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COMBINING RNA INTERFERENCE AND BIOLOGICAL CONTROL FOR THE SUSTAINABLE CONTROL OF STINK BUGS IN BRAZILIAN SOYBEAN FIELDS

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Bioscience Engineering at the Faculty of Bioscience Engeneering of Ghent University, Belgium, with co-tutelagem with the Post-Graduate Program of the Federal University of Viçosa, Brazil.

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Co-advisers: Guy Smagghe
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Abstract

CASTELLANOS, Nathaly Lara, D.Sc., Universidade Federal de Viçosa and Ghent University, December, 2019. **Combining RNA interference and biological control for the sustainable control of stink bugs in Brazilian soybean fields**. Adviser: Eugênio Eduardo de Oliveira . Co-advisers: Guy Smagghe and Olivier Christiaens.

Soybean is the most commercialized agricultural product in Brazil, and accounts for an important share of its GDP. In recent years, the sustainability of soybean production has been threatened by the over-reliance on conventional pesticide sprays as the only tool for insect pest control. The Neotropical brown stink bug, *Euschistus heros*, is the main target of those insecticide applications and an important soybean pest in South America. For a transition towards sustainability of *E. heros* control, the development of alternative control strategies that can be easily integrated into Integrated Pest Management (IPM) programs are required. Therefore, the aim of this PhD dissertation was to investigate the potential for combining RNAi technology and biological control for the sustainable control of *E. heros* in Brazilian soybean fields. Insecticide resistance is one of the main concerns when using insecticides as a single management tool. To understand the risks and to determine possible mechanisms of resistance to neonicotinoids in *E. heros*, two imidacloprid resistant strains were selected under the laboratory conditions. The first strain derived from a laboratory susceptible strain, while the other derived from a field population diagnosed with control failure. Both strains achieved significant resistance levels, but the field derived strain required only six generations of selection in comparison to the 13 generations required by the laboratory-derived strain. The resistance to imidacloprid was linked with the enhanced activity of cytochrome P450 in both imidacloprid-resistant strains. But the higher activity of these enzymes was associated

with severe fitness costs that could delay the occurrence of imidacloprid-resistant strains if preventive management practices are implemented. Among sustainable control methods, biological control using the egg parasitoid *Telenomus podisi* has the potential not only to suppress *E. heros* populations, but also to delay the evolution of insecticide resistance. Development of rearing protocols that ensure the sustained quality and efficiency of released insect agents in field is an important factor for the success of biological control programs. Thus, we investigated whether a fluctuating thermal regime can be better for the mass rearing of *T. podisi* than the constant regime that is currently used. The fluctuating thermal regime was more suitable for mass-rearing than the constant regime, as demonstrated by the increased fitness and reduced estimated costs for production. These findings suggest that parasitoid wasps reared under fluctuating regime tolerate better fluctuating temperatures that normally occur both during transport and in agricultural systems, which will increase the quality and productivity of mass-reared *T. podisi* for inundative releases. The success of augmentative biological control programs using *T. podisi* against *E. heros* will depend on the integration with other sustainable management approaches, since it is difficult to control pest populations using biological control alone. In this regard, RNA interference (RNAi) offers a selective and environmentally safe alternative for the control of this pest. Injection of target genes encoding proteins that are essential for growth and development caused significant mortality and suppression of the target transcript. dsRNA was also administered by feeding the insects with the artificial diet containing different forms of dsRNA for delivery: naked dsRNA, dsRNA with EDTA and dsRNA encapsulated in liposomes. Oral delivery of naked dsRNA resulted in lower RNAi efficiency, evidencing no clear mortality scores probably due to dsRNA degradation by the salivary secretions of *E. heros*. Furthermore, the use of EDTA and lipid-based nanoparticles improve the oral delivery of dsRNA. These formulations could have a great potential for developing novel strategies for pest management of stink bugs based on RNAi preventing the dsRNA of being degraded enzymatically by the saliva. The successful integration of RNAi-based insecticides and biological control will depend on the lack of adverse effects of RNAi to the parasitoid *T. podisi*. Consequently, the potential routes of exposure and hazards were assessed. Parasitoid wasp can be exposed to RNAi-based insecticides directly via consumption of pollen and nectar in the

adult life or indirectly during the development inside host eggs. In addition, ingestion of specific dsRNA resulted in reduced fitness and target gene expression, demonstrating that *T. podisi* adults can be physiologically exposed to insecticidal dsRNA. No adverse effects were observed in the survival, development or parasitism of *T. podisi* treated with dsRNAs targeting *E. heros* genes. Moreover, the expression levels of potential off-target genes tested were not downregulated. Although there is still much work needed to translate the research outputs of this PhD thesis into practical IPM tools for soybean production. These data support the possibility of combining RNAi-based insecticides and biological control using the parasitoid *T. podisi* inside IPM programs to improve the sustainability of *E. heros* control in the future.

Keywords: *Euschistus heros*. *Telenomus podisi*. RNAi. Insecticide resistance. Fluctuating thermal regimes.

Resumo

CASTELLANOS, Nathaly Lara, D.Sc., Universidade Federal de Viçosa e Universidade de Ghent, dezembro de 2019. **Combinação de RNA de interferência e controle biológico para o controle sustentável de percevejos em cultivos de soja no Brasil**. Orientador: Eugênio Eduardo de Oliveira. Coorientadores: Guy Smagghe e Olivier Christiaens.

A soja é o produto agrícola mais comercializado do Brasil, e representa uma parte importante do seu PIB. Nos últimos anos, a sustentabilidade da produção de soja vem sendo ameaçada pela excessiva dependência no uso de pesticidas convencionais como única forma de controle de insetos pragas. O percevejo marrom da soja, *Euschistus heros*, é o maior alvo das aplicações desses inseticidas e uma importante praga em América do Sul. A transição para o controle sustentável de *E. heros* requer o desenvolvimento de estratégias de controle alternativas que possam ser facilmente integradas a programas de Manejo Integrado de Pragas (MIP). Portanto, o objetivo desta tese de doutorado foi pesquisar a potencial combinação de RNA de interferência e controle biológico para o controle sustentável de *E. heros* em cultivos de soja no Brasil. A resistência a inseticidas é uma das principais preocupações quando o controle químico é utilizado como única ferramenta de controle. Com o fim de compreender os riscos e determinar os possíveis mecanismos de resistência a neonicotinoides, duas populações resistentes ao imidaclopride foram selecionadas em condições de laboratório. A primeira população foi selecionada a partir de uma população suscetível de laboratório, enquanto a outra foi selecionada a partir de uma população de campo diagnosticada com falha em controle. As duas populações atingiram níveis consideráveis de resistência, mas para a população proveniente do campo foram necessárias apenas seis gerações em comparação

às 13 gerações da população proveniente do laboratório. A resistência ao imidaclopride esteve associada a um incremento na atividade de citocromos P450 nas duas populações resistentes. Porém, a maior atividade dessas enzimas foi acompanhada por um severo custo no desempenho que poderia retardar a ocorrência de populações resistentes a imidaclopride uma vez implementadas medidas preventivas. Dentre as alternativas de controle sustentáveis, o controle biológico usando os parasitoides de ovos *Telenemus podisi* não só tem o potencial de reduzir as populações de *E. heros*, mas também de atrasar a evolução de resistência. O desenvolvimento de protocolos de criação que garantam a constante qualidade e eficiência dos insetos liberados em campo é um fator importante para o sucesso de programas de controle biológico. Assim, estudou-se se regimes flutuantes de temperatura são mais apropriados para a criação em massa de *T. podisi* do que os regimes constantes que são utilizados atualmente. O regime flutuante de temperatura foi mais adequado para criação em massa de *T. podisi*, evidenciado pelo incremento no desempenho e pela redução nos custos estimados de produção. Esses achados sugerem que os parasitoides criados sob o regime flutuante toleram melhor as temperaturas flutuantes que normalmente ocorrem tanto em seu transporte como nos sistemas agrícolas, refletindo-se em um aumento da qualidade e produtividade de *T. podisi* criados em massa para liberações inundativas. O sucesso dos programas de controle biológico aumentativo usando *T. podisi* contra *E. heros* dependerá da integração a outras estratégias sustentáveis de manejo, devido à dificuldade de controlar populações de pragas usando unicamente controle biológico. Neste sentido, o RNA de interferência (RNAi) oferece uma alternativa seletiva e de baixo impacto ambiental para o controle desta praga. Injeção de genes-alvo que codificam proteínas necessárias ao crescimento e desenvolvimento causaram mortalidade significativa e supressão dos transcritos do gene-alvo. O dsRNA (RNA de fita dupla) também foi administrado aos insetos por via oral usando uma dieta artificial contendo diferentes formulações de dsRNA: dsRNA puro, dsRNA com EDTA e dsRNA encapsulado em lipossomos. A administração via oral de dsRNA puro resultou numa menor eficiência do RNAi, evidenciada pela baixa mortalidade como provável consequência da degradação de dsRNA pelas secreções salivares de *E. heros*. No entanto, o uso de EDTA e nanopartículas a base de lipídeos melhoraram a administração por via oral

do dsRNA. Estas formulações possuem grande potencial para o desenvolvimento de novas estratégias de controle de percevejos baseadas em RNAi, já que evitam que o dsRNA seja degradado enzimaticamente pela saliva. A integração bem-sucedida de inseticidas baseados no RNAi e o controle biológico dependerá da ausência de efeitos adversos ao RNAi no parasitoide *T. podisi*. Consequentemente, as rotas de exposição e potenciais riscos desta tecnologia foram avaliados. Os parasitoides podem ser expostos aos inseticidas baseados em RNAi diretamente pelo consumo de pólen e néctar na vida adulta ou indiretamente durante o desenvolvimento dentro dos ovos do hospedeiro. Adicionalmente, a ingestão de dsRNA específicos reduz o desempenho e a expressão do gene-alvo, demostrando que os adultos de *T. podisi* podem ser fisiologicamente expostos ao dsRNA com atividade inseticida. Não se observaram efeitos adversos na sobrevivência, desenvolvimento ou parasitismo de *T. podisi* quando tratados com dsRNA desenhados para atingir genes de *E. heros*. Além disso, os níveis de expressão dos potenciais genes não-alvo testados não foram diminuídos. No entanto, a adoção dos resultados desta tese de Doutorado como ferramentas em programas de Manejo Integrado de Pragas na cultura da soja requer ainda mais detalhamento e ensaios. Ainda assim, esses dados suportam a possibilidade de combinar inseticidas baseados em RNAi e controle biológico usando o parasitoide *T. podisi* dentro de programas de MIP para melhorar a sustentabilidade do controle de *E. heros* no futuro.

Palavras-chave: *Euschistus heros*. *Telenomus podisi*. RNAi. Resistência a inseticidas. Regimes flutuantes de temperatura.

