	0									
	5	0	0	0	0	0	0	0	0	0	0	0	0	0								
	6	0	0	0	0	0	0	0	0	0	0	0										
	7	0	0																			
	8	0	0																			
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Remining eggs	**1**	3	0	0	0	3	0	8	0	0	0	0	0	0								
	2	0	0																			
	3	4	2	1																		
	4	0	0	0	0	1	12	0	6	0	0	10	0									
	5	0	0	0	4	6	0	2	8	0	0	12	0	0								
	6	0	0	0	0	0	0	0	0	0	0	0										
	7	1	3																			
	8	0	1																			
	9	19	1	2	12	8	0	8	0	14	4	0	8	0	6	3						
	10	0	0	0	0	0	6	0	0	0	0	8	6	13	11	4	0	10	4	0	0	1

ds*Pb*

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of eggs	**1**	15	5	1	0	1	13	0	0	0	6	5	11	8	0	7	6	18	1	0	4	0
	2	0	7	4	5	5	0	11														
	3	0	16	10																		
	4	13	5	0	4	14	0	9	0	9	0	4	0	0	0	5	0	0	0	7	0	0
	5	6	2	2	2	0	1															
	6	7	0	0																		
	7	7	17																			
	8	0	20																			
	9	7	5	7	9	13	0	0	10	0	0											
	10	0	9	14	5	11	0	3	10	4	15	8	0	8	5	0	11	5	2	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Fertilized eggs	**1**	14	4	1	0	1	10	0	0	0	6	5	10	8	0	7	6	18	1	0	4	0
	2	0	7	4	5	5	0	10														
	3	0	16	10																		
	4	0	5	0	4	14	0	9	0	9	0	3	0	0	0	5	0	0	0	7	0	0
	5	0	2	2	2	0	1															
	6	1	0	0																		
	7	0	17																			
	8	0	20																			
	9	0	0	0	5	12	0	0	10	0	0											
	10	0	2	14	5	11	0	3	10	4	15	8	0	8	5	0	11	5	2	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Unfertilized eggs	**1**	1	1	0	0	0	3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	1														
	3	0	0	0																		
	4	13	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
	5	6	0	0	0	0	0															
	6	6	0	0																		
	7	7	0																			
	8	0	0																			
	9	7	5	7	4	1	0	0	0	0	0											
	10	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Total number of nymphs	**1**	14	4	1	0	0	6	0	0	0	4	4	6	6	0	6	6	15	1	0	3	0
	2	0	0	0	0	0	0	0														
	3	0	16	10																		
	4	0	5	0	1	0	0	5	0	6	0	2	0	0	0	2	0	0	0	7	0	0
	5	0	1	1	2	0	1															
	6	0	0	0																		
	7	0	16																			
	8	0	19																			
	9	0	0	0	5	12	0	0	10	0	0											
	10	0	2	13	3	10	0	3	5	3	15	8	0	8	5	0	0	0	0	0	0	0

	Female	Days after microinjection																				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of normal nymphs	**1**	14	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0														
	3	0	16	0																		
	4	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	1	1	0	0	0															
	6	7	0	0																		
	7	0	16																			
	8	0	19																			
	9	0	0	0	0	0	0	0	0	0	0											
	10	0	2	13	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Female	Days after microinjection																			
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Number of nymphs with phenotype	**1**	0	0	0	0	0	6	0	0	0	4	4	6	6	0	6	6	15	1	0	3	0
	2	0	0	0	0	0	0	0														
	3	0	0	10																		
	4	0	0	0	1	0	0	5	0	6	0	2	0	0	0	2	0	0	0	7	0	0
	5	0	0	0	2	0	1															
	6	0	0	0																		
	7	0	0																			
	8	0	0																			
	9	0	0	0	5	12	0	0	10	0	0											
	10	0	0	0	0	10	0	3	5	3	15	8	0	8	5	0	0	0	0	0	0	0

	Female	Days after microinjection																			
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Remining eggs	**1**	0	0	0	0	1	4	0	0	0	2	1	4	2	0	1	0	3	0	0	1	0
	2	0	7	4	5	5	0	10														
	3	0	0	0																		
	4	0	0	0	3	14	0	4	0	3	0	1	0	0	0	3	0	0	0	0	0	0
	5	0	1	1	0	0	0															
	6	1	0	0																		
	7	0	1																			
	8	0	1																			
	9	0	0	0	0	0	0	0	0	0	0											
	10	0	0	1	2	1	0	0	5	1	0	0	0	0	0	0	11	5	2	0	0	0

Supplementary file S2

Days after microinjection	ds*GFP*	Standart Error	ds*Lab*	Standart Error	ds*Dfd*	Standart Error	ds*Scr*	Standart Error	ds*Exd*	Standart Error	ds*Pb*	Standart Error
3	18	1	55	2	16	1	2	0	5	0	38	2
4	17	1	74	3	5	0	11	1	16	2	25	1
5	31	1	38	2	16	2	40	2	21	1	44	3
6	13	1	56	2	6	1	20	1	18	2	14	2
7	52	2	29	2	4	1	49	2	18	2	23	2
8	32	2	65	3	7	2	43	2	15	2	20	3
9	41	2	29	3	0	0	16	1	14	2	13	2
10	15	1	66	3	9	3	18	1	4	1	21	4
11	60	3	23	2	0	0	36	2	30	2	17	1
12	40	2	23	2	11	4	31	3	14	2	11	4
13	33	3	30	2	5	2	9	1	13	3	16	3
14	58	3	63	4	5	2	7	1	17	3	5	2
15	36	3	33	3	0	0	17	1	7	1	12	2
16	9	2	42	3	2	1	13	2	0	0	17	3
17	16	2	23	2	2	1	9	1	10	0	23	5
18	12	2	10	1	3	1	10	2	4	0	3	1
19	18	2	10	1	0	0	7	1	0	0	7	2
20	25	4	4	1	6	2	4	1	0	0	4	1
21	0	0	2	0	0	0	1	0	1	0	0	0
Total eggs	526	3.90	675	5.10	97	1.14	343	3.36	207	1.93	313	2.60
Total unfetilized eggs	7	0.2	25	0.6	1	0.1	43	0.7	3	0.1	18	0.4
Total fertilized eggs	519	3.48	650	4.53	96	1.44	300	2.24	204	2.91	295	3.79

	ds*GFP*	ds*Lab*	ds*Dfd*	ds*Scr*	ds*Exd*	ds*Pb*
% of Fertilized eggs	99	96	99	87	99	94
% of Unfertilized eggs	1	4	1	13	1	6

620

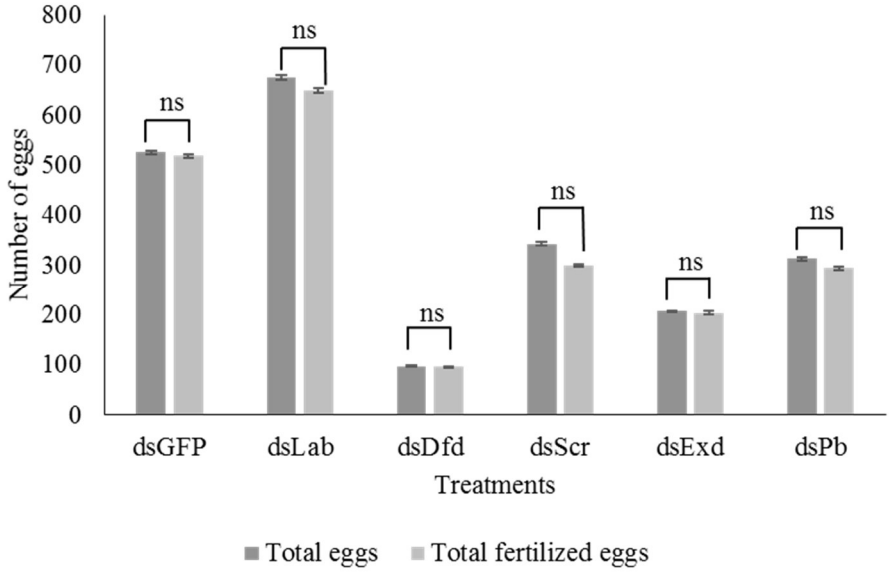


Figure 1. The brown stink bug *Euschistus heros* oviposition rates following knockdown of *labial*, *deformed*, *sex comb reduced*, *extradenticle* and *proboscipedia*. Females were microinjected with 10 µg/µl of gene-specific dsRNA. Bars represent the total number of eggs (dark grey) and the total number of fertilized eggs (light grey). The bars with different letters denote significant differences ($p<0.05$) according to a Dunn's test. ns= non-significant. Confidence bars are shown for ± SE.

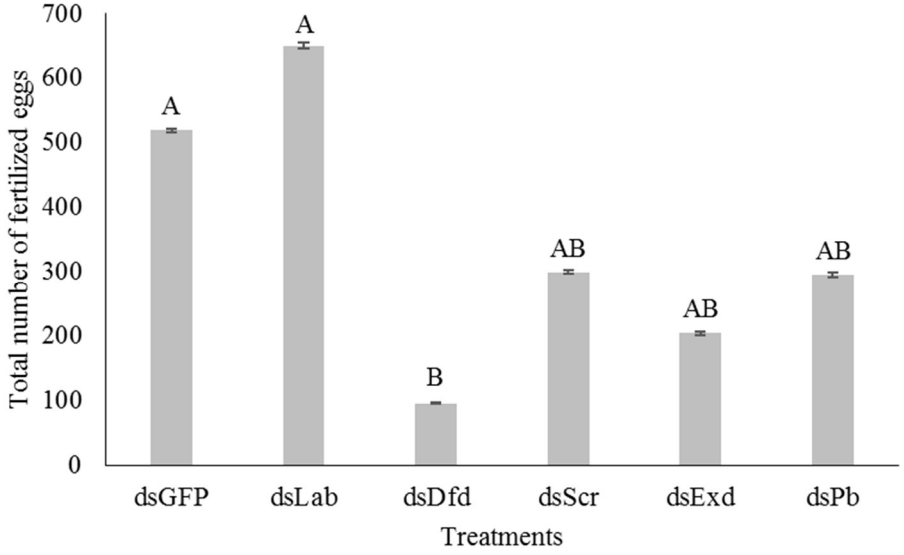


Figure 2. The brown stink bug *Euschistus heros* fertilized eggs following knockdown of *labial*, *deformed*, *sex comb reduced*, *extradenticle* and *proboscipedia*. Females were microinjected with 10 µg/µl of gene-specific dsRNA. Bars represent the total number of fertilized eggs. The bars with different letters denote significant differences ($p<0.05$) according to a Dunn's test. Confidence bars are shown for ± SE.

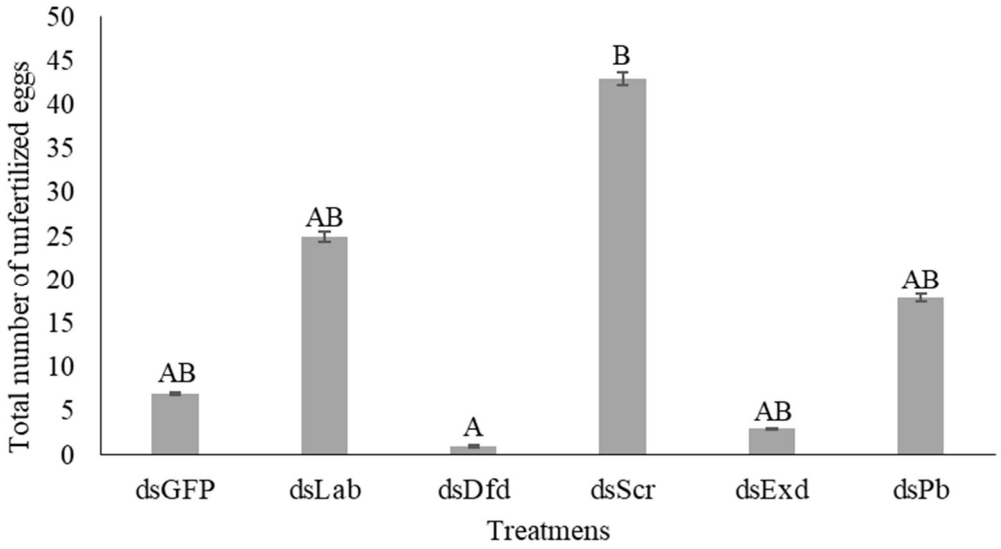


Figure 3. The brown stink bug *Euschistus heros* unfertilized eggs following knockdown of *labial*, *deformed*, *sex comb reduced*, *extradenticle* and *proboscipedia*. Females were microinjected with 10 µg/µl of gene-specific dsRNA. Bars represent the total number of unfertilized eggs. The bars with different letters denote significant differences ($p<0.05$) according to a Dunn's test. Confidence bars are shown for ± SE.

5. Manuscript 4. RNAi and CRISPR/Cas9 as functional genomic tools in the Neotropical stink bug *Euschistus heros.**

Deise Cagliari
Guy Smagghe
Moises Zotti
Clauvis Nji Tizi Taning

*Manuscript published in Insects

insects

MDPI

Communication

RNAi and CRISPR/Cas9 as functional genomics tools in the Neotropical stink bug, *Euschistus heros*

Deise Cagliari[1,2,*]; Guy Smagghe[1,*]; Moises Zotti[2]; Clauvis Nji Tizi Taning[1,*]

[1] Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium
[2] Department of Crop Protection, Molecular Entomology Laboratory, Federal University of Pelotas, Pelotas, Brazil
* Correspondence: deise.cagliari@ugent.be (D. Cagliari), guy.smagghe@ugent.be (G. Smagghe), tiziclauvis.taningnji@ugent.be (C. N. T. Taning)

Simple Summary: Understanding the biology of insect pests is an important step towards developing appropriate control strategies. In this study, a CRISPR/Cas9 gene knockout work flow was established for the first time and was together with RNAi used as tools to study gene functions in the Neotropical stink bug, *Euschistus heros*. RNAi was first employed to study the function of three genes, *abnormal wing disc* (*awd*), *tyrosine hydroxylase* (*th*), and *yellow* (*yel*). Targeting *awd* and *th* resulted in distinct malformed phenotypes such as a deformed wing or a lighter cuticle pigmentation/defects in cuticle sclerotization, respectively. However, no distinct phenotype was observed for *yel*. To further investigate the function of *yel*, a CRISPR/Cas9 gene editing protocol was developed for *E. heros*. A total of 719 eggs were microinjected with single-guide (sgRNA) and Cas9, and total of six insects hatched. Out of these six nymphs, one insect showed mutation in *yel*, but no clear phenotype was visible. Although, we were unable to generate insects with a distinct phenotype for *yel*, a successful gene editing workflow was established to complement RNAi for future functional gene studies in *E. heros*. Additionally, we provided recommendations to improve the established gene editing workflow.

Abstract: The Neotropical brown stink bug, *Euschistus heros,* is one of the most important stink bug pests in leguminous plants in South America. RNAi and CRISPR/Cas9 are important and useful tools in functional genomics, as well as in the future development of new integrated pest management strategies. Here, we explore the use of these technologies as complementing functional genomic tools in *E. heros*. Three genes, *abnormal wing disc* (*awd*), *tyrosine hydroxylase* (*th*), and *yellow* (*yel*), known to be involved in wing development (*awd*) and the melanin pathway (*th* and *yel*) in other insects, were chosen to be evaluated using RNAi and CRISPR/Cas9 as tools. First, the genes were functionally characterized using RNAi knockdown technology. The expected phenotype of either deformed wing or lighter cuticle pigmentation/defects in cuticle sclerotization was observed for *awd* and *th*, respectively. However, for *yel*, no obvious phenotype was observed. Based on this, *yel* was selected as a target for the development of a CRISPR/Cas9 workflow to study gene knockout in *E. heros.* A total of 719 eggs were injected with the Cas9 nuclease (300 ng/µl) together with the sgRNA (300 ng/µl) targeting *yel*. A total of six insects successfully hatched from the injected eggs, and one of the insects showed mutation in the target region, however, the phenotype was still not obvious. Overall, this study for the first time provides a useful CRISPR/Cas9 gene editing methodology to complement RNAi for functional genomic studies in one of the most important and economically relevant stink bug species.

Keywords: Gene knockdown, Gene knockout, Pentatomidae, Gene editing.

1. Introduction

An increase in genome and transcriptome sequence databases for non-model insects, coupled to the development of high-throughput techniques for gene expression profiling and functional characterization has made it possible to study the biology of non-model insects. This is particularly interesting for pest insect species where understanding the underlying mechanisms in their biology through functional genomics could lead to the development of potential pest control strategies. *Euschistus heros* (Hemiptera: Pentatomidae) is one of the most important stink bug species present in South America and is responsible for causing severe damage to several crops, especially soybean, mainly during the reproductive period [1–3]. The recently published transcriptome of *E. heros* [4] provides a good starting basis to explore the biology of this important pest species. However, this will require the adaptation of current available functional genomics tools, including CRISPR/Cas9 gene editing, for studies in *E. heros.*

Post-transcriptional gene silencing also known as RNA interference (RNAi) was first elucidated in 1998 [5] and has since then been widely used as a tool in the study of gene function. In the hemipteran group, RNAi has been used to study the role of genes in insect development/reproduction of several species, such as *Oncopeltus fasciatus* [6,7], *Nezara viridula* [8], *Diaphorina citri* [9], *Halyomorpha halys* [10], among others. RNAi is a highly conserved mechanism among eukaryotic organisms, in which the messenger RNA (mRNA) is cleaved by the RNAi machinery, leading to the inactivation of gene expression [11]. However, RNAi efficiency can vary between different insect groups, developmental stages or tissues [12] and due to its transient characteristic, it might not be suitable for studying some candidate genes.

On the other hand, Clustered Regularly Interspaced Palindromic Repeats/CRISPR-associated protein 9, known as CRISPR/Cas9, is a genetic tool that allows researchers to do very specific modification at a genomic level. The CRISPR/Cas9 system is formed by three main components: a molecule of approximately 21 nucleotides called CRISPR RNA (crRNA), the trans-activating CRISPR RNA (tracrRNA) and the Cas9 enzyme. The crRNA and tracrRNA form the sgRNA (single-guide RNA), which guides the Cas9 enzyme to the complementary DNA sequence in the genome, near the PAM sequence (protospacer adjacent motif) – NGG. Once the system finds the complementary region, the Cas9 endonuclease cleaves the two DNA strands, generating a double-strand break (DSB) in the target sequence [13,14]. The DSB can be repaired by two different approaches: error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR). The repair by the NHEJ can result in either deletions or insertions known as "indels", or generate nucleotide substitutions, leading to the creation of a mutant version of the target gene [15–17]. On the other hand, HDR is mainly used to generate repair based on a donor template, leading to a gene knock-in repair process [17,18].

In this study, we explored the use of RNAi and CRISPR/Cas9 as complementary functional genomics tools to elucidate the role of genes in *E. heros*. Prior to the genome editing experiments, *abnormal wing disc* (*awd*), *tyrosine hydroxylase* (*th*), and *yellow* (*yel*) were evaluated in *E. heros* by exploiting RNAi-mediated knockdown technology. *Awd* is a gene involved in wing development in several species [9,19,20], while *th* and *yel* are genes involved in the melanin pathway [21]. Based on the lack of an obvious phenotype following the knockdown of *yel*, a CRISPR/Cas9 gene editing workflow was developed for the first time for *E. heros* to complement RNAi in the study of *yel*. Additionally, we provide recommendations to further improve the gene editing workflow presented in this study for future mutagenesis studies in stink bugs.

2. Materials and Methods

2.1. Insects

A colony of *E. heros* was kept under standard mass-rearing conditions of 25 ± 2 °C, 60 ± 10% relative humidity and a light/dark photoperiod of 14:10 h at the Laboratory of Agrozoology, Ghent University.

The insects were kept in plastic boxes and fed *ad libitum* with a mixture of fresh green bean pods (*Phaseolus vulgaris* (L.)), raw shelled peanuts (*Arachis hypogaea* (L.)) and soybean seeds (*Glycine max* (L.)) [22]. The supplies were replenished every 3-days. Eggs were removed and placed in Petri dishes for five days, then transferred to plastic boxes and reared until they reached adulthood.

