**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**RNA interference as a tool for *Euschistus heros* (Fabricius, 1798)
(Hemiptera: Pentatomidae) control**

Aline Pereira Rank

Dissertation presented to obtain the degree of Master
in Science. Area: Entomology

**Piracicaba
2021**

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Agronomic Engineer

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Pentatomidae) control**

versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
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2021

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Rank, Aline Pereira

RNA interference as a tool for *Euschistus heros* (Fabricius, 1798)
(Hemiptera: Pentatomidae) control / Aline Pereira Rank. - - versão revisada
de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2021.
68 p.

Dissertação (Mestrado) - - USP / Escola Superior de
Agricultura “Luiz de Queiroz”.

1. Genes de referência 2. RNAi 3. Biotecnologia 4. Biotecnologia 5.
Segurança alimentar 6. qPCR 7. Controle de pragas I. Título

To my dear parents Vera and José, my brother Vinicius and my husband Jorge, for their affection, patience, encouragement and guidance on my personal and professional education

DEDICATION AND ACKNOWLEDGMENTS

Aos meus queridos pais Vera e José, ao meu irmão Vinicius e ao meu esposo Jorge, pelo afeto, paciência, incentivo e ensinamentos em minha formação pessoal e profissional.

DEDICO E AGRADEÇO

ACKNOWLEDGMENTS

To Professor Fernando Luís Côtoli, for his guidance, advice, support, as well as for the confidence and opportunity to carry out this work.

To my colleagues of the Insect Interactions Laboratory (ESALQ/USP), Ana Flávia, Bruna, Flávia, Diandra, Eloísa, Beatriz, Nathália, Mariane, Marcelle and Lilian for the excellent coexistence, companionship, and knowledge shared.

To Professor José Roberto Postal Neto for his support in providing access to a population of insects to conduct this work.

To my friend Lucas (Laboratory of Insect Biology, ESALQ / USP), for all the support, information and for providing the population used in our lab experiments.

To colleagues of the Laboratory of Arthropod Resistance to Pesticides (ESALQ / USP), Fernando Selmer and Aline Guidolin for their help and guidance.

To all the dear friends and colleagues of the Graduate Program in Entomology (ESALQ / USP) for the pleasant friendship and companionship.

To my family, in special to my parents Vera and José, and my brother Vinicius for their help, encouragement and contribution to my personal and professional growth.

To my husband Jorge for his unconditional love and support. Thank you for your esteem and dedication in always helping and guiding me.

To my second family “Trem Q Pula” for being a pillar in my personal and professional education. Thank you for the friendship, companionship and support at all times.

To my dear friends Bruna Baldan, Thaís Albuquerque and Nathane Maiorano for the friendship of all these years, affection and encouragement.

To all employees of the Department of Entomology and Acarology of ESALQ / USP for their dedication and services provided.

To the Luiz de Queiroz College of Agriculture for the opportunities granted during all these years.

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES Foundation) for granting the scholarship (Funding Code 001).

To the librarian Eliana Maria Garcia of the Central Library (ESALQ / USP), for the help in formatting this dissertation.

EPIGRAPH

"If I have seen a little further it is by standing on the shoulders of Giants"

Isaac Newton in a letter to his rival Robert Hooke, in 1676

"Se eu vi um pouco mais longe, é por estar nos ombros de gigantes"

Isaac Newton, em uma carta ao seu rival Robert Hooke, em 1676

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ABSTRACT

RNA interference as a tool for *Euschistus heros* (F., 1798) (Hemiptera: Pentatomidae) control

The expected increase in the world population demands greater production, efficiency and productivity. Agricultural pests are responsible for great losses in food production worldwide. So far, chemical control is the main strategy, but new, environmentally friendly strategies of pest control for the implementation of sound integrated pest management programs remains of a constant need. RNAi appears as a viable technology for controlling pests such as the bug *Euschistus heros*, which until now have restricted control methods. The technology itself is new and requires basic research in the optimization and implementation of more efficient methodologies, as well as information for correct data standardization and analysis. This study presents the analysis of five genes (*Act* - *actin*, *ART* - *actin-related protein*, *Ef-1* - *elongation factor 1*, *Hsp70* - *heat shock protein 70*, and *18s rRNA* – *18S ribosomal RNA*) for use in data normalization in RT-qPCR assays in *E. heros*. The analyzes were performed using the BestKeeper, Delta Ct, GeNorm and NormFinder platforms, and submitted to the RefFinder software for comparisons and final gene classification. The RefFinder software classified the *Act* and *Ef-1* genes as the most stable for use when comparing instars, and the *18s rRNA* and *Act* genes as the most suitable for tissue analysis of insects exposed to different nutritional conditions. We also investigated the genes involved in the RNAi pathway from an *in-house* transcriptome and selected four genes involved in key cellular events of insects as targets for RNAi. We describe the expression profiles of these genes in various tissues and during the growth of *E. heros*. We also tested different delivery strategies for double-stranded RNA (dsRNA) of the selected genes, and report insect mortality ranging from 40 to 60% depending on the selected target gene by dsRNA microinjection. We also demonstrate reduction in the mRNA abundance for all of the tested target genes, but the intensity of reduction was dependent on the target gene and the delivery strategy used. The data obtained are promising for the development of RNAi as an applied control strategy for *E. heros*.

Keywords: reference genes, RNAi, biotechnology, food security, qPCR, pest control

RESUMO

RNA de interferência como ferramenta para controle de *Euschistus heros* (F., 1798) (Hemiptera: Pentatomidae)

O esperado aumento da população mundial exige maior produção, eficiência e produtividade. As pragas agrícolas são responsáveis por grandes perdas na produção de alimentos em todo o mundo. Até agora, o controle químico é a estratégia principal, mas novas estratégias ambientalmente amigáveis de controle de pragas para a implementação de programas de manejo integrado de pragas continuam sendo uma necessidade constante. O RNAi surge como uma tecnologia viável para o controle de pragas como o *Euschistus heros*, que até agora possuem métodos de controle restritos. A tecnologia em si é nova e requer pesquisas básicas na otimização e implementação de metodologias mais eficientes, além de informações para a correta padronização e análise dos dados. Este estudo apresenta a análise de cinco genes (*Act* - actina, *ART* - proteína relacionada à actina, *Ef-1* - fator de alongamento 1, *Hsp70* - proteína de choque térmico 70 e *rRNA 18s* - RNA ribossomal 18S) para uso na normalização de dados em ensaios de RT-qPCR em *E. heros*. As análises foram realizadas nas plataformas BestKeeper, Delta Ct, GeNorm e NormFinder, e submetidas ao software RefFinder para comparações e classificação final dos genes. O software RefFinder classificou os genes *Act* e *Ef-1* como os mais estáveis para uso na comparação de ínstar, e os genes *18S rRNA* e *Act* como os mais adequados para análise de tecidos de insetos expostos a diferentes condições nutricionais. Também investigamos os genes envolvidos na via do RNAi a partir de um transcrito interno e selecionamos quatro genes envolvidos em eventos celulares importantes ao inseto como alvos para o RNAi. Descrevemos os perfis de expressão desses genes em vários tecidos e durante o crescimento de *E. heros*. Também testamos diferentes estratégias de entrega do RNA de fita dupla (dsRNA) dos genes selecionados e relatamos mortalidade de insetos variando de 40 a 60%, dependendo do gene alvo selecionado por microinjeção de dsRNA. Também demonstramos redução na abundância de mRNA para todos os genes-alvo testados, mas a intensidade da redução era dependente do gene-alvo e da estratégia de entrega usada. Os dados obtidos são promissores para o desenvolvimento de RNAi como estratégia de controle aplicada para *E. heros*.

Palavras-chave: Genes de referência, RNAi, biotecnologia, segurança alimentar, qPCR, controle de pragas

1. INTRODUCTION

An increase of 26% in the world population is expected by 2050 (Nations 2019). The expected increase in population will demand for higher investments in food production in order to optimize and increase productivity. So far, pests are one of the main obstacles for food production. It is estimated that 7.7% of the agricultural production in Brazil is lost due to damage caused directly or indirectly by agricultural pests. This reduction reflects losses of approximately US\$ 17.7 billion, equivalent to 25 million tons of food, fibers and biofuels (Oliveira et al. 2014).

Chemical control remains as the main strategy for pest control nowadays. This control is often efficient, but we already know that the use of only one control method, whatever it may be, leads to the selection of resistant individuals. Additionally, this method of control has also been linked to environmental problems as soil contamination and non-target effects. Thus, new methods of control for the use in integrated pest management strategies for a more effective, lasting and sustainable pest control are needed (Song and Swinton 2009, Panizzi 2013, de Freitas Bueno et al. 2015).

A new technology that has been addressed in studies as an alternative strategy for pest control is RNA interference or RNAi. This technique was discovered and described by Fire et al. (1998) when they promoted silencing in protein synthesis by inserting double stranded RNA (dsRNA) in the nematode *Caenorhabditis elegans*. This event had already been described in plants as "*Post-Transcriptional Genetic Silencing - PTGS*" and "Quelling" in mold (*Neurospora* spp.) (Napoli et al. 1990). For this discovery, Fire and Mello received the Nobel Prize of Physiology and Medicine in 2006.

Investigations on the use of RNAi were conducted for a variety of insect taxons, such as Diptera (Somma et al. 2002, Raza et al. 2016), Lepidoptera (Yang and Han 2014, Bento et al. 2020), Hemiptera (Kishk et al. 2017, Castellanos et al. 2019a) and Coleoptera (Baum et al. 2007, Rodrigues et al. 2017). The technique was used to observe, for example, the function of a given gene in an organism due to the physiological responses obtained in the absence of its transcription and as a pest control biotechnology (Sim and Denlinger 2009, Kim et al. 2010). The efficiency of dsRNA to silence target genes through feeding, leading to the successful control of insects was an important step to demonstrate RNAi is an amenable strategy for applied pest control (Baum et al. in 2007).

Sucking insects are pests that are more difficult to target when using molecules that do not act upon contact due to their feeding behavior, reducing the number of

molecules available for their control. In Brazil, the Neotropical brown stinkbug, *Euschistus heros* (F., 1798) (Hemiptera, Pentatomidae), is the stinkbug species that currently poses severe losses to soybean production. This insect has a sap-feeding habit leading to poor pod formation, compromising yield and indirectly facilitating infection by microorganisms. It is the main soybean pest in Brazil, Paraguay and Argentina (Silva et al. 2012, Timbó et al. 2014, Williams et al. 2017)

According to Cividanes and Parra (1994), *E. heros* reach up to eight generations per year in warmer regions. It is a polyphagous species feeding on cotton (*Gossypium hirsutum* L.), sunflower (*Helianthus annuus* L.), maize (*Zea mays*), pigeon pea (*Cajanus cajan* L. Millsp.), peanuts (*Euphorbia heterophylla* L.), and a range of alternative host plants, which facilitates the persistence of natural populations in off-season periods. It can also remain associated with the straw during the soybean off-season in the winter (Smaniotto and Panizzi 2015).