Samenvatting

CASTELLANOS, Nathaly Lara, D.Sc., Universidade Federal de Viçosa en Universiteit Gent, december, 2019. **Combinatie van RNA interferentie en biologische bestrijding voor een duurzame controle van schildwantsen in soya in Brazilië**. Promotor: Eugênio Eduardo de Oliveira. Co-promotors: Guy Smagghe en Olivier Christiaens.

Soja is het meest gecommercialiseerde landbouwproduct in Brazilië en vertegenwoordigt een belangrijk aandeel in het bruto binnenlands product van het land. De duurzame productie van soja is de laatste jaren echter onder druk komen te staan door de afhankelijkheid van chemische gewasbeschermingsmiddelen als enige middel voor de bestrijding van plaaginsecten. The schildwants *Euschistus heros*, de belangrijkste plaag in soja in Zuid-Amerika, is het belangrijkste doelwit van deze insecticiden. Voor een transitie richting duurzame bestrijding van *E. heros* is er nood aan de ontwikkeling van alternatieve strategieën die makkelijk geïntegreerd kunnen worden in geïntegreerde gewasbeschermingsprogramma's (IPM programma's). Het doel van deze thesis is om in deze context te onderzoeken of de combinatie van RNAi technologie en biologische controle potentieel kan bijdragen tot de duurzame bestrijding van *E. heros* in Zuid-Amerikaanse sojavelden. Resistentie tegen insecticiden is één van de voornaamste bezorgdheden bij een sterke afhankelijkheid van insecticiden bij plaagbestrijding. Om de risico's te begrijpen en mogelijke resistentiemechanismen tegen neonicotinoïden in *E. heros* te bepalen werden twee imidacloprid-resistente stammen geselecteerd in het laboratorium. De eerste was afkomstig van een gevoelige stam in het laboratorium, terwijl de andere afkomstig was van een veldpopulatie waartegen het insecticide een verminderde werkzaamheid had. Beide stammen ontwikkelden significante resistentie, maar bij de veldstam was slechts een selectie over 6 generaties nodig vooraleer re-

sistentie werd bekomen, terwijl in de laboratoriumstam 13 generaties nodig waren. In beide stammen werd de resistentie tegen imidacloprid gelinkt met een verhoogde activiteit van cytochroom P450 enzymen. De hogere activiteit van deze enzymen was geassocieerd met een belangrijke fitness kost die het opduiken van imidacloprid-resistente stammen kan vertragen, indien preventieve maatregelen worden geïmplementeerd. Biologische bestrijding gebruik makende van de parasitaire wesp *Telenomus podisi*, is een voorbeeld van een duurzame bestrijdingsmethode met potentieel om niet alleen de *E. heros* populaties te onderdrukken, maar ook om de opkomst van insecticide resistentie te vertragen. De ontwikkeling van kweekprotocols die een constante kwaliteit en efficiëntie van uitgezette insecten garanderen, is belangrijk voor de slaagkansen van biologische bestrijdingsprogramma's. Daarom hebben we onderzocht of fluctuerende temperatuursomstandigheden beter kunnen zijn voor de massaproductie van *T. podisi* dan het constante regime dat tot op heden wordt gebruikt. Het fluctuerende regime bleek meer geschikt te zijn voor de massaproductie dan het constante regime, aangezien het leidde tot een hogere fitness en verminderde productiekosten. Deze bevindingen suggereren dat parasitaire wespen die onder een fluctuerend temperatuursregime worden gekweekt ook beter zijn in het tolereren van fluctuerende temperaturen die voorkomen tijdens transport en op het veld. Dit zal de kwaliteit en de productiviteit van de massakweek van *T. podisi* verhogen. Aangezien het moeilijk is om plaagpopulaties te bestrijden enkel gebruik makend van biologische controle zal het succes van deze strategie afhangen van de integratie met andere duurzame bestrijdingsmethoden. In dit opzicht kan RNA interferentie (RNAi) een soort-selectief en milieuvriendelijk alternatief bieden bij de bestrijding van deze plaag. Injectie van dsRNA dat specifiek de expressie moet verhinderen van genen die coderen voor eiwitten die essentieel zijn bij de groei en ontwikkeling van het insect leidde tot significante mortaliteit en succesvolle onderdrukking van het expressieniveau. Via een artificieel dieet werd ook dsRNA gevoed aan de insecten, in drie verschillende formuleringen: naakt dsRNA, dsRNA met EDTA en dsRNA die vervat zitten in liposomen. Orale toediening van naakt dsRNA resulteerde in een lagere RNAi efficiëntie en een gebrek aan mortaliteit. De oorzaak was waarschijnlijk degradatie van dsRNA door het speeksel van *E. heros*. Gebruik van EDTA en lipide-gebaseerde nanopartikels verbeterden orale toediening van dsRNA. Deze formulerin-

gen kunnen dsRNA beschermen tegen enzymatische afbraak door het speeksel en zouden zo een groot potentieel kunnen hebben in de verdere ontwikkeling van nieuwe op RNAi gebaseerde strategieën om wantsen te bestrijden. De succesvolle integratie van biologische controle en insecticiden gebaseerd op RNAi zal enkel mogelijk zijn indien negatieve effecten van RNAi op de parasitaire wesp *T. podisi* uitblijven. Daarom werden in dit onderzoek ook de mogelijke routes van blootstelling en gevaren geëvalueerd. Parasitaire wespen kunnen blootgesteld worden aan RNAi-insecticiden op een directe manier via de consumptie van pollen en nectar gedurende het volwassen levensstadium maar ook op een indirecte manier gedurende de larvale ontwikkeling binnenin de eitjes van de gastheer. Bovendien resulteerde de orale opname van dsRNA in een verminderde fitness en doelgen expressie, wat aantoonde dat *T. podisi* adulten fysiologisch blootgesteld kunnen worden aan insecticidaal dsRNA. Geen negatieve effecten werden geobserveerd op vlak van overleving, ontwikkeling of parasitisme-activiteit van *T. podisi* na toediening van dsRNA specifiek voor *E. heros* genen. Bovendien had de orale opname van dit dsRNA geen effect op de expressie van de meest homologe doelgenen in *T. podisi*. Hoewel er nog steeds veel werk is om de bevindingen in dit onderzoeksproject te vertalen naar de praktijk ondersteunen deze data wel de mogelijkheid om RNAi-plaagbestrijding te combineren met biologische controle door de parasitaire wesp *T. podisi* binnen een IPM programma en om zo een meer duurzame bestrijding van *E. heros* te verzekeren in de toekomst.

Trefwoorden: *Euschistus heros*. *Telenomus podisi*. RNAi. Resistentie tegen insecticiden. Fluctuerende temperatuursomstandigheden

List of abbreviations

A	*A. bipunctata*	*Adalia bipunctata*
	Ago	Argonaute
	A. mellifera	*Apis mellifera*
	A. pisum	*Acyrthosiphon pisum*
	a.i.	active ingredient
B	*B. cockerelly*	*Bactericera cockerelli*
	B. germica	*Blattella germanica*
	B. mori	*Bombyx mori*
	B. tabaci	*Bemisia tabaci*
	BLAST	Basic Local Alignment Search Tool
	bp	base pairs
	Bt	*Bacillus thuringiensis*
C	*C. brunneus*	*Cylas brunneus*
	C. puncticollis	*Cylas puncticollis*
	C. septempunctata	*Coccinella septempunctata*
	cDNA	complementary DNA
	CDNB	1-chloro-2,4-dinitrobenzene
	ChUP-1	CHolesterol UPtake associated
	CI	Confidence interval
	Cr	Copulation rate
	CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
	CYP	Cytochrome P450 enzymes
D	*D. melanoganster*	*Drosophila melanoganster*
	D. v. virgifera	*Diabrotica virgifera virgifera*
	DBLS	Diazoblue Laurylsulphate Solution
	Dcr	Dicer
	dsRNA	double stranded RNA
	DvSnf7	Diabrotica virgifera virgifera Snf7
E	*E. heros*	*Euschistus heros*
	Eh	*Euschistus heros* genes
	Er	Emergence rate
F	Fd	Fecundity
	Fr	Female ratio
G	G1	Generation 1
	G2	Generation 2
	G3	Generation 3
	G4	Generation 4

List of abbreviations

	GDP	Gross Domestic Products
	GR	Glyphosate-Resistant
	GST	Glutathione-S-Transferases
H	*H. halys*	*Halyomorpha halys*
	Ha	Hatchability
	hpRNA	Hairpin RNA
I	I	trend index
	ImiGoias	Field strain collected in Santo Antonio de Goias
	ImiLabSel	Imidacloprid-resistant strain derived from Laboratory strain
	ImiRes	Imidacloprid-resistant strain derived from field strain
	ImiSusc	Imidacloprid-susceptible Laboratory strain
	IPM	Integrated Pest Management
	IR	trend index of the resistant strain
	IRM	Insecticide Resistance Management
	IS	trend index of the susceptible strain
L	*L. decemlineata*	*Leptinotarsa decemlineata*
	L. lineoralis	*Lygus lineoralis*
	L. migratoria	*Locusta migratoria*
	LT_{50}	Lethal Time 50
	LC_{50}	Lethal Concentration 50
M	miRNA	micro RNA
	mRNA	messenger RNA
N	N0	Number of individuals in the initial population
	N. lugens	*Nilaparvata lugens*
	N. viridula	*Nezara viridula*
	N. vitripennis	*Nasonia vitripennis*
	nAChR	Nicotinic acetylcholine receptor
	NCBI	National Center for Biotechnology Information
	nt	nucleotides
	NTO	Non-Target Organisms
P	PCR	Polymerase Chain Reaction
	piRNA	piwi RNA
Q	qRT-PCR	Real Time quantitative PCR
R	*R. prolixus*	*Rhodnius prolixus*
	RdRP	RNA-dependent RNA polymerase
	RISC	RNAi silencing complex
	RNAi	RNA interference
	RR	Resistance Ratio
S	SID	Systemic RNA Interference Defective
	Sil	SID-1-like
	siRNA	small interfering RNA
	Sr1	Survival from neonate to 3rd instar
	Sr2	Survival from 3rd to 5th instar
T	*T. castaneum*	*Tribolium castaneum*
	T. podisi	*Telenomus podisi*
	TMBZ	3,3′,5,5′-tetramethylbenzidine
	Tp	*Telenomus podisi* genes

Scope and outline

In Brazil, soybean is the most economically important crop and a major export product constituting 11.8 % of the nation's total export (AGROSTAT 2019). For the season 2018/2019, 35.8 million hectares were destined for the cultivation of soybean in Brazil and soybean production was estimated at 114.8 million (CONAB 2019a). This production could be even higher if the losses caused by pests were minimized. One of the most devastating sources of yield losses on Brazilian soybean fields is the damage caused by the pentatomid stink bug complex, of which the Neotropical brown stink bug, *Euschistus heros*, is the most prevalent and relevant (Sosa-Gómez et al. 2009; Panizzi 2013; De Aquino et al. 2019). In Brazil, a recent survey indicated that stink bugs are responsible for US$ 600 million of yield loss each year, despite the adoption of control measures (Oliveira et al. 2014). At present, pest control of this stink bug is mostly done by conventional use of pesticides (Tuelher et al. 2018; Marques et al. 2019; Pazini et al. 2019). However, the restricted modes of action registered to control these insects and the indiscriminate use of this control practice, will certainly lead to substantial biodiversity losses in agricultural landscapes and selection for insecticide-resistant populations, which could in turn lead to resurgence and/or outbreaks of insect pests (Szczepaniec and Raupp 2013; Macfadyen et al. 2014; Quarcoo et al. 2014; Guedes and Cutler 2014; Guedes et al. 2017). Additionally, concerns regarding the environmental impact of these conventional pesticides are increasing worldwide. Accordingly, there is a need to develop better and more sustainable strategies to manage E. heros that drastically reduces the use of conventional pesticides in Brazilian soybean fields (Laumann et al. 2018).