2.2. Target gene identification and expression profile

The protein sequences for *awd*, *th* and *yel* from the pea aphid *Acyrthosiphon pisum* (NP_001119625.1, XP_008182999.1 and XP_001948479.1, respectively) was used as query to identify homologs of the candidate genes in an own published *E. heros* transcriptome database [4], using the tBLASTn tool. We then detected the open reading frames (ORFs) in the retrieved *E. heros* homologs using the ORF Finder from National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/orffinder/). The Protein Basic Local Alignment Tool (Protein BLAST) was used for protein homology searches against the insect non-redundant protein database at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To confirm the identity of the identified genes, their protein sequences were aligned against those of other insect species using MUSCLE with default settings [23] and a phylogenetic tree was constructed using maximum likelihood with default settings in the software MEGA7 [24].

To evaluate the stage-specific expression of *awd*, *th* and *yel*, samples from different developmental stages including eggs (dissected from female ovaries, <24 h old and 7 days old), nymphs (1^{st}-, 2^{nd}-, 3^{rd}-, 4^{th}-, 5^{th}-instar) and adults (male and female) were prepared (Table S1). Briefly, total RNA was extracted using the RNeasy Mini Kit (Qiagen) and treated with DNase I (Ambion) to remove residual genomic DNA. RNA was quantified using a NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA), visualized in a 1.5% agarose gel and stored in -80°C until further use. cDNA was synthesized using a SuperScript IV kit (Invitrogen) with an oligo d(T) primer in a final volume of 20 µL, according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR) analysis was performed with a CFX 96TM real-time system and the CFX manager software (Bio-Rad, Hercules, CA, USA). qRT-PCR primers were designed using PrimerQuest Tool from IDT (https://www.idtdna.com/pages) (Table S2) and a standard curve based on a serial dilution of cDNA was done to determine the primer annealing efficiency and a melting curve analysis with a temperature range from 60 to 95 °C. The qRT-PCR reaction was done in a 20 µL-reaction system, containing 8 µL of cDNA samples, 10 µL of GoTaq qPCR Master Mix (Promega), and 1 µL of each primer (10 µM). The reactions were set-up in 96-well format Microseal PCR plates (Bio-Rad). The amplification conditions were 3 min at 95 °C followed by 39 cycles of 10 s at 95 °C and 30 s at 60 °C. After the amplification, a melting curve analysis with a temperature gradient of 0.1 °C/s from 60 to 95 °C was performed to confirm that only the specific product was amplified. The endogenous controls, *ribosomal protein 18S*, and *RPL32*, were used for normalization of the qRT-PCR data. A no-template control was also included in the assay. All experiments were performed in three biological replicates. Relative expression values of genes were calculated using the equation ratio $2^{-\Delta\Delta Ct}$ [25].

2.3 RNAi-mediated gene silencing assay

Primers were designed using the PrimerQuest Tool from Integrated DNA Technologies (IDT) (https://www.idtdna.com/pages) and T7 promoter sequences placed at the 5′-ends of both forward and reverse primers (Table S2). DNA templates were amplified using Taq DNA polymerase (Invitrogen) and cDNA as a template. *Green fluorescent protein* (GPF) was amplified from a plasmid containing the *GFP* insert (Genbank ID: NC_011521.1). The DNA templates were purified using the Wizard clean-up system (Promega, Madison, WI, USA). The dsRNAs were synthesized using the MEGAscript RNAi kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. The dsRNA was quantified on a

NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and integrity analyzed in an electrophoresis gel.

Third instar nymphs were microinjected using a microinjector (FemtoJet, Eppendorf, Hamburg, Germany), equipped with an injection needle prepared with capillary glass tubes. Each nymph was injected with 1.0 µL of a 1 µg/µL dsRNA solution, based on an established protocol [4,26]. The nymphs were anesthetized with ether for 2 min and then injected on the ventral metathoracic region, near to the hind coxa. DsRNA targeting *GFP* was used as a negative control. Twenty-six nymphs were injected per gene-specific dsRNA treatment and the experiment was repeated twice (N=52 in total). After microinjections, nymphs were put in Petri dishes containing green bean slices and kept under standard mass-rearing conditions. Insects were supplied with fresh green beans and seeds every 2-3 days. Insects were checked for phenotype under a stereomicroscope (Leica DFC295, Wetzlar, Germany).

Gene expression was measured 72 h post-microinjection. Three groups of two pooled insects/group were sampled from each gene-specific treatment group and used for the qRT-PCR measurements. This was done for the two experiment repeats (N=6 groups in total). For all samples, total RNA isolation and qRT-PCR analysis were done as described above. The qRT-PCR reactions were performed in the CFX 96TM real-time system (Bio-Rad, Hercules, CA, USA) following the steps described above.

2.4 CRISPR/Cas9 gene editing assay

The sequence for the *yel* gene was obtained from the *E. heros* transcriptome (PRJNA488833) and its open reading frame (ORF) was predicted using the ORFfinder tool from NCBI (https://www.ncbi.nlm.nih.gov/orffinder/) as mentioned above. The retrieved coding sequences were used to design a guide RNA target sequence (gRNA) that targeted both isoforms, according to the criteria: 5′-GG-(N)18-NGG-3′ [27]. The IDT Custom Alt-R CRISPR-Cas9 guide RNA design tool (https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM) was used to predict the sgRNA with the lowest potential for off-target-risk and a high on-target potential. Additionally, in the absence of a publicly available genome database for *E. heros*, potential off-targets were checked in the transcriptome using the predicted sgRNA through BLAST analysis.

Eggs were collected within a maximum of 1 h after oviposition and quickly lined up in preparation for injection. Glass slides used for microinjection were prepared as follows: two glass slides stuck together using double tape, with a 3 cm overlapping space. On this overlapping space, eggs were lined up (longitudinal orientation) against the top glass slide, quickly covered with nuclease-free water (~1.5 ml) [28] and then wrapped with plastic film with the aim to fix the eggs in position on the glass slide. An injection solution containing 300 ng/µL sgRNA and 300 ng/µL Alt-R Cas9 protein (IDT) was prepared and the eggs were injected using a microinjector (about 2 nl per egg) (FemtoJet, Eppendorf, Hamburg, Germany), equipped with a needle prepared from a capillary glass tube. Controls were injected with nuclease-free water. During injection, the needle was inclined at an angle of about 30° relative to the microscope stage. After injection, the eggs were placed into Petri dishes, underlaid by a slightly moist filter paper soaked with water + Nipagin (1%) to avoid fungal growth, especially at the injection point on the eggs. The Petri dishes were then sealed with a plastic film and returned to the incubator under standard rearing conditions as described above. The injected eggs were monitored for 8 days for nymph hatching, after which the nymphs were transferred to new Petri dishes and fed with fresh green beans *ad libitum*.

Upon hatching, the nymphs were observed and assessed under the microscope (Leica DFC295). Genomic DNA from three nymphs from the *yel* treatment and three nymphs from the control were individually extracted using QIAamp DNA Mini Kit (Qiagen) and used as a template for PCR amplification of the *yel* gene region containing the sgRNA target site (Table S2). The PCR conditions were: 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 ºC for 1 min, followed by a final extension of 10 min at 72 °C. The resulting PCR product from each *E. heros* individual was sequenced

(LGC Genomics, Berlin) to verify for mutation in *yel*. The wild-type sequence used for mutation analysis originated from control embryos injected with water.

2.5. Data analysis

The data were checked for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests. Failing these assumptions, they were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test using GraphPad Prism Software. The results of the survival bioassays were subjected to survival analysis, which was performed using Kaplan-Meier estimators (log-rank method) with SigmaPlot 12.0 (Systat Software, San Jose, CA, USA).

3. Results

3.1. RNAi-mediated knockdown for functional genomics in E. heros

Before starting with the bioassays in *E. heros*, we first confirmed the identity of the candidate genes (*awd*, *th* and *yel*) in this species through phylogenetic analysis, where they were observed to cluster on distinct branches together with their respective homologs in other insect species (Figure S1). Life stage-specific expression of *awd*, *yel* and *th* at the mRNA level was also evaluated, with the aim to select the best time-point for dsRNA treatment. Although the expression level of *awd* in the different developmental stages was more or less stable (1.34 ± 0.15) (Figure S2), its expression in the eggs dissected from the ovary was higher (7.34 ± 0.84) when compared to less than one day old laid eggs (0.42 ± 0.10). The expression level of *th* was higher in *E. heros* females (2.98 ± 0.94) compared to *E. heros* males (<0.01 ± 0.001), whereas no difference was found in its expression between the different developmental stages (0.51 ± 0.23). Similar to *th*, the expression level of *yel* was higher in females (3.47 ± 0.66) when compared to males (0.01 ± 0.004) and also eggs dissected from the ovary (0.01 ± 0.003), while it remained similar between the other developmental stages (0.59 ± 0.31). Overall, no major differences were noted in the expression profiles for *awd*, *th* and *yel* between the different nymphal stages. As such, freshly molted 3rd-instar nymphs (<24 h old) were selected for RNAi gene silencing bioassays. Out of 40 nymphs treated with ds*awd*, 18 died as 3rd-instar nymphs during the expected molting period (45%), 20 successfully molted to 5th-instar nymphs (50%) and 17 reached adulthood (43%) (Figure 1a). Moreover, 15% (6 adults) of the adults showed abnormalities in wing formation (Figure 1b) which could appear extremely shortened in some individuals (Figure S3). Out of 40 nymphs treated with ds*th*, 31 molted to the 4th-instar (78%), 16 molted to the 5th-instar (40%) and only 3 reached adulthood (8%). Moreover, defects in cuticle sclerotization, a curved abdomen, malformed antenna and legs were observed in ds*th*-treated nymphs (Figure 1b). Out of 40 ds*yel*-treated nymphs, 22 insects reached adulthood (55%) (Figure 1a). Treatment with ds*yel*, did not result in any obvious difference in cuticle development nor pigmentation when compared to the normal phenotype observed in the control (ds*GFP*) (Figure 1b). For the insects treated with ds*GFP*, 35 insects (88%) successfully molted to 4th-instar and 26 reached adulthood (65%) (Figure 1a). Treatment with ds*th* resulted in a lighter pigmentation of the nymph cuticle compared to the control (ds*GFP*) of the same life stage (Figure 1b).

To verify RNAi-mediated silencing of *awd*, *th* and *yel* in the treated insects, their transcript levels were evaluated by qRT-PCR. A respective significant reduction of 98.5, 98.4 and 91.1% in the transcript level of *awd, th* and *yel* was observed in the target gene-specific dsRNA-treated insects when compared to the control (ds*GFP*) (p<0.001) (Figure 1c, d and e).

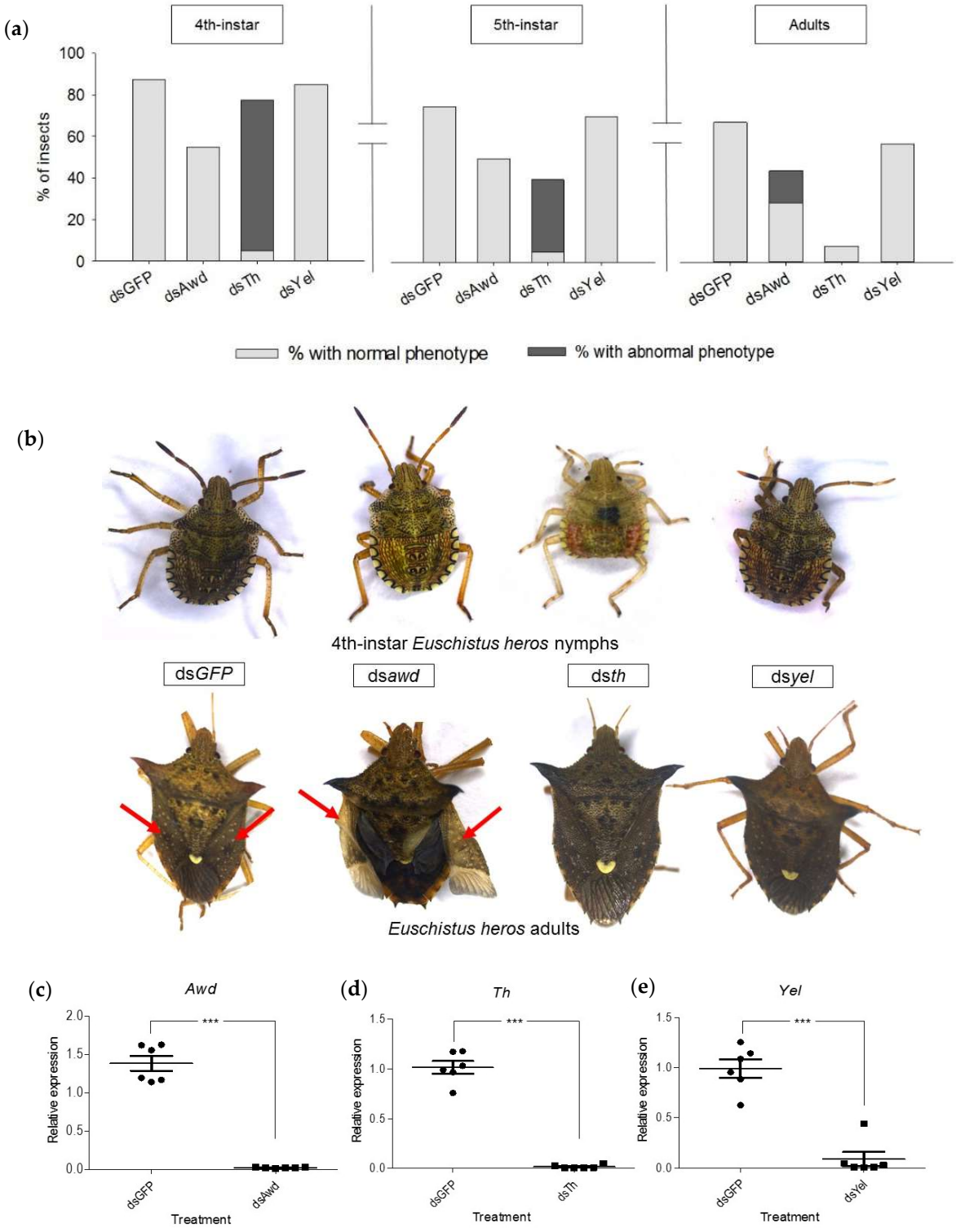


Figure 1. RNAi-mediated knockdown of 3 genes, *abnormal wing disc* (*awd*), *tyrosine hydroxylase* (*th*), and *yellow* (*yel*) in *E. heros*. (a) Percentage (%) of insects with normal phenotype and abnormal phenotype (4th- and 5th-instar nymphs and adults) following microinjection with either ds*Awd*, ds*Th* or ds*Yel*. Bars represent the mean. (b) Phenotypes in 4th-instar nymphs and adults following the treatment of 3rd-instar nymphs with either ds*Awd*, ds*Th* or ds*Yel*. The assay was conducted twice with each repeat consisting of 20 nymphs (N= 40). (c) (d) and (e) Transcript levels at 72 h after injection of 3rd-instar with ds*Awd*,

ds*Th* and ds*Yel*, respectively, compared to their respective transcript levels in the control (ds*GFP*). Three asterisks on the bar indicate a statistically significant difference ($p < 0.001$). Each sample contains 2 pooled insects. The *p*-values were calculated by unpaired t-test.

Survival of the treated insects was evaluated for 27 days. Ds*awd*-, ds*yel*- and ds*GFP*-treated groups showed similar survival levels in contrast to the ds*Th*-treated group (Holms-Sidak's statistics < 32.1, $p < 0.0001$) (Figure 2). The mortality rate of ds*awd*-, ds*yel*- and ds*GFP*-treated insects at 27 days after microinjection was 57, 45 and 35%, respectively (Holms-Sidak's statistics <3.61, $p=0.1$). The mortality rate of ds*th-t*reated insects was quite high (92%) when compared to that in the control ds*GFP*-treated insects (Holms-Sidak's statistics < 32.1, $p < 0.0001$).

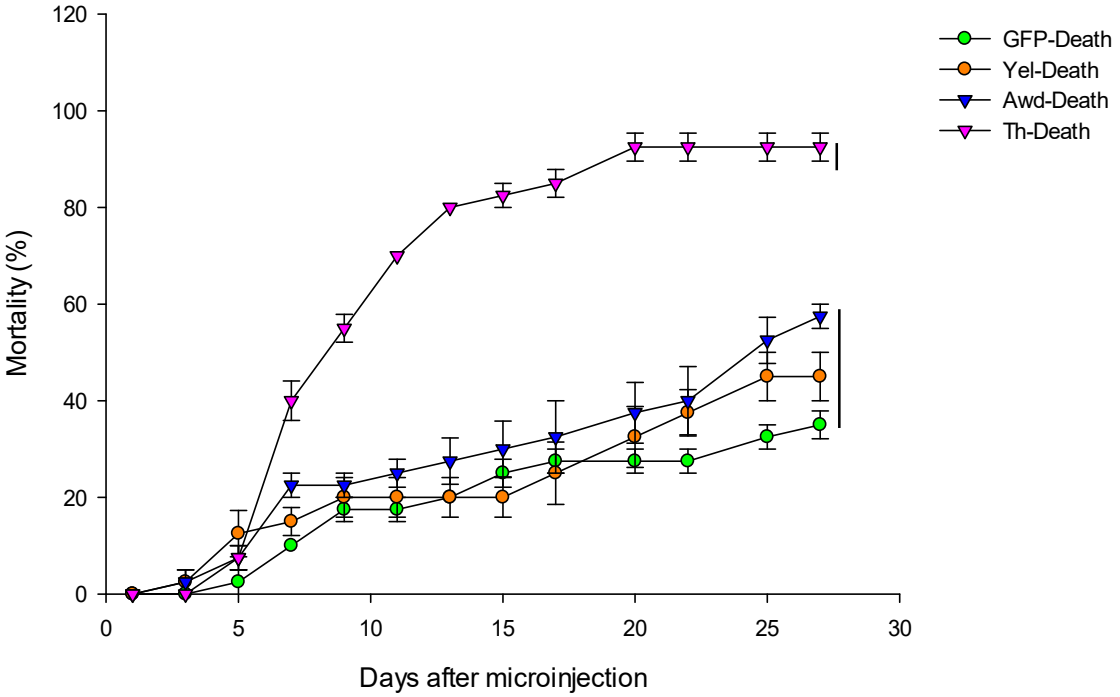


Figure 2 – Cumulative mortality of *E. heros* after microinjection of dsRNA targeting *awd*, *th* and *yel* in 3rd-instar nymphs. ds*GFP* was used as a control. The curves encompassed by the same vertical bar at the right side of the plot are not significantly different according to Holm-Sidak´s test ($p > 0.001$). The assay was conducted with two replications each consisting of 20 nymphs (N=40).

3.2 CRISPR/Cas9 gene editing for functional genomics in E. heros

A CRISPR/Cas9 workflow for gene knockout in *E. heros* was established. The entire workflow consisted of 9 steps, from egg collection through embryo injection to screening of the genotype and phenotype in hatched nymphs (1-day old), and lasted for 8 days (Figure 3).