Currently, the main management strategy available for *E. heros* is based on few insecticides. Thus, *E. heros* became a target of interest for initial studies in the implementation of the RNAi technique as a way of pest control. In 2019, Castellanos et al. (2019) published the first paper involving the silencing in *E. heros* genes where it was possible to conclude that the study and approach of the technique is worth for this pest.

Thus, we clearly see the need to better explore the resources and information available about the pest to improve this technique as a possible tool for its control. Despite these initial steps, the technology itself is very recent. Several of the physiological pathways are not fully described and little is known on how the signal spreads through tissues in insects (Cooper et al. 2019a).

Additionally, *E. heros* is a non-model insect, and only very recently the molecular information required for a more targeted development of RNAi techniques against this pest started to become available (Cagliari et al. 2020). Therefore, there are still gaps on the basic molecular information of the target pest and on the use of dsRNA to control *E. heros*, and in this work we provide additional information as a way to contribute with the filling of the gaps required to make the use of RNAi a successful strategy to control *E. heros*.

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2. SELECTION OF REFERENCE GENES FOR RT-QPCR EXPERIMENTS IN *Euschistus heros* (F., 1798) (HEMIPTERA, PENTATOMIDAE)

ABSTRACT

Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) is an extremely valuable tool used in gene expression and in molecular physiology analyses. RT-qPCR is also very sensitive and the outcome of the analysis can be easily influenced by several factors and made poorly reproducible. Thus, general procedures for standardization have been developed and are required for proper quantitative gene expression analysis. An important factor in the standardization of RT-qPCR is the selection of reliable reference genes as endogenous control for sample normalization, once their expression is stable over a range of biotic and abiotic conditions. The brown stinkbug *Euschistus heros* is currently the major stinkbug pest in soybean in South America, showing a potential status to become an invasive pest to North America. The monitoring and characterization of resistance evolution to the new chemistries available for stinkbug control and the development of molecular-based strategies for *E. heros* control rely on RT-qPCR experiments. In order to obtain reliable genes for assays with *E. heros*, we tested five candidates as reference genes for this species, *actin* (*Act*), *actin-related protein* (*ARP8*), *elongation factor 1* (*Ef-1*), *heat shock protein 70 kDa* (*Hsp70*) and *subunit 18 of the ribosomal RNA* (*18S-rRNA*). The expression levels of these genes were obtained under different endogenous and exogenous experimental factors as insect development stages, body tissues and nutritional source, following the guidelines for Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE). The data were analyzed using the BestKeeper, Delta Ct, GeNorm and NormFinder platforms, and subjected to the RefFinder software for final gene comparisons and ranking. The RefFinder software classified the *Act* and *Ef-1* genes as the most stable for use during development under the different nutritional sources and selected the *18S rRNA* and *Act* genes as the most suitable for tissue analysis.

Key-words: reference gene, normalization, gene expression, qPCR

2.1 INTRODUCTION

Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) became an important tool for molecular studies in several sciences. RT-qPCR is highly sensitive and easy to reproduce, providing highly accurate quantification of gene expression. Nevertheless, RT-qPCR accuracy relies on the strict control of interfering factors such as the quality of RNA and cDNA, the standardization of experimental procedures between treatments, and the use of reliable reference genes (Broeders et al. 2014).

Housekeeping genes were invariably selected as reference genes without additional assessments because they typically have constitutive expression, and were understood to have steady expression under a range of patho-physiological conditions, as housekeeping genes are involved with basal, essential functions for the maintenance of cell activity. However, even the most common of these genes - *actin* and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* - were shown to follow different patterns of expression between insects, stages of development, tissues, sexes, and even under different stress conditions (Bustin 2010, Derveaux et al. 2010).

The variations in the pattern of expression of housekeeping genes under different biological and experimental conditions poses a limitation to the reliability and accuracy of RT-qPCR for comparative gene expression analysis, making clear the need of proper experimentation for the selection of attested reference genes for proper normalization of RT-qPCR data according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009, Kozera and Rapacz 2013). Reference genes serve as normalizers of the mRNA fraction, ensuring that the effect observed in the expression difference given by the Cqs is derived from the distinct treatments themselves and not by errors or experimental variances on samples (Ponton et al. 2011).

Genes related to the synthesis of structural proteins, ribosomal proteins and factors related to translation are often used as reference genes (Pan et al. 2015). The selection of reliable reference genes requires the assessment and analysis of their expression by RT-qPCR under different abiotic and biotic conditions. Analysis of the expression data obtained are further compared using the Microsoft Excel-based algorithms BestKeeper (Pfaffl et al. 2004), comparative delta-Ct method (Silver et al. 2006), GeNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004), which all carry stability analysis for ranking tested genes. Gene ranking will vary from one algorithm to the

other, and the final best fit can be obtained by a joint analysis in the web-based platform RefFinder, that integrates the analyses of the four previously mentioned algorithms (BestKeeper, comparative delta-Ct method, GeNorm, and NormFinder) (Xie et al. 2012).

A recent biotechnology that is heavily dependent on the applications of RT-qPCR is the RNA interference or RNAi. RNAi is known to be a natural process occurring in eukaryotes, having the function of protecting the organism against virus infections and regulating cellular processes through transcriptional regulation (Fire et al. 1998). RNAi has been widely studied for target disease control since its discovery, but after the pioneer work of Baum et al. (2007) the interest for using this technology for agricultural pest control has increased.

Several studies have tested this technology for controlling insects to which alternative strategies of control are unavailable or that control is difficult due to the rapid evolution of resistance against the available technologies (Baum et al. 2007, Price and Gatehouse 2008, Terenius et al. 2011, Attasart et al. 2013, Haussecker 2014, Bento et al. 2020). In 2017, SmartStax Pro™ was approved by EPA (United States Environmental Protection Agency) as the first commercial plant technology based on RNAi. SmartStax Pro™ is a corn hybrid genetically modified to express dsRNA of the *essential vacuolar protein* (*Snf7*) gene, targeted to control larvae of the western corn rootworm (WCR), *Diabrotica virgifera* (LeConte, 1868) (Coleoptera: Chrysomelidae) (Zhang et al. 2017).

An insect pest that has gained attention in recent years and is of continuous concern to growers is the Neotropical brown stinkbug *Euschistus heros* (Fabricius, 1798) (Hemiptera, Pentatomidae). *Euschistus heros* causes severe damage to soybean and cotton in Brazil, Paraguay and Argentina. It is a pest that feeds on several species of alternative host plants to survive during the off-season, showing a very high biotic potential with up to eight generations/year (Cividanes and Parra 1994). Currently, the only strategy available for controlling *E. heros* is based on the few organic chemistries still in use and a new molecule recently released (Watson et al. 2011, Zhu et al. 2011). RNAi is a promising technique for the development of new strategies for sucking pests control (Soria et al. 2017, Williams et al. 2017, Castellanos et al. 2019b).

The increased interests on the development of RNAi strategies for *E. heros* control will require well-established qPCR procedures for reliable experimentation and target efficiency evaluation, but no reference genes were validated yet for RT-qPCR experiments with this species so far. Therefore, in this chapter we tested five candidate reference genes: *actin* (*Act*), *actin-related protein* (*Arp8*), *elongation factor 1* (*Ef-1*), *heat shock protein 70*

kDa (Hsp70) and the *subunit 18 of the ribosomal RNA (18S rRNA)*. These genes were analyzed by assessing their expression in different tissues and developmental stages of *E. heros* under two nutritional conditions to offer suitable reference genes for use in RT-qPCR experiments involving such conditions.

2.2 MATERIAL AND METHODS

Insect rearing

A stock colony of *E. heros* gently provided by the Biological Control Laboratory (ESALQ/USP) was kept under controlled conditions over two years following Borges et al. (2006). Part of the insects was reared on a mixture of fresh green beans pods, *Phaseolus vulgaris* (L.), and raw peanut seeds, *Arachis hypogaea* (L.), while another part was reared on a dry artificial diet (Mendoza et al. 2016). The artificial diet used was composed by grinded, lyophilized green beans (35 g), grinded peanuts (35 g), sucrose (5 g), and water (25 mL), 10,000 ppm methyl-parabenzoate, 800 ppm sorbic acid, and 7.65 ppb tetracycline (diet 11 in Mendoza et al. 2016). Water was offered in moistened cotton pads. Green beans pods, peanuts and water were replaced every two days. Two pieces of raw cotton fabric were hung on the side of the box to serve as a substrate for oviposition. The egg clusters were collected twice a week and placed in plastic boxes (6x12x12 cm) containing a moistened cotton pad to avoid egg desiccation. After hatching, nymphs were kept in rearing containers separated from adults, but fed with the same diet offered to adults.

Sample collection

Samples of *E. heros* (stages of development and tissues of adults) were collected from insects subjected to two food regimes (exogenous conditions). In one of the food regimes tested, insects were reared using fresh green beans and raw peanut seeds (Borges et al. 2006), while in the other they were offered an artificial diet (diet 11) (Mendoza et al. 2016).

Development stages

Expression of the candidate reference genes was assessed for all five instars and

adults of *E. heros*. Individuals were collected within 24 h of their eclosion (first instars) or of their molt for the instar to be sampled. Three biological replicates were collected for each stage of development. Each biological replicate of first and second instars were represented by a pool of 10 nymphs; third and fourth instars by a pool of five nymphs; and fifth instar and adults by a pool of three specimens. Samples were snap frozen in liquid nitrogen and stored in -80°C for later RNA extraction.

Insect tissues

Adults of *E. heros* were collected as previously described and subjected to dissection under sterile conditions for tissue collection. Dissections were carried under a laminar flow hood after sterilization of all tools used with ethanol and RNase away™ Decontamination Reagent (Invitrogen™). Insects were dissected in sterile saline solution and five guts, ovaries and testes were collected for each replicate. A total of three biological replicates were collected (1 replicate = tissue from 5 insects) (Pawłowicz et al. 2012). Samples were snap frozen in liquid nitrogen and stored in -80°C for later RNA extraction.