Biological control, for example by egg parasitoids, has long been considered a potential alternative to pesticidal strategies for pest management of *E. heros* (Zerbino and Panizzi 2019). Among these egg parasitoids, *Telenomus podisi* is one of the most promising. It lays its eggs inside the eggs of stink bugs, eventually leading to the death of the stink bug embryo. *T. podisi* can reach parasitism rates of 83 % under natural conditions (Corrêa-Ferreira and Moscardi 1995; Pacheco and Corrêa-Ferreira 2006; Laumann et al. 2007). The success chances of implementing biological control practices will certainly be higher if it is integrated with other sustainable management approaches (Barzman et al. 2015; Lamichhane et al. 2017; Stenberg 2017). In this perspective, molecular methods such as RNA interference (RNAi) offer great potential as a pest control

tactic and could complement the biological control within an integrated pest management (IPM) context (Thakur et al. 2016). RNAi is a posttranscriptional gene silencing mechanism in eukaryotes which has shown potential for the control of insect pests (e.g. root worms, beetles, weevils) (Joga et al. 2016; Zhang et al. 2017; Zotti et al. 2018; Bally et al. 2018; Cagliari et al. 2019). Therefore, the main goal of this PhD thesis was to investigate the potential for combining RNAi technology and biological control for the sustainable control of *E. heros* in Brazilian soybean fields.

The first Chapter of this PhD thesis summarizes the current pest status and strategies being developed to manage *E. heros* (**Chapter 1**). Host plant species, economic damage and distribution are included. Furthermore, an introduction on RNAi and its potential as a crop protection strategy is also presented.

Chapters 2 to 5 report the research conducted from 2015 until 2019 within the framework of this PhD (Figure 1). Each of these chapters centers around different pest management methods: from the potential of insecticide resistance development (**Chapter 2**), strategies to enhance the production of the biological control agent *T. podisi* (**Chapter 3**)

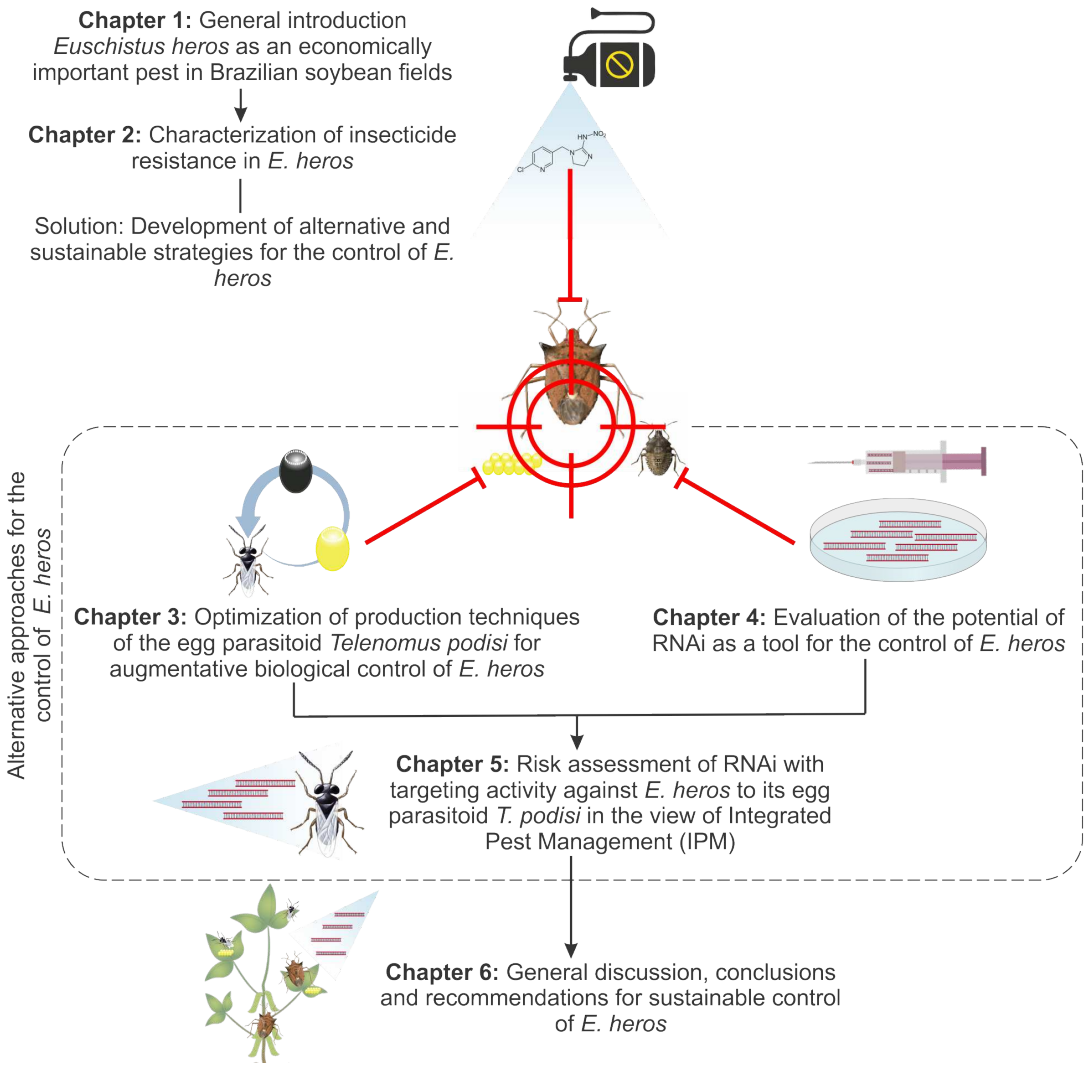


Figure 1: Schematic overview of the of the six Chapters of this PhD thesis

and the potential of using RNAi technology against *E. heros* (**Chapter 4**). In addition, we evaluated the potential risk of RNAi technology to the parasitoid wasp *T. podisi* (**Chapter 5**). Hence, these Chapters investigate two different alternatives for sustainable control of *E. heros* that can be used in combination inside IPM programs.

It is known that the sustainability of insecticides as a management tool against *E. heros* relies on the absence or scarcity of resistant populations. Therefore, **Chapter 2** explores the potential of *E. heros* to develop resistance to the neonicotinoid insecticide imidacloprid using laboratory selected strains with different genetic backgrounds. In order to have a further understanding of the process of resistance development, identification of possible biochemical mechanisms and fitness costs underlying imidacloprid resistance were also described.

Key findings in **Chapter 2** highlight the requirement for alternative management approaches to delay the occurrence of insecticide-resistant strains. Hence, **Chapter 3** examines whether fluctuating thermal regimes are more adequate for the mass-rearing of *T. podisi* than constant regimes used currently, aiming to increase performance under temperature variations experienced in field. To this end, first we measured the impact of the fluctuating regime on the life history to test the productivity of each regime. Then, we compared the thermal performance of parasitoid wasps that originated from constant and fluctuating regimes. Finally, we estimated the operative costs of production in each regime.

To evaluate whether RNAi technology can be implemented as an alternative strategy to control *E. heros*, **Chapter 4** examines the efficacy of RNAi using injection and oral delivery of double stranded RNA (dsRNA). Firstly, microinjection of dsRNA targeting genes encoding proteins that are essential for growth and development was performed to select potential target genes. After this selection, the most promising target genes were used in feeding bioassays to investigate the potential of RNAi as a control method against *E. heros*. Different formulations were tested in order to improve RNAi efficiency, including dsRNA encapsulated in liposomes and dsRNA mixed with the chelating agent EDTA.

In order to evaluate the possibility of combine RNAi-technology and biological control for the sustainable control of *E. heros*, risk assessment of RNAi to *T. podisi* is presented in **Chapter 5**. As risk comprises hazard and exposure, we first determined the susceptibility of *T. podisi* adults to dietary RNAi to verify if this parasitoid can be physiologically exposed to insecticidal dsRNA. Potential hazards to survival, parasitism, off-target gene silencing and immune stimulation were assessed in *T. podisi* adults after ingestion of high concentration of *E. heros* active dsRNAs. Furthermore, potential routes of exposure and possible developmental effects of insecticidal dsRNA were also investigated in *T. podisi* larvae.

Finally, **Chapter 6** discusses all obtained results and provides challenges and future recommendation for more sustainable management of these stink bug populations, in the context of integrated pest management in the Brazilian soybean fields.

Contents

Chapter 1

General introduction

1.1 Soybean

Soybean (*Glycine max*) is an economically very important leguminous seed crop, originating from China (Wilson 2008; Ali 2010; Orf 2016). Nowadays, soybean is the dominant oil-seed in world trade, accounting for about 59.5 % of global oilseed production (USDA 2019). It provides approximately 60 % of vegetable protein and 30 % of vegetable oil in the world (Ali 2010). Demand for soybean remains strong and continues to grow because it is used not only as an oil seed crop and feed for livestock and aquaculture, but also as a good source of protein for the human diet and as a biofuel feedstock (Wilson 2008; Masuda and Goldsmith 2009; Hartman et al. 2011). In 2017, the world's soybean production was estimated at 352.6 million tons and the area cropped with soybean was 130.9 million ha, this represents about 8 % of the world's arable land (Hartman et al. 2011; FAOSTAT 2019; USDA 2019). Currently, the predominant soybean producing countries are the United States, Brazil, and Argentina (USDA 2019). These three countries harvested 79 % of the world production in 2017 (Hartman et al. 2011; FAOSTAT 2019).

1.1.1 Soybean in Brazil

Soybeans are extensively grown in Brazil and are one of the most important agricultural commodities, accounting for an important part of its GDP (Raucci et al. 2015). The GDP of the soybean production chain reached US$ 31.6 billion in 2016, contributing significantly to the economic development of the soybean producing regions (CEPEA 2017). For the season 2018/2019, 61.7 million ha were destined for the cultivation of grains in Brazil and 58.0 % of this area was cropped with soybeans, producing a yield of around 114.8 million tons of the soybean pulses (CONAB 2019a). In 2019, Brazil exported 15.2 million tons of soy meal, almost 45 % of the total production, and 1.25 million tons of oil, about 15 % of the total volume (USDA 2019).