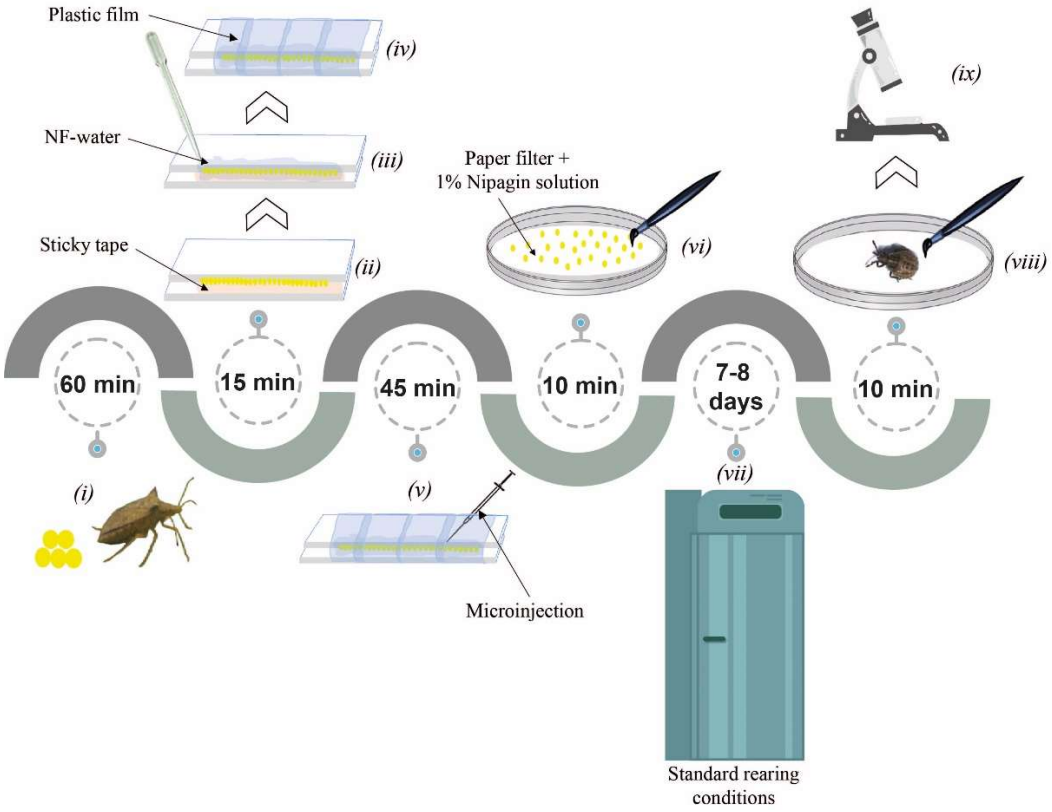


Figure 3 - CRISPR/Cas9 workflow for gene editing in *E. heros*. (i) egg collection (within 60 min after laid), (ii) careful alignment of the eggs over a sticky tape at the junction of two overlapping glass slides, (iii) soaking of eggs with nuclease-free (NF) water (1.5 ml), (iv) wrapping of the glass slides containing the eggs with plastic film to keep the eggs in place and soaked, (v) microinjection of the eggs with CRISPR/Cas9 components (within 45 min), vi) careful transfer of the injected eggs onto a filter paper slightly soaked with 1% Nipagin solution in a Petri dish, (vii) transfer Petri dishes to normal rearing conditions and check for egg hatching (between 7-8 days). viii) careful transfer of 1st-nymphs to a new Petri dish, ix) screen for mutants (genotype and phenotype). Step viii and ix can be flexible depending on the objective of the experiment.

Based on the results from the previous RNAi bioassay, where knocking down the expression of *yel* did not lead to any obvious phenotype, *yel* was selected as the target for a CRISPR/Cas9 gene knockout experiment with the aim of maybe getting a distinct visible phenotype. The *yel* gene has two isoforms in *E. heros*, hence to ensure successful disruption of the expression of both isoforms, a single-guide RNA was designed within the coding sequence for gene knockout (File S1). A total of 719 *E. heros* eggs were injected with sgRNA (300 ng/µL) and Cas9 protein (300 ng/µL), however, only 6 successfully hatched to 1st-instar nymphs (~1% hatching rate). In the control, 276 eggs were injected with water of which 28 successfully hatched into 1st-instar nymphs (10% hatching rate).

All of the hatched 1st-instar nymphs from the test group were carefully examined under the microscope. Unfortunately, no obvious distinct phenotype from the control 1st-instar nymphs was observed (Figure S4). Out of the six 1st-instar nymphs from the test group, three were randomly selected and sequenced (*Yel*-Nymph0, *Yel*-Nymph1, and *Yel*-Nymph3). The sequencing data revealed deletions in the targeted region in one of the three nymphs (Figure 4a-c). The remaining nymphs (*Yel*-Nymph2, 4 and 5) died within four days after hatching.

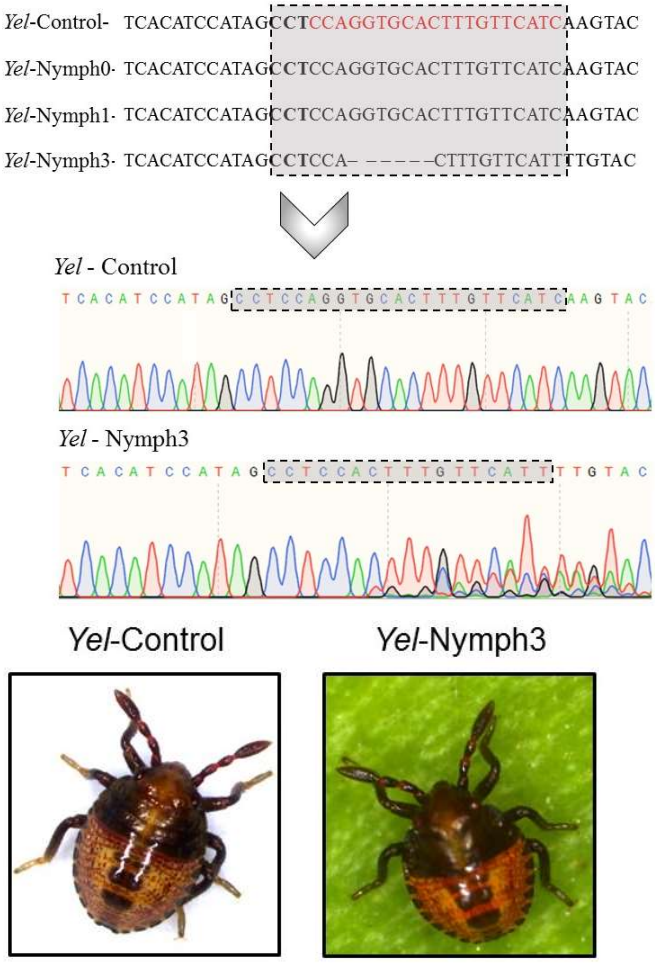


Figure 4. - Targeted mutagenesis in the *yellow* gene (*yel*) of the Brown stink bug, *Euschistus heros*. (a) DNA sequence of the control (*Yel*-Control) and test (*Yel*-nymph0, *Yel*-nymph1 and *Yel*-nymph3) insects. The boxed region highlights the guide RNA (gRNA) sequence (in red for the control) with the bolded triplet "CCT" being the reverse complement of the PAM sequence (protospacer adjacent motif) (NGG). The DNA sequence of *Yel*-nymph3 presented a mutation with an indel of 6 nucleotides located near the PAM sequence (NGG). This is typical for the Cas9 endonuclease which cleaves the DNA strands at three nucleotides upstream of the PAM sequence, while five nucleotides upstream of the PAM are defined as the seed region for target recognition. For the DNA sequences of nymph0 and nymph1, no mutation was observed. Details of the chromatogram further confirm mutation at the target region in *yel*. The occurrence of double or multiple peaks in the chromatogram of *Yel*-nymph3 (in the 3'direction from the gRNA target region) in contrast to the control, indicates mosaicism arising from different levels of somatic mutations for *yel*. (b) *Euschistus heros* nymphs (control and nymph3) with no distinct differences in phenotype.

4. Discussion

RNAi-mediated gene knockdown and CRISPR/Cas9-mediated gene knockout have been successfully used as tools for functional genomic studies in insects. However, the use of the CRISPR/Cas9 system in a non-model insect, such as *E. heros* has never been demonstrated before. In this study, we demonstrate the use of both RNAi and CRISPR/Cas9 as complementary tools in the elucidation of gene function in *E. heros*.

In a first step, RNAi-mediated knockdown experiments in *E. heros* targeting *awd* and *th* resulted in the insects having distinct malformed phenotypes, confirming the role of these genes in specific physiological processes. Treatment with ds*awd* resulted in *E. heros* adults with malformed wings which varied in severity. We hypothesize that this difference could be linked to several factors ranging from a difference in *awd* knockdown efficiency between individuals within the treated group to the length in time of the silencing signal. Nevertheless, we could confirm that *awd* is linked to wing development in *E. heros* as was also reported for other insect species such as *Drosophila melanogaster* [19], *Bombyx mori* [29], *Diaphorina citri* [9] and *Antheraea pernyi* [20]. RNAi knockdown of *th* transcripts in *E. heros* resulted in nymphs with a lighter pigmentation of the cuticle, curved abdomen, malformed antenna and lack of proper sclerotization. The *th* gene is known to be involved in the synthesis of black melanin precursors, which are in turn associated with the conversion of tyrosine to dihydroxyphenylalanine (DOPA) and dopamine (dihydroxyphenylethylamine) [21,30]. Similar to the phenotype observed in ds*th*-treated *E. heros*, knocking down *th* in the twin-spotted assassin bug *Platymeris biguttatus* also resulted in insects with a pale pigmentation of the cuticle confirming the role of *th* in the melanin pathway [21]. Contrary to *th*, knocking down *yel* did not result to any obvious change in the pigmentation of the cuticle as would be expected. Both *th* and y*el* have been reported to be involved in the melanin pathway where *yel* is required for the synthesis of DOPA and dopamine melanins [30]. Loss of *yel* in *D. melanogaster* caused a lack of melanin incorporation, resulting in a yellowish overall appearance of the cuticle [31]. Similar effects have been observed in other insects such as *B. mori* [32], *P. biguttatus* [21], while in *Tribolium castaneum*, loss of *yel* led to a slightly darker coloration of the cuticle, coupled with high mortality in the adults [33]. The transient gene silencing characteristic of RNAi can present a weakness to its use as a tool for functional genomics. For example, the time-point for injection and/or the duration of the gene silencing signal can affect the outcome of a phenotype [34]. This can range from no change in phenotype (despite gene silencing) through a mild to a strong phenotype in the target organism. As a result, RNAi might not be suitable alone as a tool for all types of functional genomic studies.

In the second part of this study, we developed and used a CRISPR/Cas9-mediated gene knockout work flow for the first time in *E. heros* to complement RNAi for functional gene studies. The *yel* gene was selected as a target for the knockout experiments based on the lack of an obvious phenotype following RNAi knockdown. The chromatogram revealed a predominant deletion of 6 nucleotides that caused an in-frame mutation in the conserved domain of *yel*, which belongs to the MRJP (Major royal jelly protein) super family (PF03022). Unfortunately, the knockout of *yel* did not lead to an obvious change in the pigmentation of the cuticle in the sequenced mutant nymph. Considering the multicolor natural appearance of *E. heros* (particularly the nymphs), it might be possible that we might have missed very subtle changes in the color of the cuticle which are not obvious with mere observation under the microscope. Also, the exact role of *yel* in body pigmentation in *E. heros* is unknown. In *B. mori*, it is hypothesized that *yel* acts together with *laccase 2* in the body pigmentation pathway [30]. This implies that in the absence of *yel*, *laccase 2* may still be functional in the melanin pathway, allowing body pigmentation. Two shortcomings of the current study are that the number of genomic loci encoding *yel* in *E. heros* are unknown and also that in the absence of next generation sequence data, solely sanger sequencing results were not conclusive to confirm heterozygosity or homozygosity of *yel* in the mutant insect. Assuming that *yel*-sgRNA targeted *yel* only on one locus, then expression from the untargeted locus could still result to a normal wild-type phenotype. In the absence of a genome for *E. heros*, we recommend southern blot analysis to confirm gene copy number. Also, the expression of a recessive

gene in a heterozygous mutant could still result in a normal phenotype. Another major challenge was the low hatchability rate of the *yel*-sgRNA injected eggs. Based on other CRISPR/Cas9 gene editing protocols developed for non-model insects, a low percentage of hatching was expected [34]. However, the percentage of hatched nymphs in the treatment with *yel*-sgRNA (~1%) was under the expected rates (≥10%), resulting in only one detected mutant. A possible explanation could be the unknown role of *yel* during embryogenesis in *E. heros*, where complete knockout of *yel* is lethal. In a similar study in the large milkweed bug *Oncopeltus fasciatus*, the knockout of the *white* gene resulted in significant embryonic mortality [35], although its homolog in *Drosophila melanogaster* is widely known to be involved in eye pigmentation [36]. Depending on the stage of embryonic development when CRISPR/Cas9 components are delivered, it is not uncommon to have a mixed population of edited (somatic mutations) and unedited cells that can result to mosaic effects in generation 0 (G_0) [37]. Furthermore, depending on the target gene and which cell population (edited versus unedited) in G_0, mosaicism can either hide or render a phenotype prominent. In the Colorado potato beetle, *Leptinotarsa decemlineata*, a range of phenotypes was observed after the vestigial gene (*vest*) was knocked out with attribution to differences in the mutation level (monoallelic, biallelic or no mutations at all) [34]. Low egg hatchability in *E. heros* following treatment with *yel*-sgRNA could also be attributed to off-target mutations in essential genes necessary for embryonic development and survival. Although *yel*-sgRNA was verified to have no potential off-targets in the transcriptome of *E. heros*, there is a still high possibility of off-targets at the genome level which could have resulted in the low hatching rates observed. The generation of a genome database for *E. heros* will greatly facilitate the use of CRISPR/Cas9 as a tool for functional genomics in this species. Nevertheless, we successfully developed and demonstrated a working CRISPR/Cas9-mediated gene editing work flow for *E. heros*, paving the way for further optimization and application.

5. Conclusions and recommendations

Until now, mainly RNAi has been explored in the research of gene function in *E. heros*. It is well known that the efficiency of RNAi-mediated gene knockdown is not always sufficient and due to this, it may not be suitable for functional analysis of some genes and in all insect species. On the other hand, the CRISPR/Cas9 system allows us to surpass some of the challenges faced using RNAi through the generation of mutant lines by a relatively simple and inexpensive method. However, this technique is time-consuming and similar to RNAi can present low efficiency in some species. Nevertheless, both tools can complement each other in functional gene studies in insects when properly applied. In our study, we successfully demonstrated that it is possible to exploit the CRISPR/Cas9 system to generate mutants in *E. heros* with room for improvement. With proper optimization following some of the recommendations provided in this study (Box 1) and some adaptations, the use of the presented CRISPR/Cas9 workflow can be exploited beyond functional gene studies to generate gene drives [15] for insect pest control.

Box 1: Some recommendations for improving CRISPR/Cas9-mediated gene editing in *E. heros*

- **Freshly laid eggs:** Cell division is a continuous process during embryonic development, hence injecting early enough (<1 h post oviposition) can reduce mosaicism.
- **Needle size:** Keep the needle opening small enough to not damage the egg while still being able to inject without requiring a high injection pressure.
- **Nuclease-free water to cover the eggs:** *E. heros* eggs have a very hard chorion, which protects them from environmental conditions. Adding water will temporarily render it soft, allowing the needle to penetrate without breaking and damaging the egg.
- **Water + Nipagin (1%) on underlying filter paper in the Petri dishes:** This will significantly reduce potential fungal growth on the eggs at the injection point.
- **Target-gene choice:** This will be dependent on the objective of the experiment. Essential genes for survival versus genes linked to non-lethal phenotypes.
- **Multiple sgRNAs:** If properly designed can significantly improve gene knockout and ease detection of mutants, based on amplicon size of the mutated gene versus the wild type
- **Ratio of Cas9:sgRNA:** Ratios other than the 1:1 ratio used in this study could improve efficiency.
- **Type of Cas enzyme:** Depending on the objective of the experiment, other Cas enzymes could be used to target specific sites in the genome (e.g. Cas12a).

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1. Phylogenetic tree for *abnormal wing disc*, *tyrosine hydroxilase* and *yellow* in *E. heros* in selected insect species. The protein sequences of the candidate genes from the neotropical stink bug *Euschistus heros* were aligned using MUCSLE with those of their homologs from other species. The phylogenetic tree was built using maximum likelihood in the software MEGA7 with default settings [24]. Figure S2 - Expression profile of *abnormal wing disc* (*awd*), *tyrosine hydroxylase* (*th*) and *yellow* (*yel*) in different life stages of *E. heros*. (a) Relative gene expression of a*wd*. (b) Relative gene expression of *th*. (c) Relative gene expression of *yel*. Values are based on three biological samples and expressed as means in every treatment. The bars with different letters denote significant differences ($p<0.05$) according to a Dunn's test. Confidence bars are shown for ± SEM. Figure S3 - *Euschistus heros* with extremely shortened wings due to treatment with dsRNA targeting the *abnormal wing disc* (*awd*). Figure S4 - *Euschistus heros* 1st-instar nymphs that hatched from eggs microinjected with *yel*-sgRNA (300 ng/μL) and Cas9 protein (300 ng/μL). *Yel*-Nymph0, 1 and 3 were sequenced to check for mutation in *yel*. *Yel*-Nymph2, 4 and 5 were kept to observe development but they died within 4 days after emergence from the eggs. File S1 - Sequences of isoforms of the yellow gene in *E. heros* and selected region for guide RNA design. Table S1 – *Euschistus heros* samples from different developmental stages used for stage-specific gene expression analysis. Table S2 – Primers used in this study, amplicon size and respective efficacy results.

Author Contributions: Conceptualization, C.N.T.T.; methodology, D.C., C.N.T.T. and G.S.; validation, D.C. and C.N.T.T.; experiments, D.C.; resources, G.S.; data curation, D.C. and C.N.T.T.; writing—original draft preparation, D.C.; writing—review and editing, C.N.T.T., M.J.Z and G.S.; supervision, C.N.T.T. and G.S.; funding acquisition, G.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) in Brazil, the Special Research Fund of Ghent University (BOF) and the Research Foundation-Flanders (FWO-Vlaanderen) in Belgium.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Panizzi, A.R.; Bueno, A.F.; Silva, F.A.C. Insetos que atacam vagens e grãos. In *Soja: manejo integrado de insetos e outros artrópodes-praga*; 2012; pp. 335–420 ISBN 978-85-7035-139-5.
2. Panizzi, A.R. Stink Bugs Growing Problems with (Hemiptera: Heteroptera: Pentatomidae): Species Invasive to the U.S. and Potential. *Am. Entomol.* **2015**, *61*, 223–233.
3. Smaniotto, L.F.; Panizzi, A.R. Interactions of Selected Species of Stink Bugs (Hemiptera: Heteroptera: Pentatomidae) from Leguminous Crops with Plants in the Neotropics. *Florida Entomol.* **2015**, *98*, 7–17, doi:10.1653/024.098.0103.
4. Cagliari, D.; Dias, N.P.; dos Santos, E.Á.; Rickes, L.N.; Kremer, F.S.; Farias, J.R.; Lenz, G.; Galdeano, D.M.; Garcia, F.R.M.; Smagghe, G.; et al. First transcriptome of the Neotropical pest Euschistus heros (Hemiptera: Pentatomidae) with dissection of its siRNA machinery. *Sci. Rep.* **2020**, *10*, 1–16, doi:10.1038/s41598-020-60078-3.
5. Fire, A.; Xu, S.; Montgomery, M.K.; Kostas, S.A.; Driver, S.E.; Mello, C.C. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **1998**, *391*, 806–811, doi:10.1038/35888.
6. Hughes, C.L.; Kaufman, T.C. RNAi analysis of deformed, proboscipedia and sex combs reduced in the milkweed bug Oncopeltus fasciatus: Novel roles for Hox genes in the Hemipteran head. *Development* **2000**, *127*, 3683–3694.
7. Liu, P.Z.; Kaufman, T.C. Hunchback is required for suppression of abdominal identity, and for proper germband growth and segmentation in the intermediate germband insect Oncopeltus fasciatus. *Development* **2004**, *131*, 1515–1527, doi:10.1242/dev.01046.
8. Riga, M.; Denecke, S.; Livadaras, I.; Geibel, S.; Nauen, R.; Vontas, J. Development of efficient RNAi in Nezara viridula for use in insecticide target discovery. *Arch. Insect Biochem. Physiol.* **2020**, 1–9, doi:10.1002/arch.21650.
9. El-Shesheny, I.; Hajeri, S.; El-Hawary, I.; Gowda, S.; Killiny, N. Silencing Abnormal Wing Disc Gene of the Asian Citrus Psyllid, Diaphorina citri Disrupts Adult Wing Development and Increases Nymph Mortality. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0065392.
10. Lu, Y.; Chen, M.; Reding, K.; Pick, L. Establishment of molecular genetic approaches to study gene expression and function in an invasive hemipteran, Halyomorpha halys. *Evodevo* **2017**, *8*, 1–11, doi:10.1186/s13227-017-0078-6.
11. Baum, J.A.; Roberts, J.K. *Progress Towards RNAi-Mediated Insect Pest Management*; 2014; Vol. 47; ISBN 0065-2806.
12. Terenius, O.; Papanicolaou, A.; Garbutt, J.S.; Eleftherianos, I.; Huvenne, H.; Kanginakudru, S.; Albrechtsen, M.; An, C.; Aymeric, J.L.; Barthel, A.; et al. RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. *J. Insect Physiol.* **2011**, *57*, 231–245, doi:10.1016/j.jinsphys.2010.11.006.
13. Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A Programmable Dual-RNA – Guided. **2012**, *337*, 816–822.
14. Doudna, J.A.; Charpentier, E. The new frontier of genome engineering with CRISPR-Cas9. *Science (80-.).* **2014**, *346*, doi:10.1126/science.1258096.
15. Taning, C.N.T.; Van Eynde, B.; Yu, N.; Ma, S.; Smagghe, G. CRISPR/Cas9 in insects: Applications, best practices and biosafety concerns. *J. Insect Physiol.* **2017**, *98*, 245–257, doi:10.1016/j.jinsphys.2017.01.007.
16. Sander, J.D.; Joung, J.K. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat. Biotechnol.* **2014**, *32*, 347–350, doi:10.1038/nbt.2842.
17. Sun, D.; Guo, Z.; Liu, Y.; Zhang, Y. Progress and prospects of CRISPR/Cas systems in insects and other arthropods. *Front. Physiol.* **2017**, *8*, 1–22, doi:10.3389/fphys.2017.00608.
18. Thurtle-Schmidt, D.M.; Lo, T.W. Molecular biology at the cutting edge: A review on CRISPR/CAS9 gene editing for undergraduates. *Biochem. Mol. Biol. Educ.* **2018**, *46*, 195–205, doi:10.1002/bmb.21108.
19. Timmons, L.; Shearn, A. Role of AWD/nucleoside diphosphate kinase in Drosophila development. *J. Bioenerg. Biomembr.* **2000**, *32*, 293–300, doi:10.1023/A:1005545214937.
20. Jiang, D.F.; Liu, Y.Q.; Li, X.S.; Shi, S.L. Characterization of the antheraea pernyi abnormal wing disc gene that may contribute to its temperature tolerance. *African J. Biotechnol.* **2010**, *9*, 7372–7378, doi:10.4314/ajb.v9i43.
21. Zhang, Y.; Li, H.; Du, J.; Zhang, J.; Shen, J.; Cai, W. Three melanin pathway genes, TH, yellow, and

aaNAT, regulate pigmentation in the twin-spotted assassin bug, Platymeris biguttatus (Linnaeus). *Int. J. Mol. Sci.* **2019**, *20*, doi:10.3390/ijms20112728.