RNA extraction and cDNA synthesis

Snap frozen samples were macerated and subjected to total RNA extraction using Trizol reagent following the manufacturer's guidelines. Briefly, one volume of chloroform was added to the samples in Trizol. Samples were vigorously mixed for 15 seconds and sat at room temperature for 3 min before centrifugation (12,000 g x 15 min x 4°C). The top layer was collected, 500 µL of ice-cold isopropanol was added, incubated for 10 min at room temperature and centrifuged as before. The pellet obtained was washed twice in 1 mL of 75% cold ethanol and recovered after centrifugation (7,500 g x 5min x 4°C). The pellet was allowed to dry at room temperature, and the RNA was resuspended in 20 µL of RNase-free water. RNA quality and quantity were verified by standard agarose gel electrophoresis and measurement of absorbance in an Epoch™ Microplate Spectrophotometer. Only RNA samples with an A260/A280 ratio ranging from 1.8 to 2.0 and an A260/A230 ratio >2.0 were stored at -80 °C and further used for cDNA synthesis.

The reverse transcription reaction of the RNA samples was performed using 1 µL of the oligo-dT15 primer available in the GoScript RT System (Promega™) kit, and a sample volume equivalent to 1.0 µg of total RNA. The reaction solution was brought to a final

volume of 5 μ L using RNase-free water. Samples were kept at 70°C for 5 min and cooled to 4°C in a thermal cycler. Then, 4 μ L of the reaction buffer, 3.75 mM of MgCl₂, 1 μ L reverse transcriptase, 0.5 mM of dNTP, RNase-free water was added to a final volume of 20 μ L and subjected to 25 °C for 5 min, 42 °C for 60 min and 70 °C for 15 min in thermocycler. The single strand cDNA obtained was stored at -80 °C until further use.

Reverse Transcription - Quantitative Polymerase Chain Reaction (RT-qPCR)

Selection and amplification of candidate reference genes

The selection of candidate genes was based on genes commonly used as reference genes for other hemipterans, such, as the *elongation factor 1 alpha (Ef-1)* for *Bemisia tabaci* (Gennadius, 1889) (Hemiptera: Aleyrodidae) (Li et al., 2013) and the *18S rRNA* for the brown marmorated stink bug, *Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae) (Mogilicherla et al. 2018). Three additional genes were selected; *actin (Act)*, *actin-related protein 8 (ARP8)*, and the *heat shock protein 70 kDa (Hsp70)*. Transcripts for each one of these genes were recovered from an in-house transcriptome of *E. heros*. Sequences obtained were analyzed in Primer Express 3.0.1 Program for primer selection. Selected primers were further checked for hairpin formation and self-dimerization using Oligo Analyzer (<https://www.idtdna.com/calc/analyzer>). Each one of the primer sets designed was used in RT-qPCR experiments using serial dilutions (500, 250, 125, 62.5 and 31.25 ng/ μ L) of single-strand cDNA (sscDNA) of *E. heros* for evaluation of their specificity through their melting curve, and their efficiency of amplification was calculated after determination of the slope of the linear regression of Ct-values against sscDNA concentration. The efficiency of amplification for each primer set was given by the equation $E = 10^{(-1 / \text{slope})}$ (Pfaffl 2001).

Table 1. Complementary information of candidate genes and primers used in RT-qPCR for the selection of reference genes for experimentation with *Euschistus heros*

Gene Symbol	Gene	Primers sequences (5'-3')	Function (putative)	Amplicon (base pairs)
<i>Act</i>	<i>Actin</i>	F: CGTCCTTCTGACTTGGGCTT	Structural (cytoskeleton)	150
		R: TCTCTCAGCAATACCGGGGA		
<i>ARP8</i>	<i>Actin related protein 8</i>	F: TGCCATTCTTGCTTGTCTTG	Chromosome organization	96
		R: GGCCCTCTTTCTCGTATCAA		
<i>Ef-1</i>	<i>Elongation factor 1</i>	F: TGCGGTGGTATTGACAAGAG	Enzymatic delivery of tRNAs to ribosome	97
		R: GTTTGTCAAGAACCCAAGCG		
<i>Hsp70</i>	<i>Heat shock protein 70 kDa</i>	F: TCTCTGCTCAGTCCCCTTCTTC	Protects stress cells, protein coating	65
		R: TTTGCTGCTTCCAATGCCTTA		
<i>18S rRNA</i>	<i>18S rRNA (Ribosomal RNA 18S)</i>	F: AATGCTGACCAAGCAACACA	Pre-rRNA process involvement	100
		R: ACTGAAAAATAGCTCGGGCA		

F - forward primer; R - reverse primer

The thermocycling conditions for assessing primer specificity and primer efficiency was 95°C for 12 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 20 s following the protocol suggested by the manufacturer (Solis Biodyne™). At the end of each PCR run, a melting curve analysis from 65 to 95°C was applied to all reactions to ensure the specificity of the amplified product. RT-qPCR of the target genes using sscDNA from each stage of development and tissue from different food sources were conducted under the same thermocycling conditions previously described, using reactions containing 500 ng of sscDNA, 2 µL 5x EvaGreen™ qPCR mix from the 5x HOT FIREPol EvaGreen™ qPCR Mix Plus (ROX) system, 160 mM of each primer (forward and reverse), and nuclease-free water to bring the reaction volume to 10 µL. All reactions were run in an Applied Biosystems ViiA™7 Real-Time PCR system (Applied Biosystems, Foster city, CA).

Gene stability analysis

The expression data obtained by RT-qPCR of all candidate genes from samples

collected from insects and tissues obtained under different conditions were analyzed using BestKeeper, DeltaCt, GeNorm and NormFinder for identification of the candidate gene holding the most stable expression trend under the different conditions samples were collected from. Gene ranking by BestKeeper is based on the geometric mean of the threshold cycle value of all reference genes grouped together (Bestkeeper index) and the pairwise correlation coefficient between each tested gene; index above 1 are considered unstable and genes obtaining such index should be avoided (Pfaffl et al. 2004). The comparative Delta Ct method ranks genes after pairwise comparisons of the average delta Ct (Cq) values, ranking genes based on the lowest standard deviation obtained (Silver et al. 2006). GeNorm calculates pair-wise variation V_n/V_{n+1} (variation between two sequential normalization factors) and generates the M value, which is the stability of the gene. Genes with M values higher than 1.5 are excluded, and the lowest M value calculated is the best candidate reference gene (Vandesompele et al. 2002). Analysis and ranking of candidate reference genes in NormFinder take into consideration the intra and intergroup variation and the stability value for each gene. The best candidate will display the least intra and intergroup variation and the lowest stability value (Andersen et al. 2004).

Since each one of these programs use different procedures in ranking genes, the gene rank each one produce holds some variation among them. Therefore, calculations from all these computational programs can be integrated in the RefFinder which allows the comparison and ranking of the candidate reference genes evaluated after the assignment of an appropriate weight to individual genes and the calculation of the geometric mean of their weights, leading to a single gene ranking (Xie et al. 2012).

2.3 RESULTS

Primers efficiency

The specificity of the primer sets used in the amplification of the target genes was confirmed by the observation of a single peak in the melting curve analysis. Primer efficiencies were 108% for the *18S rRNA* gene and 110% for the remaining genes, and the correlation coefficients (r^2) ranged between 0.984 and 0.998 (*Act*: $r^2=0.998$; *ARP8*: $r^2=0.984$; *Ef-1*: $r^2=0.992$; *Hsp70*: $r^2=0.987$; and *18S rRNA*: $r^2=0.989$).

Gene expression stability analysis

The cycle quantification (Cq) is the number of the total program cycles where the fluorescence that binds to the material is detected, so the lower this value, the greater the amount of the target material. The values obtained for the five normalizer candidate genes were used to estimate their expression stability during the six developmental stages and in three different tissues of adults, both conditions being maintained under two nutritional sources.

The raw cycle quantification (Cq) values varied from 20.926 to 24.471 for the *Act* gene, from 20.956 to 23.993 for *ARP8*, from 25.2275 to 31.106 for *Ef-1*, from 20.231 to 27.126 for *Hsp70*, and from 23.663 to 28.647 for *18S rRNA* (Figure 1).

Figure 1. Box plot representation of the Cq values obtained in RT-qPCR amplifications of five candidate reference genes *Act* (*Actin*), *ARP8* (*Actin related protein 8*), *Ef-1* (*Elongation factor -1*), *Hsp70* (*Heat shock protein 70 kDa*), *18S rRNA* (*subunit 18 of the ribosomal RNA*) tested for *Euschistus heros* in all different conditions (six stages of development, and reproductive tissues and gut of adults). The box plot shows the 25th and the 75th percentiles, asterisks represent outliers, the median expression levels (Cq) are represented by the crosses and the mean by the horizontal lines. AD : Artificial diet, ND: Natural diet

C_q values obtained for samples representing the different stages of development of *E. heros* displayed little variation among replicates and stages of development for *actin* and *ARP8*, regardless the food source used. However, a higher variation was observed particular stages of development for one or other of the food sources for the remaining reference genes tested (Figure 2).

Figure 2. Mean of Cq values obtained in RT-qPCR experiments for five candidate reference genes *Act* (*Actin*), *ARP8* (*Actin related protein 8*), *Ef-1* (*Elongation factor -1*), *Hsp70* (*Heat shock protein 70 kDa*) and *18S rRNA* (*subunit 18 of the ribosomal RNA*) tested for different stages of development of *Euschistus heros* when feeding on natural or artificial diets. Vertical bars represent standard deviations for each sample

The stability calculated for each reference gene using the Cq values obtained ranged according to the platform used (Figure 3). In general, *Actin* and *Arp8* were the genes with the lowest values of stability observed for the stages of developments feeding on both diets for most of the platforms used. But the stability values calculated for *Arp8* were quite variable depending on the insect instar (Figure 3). The joint analysis of the stability values obtained with the different platforms conducted in RefFinder resulted in a more defined ranking of the tested genes in each one of the nutritional rearing conditions tested (Figure 4). The overall ranking of the genes in an analysis considering all of the sources of variations produced a final ranking, indicating *actin* and *Ef-1* with the best rankings (Figure 5).