The more productive regions of soybeans in Brazil are currently the Midwest and South regions with a yield of 90.5 million tons (78.8 % of the total Brazilian production) (CONAB 2019a). Although soybean production started and developed in Southern Brazil, nowadays the Midwest region is responsible for 45.8 % of the Brazilian production (Cattelan and Dall'Agnol 2018; CONAB 2019a). Improved management practices, including cultivars adapted to low latitudes, allowed soybean farmers to expand into the Central region (Spehar 1995; Panizzi and Correa-Ferreira 1997), and more recently also into the North and Northeast (Bueno et al. 2017).

1.1.2 Challenges and treats to soybean production in Brazil

Given the tropical climate and the large cultivated area, Brazil faces a huge challenge to control the diseases, weeds and insect pests that affect its production fields (Godoy

et al. 2015; Marques et al. 2017; Cattelan and Dall'Agnol 2018). The set of distinct geographic regions places the country in a unique situation concerning pest management, especially considering the absence of the seasonal break provided by winter in temperate regions (Godoy et al. 2015).

Diseases

Among the diseases, Asian soybean rust caused by the fungus *Phakospora pachyrhizi* is the most aggressive disease of the crop and can cause yield losses of up to 80 % (Godoy et al. 2015, 2016). Soybean rust control has relied mainly on chemical control, with an average of three fungicide applications per soybean crop season (Godoy et al. 2016). Regulatory measures such as the prohibition of planting soybean during certain periods of the year and the use of early maturing varieties have also been adopted to manage the disease (Godoy et al. 2016; Furlan et al. 2018). Since fungicides are routinely applied for rust management, resistance has become an important issue for other foliar diseases such as target spot (*Corynespora cassiicola*) and anthracnose (*Colletotrichum truncatum*) (Wrather et al. 2010; Godoy et al. 2015).

Weeds

The species horseweed (*Conyza* spp), dayflower (*Commelina* spp.) and morning glory (*Ipomoea* spp.) are common weeds in Brazilian soybean crops (Vivian et al. 2013; Godoy et al. 2015; Lucio et al. 2019). The introduction of GR (glyphosate-resistant) transgenic soybean in 2005, contributed to standardization of weed management due to easy management and high efficiency in weed control (Vivian et al. 2013; Godoy et al. 2015). Nowadays, an area of 33.7 million ha is planted with GR transgenic soybean (97 % adoption rate) (ISAAA 2017). However, the large adoption of this technology has led to a rapid increase in the evolution of glyphosate-resistant weeds (Heap 2014; Lucio et al. 2019).

Insect pests

Soybean crops in Brazil can be affected by a wide range of insects. The most frequent insects requiring major care are defoliating insects from the Lepidoptera order, mainly from the family Noctuidae, and stink bugs (Hemiptera: Pentatomidae) (Panizzi and Correa-Ferreira 1997; Bueno et al. 2011a; Bortolotto et al. 2015). In addition, there is the occurrence of defoliating beetles (Chrysomelidae), and whitefly *Bemisia tabaci*, which can potentially cause economic damage to soybean (Bortolotto et al. 2015; Bueno et al. 2017). These insects cause direct damage (yield reduction) and indirect damage (reduce grain quality) to the soybean crop (Bueno et al. 2017). Financial losses caused by insects during crop season 2012/13 in Brazil were estimated at 5 % (US$ 1518.63 million), despite the adoption of control measures (83.67 million tons of insecticides with

the cost of US$ 1322.19 million) (Oliveira et al. 2014). In the past, the most important defoliating lepidopteran pests were the velvetbean caterpillar (*Anticarsia gemmatalis*) and the soybean looper (*Chrysodeixis includens*) (Bueno et al. 2011b; Moscardi et al. 2012). In an attempt to reduce the impact of those pests in soybean crops, the use of transgenic GR varieties expressing Cry proteins derived from the soil bacteria *Bacillus thuringiensis* (Bt) were adopted in 2013 (Bernardi et al. 2012, 2014a; Godoy et al. 2015; Marques et al. 2016, 2017). These toxins do not strongly affect all lepidopteran defoliators (Bernardi et al. 2014b; Marques et al. 2016, 2017), allowing a shift in the ecological dominance in Brazil during recent years. The armyworms of the genus *Spodoptera* are expanding the most (Bernardi et al. 2014b), but also the lesser cornstalk borer (*Elasmopalpus lignosellus*), the black cutworm (*Agrotis ipsilon*) and the cotton bollworm (*Helicoverpa armigera*) have significant potential to cause yield losses (Marques et al. 2017). Regarding the sucking insects, whitefly and stink bugs are not directly affected by the Bt toxins, and their importance has been steadily growing as pest species of soybean in recent decades (Panizzi 2013; Silva et al. 2014; Bueno et al. 2015). In Brazil, the main species of stink bugs attacking the soybean crop are the Neotropical brown stink bug (*Euschistus heros*), small green-spotted bug (*Piezodorus guildinii*), and green stink bug (*Nezara viridula*) (Corrêa-Ferreira and Azevedo 2002). Other species of the genus *Dichelops*, *Acrosternum*, *Edessa* and *Thyanta* may also occur (Panizzi et al. 2012). *N. viridula* was the most abundant stink bug species in the past, but currently, *E. heros* is considered the key pest (Sosa-Gómez et al. 2009; Panizzi 2013; Panizzi and Lucini 2016).

1.2 Neotropical brown stink bug, *E. heros*

E. heros' population density can reach 40-60 individuals m^{-1} by the end of the growing season, making this stink bug the main target of insecticides applications (Sosa-Gómez et al. 2009; Husch et al. 2018). It is a multivoltine species which can have two to six generations per year depending on the latitude (Cividanes and Parra 1994). In the laboratory, this species showed a mean development time of 38 days from eggs to adults (Figure 1.1), with an adult longevity of approximately 50 days (Costa et al. 1998). Adults are about 13 mm in length, mostly brownish in color, with the shield-shaped bodies and two horn-like lateral projections in the prothorax (Panizzi et al. 2012). When disturbed, they can secrete tiny drops of a foul-smelling liquid from the scent glands located on the metacoxae (Schaefer and Panizzi 2000). Females lay eggs on leaves, in batches of 6 to 15 (Panizzi et al. 2012). Like other Hemiptera, stink bugs show incomplete metamorphosis and go through 5 molts during their development (Panizzi et al. 2012). Upon emergence, nymphs are gregarious, remain on eggs and undergo the first ecdysis shortly after they start feeding (Panizzi et al. 2012; Souza et al. 2018). From the third instar on, nymphs become more active and disperse in the habitat (Panizzi et al. 2012; Souza et al. 2018). At this stage, their feeding activity becomes more intense,

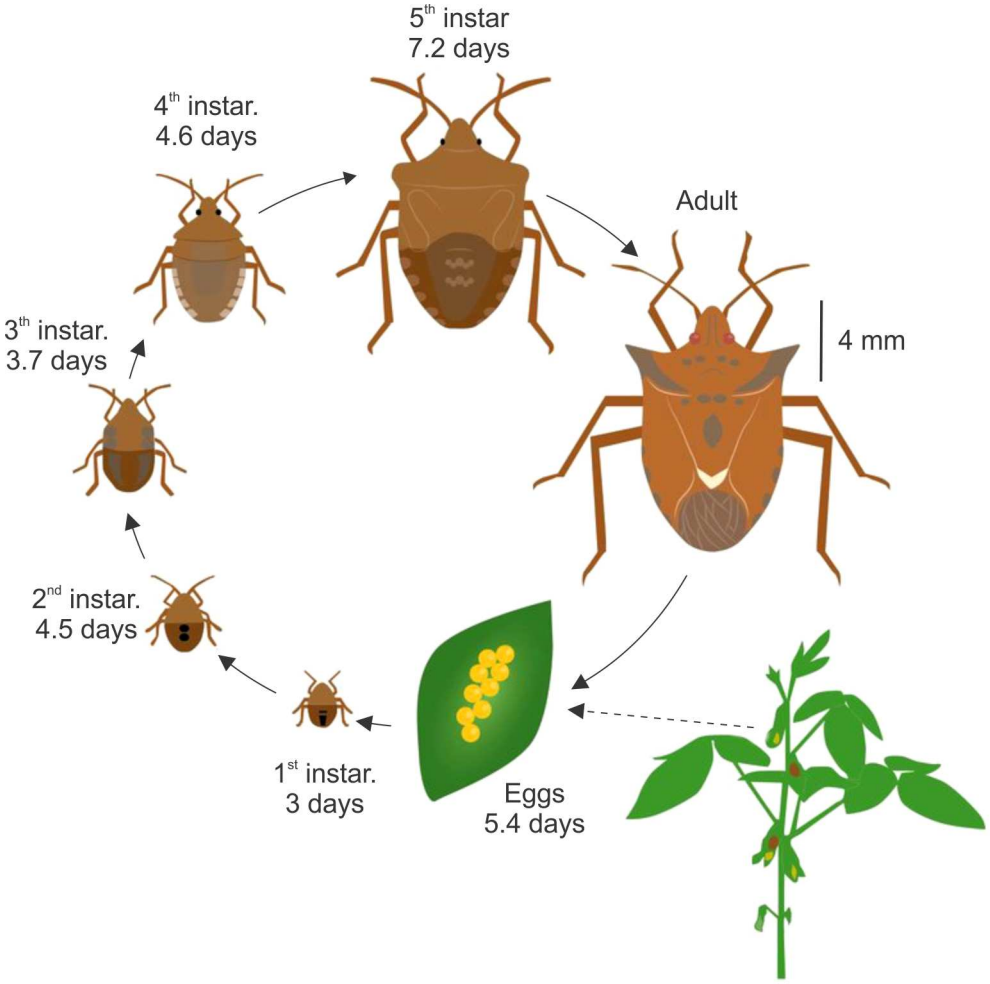


Figure 1.1: Life cycle *Euschistus heros*. Adapted from Panizzi et al. 2012

leading to damage (Panizzi et al. 2012; Bueno et al. 2013).

The relevance of *E. heros* has increased since the 1970's until recent years due to several factors; (i) expansion of area grown with soybean production to lower latitudes (North and Northeast) (CONAB 2019a); (ii) massive adoption of no-tillage cultivation (Panizzi 2013); (iii) introduction of sequential cultivation (multiple cropping), mainly soybean-corn intercrop common in 35.1 % of the area harvested with soybean (Bueno et al. 2017; CONAB 2019a); (iv) reduction of insecticide application in Bt soybean cultivars (Lüthi et al. 2015; Marques et al. 2019); (v) great adaptation to regions with high temperatures and high diversity of host plants (Cividanes and Parra 1994; Smaniotto and Panizzi 2015); (vi) higher desiccation tolerance (Panizzi and Hirose 1995); (vii) higher competitive ability (Tuelher et al. 2016; Panizzi and Lucini 2016); and (viii) reproductive diapause during the winter (Panizzi and Hirose 1995; Panizzi and Vivan 1997; Mourão and Panizzi 2002).