22. Borges, M.; Laumann, R.A.; Da Silva, C.C. a; Moraes, M.C.B.; Dos Santos, H.M.; Ribeiro, D.T. Metodologias de criação e manejo de colônias de percevejos da soja (Hemíptera - Pentatomidae) para estudos de comportamento e ecologia química. *Embrapa Recur. Genéticos e Biotecnol.* **2006**, *Documentos*, 18.
23. Capella-Gutiérrez, S.; Silla-Martínez, J.M.; Gabaldón, T. trimAl: A tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* **2009**, *25*, 1972–1973, doi:10.1093/bioinformatics/btp348.
24. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874, doi:10.1093/molbev/msw054.
25. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* **2001**, *25*, 402–408, doi:10.1006/meth.2001.1262.
26. Castellanos, N.L.; Smagghe, G.; Sharma, R.; Oliveira, E.E.; Christiaens, O. Liposome encapsulation and EDTA formulation of dsRNA targeting essential genes increase oral RNAi-caused mortality in the Neotropical stink bug Euschistus heros. *Pest Manag. Sci.* **2019**, *75*, 537–548, doi:10.1002/ps.5167.
27. Hwang, W.Y.; Fu, Y.; Reyon, D.; Maeder, M.L.; Shengdar, Q.; Sander, J.D.; Peterson, R.T.; Yeh, J.J.; Keith, J. Efficient In Vivo Genome Editing Using RNA-Guided Nucleases Woong. *Nat. Biotechnol.* **2013**, *31*, 227–229, doi:10.1038/nbt.2501.Efficient.
28. Kotwica-Rolinska, J.; Chodakova, L.; Chvalova, D.; Kristofova, L.; Fenclova, I.; Provaznik, J.; Bertolutti, M.; Wu, B.C.H.; Dolezel, D. CRISPR/Cas9 genome editing introduction and optimization in the non-model insect Pyrrhocoris apterus. *Front. Physiol.* **2019**, *10*, 1–15, doi:10.3389/fphys.2019.00891.
29. Ling, L.; Ge, X.; Li, Z.; Zeng, B.; Xu, J.; Chen, X.; Shang, P.; James, A.A.; Huang, Y.; Tan, A. MiR-2 family targets awd and fng to regulate wing morphogenesis in Bombyx mori. *RNA Biol.* **2015**, *12*, 742–748, doi:10.1080/15476286.2015.1048957.
30. Zhang, L.; Martin, A.; Perry, M.W.; van der Burg, K.R.L.; Matsuoka, Y.; Monteiro, A.; Reed, R.D. Genetic basis of melanin pigmentation in butterfly wings. *Genetics* **2017**, *205*, 1537–1550, doi:10.1534/genetics.116.196451.
31. Heinze, S.D.; Kohlbrenner, T.; Ippolito, D.; Meccariello, A.; Burger, A.; Mosimann, C.; Saccone, G.; Bopp, D. CRISPR-Cas9 targeted disruption of the yellow ortholog in the housefly identifies the brown body locus. *Sci. Rep.* **2017**, *7*, 1–9, doi:10.1038/s41598-017-04686-6.
32. Ito, K.; Katsuma, S.; Yamamoto, K.; Kadono-Okuda, K.; Mita, K.; Shimada, T. Yellow-e determines the color pattern of larval head and tail spots of the silkworm Bombyx mori. *J. Biol. Chem.* **2010**, *285*, 5624–5629, doi:10.1074/jbc.M109.035741.
33. Noh, M.Y.; Kramer, K.J.; Muthukrishnan, S.; Beeman, R.W.; Kanost, M.R.; Arakane, Y. Loss of function of the yellow-e gene causes dehydration-induced mortality of adult Tribolium castaneum. *Dev. Biol.* **2015**, *399*, 315–324, doi:10.1016/j.ydbio.2015.01.009.
34. Gui, S.; Taning, C.N.T.; Wei, D.; Smagghe, G. First report on CRISPR/Cas9-targeted mutagenesis in the Colorado potato beetle, Leptinotarsa decemlineata. *J. Insect Physiol.* **2020**, *121*, 104013, doi:10.1016/j.jinsphys.2020.104013.
35. Reding, K.; Pick, L. High efficiency CRISPR / Cas9 mutagenesis of the white gene in the milkweed bug Oncopeltus fasciatu s Katie Reding and Leslie Pick * Department of Entomology , University of Maryland * Corresponding author : lpick@umd.edu Running title : CRISPR mutagenesi. *Genetics* **2020**.
36. Mackenzie, S.M.; Brooker, M.R.; Gill, T.R.; Cox, G.B.; Howells, A.J.; Ewart, G.D. Mutations in the white gene of Drosophila melanogaster affecting ABC transporters that determine eye colouration. *Biochim. Biophys. Acta - Biomembr.* **1999**, *1419*, 173–185, doi:10.1016/S0005-2736(99)00064-4.
37. Mehravar, M.; Shirazi, A.; Nazari, M.; Banan, M. Mosaicism in CRISPR/Cas9-mediated genome editing. *Dev. Biol.* **2019**, *445*, 156–162, doi:10.1016/j.ydbio.2018.10.008.

insects

MDPI

Supplementary material

Communication

RNAi and CRISPR/Cas9 as functional genomics tools in the Neotropical stink bug, *Euschistus heros*

Deise Cagliari[1,2,*]; Guy Smagghe[1,*]; Moises Zotti[2]; Clauvis Nji Tizi Taning[1,*]

[1] Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium
[2] Department of Crop Protection, Molecular Entomology Laboratory, Federal University of Pelotas, Pelotas, Brazil
* Correspondence: deise.cagliari@ugent.be (D. Cagliari), guy.smagghe@ugent.be (G. Smagghe), tiziclauvis.taningnji@ugent.be (C. N. T. Taning)

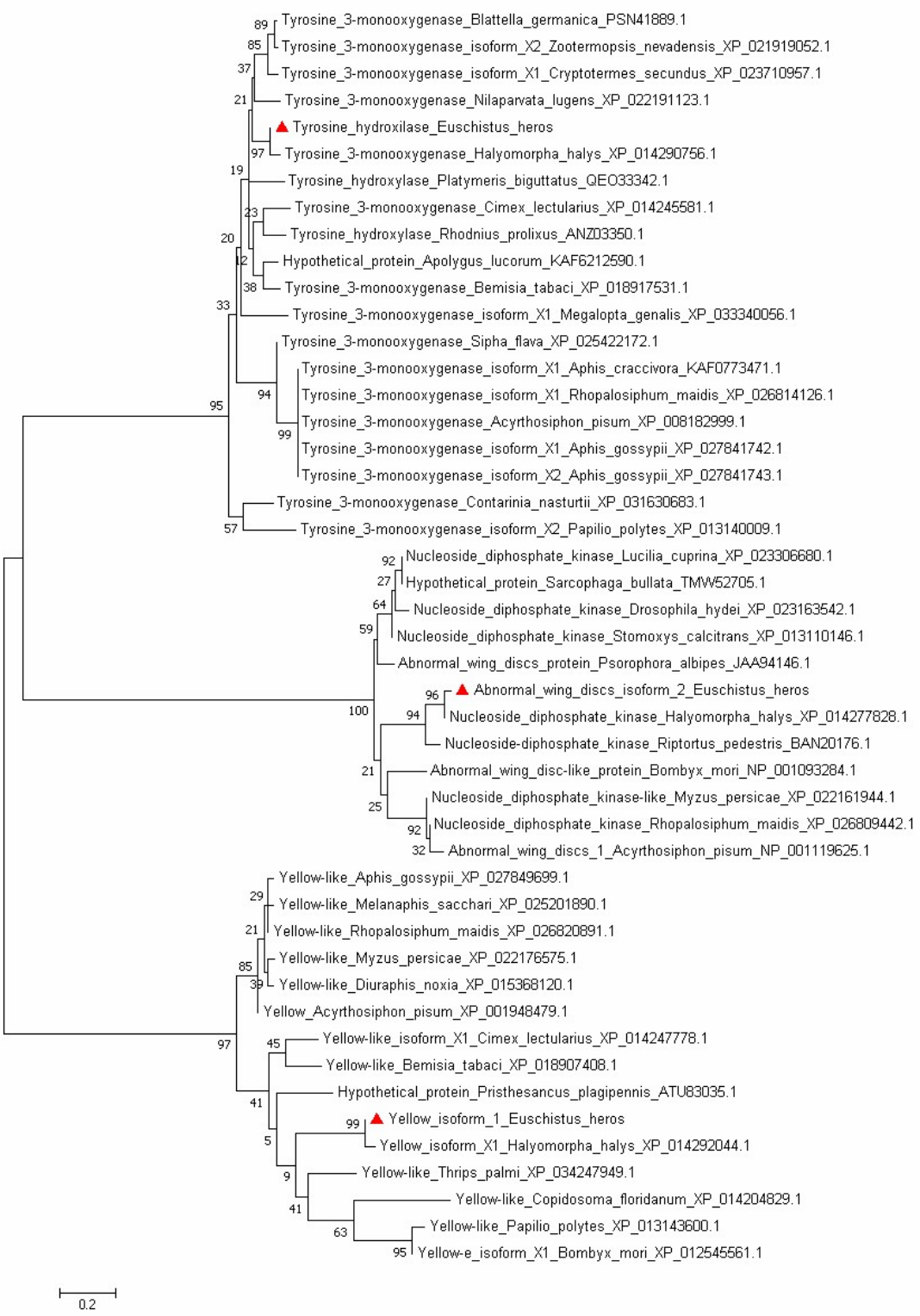


Figure S1 - Phylogenetic tree for *abnormal wing disc*, *tyrosine hydroxilase* and *yellow* in *E. heros* in selected insect species. The protein sequences of the candidate genes from the neotropical stink bug *Euschistus heros* were aligned using MUCSLE with those of their homologs from other species. The phylogenetic tree was built using maximum likelihood in the software MEGA7 with default settings.

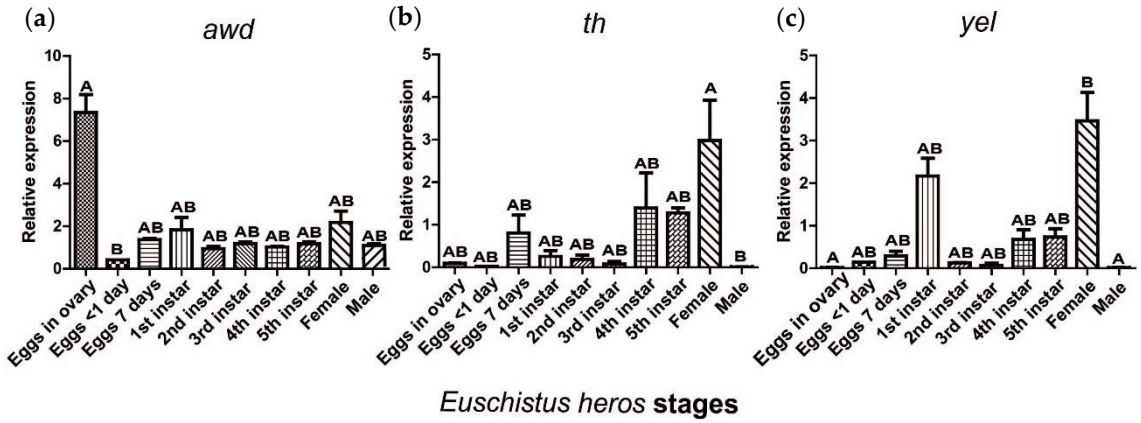


Figure S2 - Expression profile of *abnormal wing disc* (*awd*), *tyrosine hydroxylase* (*th*) and *yellow* (*yel*) in different life stages of *E. heros*. (a) Relative gene expression of a*wd*. (b) Relative gene expression of *th*. (c) Relative gene expression of *yel*. Values are based on three biological samples and expressed as means in every treatment. The bars with different letters denote significant differences ($p<0.05$) according to a Dunn's test. Confidence bars are shown for ± SEM.

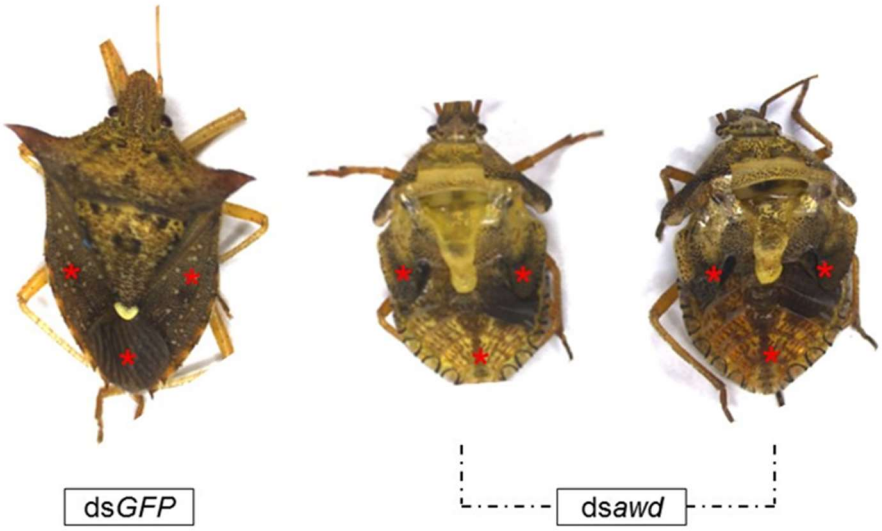


Figure S3 - *Euschistus heros* with extremely shortened wings due to treatment with dsRNA targeting the *abnormal wing disc* (*awd*). The insects treated with ds*awd* failed on changing instar. Red asterisk indicates the wings (ds*GFP*) and where they were supposed to be after changing the instar.

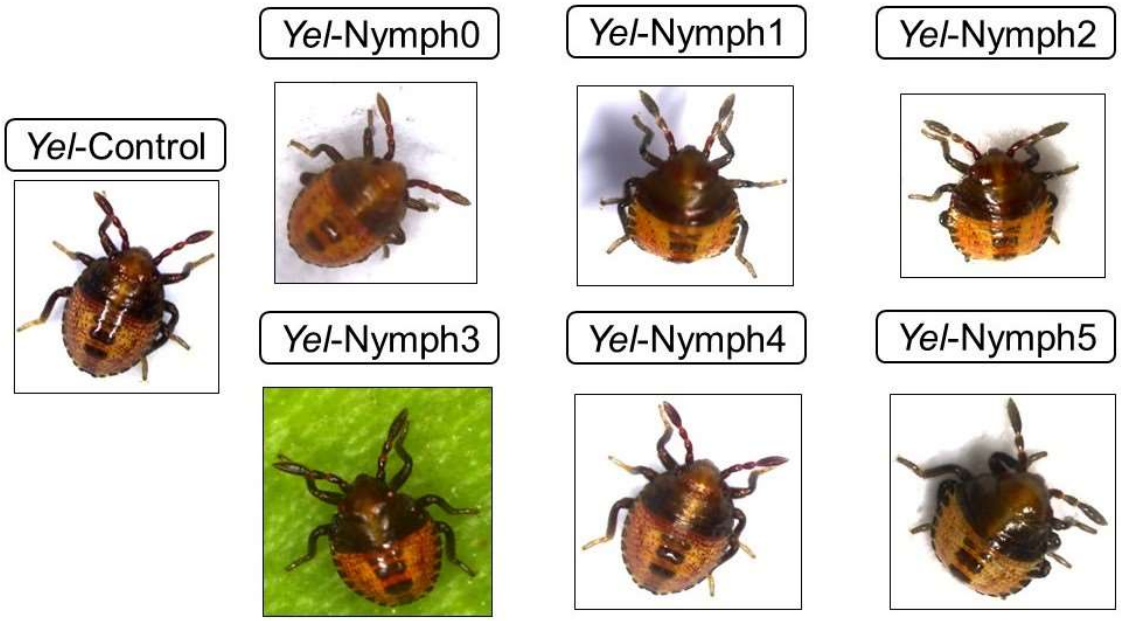


Figure S4 - *Euschistus heros* 1st-instar nymphs that hatched from eggs microinjected with *yel*-sgRNA (300 ng/µL) and Cas9 protein (300 ng/µL). *Yel*-Nymph0, 1 and 3 were sequenced to check for mutation in *yel*. *Yel*-Nymph2, 4 and 5 were kept to observe development but they died within 4 days after emergence from the eggs.

Table S1 – *Euschistus heros* samples from different developmental stages used for stage-specific gene expression analysis

Stage	*Number (N)
Eggs in ovary	25
Eggs <24 hours old	25
Eggs 7 days old	25
1st instar	10
2nd instar	10
3rd instar	5
4th instar	5
5th instar	1
Male	1
Female	1

* Pooled samples (where N>1) and this was repeated thrice

Table S2 – Primers used in this study, amplicon size and respective efficacy results.