Figure 3. Determination of gene expression stability of five candidate reference genes *Act* (*Actin*), *ARP8* (*Actin related protein 8*), *Ef-1* (*Elongation factor -1*), *Hsp70* (*Heat shock protein 70 kDa*) and *18S rRNA* (*subunit 18 of the ribosomal RNA*) for developmental stages of *Euschistus heros* under natural and artificial diet sources using BestKeeper, DeltaCt, GeNorm and NormFinder softwares. Smaller values suggest greater stability for genes

Figure 4. Ranking determined by the RefFinder software analysis for each stage of development of *Euschistus heros* maintained under artificial and natural diet sources. Smaller values suggest greater stability for genes

Figure 5. Ranking determined by the RefFinder software analysis for stages of development of *Euschistus heros* maintained under artificial and natural diet sources. Smaller values suggest greater stability for genes

To make a specific analysis of tissues of the organisms, intestines were collected from mixed adult males and females, ovaries from adult females and tests from adult males. These insects were kept during the entire cycle on an artificial or natural nutrient source forming two groups of analysis. In the graph below (Figure 6) is shown the dispersion of Cqs obtained regarding the expression of each gene in each condition. We can see a clear distinction between the different diets used in gene expression and a greater variation in the expression of the *Hsp70* gene between tissues than the other genes.

Figure 6. Expression of candidate genes in different *Euschistus heros* adult organs kept under natural and artificial diet sources. Quantitative real-time PCR was carried out using specific primers designed for each candidate genes. Vertical bars represent standard error for each sample

The stability values were calculated for each gene in different tissues using the four platforms (Figure 7). A variation in gene stability was observable depending on the tissue analyzed and nutritional resource used. The lower the stability value, the better and more suitable the gene is for use as a reference gene. Some genes resulted in stability values equal to zero, but this low stability values were not maintained in the different tissues analyzed. On the other hand, the *18S rRNA* gene displayed stable stability values in the different tissues from insects reared on the two diets used. In the same way as in developmental stages, tissues were analyzed individually and in general in RefFinder software (Figures 8 and 9).

Figure 7. Determination of gene expression stability of five candidate reference genes *Act* (*Actin*), *ARP8* (*Actin related protein 8*), *Ef-1* (*Elongation factor -1*), *Hsp70* (*Heat shock protein 70 kDa*) and *18S rRNA* (*subunit 18 of the ribosomal RNA*) for adult stage tissues of *Euschistus heros* under natural and artificial diet sources using BestKeeper, DeltaCt, GeNorm and NormFinder softwares. Smaller values suggest greater stability for genes

Figure 8. Ranking determined by the RefFinder software analysis for gut, ovaries and testes from adults of *Euschistus heros* maintained under artificial and natural diet sources. Smaller values suggest greater stability for genes

Figure 9. Ranking determined by the RefFinder software analysis for tissues in general from adults of *Euschistus heros* maintained under artificial and natural diet sources. Smaller values suggest greater stability for genes

2.4 DISCUSSION

Molecular analysis has assumed an important role in sciences since the idea that the understanding of gene expression allows proper interpretation and explanation of physiological processes governing organisms. Among the molecular analyzes, RT-qPCR is one of the most used and its characteristics of high sensitivity and efficiency make the technique essential for these understandings. The correct application of this methodology requires standardization for later data normalization and correct reading of the results (Broeders et al. 2014). In this study we addressed some experimental conditions that can often be used in molecular studies with *Euschistus heros* and that have not had any reference genes analyzed and selected so far as suitable for use.

The variation of the stability values for the candidate genes evaluated for *E. heros* reared on different food diet types when estimated using different platforms is expected as similar variation has been reported for other species under a variety of stress conditions (Kozera and Rapacz 2013, Singh et al. 2018). However, the use of several platforms associated with the use of RefFinder has been argued to provide a much more accurate selection of reliable references genes for qPCR analysis (Jacob et al. 2013).

We did observe that the stability values calculate for the candidate genes showed that some are quite stable over the period of growth of the insect, but much less stable when different tissues of the same stage of development were compared, as observed for the *18S-rRNA gene*. Unfortunately, we are unable to provide any substantiated explanation observed for the different values of stability of the reference genes assessed for *E. heros* under the experimental conditions evaluated.

Nutrition certainly plays a strong effect on the expression of these genes and on the total amount of mRNA produced in the different stages and tissues of *E. heros* analyzed. We can clearly come to this conclusion as we observed the stability values calculated for all tested genes for the first instar of *E. heros*. Nymphs of *E. heros* start feeding once they moult to the second instar, when the physiology of the insect is exposed to the range of molecules available in their diet, that can interfere with their metabolism and growth, and consequently the levels of gene expression. First instars basically ingest water and surface content associated with the chorion of the eggs they hatched (Bowling 1980). Similar results were observed even for insects that first instars are active feeders, but that samples were collected soon after eclosion, such as *Bemisia tabaci* (Kaur et al. 2017), *Spodoptera*

litura (Lu et al. 2013) and *Aphis gossypii* (Glover, 1877) (Hemiptera: Aphididae) (Ma et al. 2016).

The *18S-rRNA* gene has been reliably used as a reference gene for qPCR analysis in a diverse range of organisms under different conditions (Kim et al. 2003, Hashimoto et al. 2004, Bagnall and Kotze 2010, Kuchipudi et al. 2012). However, there are also a large number of studies indicating the expression of *18S-rRNA* gene can be affected by the experimental conditions tested, making it a less reliable normalizer for qPCR experiments (Mogal and Abdulkadir 2006, Lin and Lai 2010, Meinzer et al. 2019). Nevertheless, the *18S-rRNA* gene showed a pattern of stability in the gut, ovaries and testes of *E. heros* adults reared on different food types, corroborating the reports of the stability of this gene in tissues of *Spodoptera litura* (Lu et al. 2013), *Aphidius gifuensis* (Hymenoptera: Aphidiidae) (Gao et al. 2017), and *Apis mellifera* (Linnaeus, 1758) (Hymenoptera: Apidae) (Moon et al. 2018) exposed to nutritional stress.

Contrarily to the observations made for the *18S-rRNA* gene, the *Ef-1* gene demonstrated a high stability over different stages of development of *E. heros* reared on different food sources despite its reduce level of expression. The stability of *Ef-1* gene reported for *E. heros* is also common to a number of insects, as in *B. tabaci* (Collins et al. 2014), *Harmonia axyridis* (Pallas, 1773) (Coleoptera: Coccinellidae) (Yang et al. 2018), and *Coleomegilla maculata* (DeGeer, 1775) (Coleoptera: Coccinellidae) (Yang et al. 2015).

The *ARP8* also displayed the best ranking in the overall analysis. *Actin-related proteins* carry different functions in cellular processes, ranging from the modulation of the assembly of conventional actin to their participation as components of large proteins complexes involved in regulation of gene expression (Schafer and Schroer 1999). *Actin-related proteins* have been successfully used as normalizers for qPCR analysis, including those in which organisms are subjected to double-stranded RNA treatment (Han et al. 2019, Deng et al. 2020).

The *Hsp70* gene showed great variability of expression during the development but especially in the later stages in relation to dietary stress. These proteins have the function of reacting to stresses by preventing cell denaturation and this can be seen by the stability presented in the first instar, once first instars do not have feed. *Hsp70* are a conserved family of proteins that is expressed in different tissues, with no clear pattern of tissue-specific expression (Lindquist et al. 1988, Daugaard et al. 2007).

Actin is commonly used to normalize molecular expression studies due to its high conservation as an endogenous maintenance gene, although not a consensus for all insect

species (Ruan and Lai 2007, Majerowicz et al. 2011). In this study, the *Act* gene was considered the most stable expressed gene followed by *Ef-1* for studies involving the development of *E.heros*. For different tissues, RefFinder ranked the *18S rRNA* gene as the most stable, certainly by the regularity of the stability values obtained at different tissues in insects reared under two nutritional sources.

The present study evaluated the suitability of five genes as reference genes for RT-qPCR data normalization for *E. heros*, and indicate reference genes that are more stable for comparative gene expression analysis during growth of the insect, while others are more adequate when comparing different tissues of adults, when the insect is under different nutritional sources. This investigation is certainly a great starting point to guide future research in which RT-qPCR experiments are required with this species.

ACKNOWLEDGMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

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3. ALTERNATIVE APPROACHES OF RNAI DELIVERY FOR THE NEOTROPICAL BROWN STINKBUG *Euschistus heros* (F., 1798) (HEMIPTERA: PENTATOMIDAE)

ABSTRACT

The world population is growing steadily and food production must follow the increased demand for food supply. Pests remain the main obstacle for eliminating losses in food production in agricultural systems. Chemical control is still the main mechanism used to control pests that reduce the yield in plant production. However, the excessive use of this strategy of pest control can lead to the selection of resistant individuals and cause side-effects to non-target organisms and damage to the environment. The use of RNA interference (RNAi) appears as an alternative strategy of pest control. The technique is a tool that can be applied concurrently with other strategies of pest control, it is sustainable and promotes greater durability and efficiency of management tactics. There are still particularities of the functioning of the RNA interference machinery in several organisms that remain to be studied, such as target selection, off-target effects and efficient delivery of dsRNA, including insects. The Neotropical brown stink bug, *Euschistus heros* (F., 1798) (Hemiptera: Pentatomidae) is currently the major stinkbug pest species in Brazil due to its high damage potential to soybean, high population density, and wide geographic distribution. Additionally, the availability of a few chemistries for use in stinkbug pest control and the difficulties to control this pest, *E. heros* becomes a target of economic interest and a desirable model species for investigation. In this work, we report the genes involved in the RNAi pathway from an in-house transcriptome and selected four genes involved in the regulation of insect development and reproduction as targets of RNAi. We described the expression profiles of these genes in several tissues and during *E. heros* growth. We also tested different strategies for double-stranded RNA (dsRNA) of the selected genes to *E. heros*, and were able to obtain a mortality varying from 40 to 60% depending on the target gene selected and on the dsRNA delivery strategy used, with an interference in gene expression of up to 58%. The data obtained are promising for the development of RNAi as an applied strategy of *E. heros* control.

Key-words: biotechnology, food security, qPCR, pest control, resilient agriculture, sustainable agriculture

3.1 INTRODUCTION

Insect pests are one of the main threats to agricultural production, leading to significant losses by causing direct or indirect damage. The search for chemical molecules, biological control agents, biotechnologies for application as new strategies of pest control in integrated pest management programs are incessant. A major concern is the control of sucking pests because there is a reduced number of chemistries and alternative strategies available for their control, despite the huge economic losses the cause every year (Sosa-Gómez and da Silva 2010, Oliveira et al. 2014).