1.2.1 Distribution

E. heros is originally from the Neotropical region, with widespread distribution in South America (Figure 1.2) (Panizzi and Slansky 1985). Its estimated distribution includes several states of Brazil, Argentina and Paraguay (Saluso et al. 2011; Alves et al. 2014; Panizzi 2015; Ribeiro et al. 2016; Flores and Balbi 2018; Soares et al. 2018; Aquino et al. 2019). *E. heros* is predominant in regions with high average temperatures, living in markedly different environments including the Amazon Forest, Cerrado, Caatinga and Atlantic Forest (Cividanes and Parra 1994; Soares et al. 2018). *E. heros* distribution has expanded in the last years, raising concerns regarding a possible colonization of other areas in South, Central and North America (Panizzi 2015).

Figure 1.2: Estimated distribution of *Euschistus heros* in South America: Sources: Argentina (Saluso et al. 2011; Flores and Balbi 2018), Brazil (Alves et al. 2014; Panizzi 2015; Ribeiro et al. 2016; Soares et al. 2018; Aquino et al. 2019) and Paraguay (Soares et al. 2018).

1.2.2 Damage

E. heros is found among soybeans from the vegetative phase to the reproductive phase of soybean growth and damage pod formation at the onset of ripening (Nunes and Corrêa-Ferreira 2002; Borges et al. 2011b). Feeding injury caused by these insects affects seed production, quality, and germination potential in soybean plants (Depieri and Panizzi 2011). Both adults and nymphs feed on the developing stages of the soybeans, but the greatest yield impact is caused by the third, fourth and fifth instars and adults (Bueno et al. 2013). The damage caused by *E. heros* can reach 30 % of yield losses when no control measures are adopted (Degrande and Vivan 2012).

E. heros damages the soybean by inserting its mouthparts into the pods, injecting saliva that contains digestive enzymes and sucking-in the mixture of sap and dissolving cells (Depieri and Panizzi 2011; Silva et al. 2012). The intensity of the damage caused by stink bugs is directly related to their population level and to the plant development stage (Corrêa-Ferreira and Azevedo 2002). Stink bugs begin soybean field colonization at the end of the vegetative phase or during flowering, their populations increase from pod development reaching the highest population densities at the end of pod filling (Nunes and Corrêa-Ferreira 2002; Borges et al. 2011b). Feeding during pod development can result in dry and wrinkled pods, severely shrunken seeds and even unfilled pods (Degrande and Vivan 2012; Bueno et al. 2013). When the damage takes place during the pod filling, attacked seeds decrease in size, appear corrugated, and dark in color; show lower oil content and greater protein content (Corrêa-Ferreira and Azevedo 2002; Depieri and Panizzi 2011). Feeding punctures of stink bugs can also facilitate the contamination by plant pathogens such as the fungus *Nematospora coryli*, a causative organism of the yeast spot diseases (Corrêa-Ferreira and Azevedo 2002). Moreover, stink bug feeding may even cause physiological disturbances, like foliar retention and delayed seed maturation (Oliveira et al. 2006; Depieri and Panizzi 2011; Peiffer and Felton 2014).

1.2.3 Host plants

The polyphagous pest, *E. heros*, feeds on a wide variety of cultivated and non-cultivated plants (Table 1.1) (Panizzi and Vivan 1997; Panizzi et al. 2012; Smaniotto and Panizzi 2015). There are various records of the occurrence of *E. heros* on cotton (*Gossypium hirsutum*), corn (*Zea mays*) and sunflower (*Helianthus annuus*), despite the fact that developmental and reproduction rates are reduced in these plant in comparison to soybean (Malaguido and Panizzi 1999; Soria 2010; Borges et al. 2011b; Azambuja et al. 2013). On cotton, in particular, *E. heros* is becoming an important pest in Brazil (Soria 2010; Azambuja et al. 2013; Soria et al. 2017; Weber et al. 2018). Infestation of this stink bug can result in losses in the production of seed cotton and fiber of 18 and 25 %, respectively (Soria et al. 2017). The increasing population of *E. heros* in cotton fields is

Table 1.1: Cultivated and non-cultivated plants associated with *Euschistus heros*. Adapted from Smaniotto and Panizzi 2015

Family	Host plant	Reproductive hosts*	Reference
Amaranthaceae	*Amaranthus retroflexus*	—	Medeiros and Megier 2009
Asteraceae	*Acanthospermum hispidum*	—	Mourão andPanizzi 2002
	Helianthus annuus	—	Malaguido and Panizzi 1999
Brassicaceae	*Brassica napus*	—	Link and Grazia 1987
	Brassica oleraceae	—	Link and Grazia 1987
Euphorbiaceae	*Euphorbia heterophylla*	X	Panizzi et al. 1988
Fabaceae	*Aeschynomene rudis*	X	Link 1979
	Glycine max	X	Panizzi et al. 1977
	Lupinus albus	X	Link and Grazia 1987
	Lupinus angustifolius	—	Link and Grazia 1987
	Phaseolus vulgaris	X	Link and Grazia 1987
	Pisum sativum	X	Link and Grazia 1987
Lauraceae	*Nectandria* sp.	—	Corrêa-Ferreira and Panizzi 1982
Malpighiaceae	*Malpighia glabra*	—	Albuquerque et al. 2008
Malvaceae	*Gossypium hirsutum*	—	Soria 2010
Ranunculaceae	*Clematis dioica*	—	Corrêa-Ferreira and Panizzi 1982
Poaceae	*Sorghum bicolor*	—	Borges et al. 2011b
	Zea mays	—	Borges et al. 2011b
Salicaceae	*Casearia sylvestris*	—	Costa et al. 1999
Solanaceae	*Nicotiana tabacum*	—	Link and Grazia 1987
	Solanum mauritianum	—	Medeiros and Megier 2009
	Solanum megalochiton	—	Medeiros and Megier 2009
	Vassobia breviflora	—	Medeiros and Megier 2009

*Plants on which *E. heros* can complete development

probably caused by the fact that soybean and cotton are grown during the same season in neighboring fields, with cotton remaining in the field for longer periods (Azambuja et al. 2013; Soria et al. 2017; Weber et al. 2018).

Alternative host plants provide nutritional resources and shelter for *E. heros* during periods when soybean is unavailable in time or space, or both (Smaniotto and Panizzi 2015). When temperature and photoperiod decrease, *E. heros* adults enter in diapause, remaining on the soil under culture debris and leaf litter, without feeding and reproducing, coming out to breed on soybean plants in the following season (Panizzi and Hirose 1995; Panizzi and Vivan 1997; Mourão and Panizzi 2002). However, diapause phenomenon is not observed in lower latitudes (Central, North and Northeast Brazil) due to the higher temperatures, increasing the importance of alternative host plants (Borges et al. 2011b).

1.2.4 Strategies to control *E. heros*

Management programs for stink bugs in soybean in Brazil have relied primarily on repeated insecticide applications (Tuelher et al. 2018; Marques et al. 2019; Pazini et al. 2019). Most of the chemical applications against stink bugs target *E. heros* (Sosa-Gómez and Silva 2010). The management of this pest is a complex and difficult task because they cause economical damage at low population levels, and have intrinsic tol-

erance to the commonly used insecticides (Zerbino and Panizzi 2019). Broad-spectrum neurotoxic insecticides such as pyrethroids and organophosphates, as well as the less toxic neonicotinoids, are used against *E. heros* (AGROFIT 2019). However, the investigation of alternative methods is needed to develop efficient and sustainable control techniques to decrease and replace the use of insecticides (Laumann et al. 2018).

Among alternative control methods, biological control agents are potentially very useful in soybean integrated pest management programs (Zerbino and Panizzi 2019). Parasitoid wasps of the Scelionidae family, especially *Telenomus* spp. and *Trissolcus* spp., are very effective in controlling soybean stink bugs (Golin et al. 2011; Silva et al. 2014; Bueno et al. 2017). These parasitoids lay their eggs inside the eggs of stink bugs, killing the embryo (Bueno et al. 2012; Panizzi 2013). In the 1990s, successful biological control programs have been developed and implemented in Brazil, mainly in small areas dedicated to the production of organic soybean (Corrêa-Ferreira and Moscardi 1996; Corrêa-Ferreira and Peres 2003). For instance, inoculative releases of *Trissolcus basalis* proved to be efficient for the control of the green stink bug *N. viridula* in the northern areas of Paraná State, Brazil (Corrêa-Ferreira and Moscardi 1996). However, the tremendous increase in the cultivated areas, the reduction of cost of conventional insecticides and the low availability of these parasitoids have resulted in the abandonment of the biological control programs (Panizzi 2013; Parra 2014; Zerbino and Panizzi 2019).

Many studies on alternative strategies for its control are available, which include plant varieties that exhibit insect resistance (Souza et al. 2014, 2017; Rocha et al. 2014; Pinheiro et al. 2017), treatments to induce plant resistance (Vieira et al. 2013; Souza et al. 2014), pheromone traps for monitoring and controlling pests (Borges et al. 2011b, a), and even microbial control (Sosa-Gómez and Moscardi 1998; Sosa-Gómez 2017). Prominent among these is the use of resistant plant genotypes, offering a series of benefits from environmental and economical standpoints (Rocha et al. 2014; Pinheiro et al. 2017; Souza et al. 2017). Currently, in Brazil, few insect resistant cultivars are recommended for cultivation, including IAC-100, IAC-17, IAC-23 and IAC-24 (Veiga et al. 1999; Miranda et al. 2003a, b; Godoi et al. 2007; Pinheiro et al. 2017). However, these cultivars are only adapted to the Southeast region and are already obsolete in terms of grain yield (Pinheiro et al. 2017).

1.3 Chemical control

The effective management of insect pest in most of the world's agriculture and horticulture is dependent on conventional insecticides (Casida and Durkin 2013; Gupta et al. 2019; Nauen et al. 2019). The use of these substances in agroecosystems has enabled farmers to increase the yields of food and other agricultural products, since the use of insecticides reduces 39 % crop losses due to insects (Oerke 2006; Macfadyen et

al. 2014). In 2015, the worldwide use of insecticides was about 114.1 million tons and sales amounted to US $ 2.51 billion dollars (Casida and Bryant 2017).