	Symbol	Oligo	Primer sequence	Amplification size	Efficiency (%)	R^2
qRT-PCR						
Abnormal wing disc	qPCR-*awd*	F	TTGCTCATGGATCAGACTC	80	91.6	0.997
		R	CCATTCAACCTGCTCCTTA			
Tyrosine hydroxylase	qPCR-*th*	F	GCACTTCTGCAGGGAATA	135	90.1	0.997
		R	GCAGGCCTTAGGGTAAAT			
Yellow	qPCR-*yel*	F	CAGCCCACATACTACCAATA	146	100.0	0.998
		R	TCTCAATCACCTGCTTCC			
Ribosomal protein 18S	qPCR-*18S*	F	TACAACAAGACAACGCTCGC	150	95.7	0.998
		R	TTGCGCTCAGTGACATCTCT			
Ribosomal protein 32	qPCR-*RPL32*	F	TCAGTTCTGAGGCGTGCAT	175	90.9	0.999
		R	TCCGCAAAGTCCTCGTTCA			
dsRNA[1]						
Abnormal wing disc	ds*Awd*	F	CACTCGAGCACGTTTAGA	472	-	-
		R	CTTAGGTGTGAACCAGAGG			
Tyrosine hydroxylase	ds*Th*	F	CCATCGCTCTTACCAAACT	655	-	-
		R	AGTCTCACAGCATAGGTTTAC			
Yellow	ds*Yel*	F	TTTCACCACACATTGCTAAC	663	-	-
		R	TCCAGGAACTGCTGATTAC			
CRISPR – mutation screening						
Yellow	*yel*	F	CCTGTCTGAGGCAAATGGTT	432	-	-
		R	TGCGATGTTGATCATCCTTT			

[1]T7 sequence added in front of each primer: TAATACGACTCACTATAGGGAGA

6. General conclusions

During the last decades, agriculture has faced a huge transformation in the way growers farm the land and produce food. With the use of technology, growers increased significantly productivity, going from approximately 1,500 t/ha in the 90s to approximately 3.000 t/ha of soybean in 2016 in Brazil (CONAB 2017), for example. In the crop season 2020/2021, it is expected that Brazil will reach a planted area of almost 63 million hectares, with an increase of 4.6% of the yield compared to the previous season (CONAB 2020). In this scenario, soybean represents more than 50% of the planting area, and an expected yield of 135 million tons, putting Brazil as the major global producer of this oilseed.

Many factors can impair yields in the field, such as diseases, insect pests and weeds. To protect the field, growers rely almost exclusively on the use of chemicals. During the last years, due to the high pressure of insects, pathogens, and weeds in the field, we have been observing an increase in the use of chemicals products (Oliveira et al. 2013). Among the insects that can cause damage to crops, stink bugs are one of the main problems, especially in soybean (Panizzi et al., 2012). Among the stink bugs that can cause damage to soybean, *N. viridula*, *Piezodorus guildinii,* and *E. heros* are the most abundant ones (Hoffmann-campo et al. 2000). A considerable amount of the pesticide used in soybean fields every year is an attempt to manage these insects in the field (Panizzi 2013; Bueno et al. 2015). Due to this, alternative strategies to manage insect pests in the field are necessary and very welcome by growers and society. In

this scenario, RNAi and CRISPR appear as potential tools that can be used to design new management tools to face the problem with pest management in the field.

In this PhD dissertation, we aimed to provide relevant information regarding the i) the generation of a transcriptome dataset of genetic information, providing a complete set of free information that will help researchers to better understand this and other stink bugs. The identification of the main RNAi components present in *E. heros* as well as the confirmation of the functionality of the RNAi tool in this species; ii) an extensive literature review showing the potential uses of the RNAi tool as a non-transformative approach in the management of pests in the field; iii) the use of parental RNAi as a tool in the study on the function of genes in *E. heros*; and d) the combination of RNAi and CRISPR/Cas9 as functional genomic tools in *E. heros*. In what follows, the results are discussed and situated and the future perspectives on the use of RNAi and CRISPR in the Neotropical stink bug *E. heros* as well as other hemipteran insects are presented.

6.1 The importance of genetic information

The increasing number of genetic information help scientists to elucidate the function of genes, estimate the prevalence of genes within a population, the biology and development of a species, understand evolution, and how insecticide resistance can be selected. This data will also allow scientists to perform robust and accurate bioinformatics analyses, advancing and accelerating biological discoveries (Baxevanis 2009).

For the stink bug *E. heros*, only a small amount of genetic information is annotated and available on GenBank, from NCBI (https://www.ncbi.nlm.nih.gov/). With this in mind and knowing the importance of genetic information, in **Chapter 2**, we provide the first transcriptome of *E. heros*. A pool of different stages (eggs, 1st-, 2nd-, 3rd, 4th-, 5th-instar nymphs and adult) was prepared and sent for sequencing at the Laboratory of Functional Genomics Applied to Agriculture and Agri-Energy. The transcriptome was sequenced using the high-throughput Illumina sequencing platform (HiSeq2000). The transcriptome sequencing generated more than 126 million sequenced reads, and these were annotated in approximately 80,000 contigs (**Chapter 2**). A similar approach was used to generate the transcriptome information

of the African sweet potato weevil, *C. puncticollis* (Prentice et al. 2015), and the South-American fruit fly, *Anastrepha fraterculus* (Dias et al. 2019). In *E. heros*, a total of 60,956 hits were produced, representing 41.30% of the total transcripts (Chapter 2). Similar to this, Prentice et al. (2015) found 50% of unknown protein sequences in the transcriptome of the African sweet potato weevil, while Dias et al. (2019) found 55% of unknown protein sequences in that of the South-American fruit fly. So, even though the number of unknown proteins is high, we were able to generate a database accomplishing our objective. This work will pave the way for the generation of more and more genetic information and also the future annotation of the genome of this important species. Through the genome project iK5, scientists expect to sequence the genome of more than 5,000 species, being *E. heros* among one of the species whose DNA will be sequenced (http://i5k.github.io/arthropod_genomes_at_ncbi). This will further improve research in areas such as RNAi and CRISPR, in which genetic information is of upmost importance.

Using the information generated from transcriptome, we identified the RNAi-related genes in *E. heros*. A total of 47 genes related to the RNAi pathway - siRNA, miRNA, and piRNA – were identified (Chapter 2). These genes are related to the RNAi process of (i) dsRNA uptake, (ii) intracellular transport, (iii) dsRNA processing, (iv) RISC formation and binding process, and (v) gene silencing itself. These are the main RNAi processes to ensure a proper operation of the gene silencing machinery (Swevers et al. 2013; Prentice et al. 2015; Yoon et al. 2016). An important piece of information found in the transcriptome analysis was the absence of *sid-like* homolog genes. In eukaryote organisms, the dsRNA uptake process occurs through sid-like transmembrane proteins (Jose and Hunter 2007; Cappelle et al. 2016) or endocytosis-mediated (Ulvila et al. 2006; Cappelle et al. 2016). In the stink bug *H. halys*, when scientists analyzed the transcriptome, they were also unable to find *sid-like* homolog genes (Sparks et al. 2014). So, due to the absence of *sid-like* homolog genes, the question is, is endocytosis the main dsRNA uptake pathways in *E. heros?* This needs to be addressed in future research and will help researchers to understand more about the RNAi efficiency in stink bugs and improve the development of RNAi-based control strategies.

A total of 13 core RNAi-related genes were identified in the *E. heros* transcriptome: with 6 being related to the miRNA pathway, 3 related to the siRNA pathway, and 4 related to the piRNA pathway (Chapter 2). Among the main RNAi components, we found variants for *AGO-1* with 4 variants; *DCR-2* with 2 variants, and *AGO-2* with 2 variants. Some of these variants did not present all conserved domains, so the function of these genes in the RNAi pathways still needs to be elucidated (**Chapter 2**). Another important information found in the transcriptome is the presence of four nucleases: namely *Eri-1*, *Nibbler*, *SDN1*, and *dsRNase*. The dsRNases play an important role in the siRNA pathway, in the degradation of dsRNAs molecules. A total of 7 variants of the dsRNase were found in *E. heros,* in which 6 presented the conserved domain. It is well known what the importance is of these nucleases in RNAi efficiency across insect groups such as Hemiptera (Christiaens and Smagghe 2014; Christiaens et al. 2014; Wang et al. 2016a), Lepidoptera (Liu et al. 2012; Guan et al. 2018), and Diptera (Singh et al. 2017), and this may impact the RNAi efficiency in this species.

Still, a total of 17 intracellular factors associated with RISC, 4 genes related to the antiviral process and 3 related to intracellular transport, were identified in the Neotropical stink bug. The presence of the main RNAi-related genes in *E. heros* suggests that it has an active and functional RNAi machinery. However, it is important to note that although these genes are involved in the RNAi process in other organisms, it does not mean that they are also involved in the RNAi mechanism in *E. heros.* The real involvement of these genes needs to be further confirmed in future functional assays.

6.2. Uses of RNAi technology in the Neotropical stink bug *E. heros*

6.2.1. Gene silencing in *E. heros*

Once the main RNAi-related genes were identified in *E. heros* transcriptome, we investigated the functionality of the silencing machinery (**Chapter 2**). In other words, is the gene silence machinery activate under the supply of dsRNA in *E. heros*?

To study this, we microinjected adults with a dsRNA targeting the *V-ATPase-A* gene (**Chapter 2**). But, previous to this, we wanted to know how fast dsRNases present in the hemolymph degrades the dsRNAs molecules. Using hemolymph extracted from

E. heros adults, we showed that dsRNA was completely degraded after incubation of 120 min. Similar to this, *E. heros* saliva also degraded dsRNA after an 120 min of incubation (Castellanos et al. 2019). In *A. pisum*, the low RNAi efficiency is associated with a high dsRNase activity (Christiaens et al. 2014). This high nuclease activity in saliva and hemolymph of *E. heros* can affect RNAi efficiency and the dsRNA protection can help to protect the molecule from the activity of these enzymes. When using EDTA or liposome-encapsulated dsRNA, researchers prevented the dsRNA from being degraded enzymatically when ingested by *E. heros* (Castellanos et al. 2019).

When we injected naked dsRNA targeting the *V-ATPase-A* gene in *E. heros* adults, mortality reached 35% at 96 h post microinjection, with a reduction of 74% in the relative level of transcripts (**Chapter 2**). Similar results were found for *E. heros* nymphs, in which 30% of mortality was observed at 14 days after treatment with naked dsRNA (Castellanos et al. 2019). Third-instar larvae of *P. gossypiella* microinjected with a dsRNA targeting *V-ATPase-A* gene showed a mortality of 26% at 96 h post-microinjection (Mohammed 2016). In both cases, the authors speculated that the main reason for the low RNAi efficiency is due to the high nuclease activity. So, the main way to increase dsRNA durability inside the system is through the formulation of the molecule.

Finally, we also showed that the supply of dsRNA activates the siRNA machinery. After the microinjection with dsRNA, an upregulation of the RNAi machinery genes (*DCR-2* and *AGO-2*) was observed. In *M. sexta* and *A. fraterculus* and upregulations of the siRNA-related genes were also observed after the supply of dsRNA was also observed (Garbutt and Reynolds 2012; Dias et al. 2019).

How works the dsRNA uptake in *E. heros*? Are there efficient and effective target genes? Are these genes the same for adults and nymphs? Can we select stable and efficient carriers for the dsRNA delivery in the field to control stink bugs? These are some of the questions that still need to be answered and which will help scientists to develop RNAi as a control tool. Taken together these data provide novel and important information about the RNAi machinery and its efficiency in *E. heros*, underpinning future strategies to enhance RNAi in *E. heros* and other piercing-sucking insects important in agriculture.

6.2.2. Use of RNAi as a control tool

The use of RNAi as a control tool for pest insects, pathogens, or weeds will reach the market either through transformative methods (transgenic plants) or non-transformative methods (i.e. formulations for foliar applications, trunk injections, baits, among others). However, there are limitations on the use of RNAi-based transformative approaches, especially when it comes to society acceptance of transgenic plants expressing RNAi-based traits (**Chapter 1**).

Studies using non-transformative approaches such as foliar applications (Gong et al. 2013; de Andrade and Hunter 2016; Koch et al. 2016; San Miguel and Scott 2016; Yin et al. 2016; Gogoi et al. 2017; McLoughlin et al. 2018; Willow et al. 2020), trunk injection (Hunter et al. 2012a; Dalakouras et al. 2018), and irrigation (Li et al. 2015) have demonstrated the feasibility and efficacy of RNAi-based gene silencing through non-transformative delivery strategies (**Chapter 3**). There are other methods, such as seed coats or orchard baits that still need to be investigated and evaluated to show their feasibility. These applications confirm the great potential of RNAi as a pest management tool in a range of areas in the field of crop protection (Adeyinka et al. 2020; Fletcher et al. 2020; Mezzetti et al. 2020).

Selecting the right dsRNA delivery method will confirm the efficiency of the RNAi mechanism, varying according to the target pest, life stage, and crop (**Chapter 3**). Lepidopteran and hemipteran insects are considered more recalcitrant to RNAi, and high dsRNA concentrations are required to achieve successful gene silencing results (Terenius et al. 2011; Li et al. 2013; Christiaens and Smagghe 2014; Jain et al. 2020a), while coleopteran insects are considered more susceptible to RNAi (Baum et al. 2007; Baum and Roberts 2014; Zotti and Smagghe 2015). Mechanisms of high nuclease activity (Garbutt et al. 2013; Christiaens and Smagghe 2014; Castellanos et al. 2019) as well as uptake issues (Yoon et al. 2017) are related to the low efficiency of RNAi in these groups of insects. Choosing the right combination of target species X life stage X delivery strategy will save years of research and resources (**Chapter 3**).

Alongside, the development of more efficient dsRNA mass production systems will reduce the costs, and together with the release of new formulations will allow non-transformative RNAi to be exploited as a potential pest control strategy (Hunter et al. 2012a; de Andrade and Hunter 2016). During the last years, the cost of dsRNA

dropped significantly, from more than $ 10,000 USD/g to less than $ 2.00 USD/g in 2020, through the use of mass dsRNA production systems (Zotti et al. 2017; Dalakouras et al. 2020). But how many times the grower will need to spray the field? Under field conditions, as for chemical control approaches, RNA-based biopesticides will need periodical applications following plant growth to ensure plant protection. The use of nanotechnology plus mass dsRNA production system will allow better stability of the molecule in the field as well as the reapplication with lower costs (**Chapter 3**). In *E. heros*, the use of liposome complexes increased nymph mortality compared to naked dsRNA upon oral feeding (Castellanos et al. 2019). However, in some cases, even with the use of formulation the dsRNA molecules were unable to initiate the RNAi process. This was observe in *L. migratoria*, where liposome encapsulation was not unable to protect the dsRNA, resulting in an inefficient RNAi gene silencing (Luo et al. 2013).

The availability of more and more genetic information (**Chapter 2**) alongside with the knowledge about the use of non-transformative RNAi approaches (**Chapter 3**) will lead the development of more efficient RNA-based products to manage pest insects, pathogens, and viruses in the field. In the future, it is expected that RNAi-based pesticides will help to replace chemical pesticides in some applications or when even in combination, reducing the use of chemical pesticides in the field.

On the other side, there are still many issues involved in the use of non-transformative RNAi approaches that still need to be addressed: Is the technology going to work for all insect species, especially stink bugs? What is going to be the field concentration to target efficient control? How many applications it will demand? What is going to be the right instar to target? And one of the most important questions, how to avoid off-target effects? These are questions that still need to be addressed (Figure 8). The knowledge about this approach has significantly increased during the last years, and the scenario is positive. RNAi approaches such as concatomerization of the RNAi sequences and pyramiding RNAi will reduce dsRNA production and application cost, and will improve pest control (Jain et al. 2020a). The availability of more and more genetic information also allows more accurate bioinformatics analyses, reducing the chances of the potential off-target effects. We hope that soon we will see this

technology reaching the market to help growers to protect their crops from the damage caused by pest insects, pathogens, and weeds.

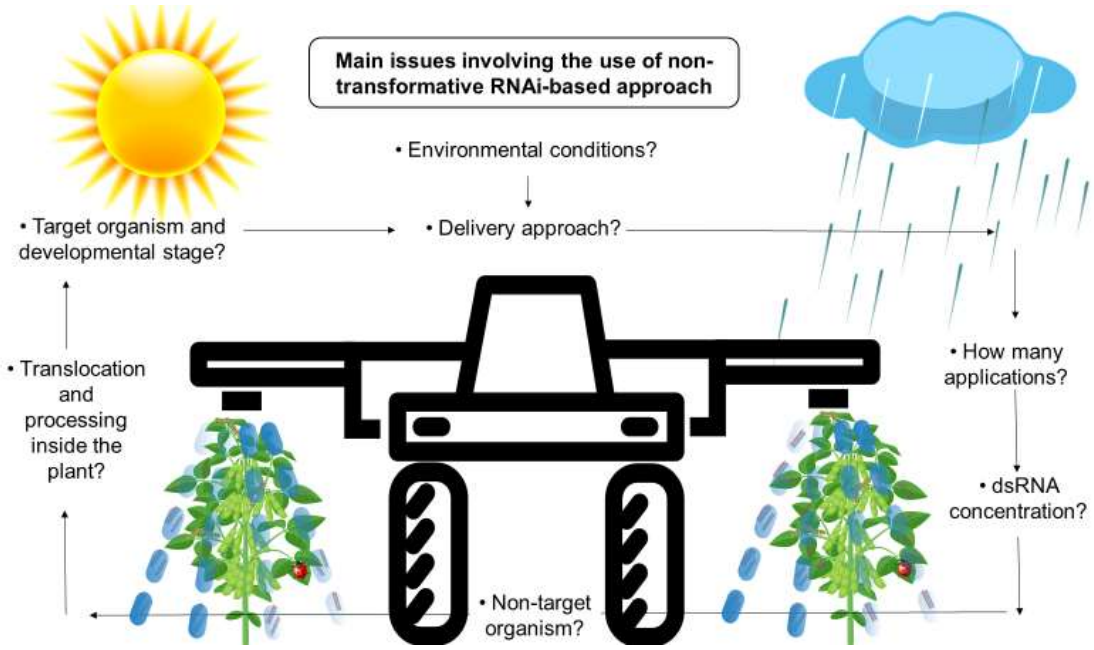


Figure 8 – Main issues related to the use of non-transformative RNAi approaches as a pest management tool. RNAi approaches that still need to be addressed: Is the technology going to work for all insect species, especially stink bugs? What is going to be the field concentration to target efficient control? How many applications it will demand? What is going to be the right instar to target? And one of the most important questions, how to avoid off-target effects? These are questions that still need to be addressed.

6.2.3. Use of RNAi as a functional genomic tool

During the last years, the progress of next-generation sequencing has allowed researchers to obtain important transcriptome data of several economically important hemipteran pests, including the stink bug *E. heros* (**Chapter 2**). With transcriptome (and also genome) data becoming more available for many insect species, research has been focusing more on the identification of gene functions in a given species (Jain et al. 2020b).

RNAi is also a promising technology in the field of functional genomics. Parental RNAi (pRNAi) as a tool to elucidate the role of genes has been explored in a range of insect species, including hemipteran ones such as bugs (Angelini et al., 2005; Hughes and Kaufman, 2000), aphids (Coleman et al. 2015) and stink bugs (Lu et al. 2017; Riga et al. 2020). Some researchers had even exploited this approach as a possible control tool for *Diabrotica virgifera* and *E. heros* (Fishilevich et al. 2016). When using pRNAi

or transgenerational RNAi, it is possible to target gene silencing in the organism and observe the phenotype in the progeny of the treated parent organism (Vélez et al. 2017).

In **Chapter 4** we showed the feasibility of pRNAi as a genomic tool in *E. heros*, showing the involvement of five target genes in *E. heros* embryonic development. In this chapter we showed the involvement of *labial (lab)*, *deformed (dfd)*, *sex comb reduced (scr)*, *extradenticle (exd)* and *proboscipedia (pb)* in *E. heros* embryonic development, and the knockdown of *dfd*, *scr* and *pb* led to an abnormal rostrum development.

In *Drosophila* (Diederich et al. 1989), *O. fasciatus* (Angelini et al. 2005) and *Apis mellifera* (Hymenoptera: Apidae) (Fleig et al. 1992), *lab, scr* and *dfd* are genes mainly expressed in the head during embryonic development. When the expression of these genes are affected, it can result in defective head formation (Merrill et al. 1989), leading to embryonic death. We evaluated the number of eggs and also the viability of the laid eggs from the treated females. The number of eggs laid per female per day was not affected by the different dsRNA-treatments, except for the ds*Dfd*-treatment, compared to the control (ds*GFP*). Similar to the hemipteran milkweed bug *O. fasciatus* (Angelini et al. 2005), *lab* was not required for normal embryo development in *E. heros*. Alongside, targeting this gene in *Drosophila* resulted in embryo lethality before hatching (Merrill et al. 1989).