A recent technology that has being intensively studied as an applied strategy for pest control is the RNA interference (RNAi). RNAi is a natural post-transcriptional regulatory mechanism of plants and animals that regulates gene expression at the mRNA level for cell control and virus protection. RNAi was discovered randomly by Fire and Mello (1998), leading to their award with the Nobel Prize for Medicine in 2006. Since its discovery RNAi has been intensively studied in gene functional analysis, but highly believed as a strategy for targeted disease control in humans, but also for agricultural pest control (Fire et al. 1998, Pai et al. 2006, Carthew and Sontheimer 2009, Camargo et al. 2016, Rodrigues et al. 2017). In short, the process of RNAi begins when a double-strand of RNA is absorbed into the cells of the organism. The double strand of RNA is then cleaved by an endoribonuclease (Dicer) in small double strands of about 21 or 22 nucleotides, the siRNAs (Elbashir et al. 2001). The siRNA produced is trapped by a complex of proteins, the RNA induced silencing complex (RISC), that will act on the siRNA in order to select a single strand of the initial double stranded siRNA, and use this single strand as a template to guide the RISC complex in locating the complementary mRNA strand available in the cytoplasm. Once the target mRNA anneals with the complementary single-strand of the siRNA and the RISC complex, the mRNA can be subjected to enzymatic hydrolysis or have interfered the translation of the mRNA by ribosome machinery (Tijsterman and Plasterk 2004).

The variability in some physiological characteristics, feeding mechanisms, digestion or metabolic pathways may promote a greater or lesser sensitivity to the treatment with dsRNA in insects, requiring the selection of target genes and insect stage of development for each target insect pest species (Scott et al. 2013). Also, other factors such as the target mRNA, size of the dsRNA to be delivered, target region in the mRNA, dsRNA

stability and the strategy for dsRNA delivery can all influence the success, applicability and efficiency of RNA interference obtained (Scott et al. 2013, Cooper et al. 2019b).

The potential in using RNAi as a strategy for pest control led to the fast development of a commercial line by Monsanto, the first genetically modified maize cultivar with RNAi technology, SMARTSTAX PROTM was approved by EPA in June of 2017. The technology targeted *Diabrotica virgifera virgifera* (LeConte, 1868) (Coleoptera: Chrysomelidae) by expressing dsRNA to silence the *Snf7* gene (vacuolar protein) although efficient (Zhang et al. 2017), proved fast the need to a more thorough understanding of the processing of dsRNA within insects, once the first reports of resistance to the dsRNA expressed in SmartStax Pro was reported after laboratory selection less than a couple of years after its release for commercialization (Khajuria et al. 2018) In fact, earlier studies already pointed by mechanisms of resistance in insects to dsRNA (Vélez et al. 2016).

In Brazil, agriculture is the major activity for the generation of commercial revenue, and soybean exportation has a relevant contribution for the positive economic balance in the country (Song and Swinton 2009, Oliveira et al. 2014). This crop is under continuous expansion in Brazil with the opening of new agricultural frontiers due to the existence of programs for the selection and development of varieties adapted to the different soil and environment conditions in the country (Elmore et al. 2001, Li et al. 2004, Manavalan et al. 2009).

The production of soybean over extensive areas, the availability of productive varieties and the practice of a highly technified agriculture placed Brazil as the world largest soybean producer, with an estimated soybean output of over 123 million tons for the 2019-2020 season (USDA 2020). Insect pests can cause severe damage to soybean, and the soybean complex of stink bugs can reduce yield production in up to 96% (Souza et al. 2016).

The brown stinkbug *Euschistus heros* (F., 1798) (Hemiptera: Pentatomidae) is the major stinkbug pest occurring in soybean fields in Brazil (Souza et al. 2016). The expansion of its distribution range to Argentina and Paraguay in the last decade (Saluso et al. 2011), increased abundance and damage to cotton (Soria et al. 2017), and the requirement of adoption of sustainable practices have maintained this pest under surveillance.

The discovery of two lineages of *E. heros* (Soares et al. 2018), the identification of genes putatively involved in the process of natural selection (Zucchi et al. 2019), and the search for RNAi-based technologies for *E. heros* control (Fishilevich, Vélez, Storer, et al.

2016a, Castellanos et al. 2019b) will require further investigations for the characterization of the molecular mechanisms involved, the testing of the efficiency of selected dsRNAs and strategies for the effectively delivery of dsRNAs.

3.2 MATERIAL AND METHODS

Rearing and maintenance of *Euschistus heros*

Insects were kept under laboratory controlled conditions ($26 \pm 1^\circ\text{C}$; $60 \pm 10\%$ RH; and 14 h of photophase) in plastic cages (21x15x8cm) (Borges et al. 2006). Nymphs and adults were fed with a mixture of fresh green bean pods, *Phaseolus vulgaris* (L.), and raw peanut seeds, *Arachis hypogaea* (L.), *ad libitum*. Water was offered on moistened cotton pads. Pods, seeds and water were replaced every two days. Two pieces of raw cotton fabric were hung on the side of the adult cages serving as a substrate for oviposition. Eggs were collected twice a week and placed in smaller plastic boxes (6 x 12 x 12 cm) containing moistened cotton pads to avoid the desiccation of the eggs.

Search and identification of transcripts belonging to the RNAi pathway in *Euschistus heros*

The identification of transcripts of *E. heros* belonging to the RNAi pathway were identified from in house transcriptomes from different tissues of *E. heros*. Transcripts were identified after a blastn search against the nr database of the National Center for Bioinformatics (NCBI). Transcripts that were identified to belong to one of the genes reported to the complete RNAi pathway were selected for further manual annotation. The nucleotide sequence of such transcripts was selected, translated using the Translate tool available in Expasy at the Swiss Institute of Bioinformatics (SIB) (<https://web.expasy.org/translate/>). The protein sequences obtained were checked for the presence of conserved regions and specific domains in each of the isoforms identified in the transcriptome available, using the tools available in . These analyzes aimed at verifying barriers and difficulties in implementing the RNAi technology to control *E. heros*. RNases that act on degrading dsRNAs have been recently identified in Hemiptera, adding another mechanism that can interfere with the delivery of dsRNAs for the applied use of RNAi (Cagliari et al. 2020).

Choice of target genes and selection of dsRNAs

The choice of target genes of *E. heros* for silencing through the delivery of dsRNA was based on their role in insect growth and development. The sequence of transcripts from the target genes selected were recovered from our in house transcriptome, manually annotated as previously described, and subjected to the prediction and selection of candidate regions for interference using the siDirect tool (<http://sidirect2.rnai.jp>) for later dsRNA production (Sætrom and Snøve 2004, Arziman et al. 2005, Naito et al. 2005).

Synthesis of double-stranded RNA (dsRNA)

RNA extraction

Samples of nymphs and adults were used for RNA extraction. Samples were snap frozen and macerated in liquid nitrogen for the complete homogenization of their tissues using containers and materials treated with ethanol and RNase away TM Decontamination Reagent (Invitrogen TM). Aliquotes of the macerated tissues were then transferred to TrizolTM (Invitrogen TM) for tissue lysis. Afterwards, 200 µL of chloroform was added, the sample was vigorously mixed for 15 s, allowed to sit at room temperature for three min, and then centrifuged at 12,000 g for 15 minutes at 4 °C. The aqueous phase was collected, added with 500 µL of ice-cold isopropanol (100%), incubated for 10 min at room temperature, and centrifuged again (12,000 g x 10 min x 4 °C). The pellet obtained was washed with 1 mL of ice-cold 75% ethanol and recovered after centrifugation (7,500 g x 5 min x 4 °C). The pellet obtained was allowed to air dry in room temperature and was later recovered in 20 µL of RNase-free water. The quality and quantity of the total RNA obtained was verified by standard agarose gel electrophoresis containing 0.5 µg/mL ethidium bromide in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH = 8.2) at 90 V (Sambrook and Russel 2001), and reading of absorbance in an Epoch TM Microplate Spectrophotometer before being stored at -80 °C until further use.

cDNA synthesis

The reverse transcription reaction of the RNA samples was performed using 1 µL of the oligo-dT₁₅ primer available in the GoScriptTM RT System (Promega TM) kit, and a volume equivalent to 4.0 µg of total RNA, supplemented to 5 µL of free water of RNases. The samples were kept at 70 °C for 5 min and cooled to 4 °C in a thermal cycler. Then, 4

μL 5x Reaction buffer, 3.75 mM of MgCl₂, 1 μL reverse transcriptase, 0.5mM of dNTP and the required volume of RNase free water to complete 20 μL of the total reaction volume. The samples were cycled at 25 °C for 5 min, 42 °C for 60 min and 70 °C for 15 min in thermocycler, and stored at -20 °C for double-strand cDNA synthesis.

Polymerase chain reaction (PCR)

Sequences of each selected target gene were used for primer design, and primers were analyzed using the Oligo Analyzer (<https://www.idtdna.com/calc/analyzer>) (Table 2).

Table 2. Target genes selected for RNAi silencing experiments in *Euschistus heros*

Genes	Amplicon size (bp)	Primers
<i>Aquaporin</i>	300	F: ACGATGGGTTCTCGGACT R: GCTGGAAGACGGTGTAGAAG
<i>Brahma</i>	381	F: CGGGACAGTACATGTCTGA R: TAACGGTGGAGGCTTGTT
<i>Laccase 2</i>	505	F: AGCTCCACCAATATCTCAGAT R: ATCTCCACATCGTGGGAA
<i>V₀ domain of H⁺ vacuolar ATPase (v0-ATPase)</i>	464	F: CCTCTCTATTATCGCCGCA R: ACGTACAAGTGACCCTCA

PCR reactions were established following the GoTaq Flexi DNA Polymerase kit protocol (Promega™). We used 5 μl 5x Green GoTaq buffer, 1.5 μl MgCl₂ (25 mM), 0.5 μL dNTPs (10 mM), 0.16 μl of each of primer (10 μM), 0.125 μL of GoTaq DNA Polymerase, 1 μL of the single-strand cDNA synthesis reaction as template, and nuclease-free water to 25 μL reaction volume. Reactions were ran at 95 °C for 2 min (1 cycle); 95 °C for 45 s, *Ta* based on of each primer set for 45 s, 72 °C for 1 min (35 cycles); followed by 1 cycle at 72 °C for 5 min. PCR reactions were subjected to standard 1.5% agarose gel electrophoresis for verification of the successful amplicon amplification by verification of amplicons of the expected sizes (Sambrook and Russel 2001). Final confirmation of the successful amplification was obtained after purification and Sanger sequencing of the amplicons obtained. Ten-μL of each PCR reaction was then treated with exonuclease (0.33 μL of Exo-ExoI) and shrimp alkaline phosphatase (0.33 μL of SAP-Fastap) for elimination of nucleotide and primer residues, using a thermocycler set at 37 °C for 30 min followed by 15 min at 80 °C. PCR products were then subjected to bi-directional Sanger sequencing.