As mentioned in the previous section, *E. heros* management is still heavily dependent on insecticides. Until 2004, this species was managed exclusively with chemicals, mainly organophosphates and the cyclodiene organochlorine endosulfan (Sosa-Gómez and Silva 2010; Panizzi et al. 2012). Since the early 1990s, control failures with endosulfan have been observed on neotropical brown stink bug populations, probably caused by formulation problems (Sosa-Gómez and Omoto 2012). In 1999, decreased susceptibility of *E. heros* to the organophosphate methamidophos (Sosa-Gomez et al. 2001) was found in the State of São Paulo; and more recently a higher number of resistant genotypes was observed in the State of Paraná (Sosa-Gómez and Silva 2010). Currently, the use of some organophosphates and endosulfan is banned (Sosa-Gómez and Omoto 2012; Rocha et al. 2014) and neonicotinoids and pyrethroids have become the most common insecticides used to control the *E. heros* (Sosa-Gómez and Silva 2010; Hegeto et al. 2015). In total, 47 commercial products and 16 active ingredients are registered with the Brazilian Ministry of agriculture, including eight pyrethroids, four organophosphates, three neonicotinoids and one carbamate (Table 1.2) (AGROFIT 2019). Most

Table 1.2: Active ingredients registered with the Brazilian Ministry of agriculture for the control of *Euschistus heros*. Source: AGROFIT 2019

Group	**Active ingredient**	**Products registered**	
		Isolated	Mixture
Carbamates	Carbosulfan		1
Neonicotinoids	Acetamiprid	1	4
	Imidacloprid	1	2
	Thiamethoxam		8
Organophosphates	Acephate	19	
	Chlorpyrifos	2	
	Fenitrothion	1	1
	Malathion	1	
Pyrethroids	Alpha-cypermethrin		2
	Beta-cyfluthrin		1
	Bifenthrin		5
	Cypermethrin	2	4
	Esfenvalerate		1
	Fenpropathrin		1
	Lambda-cyhalothrin	1	3
	Zeta-cypermethrin	1	
Mixtures			
Neonicotinoid + Pyrethroid	Acetamiprid + Alpha-cypermethrin		2
	Acetamiprid + Bifenthrin		1
	Acetamiprid + Fenpropathrin		1
	Imidacloprid + Beta-Cyfluthrin		1
	Imidacloprid +Bifenthrin		1
	Thiamethoxam + Cypermethrin		2
	Thiamethoxam + Lambda-cyhalothrin		3
Pyrethroid + Carbamate	bifenthrin + carbosulfan		1
Pyrethroid + Organophosphate	Fenpropathrin + Fenitrothion		1
Pyrethroids	Bifenthrin + Cypermethrin		2
Neonicotinoid + Fungicides	Thiamethoxam + Cyproconazole		3

neonicotinoids and pyrethroids registered are often used as commercial mixture formulations (Marques et al. 2019; Pazini et al. 2019). These mixtures allow broader spectrum of action, targeting different toxicological sites of the pest (Larson et al. 2014; Gazziero 2015).

1.3.1 Effectiveness of insecticides against *E. heros*

The mixtures of neonicotinoids and pyrethroids are the most efficient products for the control of *E. heros*, not only in terms of control efficiency but also in productivity and reduction of seed damage (Roggia et al. 2018; Marques et al. 2019; Pazini et al. 2019). The highest control efficiency was obtained with the combination of lambda-cyhalothrin + thiamethoxam (Roggia et al. 2018; Marques et al. 2019; Pazini et al. 2019). Other mixtures as lambda-cyhalothrin + imidacloprid, bifenthrin + imidacloprid, fenpropathrin + acetamiprid were also highly effective for the control of *E. heros* (Roggia et al. 2018; Pazini et al. 2019).

Although less efficient than mixtures, most of isolated active ingredients used for the control of this stink bug surpasses the minimum efficacy threshold of 80 % required by Brazilian regulations (Santos et al. 2015; Pitta et al. 2018; Roggia et al. 2018; Marques et al. 2019; Pazini et al. 2019). However, isolated insecticides showed different performances depending on the collection site (Pitta et al. 2018; Roggia et al. 2018; Tuelher et al. 2018; Marques et al. 2019; Pazini et al. 2019). For instance, the neonicotinoid with the highest control efficiency was thiamethoxan in stink bugs collected in Mato Grosso (Pitta et al. 2018), but have the lower efficiency in insects collected in Rio Grande do Sul (Marques et al. 2019). This could indicate possible differences in the susceptibility to insecticides in the soybean-producing regions in Brazil. An area-wide spatial survey of the likelihood of insecticide control failure among populations of *E. heros* in Goias can support this hypothesis (Tuelher et al. 2018). In this study, small but significant likelihood of control failure was observed for the insecticides beta-cyfluthrin in the southwest region and imidacloprid in the northeast regions of the state (Tuelher et al. 2018). Such a finding indicates that control failure to both compounds is uncorrelated, and is probably caused by differences in insecticides use pattern (Tuelher et al. 2018).

1.3.2 Overuse of insecticides

While chemical control of *E. heros* continues to be effective, high frequency of sprays and high doses of insecticides are currently used by the soybean growers, compromising the sustainability of this practice (Panizzi 2013; Bueno et al. 2013, 2015; Bortolotto et al. 2015). Insecticide applications have reached an average of four to six sprays per crop cycle in recent years (Panizzi 2013; Bueno et al. 2013, 2015; Bortolotto et al. 2015). The increased use of insecticides on soybeans is mainly a consequence of the use of preventive (prophylactic) applications based on the calendar, without performing

sampling of the pest density (Panizzi 2013; Bueno et al. 2013, 2015; Bortolotto et al. 2015). It is important to highlight that those preventive applications do not increase yield, seed quality or the net income, generally raise the production costs and increase the problems associated with insecticide use (Bueno et al. 2015).

Moreover, in an attempt to reduce the operational cost of application of pesticides, many farmers started to use insecticides mixed with herbicides at the post-emergence or fungicides at the beginning of the crop development (Bortolotto et al. 2015; Bueno et al. 2015). However, this is not necessarily the best timing since insects, pathogens and/or weeds do not occur simultaneously, and insecticide application before the reproductive period have intensified problems of suppression of natural enemies and selection pressure on stink bug populations (Sosa-Gómez and Silva 2010; Bortolotto et al. 2015).

1.3.3 Risks of insecticides

Unlike highly toxic insecticides used before 1970s, the insecticides used currently are more selective and less persistent due to requirements for improved environmental and toxicological profiles (Sparks 2013). However, the overuse of these substances can still cause diverse adverse effects including: (i) high insecticide loads in the environment (Weston et al. 2005; Hladik et al. 2018; Kasambala Donga and Eklo 2018) and on farm products (Quarcoo et al. 2014; Chang et al. 2018); (ii) biodiversity losses in agricultural and natural landscapes (Relyea 2005; Geiger et al. 2010; Macfadyen et al. 2014; Stehle and Schulz 2015), this decline in the biodiversity can also affect the delivery of ecosystem services such as biological pest control (Roubos et al. 2014; Macfadyen et al. 2015; Hill et al. 2017) and crop pollination (Barbosa et al. 2015; Straub et al. 2016; Woodcock et al. 2016); (iii) human health problems associated with exposure to insecticides (Kolaczinski and Curtis 2004; Han et al. 2018; Zhang et al. 2018); (iv) resurgence and secondary pests outbreaks (Szczepaniec and Raupp 2013; Guedes and Cutler 2014; Hill et al. 2017); and (v) selection for insecticide-resistant populations (Feyereisen et al. 2015; Sparks and Nauen 2015; Nauen et al. 2019). These adverse effects not only compromise the sustainability of chemical control, but also its profitability due to the hidden cost associated with the impacts on human health and the environment (Bourguet and Guillemaud 2016).

1.3.4 Insecticide resistance

Insecticide resistance is one of the main concerns to long term sustainability for chemical control of insect pests, with close to 600 species of insects now described with resistance to at least one insecticide (Bass and Field 2011; Sparks and Nauen 2015; Alyokhin and Chen 2017; Pavlidi et al. 2018; Nauen et al. 2019). Resistance is a genetic change in response to insecticide selection, characterized by a decrease in susceptibility of a pest population (Guedes 2017; Alyokhin and Chen 2017; Nauen et al. 2019). This

phenomenon has the potential to cause control failure, due to a significant reduction of efficacy of an insecticide product used at its recommended dose/concentration but not reaching an expected control level (Guedes 2017).

Increases in the use of insecticides for the control of the Neotropical stink bug might inevitably lead to a mounting selection pressure for resistance, as reflected by the risk of control failure to imidacloprid and beta-cyfluthrin (Tuelher et al. 2018).

Insecticide resistance mechanisms

Insects can evolve resistance to insecticides through different mechanisms such as target site insensitivity, oxidative detoxification enhancement, a decreased cuticular penetration and taste aversion-related behavioral resistance (Montella et al. 2012; Feyereisen et al. 2015; Zalucki and Furlong 2017). Mechanism of resistance are traditionally classified as toxicodynamic and toxicokinetic (Feyereisen et al. 2015). Toxicodynamic mechanisms are changes in the interaction between the insecticide and its target receptor (i.e., target site insensitivity), which render it less sensitive to the toxic effects of the insecticide (Bass and Field 2011; Feyereisen et al. 2015). Toxicokinetic mechanisms describe the disposition of the insecticide through the organism, including decreased penetration, increased production of detoxifying enzymes that can break down or sequester the insecticide, or increased excretion of the insecticide (Bass and Field 2011; Feyereisen et al. 2015). Among these mechanisms of resistance, the metabolic detoxification is considered the most frequently encountered mechanism (Zhang et al. 2016a; Pavlidi et al. 2018). Typically, the metabolic detoxification in insects occurs via three major phases (Yang et al. 2013b). The phase I or functionalization step results in the addition of a functional group to the insecticidal molecule (i.e., oxidation or reduction) or hydrolysis (i.e., hydrolysis ester bonds), making it hydrophilic and therefore a substrate more adequate for phase II enzymes (Reyes et al. 2011; Heidel-Fischer and Vogel 2015). In phase II or conjugation, an endogen molecule (i.e., glutathione) is added to the functionalized or original insecticide, the conjugated molecule is more hydrophilic and can be easily excreted or sequestered (Reyes et al. 2011; Heidel-Fischer and Vogel 2015). Lastly, in phase III, transmembrane transports actively pump the conjugated molecules out of the cell (Yang et al. 2013b). The three main enzyme families implicated in the metabolic resistance are the cytochrome P450s and the esterases in phase I and the glutathione S-transferases in phase II (Reyes et al. 2011; Bass and Field 2011; Ffrench-Constant 2014).

1.4 Biological control

Biological control is the use of a population of a natural enemy, also known as biological control agent, to reduce the population density of a pest (Waage and Greathead 1988; Cock et al. 2010; van Lenteren 2012; van Lenteren et al. 2018). Augmentative

biological control refers to periodic releases of large numbers of mass-reared natural enemies to provide immediate suppression of the pest (Cock et al. 2010; Lorito et al. 2010; Parnell et al. 2016; van Lenteren et al. 2018). Augmentative biological control is commercially applied on more than 30 million ha in various cropping systems worldwide, including fruit and vegetable crops, cereals, maize, cotton, sugarcane, soybean, grapes and many greenhouse crops (van Lenteren et al. 2018).