On the other hand, the treatment with ds*Lab, dsDfd*, ds*Scr* or ds*Exd* significantly affected egg hatching. The nymphs that hatched from the ds*Exd- and* ds*Lab*-treatment showed no apparent change in phenotype, while the nymphs from *dsDfd*, ds*Scr* and ds*Pb*-treatment showed an abnormal rostrum development (**Chapter 4**). The offspring from ds*Scr*- and ds*Pb*-treated females showed a bifurcated rostrum with a leg-like structure, while the offspring from ds*Dfd* presented two short and curled structures posterior of the rostrum. Similar results were described in *O. fasciatus* (Hughes and Kaufman 2000). These researchers found that the depletion of *scr* and *pb* led to abnormal development of the labium to a leg-like structure. In *N. viridula* (Hemiptera: Pentatomidae), silencing of *scr* led to a phenotype similar to the one found in *E. heros* offspring, which further confirms the involvement of this gene in rostrum development (Riga et al. 2020).

Exploiting the biological rules of critical genes will further enhance the development of RNAi-based biopesticide (Jain et al. 2020b). Moreover, RNAi has been exploited to determine the roles of genes in diverse species, providing important insight into the development and evolutionary processes that have modeled the gene functions in insects (Bellés 2010). All this is increasing our current knowledge on the development, biology and reproductive characteristics in insects through the study of functional genomics and this will, in turn, facilitate the further development of regulations that will allow the transfer of this technology to the field (Scolari et al. 2014).

6.3. Use of CRISPR in the stink bug *E. heros*

Until now, mainly RNAi has been explored in the research of gene function in non-model insects. It is well known that the efficiency of RNAi gene-silencing is not always sufficient, and due to this, it may not be suitable for functional analysis of some genes in some insect species. On the other hand, the CRISPR/Cas9 system allows us to surpass those problems by generating mutant lines by a relatively simple and inexpensive method. However, this technique is time-consuming and, as well as RNAi, can present low efficiency in some species depending on the target site.

In **Chapter 5,** we demonstrated that it is possible to generate knockout mutants in *E. heros* using the CRISPR/Cas9 system. Three genes were selected, namely *abnormal wing disc* (*awd*), *tyrosine hydroxylase* (*th*), and *yellow* (*yel*), and we first evaluated the phenotype using the RNAi tool. *Awd* is known to be involved in wing development (Timmons and Shearn 2000; Jiang et al. 2010; El-Shesheny et al. 2013; Ling et al. 2015), while *yel* and *th* are involved in the melanin pathway (Zhang et al. 2017b). Knockdown of *awd* and *th* resulted in insects with malformed wings and disruption in cuticle pigmentation, respectively (**Chapter 5**). On the other hand, the knockdown of *yel* did not result in insects with a clear phenotype. Due to this, a CRISPR/Cas9-mediated workflow was developed to study the function of these genes in *E. heros*.

To do this, a total of 719 eggs were injected with the Cas9 nuclease (300 ng/µl) and sgRNA (300 ng/µl) targeting the *yel* gene (**Chapter 5**). Out of this, six insects successfully hatched from the injected eggs, and one of the insects showed a mutation in the target region, however, the phenotype was not clear. Two important issues need

attention: the number of genomic locus encoding *yel* as well as the heterozygosis/homozygosis of the *yel* loci. In *E. heros*, the number of genomic loci encoding for *yel* is unknown, and if *yel*-sgRNA targeted only one of the *yel* locus, then expression from the untargeted locus could still generate a normal wild-type phenotype. On the other hand, the expression of a recessive gene in a heterozygous mutant could still result in a normal phenotype, and solely the results from sanger sequencing were not conclusive to confirm heterozygosity or homozygosity of *yel* in the mutant insect. Still, the exact role of *yel* in body pigmentation in *E. heros* is unknown. In *B. mori*, it is hypothesized that *yel* acts together with *laccase 2* in the body pigmentation pathway (Zhang et al. 2017b). So, in the absence of *yel*, *laccase 2* may still be functional in the melanin pathway, allowing body pigmentation.

Alongside with the absence of a clear phenotype, low egg hatchability in *E. heros* following treatment with *yel*-sgRNA also plays an important role. When *yel*-sgRNA was verified at the transcript level, no potential off-targets were found. However, there are still high possibilities of off-target effects at the genome level, leading to the low hatching rates observed in the experiment.

Even not being able to generate insects with clear phenotype, we show that it is possible to use CRISPR/Cas9 in the stink bug *E. heros*. The main issues that remain are related to the design of the sgRNA; the right ratio of Cas9:sgRNA; the right time for injection in the embryos and the size of the needles. With proper optimization and some adaptations, the use of the CRISPR/Cas9 workflow can be exploited beyond functional gene studies to generate gene drives for insect pest control (**Chapter 5**).

6.4. Future perspectives for RNAi and CRISPR/Cas9

In the field, growers still rely heavily on the widespread use of conventional chemical pesticides to protect crops against insects, pathogens, and weeds, and large amounts of these products are applied every year. Sustainable production of soybean and other crops will not depend only on the management of *E. heros*, but on the IPM programs addressing multiple pests taking into account management, business, and sustainability aspects (Kennedy 2008; Barzman et al. 2015; Dara 2019)

Therefore, the development of novel management tools that are more sustainable and less detrimental to the environment are needed. In this line, RNAi and

CRISPR/Cas9 appear as promising tools. A large number of studies have shown the feasibility and efficacy of RNAi-based approaches as well as CRISPR/Cas9, with the authorization of RNAi-based corn (MON87411; SmartStax Pro) for commercialization already (Head et al. 2017; Zotti et al. 2017; Arpaia et al. 2020).

The risk-assessment and regulation of RNAi- and CRISPR-based products will play an important role in the release of these technology for growers. Due to the characteristic of the dsRNA and sgRNA molecules, the risk-assessments need to be assessed according in a case-by-case basis (Fishilevish et al. 2016; Taning et al. 2019). For instance, when carrying out a risk assessment for *DvSnf7* RNA, scientist found *DvSnf7* activity in a group of beetles within the Galeruciane subfamily of Chrysomelidae (Bachman et al. 2016). Similar to this, when studying the nontarget effects of *inhibitor of apoptosis* (*iap*) gene, no significant nontarget effects of ds*IAP* were observed even among closely related insects such as stink bugs, *N. viridula*, *H. halys*, and *M. histrionica*, with substantial sequence similarity among *iap* genes (Chereddy et al. 2020). This also impairs the use of the CRISPR/Cas9 system, in which off-target effects can arise from the sgRNA binding to undesired places (Fu et al. 2013; Sander and Joung 2014; Schaefer et al. 2017). Studies such as the one conduced for RNAi together with the improvement of bioinformatic analyses provides a guidance for future RNAi and CRISPR risk analysis.

Regarding the regulation, RNAi-based transformed plants will undergo GMO regulatory procedures to enable authorization (Arpaia et al. 2020; Papadopoulou et al. 2020). This process will demand time and a large amount of resources to get approvals for commercialization. On the other hand, RNAi-based pesticides will be differently regulated when compared to transgenic plants. However, this will also be an expensive process that will demand time and resources, but will have a better acceptance from society. In the US, the RNAi-based products will undergo EPA regulation under the Biochemical Pesticides mode of action, while in the European Union, there is no specific class for registering this type of product yet (Mendelsohn, et al. 2020). In Brazil, RNAi products will undergo MAPA registration process, but also, there is no guideline for RNAi-based products yet. But it is expected that soon, we will be able to see these kind of products in the market (Das and Sherif 2020). For CRISPR-edited organisms, we have the case of the CRISPR-edited mushroom, which in 2016 escaped the US

regulation, falling outside GMO legislation because it did not contain foreign DNA (Kim and Kim 2016). This indicates that the CRISPR-edited crops are not going under the same strict regulations as traditional GM crops, depending on the DNA edition done (Wang et al. 2019). However, we need to keep in mind that this will not apply in the case of gene-drives, in which an edited population of insects can alter a whole ecosystem (Taning et al. 2017).

To achieve effective and durable solutions for pest management in the field, researchers, growers, pesticide and seed industries, as well as the government play an important role in the process (Anderson et al. 2019). Only with the cooperation among the fields, it will be possible to find new solutions, implement them and highlight the importance of these approaches in a sustainable soybean production system (Lamichhane et al. 2016; Anderson et al. 2019).

6.5. Conclusion

The development of new control tools for the sustainable management of *E. heros* in an attempt to solve problems related to the overuse of pesticides is necessary. In the front line of this, research needs to provide information about the biology, development, and behavior of insects. The genetic information provided by this PhD and overall results including the validation of the RNAi machinery and the use of RNAi and CRISPR/Cas9 approaches as functional genomic tools. This suggests the potential of these tools in functional genomic studies as well as in the future development of new tools for the management of *E. heros* and other stink bugs in the field.

Referências

ADEYINKA, O. S.; RIAZ, S.; TOUFIQ, N.; et al. Advances in exogenous RNA delivery techniques for RNAi-mediated pest control. **Molecular Biology Reports**, v. 47, n. 8, p. 6309–6319, 2020.

AGRAWAL, N.; DASARADHI, P. V. N.; MOHAMMED, A.; et al. RNA Interference: biology, mechanism, and applications. **Microbiology and molecular biology reviews**, v. 67, n. 4, p. 657–685, 2003.

ALBRIGHT III, V. C.; WONG, C. R.; HELLMICH, R. L.; COATS, J. R. Dissipation of double-stranded RNA in aquatic microcosms. **Environmental Toxicology and Chemistry**, v. 36, n. 5, p. 1249–1253, 2017.

ALPHEY, L. Can CRISPR-Cas9 gene drives curb malaria? **Nature Biotechnology**, v. 34, n. 2, p. 149–150, 2016. Nature Publishing Group.

ANDERSON, J. A.; ELLSWORTH, P. C.; FARIA, J. C.; et al. Genetically engineered crops: Importance of diversified integrated pest management for agricultural sustainability. **Frontiers in Bioengineering and Biotechnology**, v. 7, p. 1–14, 2019.

DE ANDRADE, E. C.; HUNTER, W. B. RNA Interference – natural gene-based technology for highly specific pest control (HiSPeC). **RNA Interference**. p.391–409, 2016.

ANGELINI, D. R.; LIU, P. Z.; HUGHES, C. L.; KAUFMAN, T. C. Hox gene function and interaction in the milkweed bug *Oncopeltus fasciatus* (Hemiptera). **Developmental Biology**, v. 287, n. 2, p. 440–455, 2005.

ARIMATSU, Y.; KOTANI, E.; SUGIMURA, Y.; FURUSAWA, T. Molecular characterization of a cDNA encoding extracellular dsRNase and its expression in the silkworm, *Bombyx mori*. **Insect Biochemistry and Molecular Biology**, v. 37, n. 2, p. 176–183, 2007.

ARONSTEIN, K.; PANKIW, T.; SALDIVAR, E. SID-I is implicated in systemic gene silencing in the honey bee. **Journal of Apicultural Research**, v. 45, n. 1, p. 20–24, 2006.

ARPAIA, S.; CHRISTIAENS, O.; GIDDINGS, K.; et al. Biosafety of GM crop plants expressing dsrna: data requirements and EU regulatory considerations. **Frontiers in Plant Science**, v. 11, p. 1–13, 2020.

BACHMAN, P.; FISCHER, J.; SONG, Z.; URBANCZYK-WOCHNIAK, E.; WATSON, G. Environmental fate and dissipation of applied dsRNA in soil, aquatic systems, and plants. **Frontiers in Plant Science**, v. 11, p. 1–6, 2020.

BACHMAN, P. M.; HUIZINGA, K. M.; JENSEN, P. D.; et al. Ecological risk assessment for *DvSnf7* RNA: A plant-incorporated protectant with targeted activity against western corn rootworm. **Regulatory Toxicology and Pharmacology**, v. 81, p. 77–88, 2016. Elsevier Ltd.

BARZMAN, M.; BÀRBERI, P.; BIRCH, A. N. E.; et al. Eight principles of integrated pest management. **Agronomy for Sustainable Development**, v. 35, n. 4, p. 1199–1215, 2015.

BAUM, J. A.; BOGAERT, T.; CLINTON, W.; et al. Control of coleopteran insect pests through RNA interference. **Nature Biotechnology**, v. 25, n. 11, p. 1322–1326, 2007.

BAUM, J. A.; ROBERTS, J. K. **Progress Towards RNAi-Mediated Insect Pest Management**. In: Progress Towards RNAi-Mediated Insect Pest Management. Academic Press, p. 249–295, 2014.

BAXEVANIS, A. D. The importance of biological databases in biological discovery. **Current Protocols in Bioinformatics**, n. 27, p.1-8, 2009.

BELLÉS, X. Beyond *Drosophila*: RNAi in vivo and functional genomics in insects. **Annual Review of Entomology**, v. 55, p. 111–128, 2010.

BERNSTEIN, E.; CAUDY, A. A.; HAMMOND, S. M.; HANNON, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. **Nature**, v. 409, p. 363–366, 2001.

BEUMER, K. J.; TRAUTMAN, J. K.; MUKHERJEE, K.; CARROLL, D. Donor DNA utilization during gene targeting with zinc-finger nucleases. **G3: Genes, Genomes, Genetics**, v. 3, n. 4, p. 657–664, 2013.

BIRMINGHAM, A.; ANDERSON, E. M.; REYNOLDS, A.; et al. 3’ UTR seed matches, but not overall identity, are associated with RNAi off-targets. **Nature methods**, v. 3, n. 3, p. 199–204, 2006.

BORGES, M.; LAUMANN, R. A.; DA SILVA, C. C. A; et al. Metodologias de criação e manejo de colônias de percevejos da soja (Hemíptera - Pentatomidae) para estudos de comportamento e ecologia química. **Embrapa Recursos Genéticos e Biotecnologia,** v. Documentos, p. 18, 2006.

BORTOLOTTO, O. C.; BUENO, A. DE F.; STOPA, Y. K.; SILVA, G. V.; QUEIROZ, A. P. Development of dichelops melacanthus and its egg parasitoid *Telenomus podisi* reared on *Bt*-soybean MON 87701 x MON 89788 and its near conventional isoline under different temperatures. **Anais da Academia Brasileira de Ciencias**, v. 88, n. 2, p. 1023–1034, 2016.

BRADDICK, D.; RAMAROHETRA, R. F. **Emergent challenges for CRISPR:**

biosafety, biosecurity, patenting, and regulatory issues. In: Genome Engineering via CRISPR-Cas9 System. p. 281-307, 2020.

BRUNET, E.; SIMSEK, D.; TOMISHIMA, M.; et al. Chromosomal translocations induced at specified loci in human stem cells. **Proceedings of the National Academy of Sciences**, v. 106, n. 26, p. 10620–10625, 2009.

BUENO, A. D. F.; BORTOLOTTO, O. C.; CRISTINA, R.; et al. Efeitos do aquecimento global sobre pragas de oleaginosas. In: **Aquecimento Global e Problemas Fitossanitários**. p.280–347, 2017.

BUENO, A. DE F.; CEOLIN BORTOLOTTO, O.; POMARI-FERNANDES, A.; FRANÇA-NETO, J. DE B. Assessment of a more conservative stink bug economic threshold for managing stink bugs in Brazilian soybean production. **Crop Protection**, v. 71, p. 132–137, 2015.

BURT, A.; KOUFOPANOU, V. Homing endonuclease genes: The rise and fall and rise again of a selfish element. **Current Opinion in Genetics and Development**, v. 14, n. 6, p. 609–615, 2004.

CAGLIARI, D.; SANTOS, E. A. DOS; DIAS, N.; SMAGGHE, G.; ZOTTI, M. **Nontransformative strategies for RNAi in Crop Protection**. In: Modulating Gene Expression - Abridging the RNAi and CRISPR-Cas9 Technologies. v. 2, p.1–18, 2018.

CAPPELLE, K.; DE OLIVEIRA, C. F. R.; VAN EYNDE, B.; CHRISTIAENS, O.; SMAGGHE, G. The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. **Insect Molecular Biology**, v. 25, n. 3, p. 315–323, 2016.

CARTHEW, R. W. S. J. E. Origins and mechanisms of miRNAs and siRNAs. **National Institutes of Health**, v. 136, n. 4, p. 642–655, 2009.

CASACUBERTA, J. M.; DEVOS, Y.; DU JARDIN, P.; et al. Biotechnological uses of RNAi in plants: Risk assessment considerations. **Trends in Biotechnology**, v. 33, n. 3, p. 145–147, 2015.

CASTELLANOS, N. L.; SMAGGHE, G.; SHARMA, R.; OLIVEIRA, E. E.; CHRISTIAENS, O. Liposome encapsulation and EDTA formulation of dsRNA targeting essential genes increase oral RNAi-caused mortality in the Neotropical stink bug *Euschistus heros*. **Pest Management Science**, v. 75, n. 2, p. 537–548, 2019.

CHAMPER, J.; BUCHMAN, A.; AKBARI, O. S. Cheating evolution: Engineering gene drives to manipulate the fate of wild populations. **Nature Reviews Genetics**, v. 17, n. 3, p. 146–159, 2016.

CHAPMAN, E. J.; CARRINGTON, J. C. Specialization and evolution of endogenous small RNA pathways. **Nature Reviews Genetics**, v. 8, n. 11, p. 884–896, 2007.

CHEN, J.; ZHANG, D.; YAO, Q.; et al. Feeding-based RNA interference of a trehalose phosphate synthase gene in the brown planthopper, *Nilaparvata lugens*. **Insect Molecular Biology**, v. 19, n. 6, p. 777–786, 2010.

CHRISTIAENS, O.; DZHAMBAZOVA, T.; KOSTOV, K.; et al. Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi-

based GM plants. **EFSA Supporting Publications**, v. 15, n. 5, 2018.

CHRISTIAENS, O.; SMAGGHE, G. The challenge of RNAi-mediated control of hemipterans. **Current Opinion in Insect Science**, v. 6, p. 15–21, 2014.

CHRISTIAENS, O.; SWEVERS, L.; SMAGGHE, G. DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. **Peptides**, v. 53, p. 307–314, 2014.

COLEMAN, A. D.; WOUTERS, R. H. M.; MUGFORD, S. T.; HOGENHOUT, S. A. Persistence and transgenerational effect of plant-mediated RNAi in aphids. **Journal of Experimental Botany**, v. 66, n. 2, p. 541–548, 2015.

CONAB. Compêndio de Estudos Conab/Companhia Nacional de Abastecimento. **Conab**, v. 10, p. 35, 2017.

CONAB. Acompanhamento da safra brasileira 2019/2020. **Acompanhamento da Safra Brasileira de Grãos 2019/2020**, v. 8, p. 1–29, 2020.

CORRÊA-FERREIRA, B. S.; ALEXANDRE, T. M.; PELLIZZARO, E. C.; MOSCARDI, F.; BUEN, A. DE F. Práticas de manejo de pragas utilizadas na soja e seu impacto sobre a cultura. **Circular técnica/Embrapa CNPSo,** p. 16, 2010.

CORRÊA-FERREIRA, B. S.; PANIZZI, A. R. Percevejos da Soja e seu Manejo. **Circular técnica/Embrapa CNPSo, ISSN 0100-6703 n.24**, p. 45, 1999.

COSTA, M. L. M. M.; BORGES, M.; VILELA, E. F. Biologia Reprodutiva de *Euschistus heros* (F .) (Heteroptera : Pentatomidae). **Anais da Sociedade Entomológica do Brasil**, v. 27, n. 4, p. 559–568, 1998.

COURTIER-ORGOGOZO, V.; MORIZOT, B.; BOËTE, C. Agricultural pest control with CRISPR -based gene drive: time for public debate . **EMBO reports**, v. 18, n. 6, p. 878–880, 2017.

DALAKOURAS, A.; JARAUSCH, W.; BUCHHOLZ, G.; et al. Delivery of hairpin RNAs and small RNAs into woody and herbaceous plants by trunk injection and petiole absorption. **Frontiers in Plant Science**, v. 9, p. 1–11, 2018.

DALAKOURAS, A.; WASSENEGGER, M.; DADAMI, E.; et al. Genetically modified organism-free RNA interference: exogenous application of RNA molecules in plants1[open]. **Plant Physiology**, v. 182, n. 1, p. 38–50, 2020.

DALAZEN, G.; CURIOLETTI, L. E.; CAGLIARI, D.; STACKE, R. F.; GUEDES, J. V. C. Hairy fleabane as a source of major insect pests of soybean. **Planta Daninha**, v. 34, n. 3, 2016.

DARA, S. K. The new Integrated Pest Management paradigm for the modern age. **Journal of Integrated Pest Management**, v. 10, n. 1, 2019.