The sequencing quality was verified using FinchTV v1.4.0 (Geospiza Inc.), and the final sequences recovered were aligned against the transcript sequences available using the Mega7 TM software (Kumar et al., 2016).

Synthesis of dsRNA

Synthesis of dsRNA products required the 5'-end flanking to gene specific primers with the T7 polymerase promoter sequence (TAATACGACTCACTATAGGG) for their use in the amplification of ds-cDNA for each target gene as earlier described. The ds-cDNA obtained for each one of the selected target genes - *Aquaporin*, *Brahma*, *Laccase 2* and *v0-ATPase*, were once again subjected to sequencing as earlier described, and used in the synthesis of dsRNA using the T7 RibomaxTM Express Large-Scale RNA Production System kit (PromegaTM). The reaction contained 10 µL Ribomax T7 2x Buffer, 8 µL of ds-cDNA of the target gene equivalent to 1 µg of DNA, and 2 µL of Enzyme mix T7 express. The reaction mixture was gently mixed and incubated at 37 °C for 30 min. Afterwards, single strand RNA and dsDNA were eliminated by adding 1 µL of RNase and 1 µL of RQDNase followed by sample exposure at 37 °C for 30 min. Samples were then subjected to quantitation in a NanoDrop TM 2000/2000c Spectrophotometer (Thermo Scientific TM).

The synthesis of control dsRNA followed the same procedure using the green fluorescent protein gene (GFP) cloned in a plasmid and used in the transformation of *Escherichia coli* cells, which were available in the laboratory. Transformed *E. coli* colonies were selected from solid Luria Bertani (LB) medium (Sigma AlderichTM), and cultivated in LB broth for plasmid extraction using a standard procedure (Sambrook J and Russel 2012). The plasmid DNA obtained was recovered in TE buffer, quantified and stored at -20 °C. Plasmid DNA was subjected to PCR amplification using a set of primers adapted to T7 sequences as before. An amplicon of 400 base pairs was cut from the gel and the DNA was extracted using Ultrafree-DA Centrifugal Filter Unit (MilliporeTM), which was PCR amplified using the same conditions as before. The PCR product obtained was quantified and used in reactions for the synthesis of dsRNA using the T7 Ribomax kit, as earlier described.

Secondary structure analysis

The secondary structure of the mRNA can affect the annealing of the guiding siRNA associated with the RISC complex, leading to ineffective RNA interference. The analysis of the predicted secondary structure of the target mRNA helps in the identification of suitable candidate regions for targeted interference. Thus, sequences of the mRNA of the selected target genes of *E. heros* were subjected to analysis for prediction of their secondary structure using web-based tools (<http://rna.urmc.rochester.edu/RNAstructureWeb>), which result in the prediction of the 20 candidate secondary structures that can be formed with the least energy expenditure (Bellaousov et al. 2013). The predicted secondary structures were analyzed for verification of the structure of region targeted for interference, but we only represented the least energy expensive secondary structure of the mRNA for illustration.

Differential expression of target genes in different stages and tissues of *Euschistus heros*

Sample collection

Samples of the different stages of growth and development of *E. heros* were collected within 24 h of their eclosion (first instars) or of their molt to the instar/stage to be sampled. Three biological replicates were collected for each stage of development. Each biological replicate of first and second instars were represented by a pool of 10 nymphs; third and fourth instars by a pool of five nymphs; and fifth instar and adults by a pool of three specimens. Samples were snap frozen in liquid nitrogen and stored in -80°C for later RNA extraction.

For tissue analysis, adults of *E. heros* were collected within 24 h after molting and subjected to dissection under sterile conditions. Dissections were carried under a laminar flow hood after sterilization of all tools used with ethanol and RNase away™ Decontamination Reagent (Invitrogen™). Insects were dissected in sterile saline solution and samples of gut, ovaries and testicles were collected. A total of three biological replicates were collected (1 replicate = tissue from 5 insects) (Pawłowicz et al. 2012). Samples were snap frozen in liquid nitrogen and stored in -80°C for later RNA extraction.

RNA extraction, cDNA synthesis

Samples stored at -80°C were subjected to RNA extraction as previously described. Total RNA samples obtained were used in the synthesis of single-strand cDNA (sscDNA) using reverse transcriptase also as earlier described. Samples were stored at -80 °C until further use. A mock sample composed of a pool of subsamples from all instar and tissues was made and used in the evaluation of primer specificity and efficiency. This mock sscDNA sample was serially diluted (10^{-1} , 10^{-2} , 10^{-3} and 10^{-4}) and used in RT-qPCR amplifications with primers set for each target gene. The C_q values obtained were plotted against the sscDNA dilutions and subjected to linear regression using the tools available in an Applied Biosystems ViiA™7 Real-Time PCR system (Applied Biosystems, Foster city, CA). Amplification efficiencies for each gene were calculated according to the following equation: $E = 10^{(-1 / \text{slope})}$ (Pfaffl 2001). Primer specificity was analyzed by the verification of the melting curve. RT-qPCR analyses were run using technical triplicates for each biological sample.

Table 3. Primer sets used in RT-qPCR reactions for analysis of the expression profile of target genes of *Euschistus heros* selected for RNAi experiments

Genes	Amplicon size (bp)	Primers
<i>Aquaporin</i>	96	F:TCTTTGGTGGTTTTCTCGGA R:TGCTTACATCAGCATTGTC
<i>Brahma</i>	98	F: GTTAAAACGCCCAATGAAGC R: CACTGCCTTCCTCAGTCTCC
<i>Laccase 2</i>	63	F:GCCTTCCTCAAGGAGTTGTTCTT R:CATCATTCTTGGTTGGTTGCA
<i>v₀ domain of H⁺ vacuolar ATPase (v₀-ATPase)</i>	100	F:CCTCAGCTTGTGTGTCGTGT R:GGCCAAAACATGGAGATCA

Quantitative real-time PCR was performed using 5x HOT FIREPol EvaGreen™ qPCR Mix Plus (ROX) - Solis Biodyne on an Applied Biosystems ViiA™7 Real-Time PCR system (Applied Biosystems, Foster city, CA). The reactions were performed using 500 ng of cDNA template, 2 µL 5x EvaGreen™ qPCR mix, 160 pM of each primer (forward and reverse), and nuclease-free water up to complete the 10 µL final reaction volume. Samples were thermocycled at 95°C for 12 min (1x), followed by 40 cycles at 95°C for 15s, 60°C for 20s and 72°C for 20s. At the end of each PCR run, a melting curve

analysis from 65 to 95°C was applied to all reactions to ensure the specificity of the amplified product.

Bioassays

Effect of dsRNA treatment on *Euschistus heros* mortality

Biological assays to identify phenotypic effects in insects microinjected with dsRNA to target four selected genes of *E. heros* were conducted by microinjecting 1000 ng/μL of dsRNA diluted in 8% Lipofectamine 2000 Transfection Reagent (Thermo Fisher™) in a final volume of 0.6 μL. Borosilicate filament needles were made in the Flaming/Brown Micropipette Puller Model P-1000 (Sutter Instrument), and injections were carried out using the Pneumatic Picopump Model PV820 / PV830 (World Precision Instruments™). Microinjections were done at the abdomen of newly molted fourth instars. After injection, insects were kept under controlled laboratory conditions and reared as initially described. Insects microinjected with dsRNA of GFP and 0.8% NaCl saline solution were used as negative controls. Each treatment consisted of four biological replicates, and each biological replication represented a pool of 10 fourth instars, totaling 40 insects microinjected in each treatment. Mortality was assessed 7 days after microinjection, and data collected was subjected to analysis of variance (ANOVA), and averages compared against the control treatment using Bonferroni test ($p < 0.05$), using the XLSTAT software (Wu and Liang 2009).

Testing dsRNA delivery strategies - gene expression assessment

The efficiency of RNAi when using different strategies of dsRNA delivery was assessed by RT-qPCR. The following dsRNA delivery strategies were evaluated: microinjection, droplet feeding, nebulization, continuous feeding on wet cotton added of dsRNA solution and artificial diet feeding. dsRNA of the target mRNAs and the two negative controls (dsRNA of GFP and 0.8% saline water) were delivered to fourth instar of *E. heros* in a Lipofectamine 2000 Transfection Reagent (Thermo Fisher™) solution. Lipofectamine has been proven to make the reaction and action of dsRNA more efficient and long lasting in *E. heros* (Castellanos et al. 2019b).

Microinjections

Newly molted fourth instars of *E. heros* were injected with 0.6 μ L of a dsRNA solution containing 1 μ g dsRNA/ μ L at the abdomen. Borosilicate filament needles were made using a Flaming/Brown Micropipette Puller Model P-1000 (Sutter Instrument) equipment, and microinjections were carried out using the Pneumatic Picopump Model PV820 / PV830 device (World Precision InstrumentsTM). After injection, insects were maintained in rearing cages under controlled conditions and fed as earlier described. Three biological replicates of ten insects were collected 24h and 48h after being subjected to microinjection.

Drop feeding

Delivery of dsRNA through drop feeding assay was obtained by adding the dsRNA solution to a drop of 10% sucrose solution that was made available to insects. Fourth instar nymphs were fasted of food and water for 12 h, and then five nymphs were placed in a Petri dish containing two drops of 2 μ L of 10% sucrose solution containing 250ng/ μ L of dsRNA over a piece of ParafilmTM. The nymphs left feeding on the droplet. 30 minutes later they were fed with artificial diet. Three biological replicates of five insects were taken 24h and 48h after exposure to the droplets, were frozen in liquid nitrogen and stored in an ultra-freezer.

Nebulization

dsRNA delivery through nebulization was obtained by nebulizing 3 mL of nuclease-free water containing 5 μ g of dsRNA using a residential inhaler type nebulizer connected to a 10x10x15cm plastic cages without openings consolidating in our nebulization chamber. Nebulization was carried for about 40 min at 2 min intervals to avoid heating of the sample and condensation within the nebulization chamber in three biological samples containing 10 fourth instars. At the end of the nebulization, each biological sample were transferred to rearing cages and kept under controlled rearing conditions as earlier described. Three biological replicates were taken 24h and 48h after exposure to the nebulization, were frozen in liquid nitrogen and stored in an ultra-freezer.