Biological control agents of insect pests include predators, parasitoids, as well as entomopathogenic nematodes and microbes, and their use carries great pest management potential because of their multiple economic and environmental benefits (Gurr and You 2016; Stenberg 2017). Some of the advantages of the use of biological control compared with insecticides include: (i) safety for the applicant and food supply due to reduced effects on human health (Cock et al. 2010; van Lenteren 2012); (ii) the impacts on beneficial and other non-target organisms (NTOs) depend on the host range of the natural enemy, but normally are reduced in comparison with broad-spectrum insecticides (van Lenteren 2012; Kaser and Heimpel 2015); (iii) no harvesting interval or waiting period after release of agents, allowing continuous harvesting without danger to the health of personnel (Van Lenteren and Bueno 2003); (iv) no phytotoxic damage to plants, that might result in plant growth inhibition (Van Lenteren and Bueno 2003; De Buck and Beerling 2007); (v) there are few examples of evolved resistance to introduced parasitoids or predators, since the selection pressure is low and these biological control agents co-evolve with a pest and thereby counteract resistance developing in the latter (Goldson et al. 2014; Tomasetto et al. 2017); (vi) the benefit/cost ratio of biological control is similar, but higher if the hidden costs of insecticides are considered (Cock et al. 2010; van Lenteren 2012; Naranjo et al. 2015; Bourguet and Guillemaud 2016); (vii) the development cost of a product for biological control are a fraction of those for chemical control due to the higher success ratio of discovery of new biological control agents (van Lenteren 2012; Sparks 2013).

However, augmentative biological control also has drawbacks that can explain the modest and inconsistent use of this pest control tactic. Firstly, in most of the cases augmentative biological control lacks the efficiency to reduce the pest population below the economic injury levels (Collier and Van Steenwyk 2004). The lack of effectiveness to suppress the pests may result from several different ecological limitations, including mortality, predation and adverse environmental conditions (Collier and Van Steenwyk 2004; Sørensen et al. 2012; Parra 2014; Terblanche 2014). Consequently, integrating augmentative releases with other pest management practices is of vital importance to overcome these limitations (Collier and Van Steenwyk 2004; Parra 2014; Lamichhane et al. 2017; Stenberg 2017). Second, the intensive use of biological control agents in augmentation programs has led to growing environmental concerns (De Clercq 2002). The major environmental concerns include the irreversibility of introductions, potential of host switching, dispersal to non-agricultural areas, negative food web effects such

as competition or displacement of natural species (Roitberg et al. 2001; De Clercq 2002; Louda et al. 2003; Van Lenteren et al. 2003; Barratt et al. 2010; Driesche et al. 2017). In fact, traits viewed as advantageous for biological control, such as capacity of self-replication, rapid increase, and high dispersal, are also traits that enhance the probability of unexpected ecological impacts (Louda et al. 2003; Barratt et al. 2010). Moreover, there is a general consensus that the potential risk to cause adverse effects is higher when generalist species are employed (Roitberg et al. 2001; De Clercq 2002; Kaufman and Wright 2017). Therefore, biological control programs should increase the emphasis on selecting biological control agents that are highly effective for reducing the population of the pest, but that are unlikely to impact NTOs (Barratt et al. 2010).

1.4.1 Natural enemies of *E. heros*

Stink bugs have a diverse set of natural enemies in the Neotropics that can be used as biological control agents (Table 1.3) (Zerbino and Panizzi 2019). Among them, hymenopteran egg parasitoids are probably the most important due to biological attributes and behavioral responses that could lead to density dependent parasitism and efficient regulation of stink bug populations (Corrêa-Ferreira and Moscardi 1995, 1996; Bueno et al. 2012; Zerbino and Panizzi 2019). Nine species of egg parasitoids have been reported for *E. heros* eggs, including *T. podisi* and *Trissolcus* spp. (Hymenoptera: Scelionidae) that have been used successfully for biological control of stink bugs by mass or inoculative releases (Corrêa-Ferreira and Moscardi 1995; Panizzi and Correa-Ferreira 1997; Medeiros et al. 1998; Margaría et al. 2009; Paz-Neto et al. 2015; Zerbino and Panizzi 2019). In addition to egg parasitoids, four species of flies (Diptera: Tachinidae) and micro-Hymenoptera (Encyrtidae) parasitize adults and nymphs of *E. heros* (Panizzi and Correa-Ferreira 1997; Corrêa-Ferreira et al. 1998; Turchen et al. 2016b; Aquino et

Table 1.3: Natural enemies of *Euschistus heros*. Adapted from Zerbino and Panizzi 2019

Order/ Family	Biological control agent	*E. heros* life stages attacked	Reference
Hymenoptera			
Scelionidae	*Telenomus podisi*	Egg	Corrêa-Ferreira and Moscardi 1995
	Trissolcus basalis	Egg	Corrêa-Ferreira and Moscardi 1995
	Trissolcus urichi	Egg	Corrêa-Ferreira and Moscardi 1995
	Trissolcus brochymenae	Egg	Corrêa-Ferreira and Moscardi 1995
	Trissolcus teretis	Egg	Panizzi and Correa-Ferreira 1997
	Gryon obesum	Egg	Corrêa-Ferreira and Moscardi 1995
Eurytomidae	*Neorileya* sp.	Egg	Corrêa-Ferreira and Moscardi 1995
Eupelmidae	*Eupelmus* sp.	Egg	Medeiros et al. 1998
Encyrtidae	*Ooencyrtus* sp.	Egg	Corrêa-Ferreira and Moscardi 1995
	Hexacladia smithii	Adult/ late nymphs	Corrêa-Ferreira et al. 1998
Diptera			
Tachinidae	*Gymnoclytia paulista*	Adult/ late nymphs	Panizzi and Correa-Ferreira 1997
	Hyalomyodes sp.	Adult/ late nymphs	Aquino et al. 2019
	Phasia sp.	Adult/ late nymphs	Aquino et al. 2019
	Trichopoda giacomelli	Adult/ late nymphs	Panizzi and Correa-Ferreira 1997

al. 2019).

1.4.2 Stink bug egg parasitoid *T. podisi*

Among the different natural enemies that might be used as biological control agent of *E. heros*, the egg parasitoid *T. podisi* stands out (Silva et al. 2006; Bortolotto et al. 2016; Queiroz et al. 2018). *T. podisi* is an egg parasitoid of several stink bugs in North and South America, including both herbivorous and predatory species (Borges et al. 2003; Koppel et al. 2009). This parasitoid is considered quasi-gregarious since it develops in host eggs that are laid in patches (Figure 1.3) (Koppel et al. 2009; Abram et al. 2017). Like many other Hymenoptera, *T. podisi* is haplodiploid, so unfertilized eggs (haploid) result in males, while fertilized eggs (diploid) result in females (Borges et al. 2003; Silva et al. 2018a). Male *T. podisi* emerge approximately one day before females, remain on the host egg mass, and mate with sibling females as they emerge (Yeargan 1980). Females of *T. podisi* usually mark their host eggs after oviposition to reduce superparasitism, and only one parasitoid develops and emerges for each host egg (Okuda and Yeargan 2015).

T. podisi is the most abundant egg parasitoid of *E. heros*, parasitizing up to 80 % of stink bug egg masses under natural conditions (Pacheco and Corrêa-Ferreira 2006). Although polyphagous, *T. podisi* prefers *E. heros* eggs and has shown a great performance after inundative releases across approximately 30000 soybean ha in South America (van

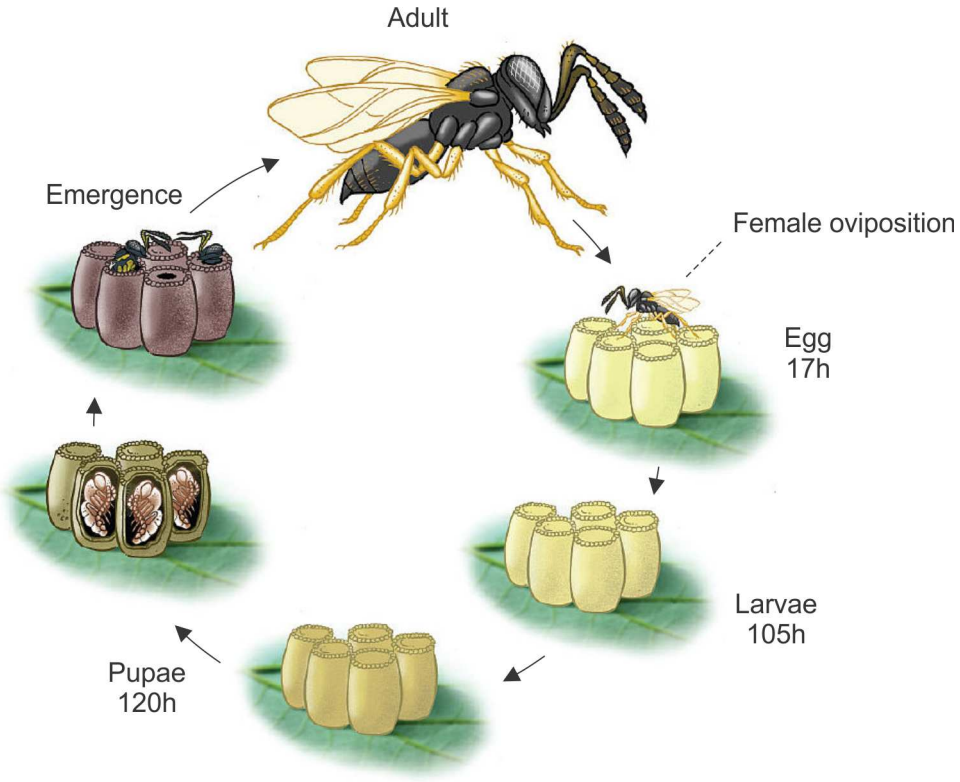


Figure 1.3: Life cycle of the egg parasitoid *Telenomus podisi*. Adapted from Koppert 2019

Lenteren et al. 2018; Queiroz et al. 2018). The success of its parasitism can be attributed to high searching abilities and reproductive rates that lead to positive host-density responsiveness (Orr 1988).*T. podisi* is attracted to its primary host, *E. heros*, by chemical cues including sex pheromones, defensive compounds from the methatoracic gland, and cuticular compounds (Borges et al. 1999, 2003; Silva et al. 2006), plant volatiles induced by feeding or oviposition (Moraes et al. 2005, 2008; Michereff et al. 2011, 2013, 2016), vibratory signals used for sexual communication (Laumann et al. 2007, 2011), and visual cues (Aquino et al. 2012). Besides presenting desirable characteristics for the biological control, these parasitoids can be reared easily on small-scale in the laboratory using eggs of *E. heros* (Peres and Corrêa-Ferreira 2004). However, as a polyphagous species, *T. podisi* parasites eggs of a variety of stink bugs, including several agricultural pests as well as predatory stink bugs like *Podisus nigrispinus* and *Podisus maculaventris* (Tognon et al. 2014; Queiroz et al. 2018). Therefore, there is a strong potential impact of T. podisi on the eggs of these predatory stink bugs, inducing significant levels of egg mortality, and thus impairing the pest suppression that they provide (Torres et al. 1997). Moreover, *T. podisi* may also impact herbivorous species that are present in natural ecosystems. For instance, the egg parasitoid *Trissolcus basalis* was implicated in the decline of the native Hawaiian "koa bug" *Coleotichus blackburniae* after been introduced into Hawaii against the invasive pest *N. viridula* (Louda et al. 2003; Driesche et al. 2017). In addition to the risks for non-target pentatomids, there are other challenges to apply biological control with *T. podisi* to soybean fields.