DAS, P. R.; SHERIF, S. M. Application of exogenous dsRNAs-induced RNAi in agriculture: challenges and triumphs. **Frontiers in Plant Science**, v. 11, n. June, 2020.

DENECKE, S.; SWEVERS, L.; DOURIS, V.; VONTAS, J. How do oral insecticidal compounds cross the insect midgut epithelium? **Insect Biochemistry and Molecular**

Biology, v. 103, p. 22–35, 2018.

DIAS, N.; CAGLIARI, D.; KREMER, F. S.; et al. The South american fruit fly: an important pest insect with RNAi-sensitive larval stages. **Frontiers in Physiology**, v. 10, n. June, 2019.

DIEDERICH, R. J.; MERRILL, V. K.; PULTZ, M. A.; KAUFMAN, T. C. Isolation, structure, and expression of labial, a homeotic gene of the Antennapedia Complex involved in *Drosophila* head development. **Genes & development**, v. 3, n. 3, p. 399–414, 1989.

DILLIN, A. The specifics of small interfering RNA specificity. **Proceedings of the National Academy of Sciences**, v. 100, n. 11, p. 6289–6291, 2003.

DOUDNA, J. A.; CHARPENTIER, E. The new frontier of genome engineering with CRISPR-Cas9. **Science**, v. 346, n. 6213, 2014.

DRURY, D. W.; DAPPER, A. L.; SINIARD, D. J.; ZENTNER, G. E.; WADE, M. J. CRISPR/Cas9 gene drives in genetically variable and nonrandomly mating wild populations. **Science Advances**, v. 3, n. 5, p. 1–8, 2017.

DUBELMAN, S.; FISCHER, J.; ZAPATA, F.; et al. Environmental fate of double-stranded RNA in agricultural soils. **PLoS ONE**, v. 9, n. 3, p. e93155, 2014.

EL-SHESHENY, I.; HAJERI, S.; EL-HAWARY, I.; GOWDA, S.; KILLINY, N. Silencing *abnormal wing disc* gene of the asian citrus psyllid, *Diaphorina citri* disrupts adult wing development and increases nymph mortality. **PLoS ONE**, v. 8, n. 5, 2013.

ESVELT, K. M.; SMIDLER, A. L.; CATTERUCCIA, F.; CHURCH, G. M. Concerning RNA-guided gene drives for the alteration of wild populations. **eLife**, v. 3, n. July2014, p. 1–21, 2014.

FEINBERG, E. H.; HUNTER, C. P. Transport of dsRNA into cells by the transmembrane protein SID-1. **Science**, v. 301, n. 5639, p. 1545–1547, 2003.

FERREIRA, R.; GATTO, F.; NIELSEN, J. Exploiting off-targeting in guide-RNAs for CRISPR systems for simultaneous editing of multiple genes. **FEBS Letters**, v. 591, n. 20, p. 3288–3295, 2017.

FIRMINO, A. A. P.; DE FONSECA, F. C. A.; DE MACEDO, L. L. P.; et al. Transcriptome analysis in cotton boll weevil (*Anthonomus grandis*) and RNA interference in insect pests. **PLoS ONE**, v. 8, n. 12, p. 1–15, 2013.

FISCHER, J. R.; ZAPATA, F.; DUBELMAN, S.; et al. Characterizing a novel and sensitive method to measure dsRNA in soil. **Chemosphere**, v. 161, p. 319–324, 2016.

FISCHER, J. R.; ZAPATA, F.; DUBELMAN, S.; et al. Aquatic fate of a double-stranded RNA in a sediment water system following an over-water application. **Environmental Toxicology and Chemistry**, v. 36, n. 3, p. 727–734, 2017.

FISHILEVICH, E.; V??LEZ, A. M.; KHAJURIA, C.; et al. 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*). **Insect Biochemistry and Molecular Biology**, v. 71, p. 58–71, 2016.

FLEIG, R.; WALLDORF, U.; GEHRING, W. J.; SANDER, K. Development of the Deformed protein pattern in the embryo of the honeybee *Apis mellifera* L. (Hymenoptera). **Archives of Developmental Biology**, v. 201, n. 4, p. 235–242, 1992.

FLETCHER, S. J.; REEVES, P. T.; HOANG, B. T.; MITTER, N. A perspective on RNAi-based biopesticides. **Frontiers in Plant Science**, v. 11, p. 1–10, 2020.

FU, Y.; FODEN, J. A.; KHAYTER, C.; et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. **Nature Biotechnology**, v. 31, n. 9, p. 822–826, 2013.

GALDEANO, D. M.; BRETON, M. C.; LOPES, J. R. S.; FALK, B. W.; MACHADO, M. A. Oral delivery of double-stranded RNAs induces mortality in nymphs and adults of the Asian citrus psyllid, *Diaphorina citri*. **Plos One**, v. 12, n. 3, p. e0171847, 2017.

GANTZ, V. M.; BIER, E. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. **Science**, v. 348, n. 6233, p. 442–444, 2015.

GANTZ, V. M.; JASINSKIENE, N.; TATARENKOVA, O.; et al. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. **Proceedings of the National Academy of Sciences**, v. 112, n. 49, p. e6736–e6743, 2015.

GARBUTT, J. S.; BELLÉS, X.; RICHARDS, E. H.; REYNOLDS, S. E. Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: Evidence from *Manduca sexta* and *Blattella germanica*. **Journal of Insect Physiology**, v. 59, n. 2, p. 171–178, 2013.

GARBUTT, J. S.; REYNOLDS, S. E. Induction of RNA interference genes by double-stranded RNA; implications for susceptibility to RNA interference. **Insect Biochemistry and Molecular Biology**, v. 42, p. 621–628, 2012.

GASIUNAS, G.; BARRANGOU, R.; HORVATH, P.; SIKSNYS, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. **Proceedings of the National Academy of Sciences of the United States of America**, v. 109, n. 39, p. 2579–2586, 2012.

GHOSH, S. K. B.; HUNTER, W. B.; PARK, A. L.; GUNDERSEN-RINDAL, D. E. Double strand RNA delivery system for plant-sap-feeding insects. **Plos One**, v. 12, n. 2, p. e0171861, 2017.

GOGOI, A.; SARMAH, N.; KALDIS, A.; PERDIKIS, D.; VOLOUDAKIS, A. Plant insects and mites uptake double-stranded RNA upon its exogenous application on tomato leaves. **Planta**, v. 246, n. 6, p. 1233–1241, 2017.

GONG, L.; CHEN, Y.; HU, Z.; HU, M. Testing insecticidal activity of novel chemically synthesized siRNA against *Plutella xylostella* under laboratory and field conditions. **PLoS ONE**, v. 8, n. 5, p. 1–8, 2013.

GUAN, R.-B.; LI, H.-C.; FAN, Y.-J.; et al. A nuclease specific to lepidopteran insects suppresses RNAi. **Journal of Biological Chemistry**, v. 293, n. 16, p. 6011–6021, 2018.

GUEDES, R. N. C. Insecticide resistance, control failure likelihood and the First Law

of Geography. **Pest Management Science**, v. 73, n. 3, p. 479–484, 2017.

GUEDES, R. N. C.; CUTLER, G. C. Insecticide-induced hormesis and arthropod pest management. **Pest Management Science**, v. 70, n. 5, p. 690–697, 2014.

GUI, S.; TANING, C. N. T.; WEI, D.; SMAGGHE, G. First report on CRISPR/Cas9-targeted mutagenesis in the Colorado potato beetle, *Leptinotarsa decemlineata*. **Journal of Insect Physiology**, v. 121, p. 104013, 2020.

GULLAN, P. J.; CRANSTON, P. S. Manejo de Pragas. In: **Insetos - Fundamentos da Entomologia**. Rio de Janeiro: Roca, p. 313-340, 2019.

HAMMOND, A.; GALIZI, R.; KYROU, K.; et al. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. **Nature Biotechnology**, v. 34, n. 1, p. 78–83, 2016.

HEAD, G. P.; CARROLL, M.; EVANS, S.; et al. Evaluation of SmartStax and SmartStax PRO Maize against Western Corn Rootworm and Northern Corn Rootworm: Efficacy and Resistance Management Evaluation. **Pest Management Science**, v. 73, n. 9, p. 1883–1899, 2017.

HELER, R.; SAMAI, P.; MODELL, J. W.; et al. Cas9 specifies functional viral targets during CRISPR-Cas adaptation. **Nature**, v. 519, n. 7542, p. 199–202, 2015.

HOFFMANN-CAMPO, C. B.; CORRÊA-FERREIRA, B. S.; OLIVEIRA, L. J.; et al. Pragas da soja no brasil e seu manejo integrado. **Circular técnica/Embrapa soja, ISSN 1516-7860; n.30**, p. 70, 2000.

HONG, S. W.; JIANG, Y.; KIM, S.; LI, C. J.; LEE, D. Target gene abundance contributes to the efficiency of siRNA-mediated gene silencing. **Nucleic Acid Therapeutics**, v. 24, n. 3, p. 192–198, 2014.

HRYCAJ, S.; CHESEBRO, J.; POPADIC, A. Functional analysis of *Scr* during embryonic and post-embryonic development in the cockroach, *Periplaneta americana*. **Developmental Biology**, v. 341, n. 1, p. 324–334, 2010.

HUGHES, C. L.; KAUFMAN, T. C. RNAi analysis of deformed, proboscipedia and sex combs reduced in the milkweed bug *Oncopeltus fasciatus:* Novel roles for Hox genes in the Hemipteran head. **Development**, v. 127, n. 17, p. 3683–3694, 2000.

HUNTER, W B; GLICK, E.; PALDI, N.; BEXTINE, B. R. Advances in RNA interference: dsRNA treatment in trees and grapevines for insect pest suppression. **Southwestern Entomologist**, v. 37, n. 1, p. 85–87, 2012.

HUNTER, W. B.; GLICK, E.; PALDI, N.; BEXTINE, B. R. Advances in RNA interference: dsRNA treatment in trees and grapevines for insect pest suppression. **Southwestern Entomologist**, v. 37, n. 1, p. 85–87, 2012.

HUVENNE, H.; SMAGGHE, G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. **Journal of Insect Physiology**, v. 56, n. 3, p. 227–235, 2010.

JACKSON, A. L.; LINSLEY, P. S. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. **Nature Reviews Drug Discovery**,

v. 9, n. 1, p. 57–67, 2010.

JAIN, R. G.; ROBINSON, K. E.; ASGARI, S.; MITTER, N. Current scenario of RNAi - based hemipteran control. **Pest Management Science**, 2020.

JAIN, R. G.; ROBINSON, K. E.; FLETCHER, S. J.; MITTER, N. RNAi-based functional genomics in hemiptera. **Insects**, v. 11, n. 9, p. 1–22, 2020.

JIANG, D. F.; LIU, Y. Q.; LI, X. S.; SHI, S. L. Characterization of the antheraea pernyi abnormal wing disc gene that may contribute to its temperature tolerance. **African Journal of Biotechnology**, v. 9, n. 43, p. 7372–7378, 2010.

JINEK, M.; CHYLINSKI, K.; FONFARA, I.; et al. A programmable dual-RNA - guided DNA endonuclease in adaptive bacterial immunity. **Science,** v. 337, p. 816–822, 2012.

JOAQUIM, M. E. S.; BELCHIOR, G. G.; JOSÉ, M. O. DE M. A.; et al. Dissipation of DvSnf7 double-stranded RNA in Brazilian soils. **Agricultural & Environmental Letters**, v. 4, n. 1, p. 1–4, 2019.

JOGA, M. R.; ZOTTI, M. J.; SMAGGHE, G.; CHRISTIAENS, O. RNAi Efficiency, systemic properties, and novel delivery methods for pest insect control: What we know so far. **Frontiers in Physiology**, v. 7, n. 553, p. 1–14, 2016.

JOSE, A. M.; HUNTER, C. P. Transport of Sequence-Specific RNA Interference Information Between Cells. **Annual Review of Genetics**, v. 41, n. 1, p. 305–330, 2007.

KARLIKOW, M.; GOIC, B.; MONGELLI, V.; et al. *Drosophila* cells use nanotube-like structures to transfer dsRNA and RNAi machinery between cells. **Scientific Reports**, v. 6, p. 1–9, 2016.

KENNEDY, G. G. **Integration of Insect-Resistant Genetically Modified Crops within IPM Programs**. In: Integration of Insect-Resistant Genetically Modified Crops within IPM Programs, p. 1–26, 2008.

KETTING, R. F. The Many Faces of RNAi. **Developmental Cell**, v. 15, n. 2, p. 148-161, 2011.

KHAJURIA, C.; IVASHUTA, S.; WIGGINS, E.; et al. Development and characterization of the first dsRNA-resistant insect population from western corn rootworm, *Diabrotica virgifera virgifera* LeConte. **PLoS ONE**, v. 13, n. 5, p. 1–19, 2018.

KHAN, A. A.; BETEL, D.; MILLER, M. L.; et al. Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs. **Nature Biotechnology**, v. 27, n. 6, p. 5, 2009.

KIM, J.; KIM, J. S. Bypassing GMO regulations with CRISPR gene editing. **Nature Biotechnology**, v. 34, n. 10, p. 1014–1015, 2016.

KOBAYASHI, I.; TSUKIOKA, H.; KÔMOTO, N.; et al. SID-1 protein of *Caenorhabditis elegans* mediates uptake of dsRNA into *Bombyx cells*. **Insect Biochemistry and Molecular Biology**, v. 42, n. 2, p. 148–154, 2012.

KOCH, A.; BIEDENKOPF, D.; FURCH, A.; et al. An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage

and is controlled by the fungal silencing machinery. **PLOS Pathogens**, v. 12, n. 10, p. e1005901, 2016.

KOONIN, E. V; MAKAROVA, K. S. CRISPR-Cas: an adaptive immunity system in prokaryotes. **Biology Reports**, v. 1, p. 1–6, 2009.

KOONIN, E. V; MAKAROVA, K. S. CRISPR-Cas: Evolution of an RNA-based adaptive immunity system in prokaryotes. **RNA Biology**, v. 10, n. 5, p. 679–686, 2013.

KUSS-ROGGIA, R. C. R. **Distribuição espacial e temporal de percevejos da soja e comportamento de *Piezodorus guildinii* (Westwood, 1837) (Hemiptera: Pentatomidae) na soja (Glycine max (L.) Merrill) ao longo do dia.** Tese (Tese de doutorado em Agronomia) - Universidade Federal de Santa Maria, 128p., 2009.

LAMICHHANE, J. R.; AUBERTOT, J. N.; BEGG, G.; et al. Networking of integrated pest management: A powerful approach to address common challenges in agriculture. **Crop Protection**, v. 89, p. 139–151, 2016.

LI, H.; GUAN, R.; GUO, H.; MIAO, X. New insights into an RNAi approach for plant defence against piercing-sucking and stem-borer insect pests. **Plant Cell and Environment**, v. 38, n. 11, p. 2277–2285, 2015.

LI, J.; WANG, X. P.; WANG, M. Q.; MA, W. H.; HUA, H. X. Advances in the use of the RNA interference technique in Hemiptera. **Insect Science**, v. 20, n. 1, p. 31–39, 2013.

LING, L.; GE, X.; LI, Z.; et al. MiR-2 family targets awd and fng to regulate wing morphogenesis in *Bombyx mori*. **RNA Biology**, v. 12, n. 7, p. 742–748, 2015.

LINK, D.; GRAZIA, J. Pentatomídeos da região central do Rio Grande do Sul (Heteroptera). , v. 16, n. 1, p. 115–129, 1987.

LIU, J.; SWEVERS, L.; IATROU, K.; HUVENNE, H.; SMAGGHE, G. *Bombyx mori* DNA/RNA non-specific nuclease: Expression of isoforms in insect culture cells, subcellular localization and functional assays. **Journal of Insect Physiology**, v. 58, n. 8, p. 1166–1176, 2012.

LIU, Q.; RAND, T. A.; KALIDAS, S.; et al. R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. **Science**, v. 301, n. 5641, p. 1921–1925, 2003.

LOMATE, P. R.; BONNING, B. C. Distinct properties of proteases and nucleases in the gut, salivary gland and saliva of southern green stink bug, *Nezara viridula*. **Scientific Reports**, v. 6, p. 27587, 2016.

LU, Y.; CHEN, M.; REDING, K.; PICK, L. Establishment of molecular genetic approaches to study gene expression and function in an invasive hemipteran, *Halyomorpha halys*. **EvoDevo**, v. 8, n. 1, p. 1–11, 2017. BioMed Central.

LUNDGREN, J. G.; DUAN, J. J. RNAi-based insecticidal crops: Potential effects on nontarget species. **BioScience**, v. 63, n. 8, p. 657–665, 2013.

LUNDGREN, J. G., JURAT-FUENTES, J. L. **The physiology and ecology of host defense against microbial invaders.** In: Insect Pathology. p. 460-480, 2012.

LUO, Y.; WANG, X.; WANG, X.; et al. Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding. **Insect Molecular Biology**, v. 22, n. 5, p. 574–583, 2013.

MA, S.; ZHANG, S.; WANG, F.; et al. Highly efficient and specific genome editing in silkworm using custom TALENs. **PLoS ONE**, v. 7, n. 9, p. 1–7, 2012.

MACFADYEN, S.; HARDIE, D. C.; FAGAN, L.; et al. Reducing insecticide use in broad-acre grains production: An Australian study. **PLoS ONE**, v. 9, n. 2, 2014.

MARTIN JINEK; KRZYSZTOF CHYLINSKI; INES FONFARA; et al. A Programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. **Science**, v. 337, n. 6096, p. 816–821, 2012.

MATRANGA, C.; TOMARI, Y.; SHIN, C.; BARTEL, D. P.; ZAMORE, P. D. Passenger-strand cleavage facilitates assembly of siRNA into AGO2-containing RNAi enzyme complexes. **Cell**, v. 123, n. 4, p. 607–620, 2005.

MCLOUGHLIN, A. G.; WYTINCK, N.; WALKER, P. L.; et al. Identification and application of exogenous dsRNA confers plant protection against *Sclerotinia sclerotiorum* and *Botrytis cinerea*. **Scientific Reports**, v. 8, n. 1, p. 1–14, 2018.

MEDEIROS, L.; AMEGIER, G. A. Ocorrência e desempenho de *Euschistus heros* (F .) (Heteroptera : Pentatomidae) em plantas hospedeiras alternativas no Rio Grande do Sul. **Neotropical Entomology**, v. 38, n. 4, p. 459–463, 2009.

MEISTER, G.; TUSCHL, T. Mechanisms of gene silencing by double-stranded RNA. **Nature**, v. 431, n. September, p. 343–349, 2004.

MELLO, C. C.; CONTE, J. D. Revealing the world of RNA interference. **Nature**, v. 431, p. 338–342, 2004.

MENDELSOHN, M. L. et al. Summary of discussions from the 2019 OECD Conference on RNAi based pesticides. **Frontiers in Plant Science**, n. 11, p. 740, 2020.

MERRILL, V. K. L.; DIEDERICH, R. J.; TURNER, F. R.; KAUFMAN, T. C. A genetic and developmental analysis of mutations in labial, a gene necessary for proper head formation in *Drosophila melanogaster*. **Developmental Biology**, v. 135, n. 2, p. 376–391, 1989.

MEZZETTI, B.; SMAGGHE, G.; ARPAIA, S.; et al. RNAi: What is its position in agriculture? **Journal of Pest Science**, v. 93, n. 4, p. 1125–1130, 2020.

MITTER, N.; WORRALL, E. A.; ROBINSON, K. E.; et al. Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. **Nature Plants**, v. 3, n. 2, p. 16207, 2017.

MIYOSHI, K.; MIYOSHI, K.; TSUKUMO, H.; et al. Slicer function of *Drosophila* Argonautes and its involvement in RISC formation. **Genes & Development**, v. 2, p. 2837–2848, 2005.

MOHAMMED, A. M. A. RNAi-based silencing of genes encoding the vacuolar-ATPase subunits A and C in pink bollworm (*Pectinophora gossypiella*). **African Journal of Biotechnology**, v. 15, n. 45, p. 2547–2557, 2016.

MONGELLI, V.; SALEH, M. Bugs Are Not to Be Silenced : Small RNA Pathways and Antiviral Responses in Insects. **Annual Review of Virology**, n. 3, v. 1, p. 573-589, 2016.