Continuous feeding on wet cotton pads

This test was done using 10 µg of dsRNA, 2 µL of Lipofectamine™ in 1 mL of nuclease-free water that was offered in a 1.5mL vial tapped with small cotton pads to allow nymphs to feed on. Three biological replicates of ten insects were collected 24 h and 48 h after the setup of the experiment. Insects were collected and snap frozen in liquid nitrogen and stored in an ultra-freezer.

Delivery of dsRNA on an artificial diet

For the next assay, artificial diet (Mendoza et al. 2016) was diced in cubes of 0.5cm x 0.5cm x 0.5cm. Two cubes were placed in plastic Petri dishes with five nymphs on 4th instar. In this case, 2 µL of a solution containing 1000 ng dsRNA/µL in 8% Lipofectamine™ were applied to a cube of the dry artificial diet. The artificial diet was then offered to newly molted fourth instars within Petri dishes, and nymphs were allowed to feed. Three biological replicates of ten insects were collected 24h and 48h after start feeding on the artificial diet, frozen in liquid nitrogen and stored in an ultra-freezer.

Samples taken from all treatments were subjected to RNA extraction and sscDNA synthesis as earlier described. sscDNA was then used for the evaluation of gene expression of the target genes by quantitative RT-qPCR analysis, and the efficiency of RNAi was compared within each strategy of dsRNA delivery tested. Ct values were used to determine differences in gene expression using the Delta Delta Ct method (Livak and Schmittgen, 2001), using the *Ef-1* gene of *E. heros* as a reference gene for sample normalization. Nymphs treated with saline solution and GFP dsRNA were used as negative control. After determining the gene expression values, dsRNA treatments including GFP and target genes were compared individually with saline water control. The data were submitted to analysis of variance (ANOVA) and the mean values of the treatments were compared using the Bonferroni test ($p < 0.05$), using the XLSTAT statistical software (Wu and Liang 2009).

3.3 RESULTS

Search and identification of transcripts belonging to the RNAi pathway in *Euschistus heros*

We identified 161 transcripts belonging to 26 of genes that have been described to participate in the RNAi pathway by searching our in-house transcriptome library of *E. heros*. (Table 4). Complementing the first work with analysis of the *Euschistus heros* transcriptome, in this analysis it was possible to identify the SMG gene and the RISC-loading complex (Cagliari et al. 2020).

Table 4. Genes involved with the RNA interference machinery in *Euschistus heros*, identified after searching and manual annotation of transcripts available in an *in-house* transcriptome library.

General function	Gene	Number of Transcripts	Main specific domains
dsRNA uptake	<i>Scavenger</i>	15	DcpS; CD36
	<i>clathrin heavy chain (Chc)</i>	17	Clathrin; CLH
	<i>Epsin 2 (Epn2)</i>	4	ENTH
	<i>Gap junction protein (Innexin2)</i>	1	Innexin
miRNA	<i>Tarbp2</i>	13	DSRM; dsrm
	<i>Dicer</i>	8	RIBOc; Ribonuclease_3
	<i>Argonaute 3</i>	1	PAZ; Piwi
	<i>Exportin-5</i>	4	IBN_N
siRNA	<i>Dicer</i>	2	RIBOc; Ribonuclease_3
	<i>Argonaute 2</i>	9	PAZ_argonaute_like; Piwi
	<i>SMG</i>	11	PIN_Smg5-like; EST1_DNA_bind; EST1
piRNA	<i>Argonaute 3</i>	1	PAZ; Piwi
	<i>Aubergine</i>	6	PAZ; Piwi
	<i>Piwi</i>	1	Piwi
RISC complex	<i>Tudor-SN</i>	15	TUDOR
	<i>Translin</i>	3	Translin
	<i>maelstrom</i>	2	HMG; HMG-box
	<i>PRP16. mut6 homolog</i>	1	HA2
	<i>Clp1 homolog (kinase)</i>	8	CLP1_P
	<i>GLD-1 homolog</i>	4	NT_PAP_TUTase; PAP_assoc
	<i>Staufen</i>	7	DSRM; dsrm
	<i>Gawky</i>	2	RRM; UBA
	<i>Zinc finger ADRI</i>	1	DUF241
Nucleases	<i>Exoribonuclease 1 (Eri1)</i>	10	Glyco_trans_2_3
Antivirals	<i>Beta 1.4-mannosyltransferase (egh)</i>	1	BcsA
Intracellular transport	<i>Small Rab GTPases</i>	14	FYVE; FYVE_RABE_unchar

mRNA secondary structure analysis

Analysis of the secondary structure of target mRNAs for RNAi interference yielded structures that ranged in their complexity depending on the target mRNA analyzed, even when focusing on the structures that require the least amount of energy. mRNA secondary structure can be arranged in different ways (Luo and Chang 2004, Bellaousov et al. 2013), but the regions selected for RNAi in the sequence of the four targeted mRNAs when analyzing the 20 least energy expensive secondary structures were shown not to participate of hairpins or be part of local structures that could limit the annealing of the guiding siRNA and RISC complex (see Appendix).

Differential gene expression among stages and tissues

Primer efficiency and specificity

The primers set to amplify the *aquaporin* gene had an efficiency of 98% ($R^2 = 0.994$), *brahma* gene 103% ($R^2 = 0.991$), *laccase 2* 110% ($R^2 = 0.994$), and *v0-ATPase* e 105% ($R^2 = 0.991$). Melting curve analysis produced one single peak, proving their specificity in target amplification.

Quantitative reverse-transcribed real-time PCR (RT-qPCR)

Comparative analysis of gene expression of the selected target genes in the different instars and in adults of *E. heros* demonstrated the expression of *aquaporin* and *brahma* was reduced in the growth stages following the first instar (Figure 10). The expression levels of *aquaporin* from second to fifth instar was less than half of the expression observed in the first instar, with a slight recovery of the expression levels in the adult stage. *Brahma* also had a lower expression level after first instar, but with a completely different trend than that observed for *aquaporin*. In the case of *brahma*, the expression levels reduced progressively from first to fifth instar, demonstrating once again a slight recovery at the adult stage (Figure 10). *Laccase 2* and *v0-ATPase* had an expression profile opposed to that observed for *aquaporin* and *brahma*. In both cases, the expression of these genes progressively increased from first to fourth instar, which was followed by a slight decrease thereafter (Figure 10).

Figure 10. Fold change of *Aquaporin*, *Brahma*, *Laccase 2* and *v0-ATPase* genes during the development of *Euschistus heros*. Each gene is analyzed separately and using its expression in the first instar as a standard. The bars represent the standard errors.

Analysis of the expression of the selected genes in ovaries and testes when compared to the expression in the gut resulted in the observation of a lower expression of *aquaporin* in the ovaries, only half of the expression observed in the gut, but higher expression of *brahma* in the reproductive tissues. No differences in fold-change were detected in the expression levels of *Laccase 2* and *v0-ATPase* in the tissues analyzed (Figure 11).

Figure 11. Fold change of *Aquaporin*, *Brahma*, *Laccase 2* and *V0-ATPase* genes on tissues of *Euschistus heros*. Each gene is analyzed separately and using its expression in the gut as a standard. The bars represent the standard errors.

Bioassays

Effect of dsRNA treatment on *Euschistus heros* mortality

Mortality of microinjected fourth instars of *E. heros* with dsRNA of the selected target genes resulted in insect mortality that ranged from 40 to 60% after seven days of treatment. dsRNA microinjection induced significant mortality for three out of the four gene candidates selected. Microinjection of *v0 ATPase*-dsRNA to led the highest insect mortality, followed by *brahma*- and *laccase 2*-dsRNAs (Figure 12).

Figure 12. Mortality of *Euschistus heros* seven days after microinjection of fourth instars with saline (control), *GFP*, *aquaporin*, *brahma*, *laccase 2* and *v0-ATPase* dsRNAs. Asterisks indicate treatments were significantly different from the saline water as a control according to the Bonferroni test ($p < 0.05$).

Gene expression assay

The availability of each mRNA targeted for RNAi was dependent on the strategy used for the delivery of the dsRNA, but all targeted mRNAs had their abundance reduced in insects that were exposed to dsRNA in at least one of the strategies of delivery tested (Figure 12). dsRNA delivery through nebulization led to a reduction in the mRNA

availability of three out of the four target mRNAs selected, particularly 24h after dsRNA exposure. But all targeted mRNA had reduced levels when dsRNAs were microinjected. In general, mRNA abundance was recovered 48h after insect exposure to dsRNA (Figure 12). The highest reduction in mRNA abundance was observed for insects exposed to *aquaporin*-dsRNA by nebulization. In this case, *aquaporin* mRNA was close to zero 24 and 48 h after *aquaporin*-dsRNA exposure (Figure 12). Three of the targeted mRNAs (*v0-ATPase*, *brahma* and *aquaporin*) had their abundance increased when exposed to dsRNA, but the increased response in mRNA levels to dsRNA exposure depended on the method used for dsRNA delivery and the time after exposure the insect was analyzed. Very conspicuous increases in mRNA abundance were observed in insects treated with *aquaporin*-dsRNA and *v0-ATPase*-dsRNA, when dsRNA was respectively delivered through feeding on a cotton (*Aquaporin*) and nebulization (*v0-ATPase*) (Figure 12).

Aquaporin gene

Figure 13. Gene expression with 24h and 48h after delivery of GFP dsRNA solution and *Aquaporin*-dsRNA compared to saline water. The treatments are compared to the water-based treatment with expression equivalent to 1 (data not shown). Bars represent standard errors.

Table 5. ANOVA and Tukey test ($p < 0.05$) between delivery methods using *Aquaporin*-dsRNA

Delivery method	Means	Standard error	Inferior limit (95%)	Superior limit (95%)	Groups			
Nebulization	0.045	0.084	-0.123	0.213	A			

Microinjection	0.662	0.084	0.494	0.830		B		
Droplet feeding	0.857	0.084	0.689	1.025		B	C	
Artificial diet feeding	1.108	0.084	0.941	1.276			C	
Cotton feeding	1.468	0.084	1.300	1.636				D

Table 6. ANOVA and Tukey test ($p < 0.05$) between 24 and 48 hours analysis of all delivery methods of *Aquaporin-dsRNA*

Hours after delivery	Means	Standard error	Inferior limit (95%)	Superior limit (95%)	Groups	
24	0.712	0.053	0.606	0.819	A	
48	0.944	0.053	0.838	1.050		B

Brahma gene

Figure 14. Gene expression with 24h and 48h after delivery of GFP dsRNA solution and *Brahma*-dsRNA compared to saline water. The treatments are compared to the water-based treatment with expression equivalent to 1 (data not shown). Bars represent standard errors.

Table 7. ANOVA and Tukey test ($p < 0.05$) between delivery methods using *Brahma-dsRNA*

Delivery method	Means	Standard error	Inferior limit (95%)	Superior limit (95%)	Groups	
Nebulization	0.537	0.120	0.298	0.776	A	
Microinjection	0.577	0.120	0.337	0.816	A	
Droplet feeding	1.068	0.120	0.829	1.308		B
Artificial diet feeding	1.182	0.120	0.943	1.421		B

Cotton feeding	1.202	0.120	0.963	1.441		B
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Table 8. ANOVA and Tukey test ($p < 0.05$) between 24 and 48 hours analysis of all delivery methods of *Brahma-dsRNA*

Hours after delivery	Means	Standard error	Inferior limit (95%)	Superior limit (95%)	Groups	
24	0.792	0.076	0.641	0.943	A	
48	1.034	0.076	0.883	1.185		B

Laccase 2 gene

Figure 15. Gene expression with 24h and 48h after delivery of GFP dsRNA solution and *Laccase 2*-dsRNA compared to saline water. The treatments are compared to the water-based treatment with expression equivalent to 1 (data not shown). Bars represent standard errors.

Table 9. ANOVA and Tukey test ($p < 0.05$) between delivery methods using *Laccase-dsRNA*

Delivery method	Means	Standard error	Inferior limit (95%)	Superior limit (95%)	Groups		
Nebulization	0.375	0.036	0.303	0.447	A		
Microinjection	0.429	0.036	0.357	0.501	A		
Droplet feeding	0.726	0.036	0.654	0.799		B	
Artificial diet feeding	0.997	0.036	0.924	1.069			C
Cotton feeding	1.033	0.036	0.960	1.105			C

Table 10. ANOVA and Tukey test ($p < 0.05$) between 24 and 48 hours analysis of all delivery methods of *Laccase 2-dsRNA*

Hours after delivery	Means	Standard error	Inferior limit (95%)	Superior limit (95%)	Groups	
24	0.651	0.023	0.605	0.697	A	
48	0.773	0.023	0.727	0.818		B

V0-ATPase gene

Figure 16. Gene expression with 24h and 48h after delivery of GFP dsRNA solution and *V0-ATPase*-dsRNA compared to saline water. The treatments are compared to the water-based treatment with expression equivalent to 1 (data not shown). Bars represent standard errors.

Table 11. ANOVA and Tukey test ($p < 0.05$) between delivery methods using *V0-ATPase-dsRNA*

Delivery method	Means	Standard error	Inferior limit (95%)	Superior limit (95%)	Groups	
Nebulization	0.681	0.055	0.571	0.791	A	
Microinjection	0.724	0.062	0.601	0.847	A	
Droplet feeding	0.749	0.055	0.639	0.859	A	
Artificial diet feeding	0.877	0.055	0.767	0.987	A	
Cotton feeding	2.050	0.055	1.940	2.160		B

Table 12. ANOVA and Tukey test ($p < 0.05$) between 24 and 48 hours analysis of all delivery methods of *V0-ATPase-dsRNA*

Hours after delivery	Means	Standard error	Inferior limit (95%)	Superior limit (95%)	Groups	
24	0.762	0.037	0.689	0.835	A	
48	1.270	0.035	1.201	1.340		B

3.4 DISCUSSION

We demonstrated *E. heros* has a diverse repertoire of genes that participate in the machinery of RNA interference, indicating how complex the understanding of this system is for the development of rational strategies of control based on the exploitation of the RNAi process. We identified 26 genes involved in RNAi by exploring a transcriptome library available in our laboratory, increasing the number of known genes reported for this species, by adding the *SMG* and *tarbp2* genes (Cagliari *et al.* 2020).

SMG prevents mRNA translation by binding to the 3-untranslated region of target mRNAs acting as an important translation regulator (Fukuhara *et al.* 2005, Glavan *et al.* 2006, Jonas *et al.* 2013). *tarbp2* is a component of the RISC complex that acts on the processing of precursor RNAs to mature miRNAs for the posterior loading of the protein argonaute (Haase *et al.* 2005, Redfern *et al.* 2013). The high number of transcripts identified in the transcriptome of *E. heros* that participate in post-transcriptional regulation will be better understood once the genome of this species can be assessed for further analysis.

We also demonstrated the efficiency in RNAi in *E. heros* by delivering high amounts of dsRNA using different strategies can be very low even for target mRNA that have a secondary structure that is not limiting to the binding of the guiding strand of the siRNA and the RISC complex. Efficiency of dsRNA delivery systems is often considered a possible reason to explain the reduced efficacy on the activation and elimination of target mRNAs (Christiaens and Smagghe 2014, Joga *et al.* 2016). But this is commonly related with the participation of RNA degrading enzymes that are released in the hemolymph or into the lumen of the digestive system (Gu and Knipple 2013, Lomate and Bonning 2016, 2018). In *E. heros*, the presence of the enzymes Eri-1, Nibbler, SDN1, and dsRNase have been reported, however only dsRNase found with seven isoforms could certainly be linked to the RNAi pathway. DsRNA stability assays in hemolymph demonstrated an initial degradation with ten minutes of exposure, increasing up to 120 minutes and having a total degradation after two hours in contact with watery saliva (Castellanos *et al.* 2019). The participation of such enzymes in the degradation of the delivered dsRNA to *E. heros* would also explain the fast recovery observed in the levels of mRNA after 48 h of dsRNA exposure, even in cases in which a reduction in mRNA was obtained after 24 h of exposure.

The increased abundance in mRNA in insects exposed to aquaporin-dsRNA delivered by feeding on wet cotton pads, brahma-dsRNA by droplet feeding and v0-

ATPase-dsRNA by nebulization could represent a general compensatory response produced by the insect due to the degradation of mRNAs that are required for their proper functioning as suggested by others (Gu and Knipple 2013), even though we cannot argue on why such responses were only obtained when using particular strategies for dsRNA delivery.

However, we did see differences in the efficiency of gene silencing even when adopting strategies that avoid the delivery of dsRNA in the hemolymph or gut of *E. heros*, such as the delivery of dsRNA through nebulization.

Despite the fact that *E. heros* may carry active RNA-degrading enzymes in the hemolymph, we demonstrated that the delivery of dsRNA by microinjection successfully caused significant mortality when targeting mRNA from the *vATPase* (Fishilevich, Vélez, Storer, et al. 2016b, Basnet and Kamble 2018a, Sato et al. 2019), *laccase* (Matsumoto and Hattori 2016, Zhang et al. 2018) and *brahma* genes (Basnet and Kamble 2018b), even when low levels of interference were obtained, as observed in many different insects. We also had expectations that the interference with the abundance of *aquaporin*-mRNA would cause a great distress in water balancing in *E. heros*, and induce high mortality as reported to other species (Raza et al. 2016, Luo et al. 2017).

Aquaporin is an important protein involved in water movement during excretion, but aquaporin has also been shown to be a large family of proteins, probably playing different functions depending on structure and tissue localization (Spring et al. 2009, MingXing et al. 2017, Ishibashi et al. 2020). In the nematode *Caenorhabditis elegans* (Maupas, 1900) (Rhabditida: Rhabditidae), aquaporins were shown not to be required in the process of water regulation (Huang et al. 2007). Regardless the multiple roles aquaporins may have by acting as a membrane protein in insects, our data clearly demonstrated a very high effect in mRNA abundance was obtained by delivering dsRNA through nebulization. We cannot speculate on why this delivery strategy had such a strong effect in down-regulating *aquaporin* mRNA, but not the mRNA of the remaining target genes tested. Additionally, we cannot speculate if such level of regulation would have led insects to fail to complete their development or to present any altered phenotype, once nebulized insects were only subjected to gene expression analysis.

The tissue expression profile of the target genes was pretty much the same for *laccase* and *vATPase*, and changes in *aquaporin* and *brahma* were basically observed in the ovaries. The higher expression of *brahma* in the ovaries of *E. heros* corroborates to the importance of this gene to the ovaries, as RNAi experiments in which *brahma*-dsRNA were

delivered to *E. heros* and *D. virgifera* resulted in adults with underdeveloped ovaries and a strong reduction in female fecundity (Fishilevich, Vélez, Khajuria, et al. 2016). The expression of *aquaporin* in *E. heros* ovaries was half of the expression observed in the gut and in the tests. As discussed earlier, we cannot provide any explanation at this point for the observed pattern of expression due to the various functions aquaporins can have.

In conclusion, we were able to demonstrate that RNAi can be efficiently used to control *E. heros*, as we were able to obtain significant increase in mortality by microinjecting insects with dsRNA. Microinjection is a widely used strategy for dsRNA delivery, and despite the several issues that may affect the efficiency of dsRNA delivery by microinjection it has been a successful tool for gene function determination (Arakane et al. 2005, Yu et al. 2013, Fabrick et al. 2014, Lenaerts et al. 2017). Nevertheless, is far from being a suitable strategy for dsRNA for applied purposes of pest control. We also demonstrate dsRNA can be successfully delivered to *E. heros* by using alternative strategies, and efficiently impact the target mRNA abundance. These alternative strategies are promising, but additional studies must be further conducted as the efficiency to interfere with mRNA levels was affected by the target gene. Finally, we believe our data will serve as the basis for additional studies focused on the development of RNAi-based strategies for agricultural pest control.

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APPENDIX

Appendix A. Secondary structure of *Aquaporin* protein. Energy expenditure = -273 cal/mol. Yellow outlined represents the synthesized dsRNA, in red it represents the regions most conducive to silencing.

Appendix B. Secondary structure of *Brahma* protein. Energy expenditure = -532.4 cal/mol. Yellow outlined represents the synthesized dsRNA, in red it represents the regions most conducive to silencing.

Appendix C. Secondary structure of *Laccase 2* protein. Energy expenditure = - 829.4cal/mol. Yellow outlined represents the synthesized dsRNA, in red it represents the regions most conducive to silencing.

Appendix D. Secondary structure of *v0-ATPase* protein. Energy expenditure = -322.0 cal/mol. Yellow outlined represents the synthesized dsRNA, in red it represents the regions most conducive to silencing.