OLIVEIRA, C. M.; AUAD, A. M.; MENDES, S. M.; FRIZZAS, M. R. Economic impact of exotic insect pests in Brazilian agriculture. **Journal of Applied Entomology**, v. 137, n. 1–2, p. 1–15, 2013.

PACHECO, D. J. P.; CORRÊA-FERREIRA, B. S. Parasitismo de Telenomus podisi Ashmead (Hymenoptera: Scelionidae) em populações de percevejos pragas da soja. **Anais da Sociedade Entomológica do Brasil**, v. 29, n. 2, p. 295–302, 2000.

PANIZZI, A. R. FORUM Suboptimal nutrition and feeding behavior of hemipterans on less preferred plant food sources. **Anais da Sociedade Entomológica do Brasil**, v. 29, n. 29l, p. 1–12, 2000.

PANIZZI, A. R. History and Contemporary Perspectives of the Integrated Pest Management of Soybean in Brazil. **Neotropical Entomology**, v. 42, n. 2, p. 119–127, 2013.

PANIZZI, A. R. Stink bugs growing problems with (Hemiptera: Heteroptera: Pentatomidae): species invasive to the U.S. and potential. **American Entomologist**, v. 61, p. 223–233, 2015.

PANIZZI, A. R.; BUENO, A. F.; SILVA, F. A. C. Insetos que atacam vagens e grãos. **Soja: manejo integrado de insetos e outros artrópodes-praga**. p.335–420, 2012.

PANIZZI, A. R.; CORRÊA, B. S.; GAZZONI, D. L.; et al. Insetos da Soja no Brasil. **Boletim Técnico**, , n. 1, p. 20, 1977.

PANIZZI, A. R.; MCPHERSON, J. E.; JAMES, D. G.; HAVAHERY, M.; MCPHERSON, R. M. Stink Bugs (Pentatomidae). In: C. W. . SCHAEFER; A. R. PANIZZI (Orgs.); **Heteroptera of economic importance**. p.421–473, 2000. Boca Raton: CRC.

PAPADOPOULOU, N.; DEVOS, Y.; ÁLVAREZ-ALFAGEME, F.; LANZONI, A. Risk assessment considerations for genetically modified RNAi plants : EFSA ' s Activities and Perspective. , v. 11, n. April, p. 1–8, 2020.

PARKER, K. M.; BARRAGÁN BORRERO, V.; VAN LEEUWEN, D. M.; et al. Environmental Fate of RNA Interference Pesticides: Adsorption and Degradation of Double-Stranded RNA Molecules in Agricultural Soils. **Environmental Science and Technology**, v. 53, n. 6, p. 3027–3036, 2019.

PERERA, O. P.; SHELBY, K. S.; POPHAM, H. J. R.; et al. Generation of a transcriptome in a model lepidopteran pest, *Heliothis virescens*, using multiple sequencing strategies for profiling midgut gene expression. **PLoS ONE**, v. 10, n. 6, p. 1–28, 2015.

PETRICK, J. S.; BROWER-TOLAND, B.; JACKSON, A. L.; KIER, L. D. Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: A scientific review. **Regulatory Toxicology and Pharmacology**, v. 66, n. 2, p. 167–176, 2013.

PETRICK, J. S.; FRIERDICH, G. E.; CARLETON, S. M.; et al. Corn rootworm-active RNA DvSnf7: Repeat dose oral toxicology assessment in support of human and mammalian safety. **Regulatory Toxicology and Pharmacology**, v. 81, p. 57–68, 2016.

PINHEIRO, D. H.; VÉLEZ, A. M.; FISHILEVICH, E.; et al. Clathrin-dependent endocytosis is associated with RNAi response in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. **Plos One**, v. 13, n. 8, p. e0201849, 2018.

PREALL, J. B.; SONTHEIMER, J. E. RNAi: RISC Gets Loaded. **Cell**, v. 123, n. 4, p. 543–545, 2005.

PRENTICE, K.; CHRISTIAENS, O.; PERTRY, I.; et al. RNAi-based gene silencing through dsRNA injection or ingestion against the African sweet potato weevil *Cylas puncticollis* (Coleoptera: Brentidae). **Pest Management Science**, v. 73, n. 1, p. 44–52, 2016.

PRENTICE, K.; PERTRY, I.; CHRISTIAENS, O.; et al. Transcriptome analysis and systemic RNAi response in the African sweetpotato weevil (*Cylas puncticollis*, Coleoptera, Brentidae). **PLoS ONE**, v. 10, n. 1, p. 1–18, 2015.

QUEIROZ, A. P.; TAGUTI, E. A.; BUENO, A. F.; GRANDE, M. L. M.; COSTA, C. O. Host Preferences of *Telenomus podisi* (Hymenoptera: Scelionidae): Parasitism on Eggs of Dichelops melacanthus, *Euschistus heros*, and Podisus nigrispinus (Hemiptera: Pentatomidae). **Neotropical Entomology**, v. 47, n. 4, p. 543–552, 2018.

RAMON, M.; DEVOS, Y.; LANZONI, A.; et al. RNAi-based GM plants: Food for thought for risk assessors. **Plant Biotechnology Journal**, v. 12, n. 9, p. 1271–1273, 2014.

RIGA, M.; DENECKE, S.; LIVADARAS, I.; et al. Development of efficient RNAi in *Nezara viridula* for use in insecticide target discovery. **Archives of Insect Biochemistry and Physiology**, v. 103, n. 3, p. e21650, 2020.

ROBBINS, M.; JUDGE, A.; MACLACHLAN, I. SiRNA and innate immunity. **Oligonucleotides**, v. 19, n. 2, p. 89–101, 2009.

ROBERTS, A. F.; DEVOS, Y.; LEMGO, G. N. Y.; ZHOU, X. Biosafety research for non-target organism risk assessment of RNAi-based GE plants. **Frontiers in Plant Science**, v. 6, p. 1–9, 2015.

RODE, N. O.; ESTOUP, A.; BOURGUET, D.; COURTIER-ORGOGOZO, V.; DÉBARRE, F. Population management using gene drive: molecular design, models of spread dynamics and assessment of ecological risks. **Conservation Genetics**, v. 20, n. 4, p. 671–690, 2019.

ROMEIS, J.; RAYBOULD, A.; BIGLER, F.; et al. Deriving criteria to select arthropod species for laboratory tests to assess the ecological risks from cultivating arthropod-resistant genetically engineered crops. **Chemosphere**, v. 90, n. 3, p. 901–909, 2013.

RUNO, S.; ALAKONYA, A.; MACHUKA, J.; SINHA, N. RNA interference as a resistance mechanism against crop parasites in Africa: A “Trojan horse” approach. **Pest Management Science**, v. 67, n. 2, p. 129–136, 2011.

SAJWAN, S.; TAKASU, Y.; TAMURA, T.; et al. Efficient disruption of endogenous

Bombyx gene by TAL effector nucleases. **Insect Biochemistry and Molecular Biology**, v. 43, n. 1, p. 17–23, 2013.

SALEH, M.; RIJ, R. P. VAN; HEKELE, A.; et al. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. **Cell**, v. 8, n. 8, p. 793–802, 2006.

SALUSO, A.; XAVIER, L.; SILVA, F. A. C.; PANIZZI, A. R. An invasive pentatomid pest in Argentina: Neotropical brown stink bug, *Euschistus heros* (F.) (Hemiptera: Pentatomidae). **Neotropical Entomology**, v. 40, n. 6, p. 704–705, 2011.

SALVADOR, R.; PRÍNCIPI, D.; BERRETTA, M.; et al. Transcriptomic survey of the midgut of *Anthonomus grandis* (Coleoptera: Curculionidae). **Journal of Insect Science**, v. 14, n. 1, p. 1–9, 2014.

SAN MIGUEL, K.; SCOTT, J. G. The next generation of insecticides: DsRNA is stable as a foliar-applied insecticide. **Pest Management Science**, v. 72, n. 4, p. 801–809, 2016.

SANDER, J. D.; JOUNG, J. K. CRISPR-Cas systems for editing, regulating and targeting genomes. **Nature Biotechnology**, v. 32, n. 4, p. 347–350, 2014.

SANTOS, M. F.; SANTOS, R. L.; TOMÉ, H. V. V.; et al. Imidacloprid-mediated effects on survival and fertility of the Neotropical brown stink bug *Euschistus heros*. **Journal of Pest Science**, v. 89, n. 1, p. 231–240, 2016.

SCHAEFER, K. A.; WU, W.; COLGAN, D. F.; et al. Unexpected mutations after CRISPR – Cas9 editing in vivo Digenome-seq web tool for profiling CRISPR specificity. **Nature Methods**, v. 14, n. 6, p. 547–548, 2017.

SCHUSTER, S.; MIESEN, P.; VAN RIJ, R. P. Antiviral RNAi in insects and mammals: Parallels and differences. **Viruses**, v. 11, n. 5, 2019.

SCOLARI, F.; GOMULSKI, L. M.; GABRIELI, P.; et al. How functional genomics will impact fruit fly pest control: The example of the Mediterranean fruit fly, *Ceratitis capitata.* **BMC Genetics**, v. 15, p. S11, 2014.

SINGH, I. K.; SINGH, S.; MOGILICHERLA, K.; SHUKLA, J. N.; PALLI, S. R. Comparative analysis of double-stranded RNA degradation and processing in insects. **Scientific Reports**, v. 7, n. 1, p. 1–12, 2017.

SIOMI, H.; SIOMI, M. C. On the road to reading the RNA-interference code. **Nature**, v. 457, n. 7228, p. 396–404, 2009.

SMANIOTTO, L. F.; PANIZZI, A. R. Interactions of Selected Species of Stink Bugs (Hemiptera: Heteroptera: Pentatomidae) from Leguminous Crops with Plants in the Neotropics. **Florida Entomologist**, v. 98, n. 1, p. 7–17, 2015.

SOARES, P. L.; CORDEIRO, E. M. G.; SANTOS, F. N. S.; OMOTO, C.; CORREA, A. S. The reunion of two lineages of the Neotropical brown stink bug on soybean lands in the heart of Brazil. **Scientific Reports**, v. 8, n. 1, p. 1–12, 2018.

SORIA, M. F.; DEGRADE, P. E.; PANIZZI, A. R. Algodão invadido. **Revista Cultivar**, v. 11, p. 274, 2007.

SORIA, M. F.; THOMAZONI, D.; DEGRANDE, P. E.; KODAMA, E. Neotropical brown stink bug *Euschistus heros* (fabr., 1798) attack on bt-cotton bolls cultivated in Brazilian savannah. Proceedings, Beltwide Cotton Conferences. **Anais...** . p.978–984, 2010.

SOSA-GÓMEZ, D.; CÔRREA-FERREIRA, B.; HOFMANN-CAMPO, C.; et al. Manual de identificação de insetos e outros invertebrados da cultura da soja. 2010.

SOSA-GÓMEZ, D. R.; SILVA, J. J. DA. Neotropical brown stink bug (*Euschistus heros*) resistance to methamidophos in Paraná, Brazil. **Pesquisa Agropecuária Brasileira**, v. 45, n. 1, p. 767–769, 2010.

SOSA-GÓMEZ, D. R.; DA SILVA, J. J.; LOPES, I. D. O. N.; et al. Insecticide susceptibility of *Euschistus heros* (Heteroptera: Pentatomidae) in Brazil. **Journal of economic entomology**, v. 102, n. 3, p. 1209–1216, 2009.

SPARKS, M. E.; SHELBY, K. S.; KUHAR, D.; GUNDERSEN-RINDAL, D. E. Transcriptome of the invasive brown marmorated stink bug, *Halyomorpha halys* (Sta˚l) (Heteroptera: Pentatomidae). **PLoS ONE**, v. 9, n. 11, p. e111646, 2014.

SUN, D.; GUO, Z.; LIU, Y.; ZHANG, Y. Progress and prospects of CRISPR/Cas systems in insects and other arthropods. **Frontiers in Physiology**, v. 8, p. 1–22, 2017.

SWEVERS, L.; HUVENNE, H.; MENSCHAERT, G.; et al. Colorado potato beetle (Coleoptera) gut transcriptome analysis: Expression of RNA interference-related genes. **Insect Molecular Biology**, v. 22, n. 6, p. 668–684, 2013.

TAN, J.; LEVINE, S. L.; BACHMAN, P. M.; et al. No impact of DvSnf7 RNA on honey bee (*Apis mellifera* L.) adults and larvae in dietary feeding tests. **Environmental Toxicology and Chemistry**, v. 35, n. 2, p. 287–294, 2016.

TANING, C. N. T.; VAN EYNDE, B.; YU, N.; MA, S.; SMAGGHE, G. CRISPR/Cas9 in insects: Applications, best practices and biosafety concerns. **Journal of Insect Physiology**, v. 98, p. 245–257, 2017.

TERENIUS, O.; PAPANICOLAOU, A.; GARBUTT, J. S.; et al. RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. **Journal of Insect Physiology**, v. 57, n. 2, p. 231–245, 2011.

THURTLE-SCHMIDT, D. M.; LO, T. W. Molecular biology at the cutting edge: A review on CRISPR/CAS9 gene editing for undergraduates. **Biochemistry and Molecular Biology Education**, v. 46, n. 2, p. 195–205, 2018.

TIMMONS, L.; SHEARN, A. Role of AWD/nucleoside diphosphate kinase in *Drosophila* development. **Journal of Bioenergetics and Biomembranes**, v. 32, n. 3, p. 293–300, 2000.

TOMARI, Y.; DU, T.; ZAMORE, P. D. Sorting of *Drosophila* small silencing RNAs. **Cell**, v. 130, n. 2, p. 299–308, 2007.

TOMARI, Y.; ZAMORE, P. D. Perspective: machines for RNAi. **Genes & Development**, v. 19, p. 517–529, 2005.

TOMOYASU, Y.; MILLER, S. C.; TOMITA, S.; et al. Exploring systemic RNA interference in insects: A genome-wide survey for RNAi genes in *Tribolium*. **Genome**

Biology, v. 9, n. 1, p. 1–22, 2008.

TUELHER, E. S.; DA SILVA, É. H.; RODRIGUES, H. S.; et al. Area-wide spatial survey of the likelihood of insecticide control failure in the neotropical brown stink bug *Euschistus heros*. **Journal of Pest Science**, v. 91, n. 2, p. 849–859, 2018.

ULVILA, J.; PARIKKA, M.; KLEINO, A.; et al. Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in *Drosophila* S2 cells. **Journal of Biological Chemistry**, v. 281, n. 20, p. 14370–14375, 2006.

UPADHYAY, S. K.; CHANDRASHEKAR, K.; THAKUR, N.; et al. RNA interference for the control of whiteflies (*Bemisia tabaci*) by oral route. **Journal of Biosciences**, v. 36, n. 1, p. 153–161, 2011.

VÉLEZ, A. M.; FISHILEVICH, E. The mysteries of insect RNAi: A focus on dsRNA uptake and transport. **Pesticide Biochemistry and Physiology**, v. 151, p. 25–31, 2018.

VÉLEZ, A. M.; FISHILEVICH, E.; MATZ, N.; et al. Parameters for successful parental RNAi as an insect pest management tool in western corn rootworm, *Diabrotica virgifera virgifera*. **Genes**, v. 8, n. 7, p. 1–18, 2017.

VÉLEZ, A. M.; KHAJURIA, C.; WANG, H.; NARVA, K. E.; SIEGFRIED, B. D. Knockdown of RNA interference pathway genes in western corn rootworms (*Diabrotica virgifera virgifera* Le Conte) demonstrates a possible mechanism of resistance to lethal dsRNA. **PLoS ONE**, v. 11, n. 6, 2016.

WANG, K.; PENG, Y.; PU, J.; et al. Variation in RNAi efficacy among insect species is attributable to dsRNA degradation *in vivo*. **Insect Biochemistry and Molecular Biology**, v. 77, p. 1–9, 2016.

WANG, T.; ZHANG, H.; ZHU, H. CRISPR technology is revolutionizing the improvement of tomato and other fruit crops. **Horticulture Research**, v. 6, n. 1, 2019.

WATANABE, T.; OCHIAI, H.; SAKUMA, T.; et al. Non-transgenic genome modifications in a hemimetabolous insect using zinc-finger and TAL effector nucleases. **Nature Communications**, v. 3, p. 1017–1018, 2012.

WATSON, J. D.Técnicas de Biologia molecular. In: **Biologia molecular do gene**. Porto Alegre: Artmed, p. 147-190, 2015.

WHANGBO, J. S.; HUNTER, C. P. Environmental RNA interference. **Trends in Genetics**, v. 24, n. 6, p. 297–305, 2008.

WHITFIELD, R.; ANASTASAKI, A.; TRUONG, N. P.; et al. Efficient binding, protection, and self-release of dsRNA in soil by linear and star cationic polymers. **ACS Macro Letters**, v. 7, n. 8, p. 909–915, 2018.

WHYARD, S.; SINGH, A. D.; WONG, S. Ingested double-stranded RNAs can act as species-specific insecticides. **Insect Biochemistry and Molecular Biology**, v. 39, n. 11, p. 824–832, 2009.

WILLOW, J.; SOONVALD, L.; SULG, S.; et al. First evidence of bud feeding-induced RNAi in a crop pest via exogenous application of dsRNA. **Insects**, v. 11, n. 11, p. 1–

8, 2020.

WU, K.; CAMARGO, C.; FISHILEVICH, E.; et al. Distinct fitness costs associated with the knockdown of RNAi pathway genes in western corn rootworm adults. **PLoS ONE**, 2017.

WYNANT, N.; SANTOS, D.; VANDEN BROECK, J. Biological mechanisms determining the success of RNA interference in insects. **International Review of Cell and Molecular Biology,** v. 312, p. 139-67, 2014.

XUE, W. H.; XU, N.; YUAN, X. B.; et al. CRISPR/Cas9-mediated knockout of two eye pigmentation genes in the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae). **Insect Biochemistry and Molecular Biology**, v. 93, n. 2018, p. 19–26, 2018.

YATES, A. D. **RNA interference as a tool for the functional analysis of genes in the Colorado Potato Beetle, *Leptinotarsa decemlineata* (Say).** 2014. Thesis (Dissertations and Student Research in Entomology) - Department of Entomology, University of Nebrasca-Lincoln, 2014.

YE, Z.-F.; LIU, X.-L.; HAN, Q.; et al. Functional characterization of PBP1 gene in *Helicoverpa armigera* (Lepidoptera: Noctuidae) by using the CRISPR/Cas9 system. **Scientific Reports**, v. 7, n. 1, p. 8470, 2017.

YIN, C.; SHEN, G.; GUO, D.; et al. InsectBase: A resource for insect genomes and transcriptomes. **Nucleic Acids Research**, v. 44, p. D801–D807, 2016.

YOON, J.-S.; SHUKLA, J. N.; GONG, Z. J.; MOGILICHERLA, K.; PALLI, S. R. RNA interference in the Colorado potato beetle, *Leptinotarsa decemlineata*: Identification of key contributors. **Insect Biochemistry and Molecular Biology**, v. 78, p. 78–88, 2016.

YOON, J. S.; GURUSAMY, D.; PALLI, S. R. Accumulation of dsRNA in endosomes contributes to inefficient RNA interference in the fall armyworm, *Spodoptera frugiperda*. **Insect Biochemistry and Molecular Biology**, v. 90, p. 53–60, 2017.

ZHANG, J.; KHAN, S. A.; HECKEL, D. G.; BOCK, R. Next-generation insect-resistant plants: RNAi-mediated crop protection. **Trends in Biotechnology**, v. 35, p. 871–882, 2017.

ZHANG, L.; MARTIN, A.; PERRY, M. W.; et al. Genetic basis of melanin pigmentation in butterfly wings. **Genetics**, v. 205, n. 4, p. 1537–1550, 2017.

ZOTTI, M. J.; SMAGGHE, G. RNAi Technology for Insect Management and Protection of Beneficial Insects from Diseases: Lessons, Challenges and Risk Assessments. **Neotropical Entomology**, v. 44, n. 3, p. 197–213, 2015.

ZOTTI, M.; DOS SANTOS, E. A.; CAGLIARI, D.; et al. RNAi technology in crop protection against arthropod pests, pathogens and nematodes. **Pest Management Science**, v. 74, n. 6, p. 1239–1250, 2017.