1.4.3 Challenges for implementation of biological control of *E. heros* with *T. podisi*

Biological control programs using *T. podisi* against *E. heros* involve the mass-rearing and the release of the parasitoids, as well as the effectiveness for the suppression of the pest, reduction of crop damage and yield increase (Bueno et al. 2012; Lamichhane 2017). Therefore, these programs are inextricably dependent on the producers of biological control agents, and the cost, availability and quality of natural enemies they produce (Warner and Getz 2008; van Lenteren 2012). Recently, the use of *T. podisi* for the control of several Pentatomid species, including *E. heros*, was approved by the Ministry of Agriculture, Livestock, and Supply (MAPA register number 43919). To receive the approval, Koppert Biological Systems provided information regarding the efficiency, quality control, potential risks to human health and the environment in accordance with the correspondent regulations (MAPA 2006). Then, eggs parasited by *T. podisi* will be available for augmentative releases in the soybean crop season 2019/2020. However, the success of biological control using *T. podisi* faces many challenges and potential obstacles, including; (i) development of suitable rearing regimes that ensure field performance; (ii) development of artificial diets that facilitate the rearing of its host *E. heros* on a large scale; (iii) development of appropriate monitoring, storage and release meth-

ods compatible with farmers practices; and (iv) reduction of insecticide use that benefit establishment of parasitoid population in the field (Parra 2010, 2014; Sørensen et al. 2012; Panizzi 2013; Terblanche 2014; Zerbino and Panizzi 2019).

The need for ensuring sustained quality and efficiency of released insect agents is an important factor for the success of biological control programs, since poorly performing natural enemies in the field can result in control failures (Lenteren 2009; Parra 2010, 2014; Cock et al. 2010; Sørensen et al. 2012). Field performance depends on the ability of the parasitoids to effectively locate and oviposit in their hosts, as well as the ability of eggs and larvae to overcome or evade the host's immune defenses (Jeffs and Lewis 2013). These processes are frequently temperature dependent, thereby, the thermal performance of mass-reared insects facing novel environments upon release in the field has long been considered a challenge for mass-reared biological control agents (Sørensen et al. 2012; Terblanche 2014). In the case of *T. podisi*, parasitized eggs, in the parasitoid pupae stage, are transported unprotected or inside capsules of stiff cardboard through parcel delivery services from rearing facilities to the field. During this process and after parasitoid pupae release in the field, the parasitoids are exposed to drastic temperature changes, that can impair the survival and parasitism efficiency (Parra 2014). Therefore, development of mass rearing techniques for breeding better field adapted parasitoids will benefit biological control programs using *T. podisi*.

Secondly, soybean is extensively cultivated in Brazil, and the size of soybean fields for a single grower can reach 20000 - 30000 and even 100000 ha (Parra 2014). Consequently, the implementation of biological control in such large areas require larger production of *T. podisi*i to meet the market demand for biological control agents (Bueno et al. 2012; van Lenteren 2012; Parra 2014). As production of this parasitoid is still dependent on the production of the stink bug host for the supply of eggs (Strand et al. 1988; Volkoff et al. 1992; Shirazi 2006). The use of suitable artificial diets for the rearing of *E. heros*, as well as the automation and mechanization of rearing, can facilitate mass production, lower cost of production and made biological control a viable commercial option for large scale crops (Panizzi 2013; Parra 2014; Lacey et al. 2015; Zerbino and Panizzi 2019).

Apart from quality and cost of the pest control strategy, the implementation of biological control from the farmer point of view depends also on labor and supply requirements (De Buck and Beerling 2007; Lefebvre et al. 2014). Therefore, some improvements should be done in release, shipment and storage of *T. podisi* to make it compatible with the farmer's equipment and production practices (De Buck and Beerling 2007; Lefebvre et al. 2014; Lamichhane et al. 2017; Barratt et al. 2018). Moreover, in order to release natural enemies at the right time in large areas, there is a need to develop monitoring techniques for *E. heros* using pheromones or remote sensing (Parra 2014).

Lastly, the current extensive use of insecticides in soybean crops in Brazil results in unbalanced systems, where the introduced natural enemies cannot be established

(Parra 2014; Ponisio et al. 2015; Michaud 2018). The chances of survival and reproduction of multiple generations following a single release will ultimately depend on the reduction of pesticide use, since most of the insecticides used currently in soybean are harmful to *T. podisi* (Turchen et al. 2016a; Silva et al. 2018b; Zantedeschi et al. 2018; Stecca et al. 2018; Feltrin-Campos et al. 2018; Pazini et al. 2019). Strategies like using selective pesticides, reducing doses and application frequencies, timing insecticide applications in periods of low activity of the natural enemies, and even use of selective insect resistant genetically modified plants can reduce the negative effect on natural enemies (Roubos et al. 2014; Gurr and You 2016).

1.4.4 Biological control and integrated pest management

Augmentative biological control programs using *T. podisi* against *E. heros* must be implemented as one contributive component of integrated pest management (IPM), since it is difficult to control pest populations using biological control alone (Parra 2014; Lamichhane et al. 2017; Stenberg 2017). IPM is a decision-based process involving coordinated use of multiple tactics for optimizing the control of all classes of pests (insects, pathogens, weeds, vertebrates) in an ecologically and economically sound manner (Ehler 2006; Roubos et al. 2014). The combination of control tactics into management strategies generates more effective and sustainable results than single tactic approaches (Ehler 2006; Lefebvre et al. 2014; Barzman et al. 2015; Lamichhane et al. 2016). These tactics include biological, cultural, mechanical, and behavioral (pheromones) methods, insect resistant cultivars, conventional insecticides and, more recently transgenic plants (Parra 2014).

1.5 RNA interference (RNAi)

In the context of IPM, RNA interference (RNAi) technology can play an important role as a non-chemical pesticide tactic in IPM programs for the control of *E. heros* in soybean (Thakur et al. 2016). RNAi refers to a set of molecular processes in which small RNAs regulate the expression of specific nucleic acids via mRNA degradation, repression of translation, or even long term expression changes through chromatin remodeling (Obbard et al. 2009; Ha and Kim 2014; Zotti et al. 2018; Vogel et al. 2019). RNAi is a conserved mechanism present in most eukaryotic organisms, including unicellular protists, fungi, plants and animals albeit with different roles (Vodovar and Saleh 2012). In insects, for instance, RNAi have been found to participate in multiple biological processes, including development, metabolism, circadian rhythm, insect immunity against viruses, and transposon activity suppression (Xue et al. 2012; Wynant et al. 2014b; Zhang et al. 2017).

There are three small RNA silencing pathways in insects: small interfering RNA

(siRNA), micro RNA (miRNA) and Piwi interacting RNA (piRNA) (Ghildiyal and Zamore 2009; Lucas and Raikhel 2013; Ha and Kim 2014; Vogel et al. 2019). Although these pathways share some characteristics and cross-talk between the pathways can occur, they largely differ in their origin, substrates, structure, effector proteins, modes of target regulation and role in distinct biological processes (Ghildiyal and Zamore 2009; Vogel et al. 2019). miRNAs are genome-encoded small RNAs ($\sim$22 nucleotides-nt) that regulate the gene expression through translational inhibition and/or mRNA degradation (Obbard et al. 2009; Vodovar and Saleh 2012; Lucas and Raikhel 2013). miRNAs constitute the most evolutionary conserved class of small RNAs and play critical role in the in development and regulation of cellular processes (Vodovar and Saleh 2012; Lucas and Raikhel 2013). siRNAs ($\sim$21 nt) originated from perfectly base-paired long double-stranded RNAs (dsRNAs) derived from endogenous loci or foreign genetic material (Obbard et al. 2009; Vodovar and Saleh 2012; Ha and Kim 2014). siRNAs are mostly involved in defense against parasitic nucleic acid elements, such as retrotransposons or viruses, but also participate in heterochromatin maintenance (Vodovar and Saleh 2012; Lucas and Raikhel 2013; Meister 2013). piRNAs (24–30 nt) protect the germline from double-stranded DNA breaks and insertion mutagenesis by silencing transposons (Czech and Hannon 2016; Lewis et al. 2018).

Among RNAi pathways, the siRNA pathway has become a widely used research tool for reverse genetics (Blandin et al. 2002; Kurscheid et al. 2009; Santos et al. 2014) and functional genomics (Bellés 2010; Noh et al. 2012; Weiner et al. 2017; Homem and Davies 2018), since this pathway can be triggered by exogenous dsRNAs. Furthermore, the ability to suppress expression in a sequence-specific manner has created numerous opportunities for the development of novel therapeutic strategies for many human diseases (Mansoori et al. 2014; Gomes et al. 2015; Bobbin and Rossi 2016; Rupaimoole and Slack 2017) and innovative RNAi-based approaches for crop protection (Tang et al. 2007; Auer and Frederick 2009; Joga et al. 2016; Zhang et al. 2017; Zotti et al. 2018; Bally et al. 2018; Rosa et al. 2018; Cagliari et al. 2019; Dalakouras et al. 2019).

1.5.1 The siRNA pathway

In insects, the RNAi pathways have been mainly studied in the model organism *Drosophila melanogaster*. In this insect, the siRNA pathway is divided in three steps, namely dicing, loading and slicing (Figure 1.4). The first step, dicing, starts when long dsRNA is introduced into the cells, where dsRNA is recognized and processed by a ribonuclease III enzyme known as Dicer-2 (Dcr-2) (Vodovar and Saleh 2012; Aronstein et al. 2012). Dcr-2 cleavages at the ends of the dsRNA, making successive cleavages to generate $\sim$21 nt siRNAs duplexes (Siomi and Siomi 2009; Meister 2013; Baum and Roberts 2014). Those siRNA duplexes have 2-3 nt overhangs at the 3' ends of both strands, that allow them to be recognized by the enzymes from the RNA machinery (Wynant et al. 2014b; Mongelli and Saleh 2016; Sinha et al. 2018).

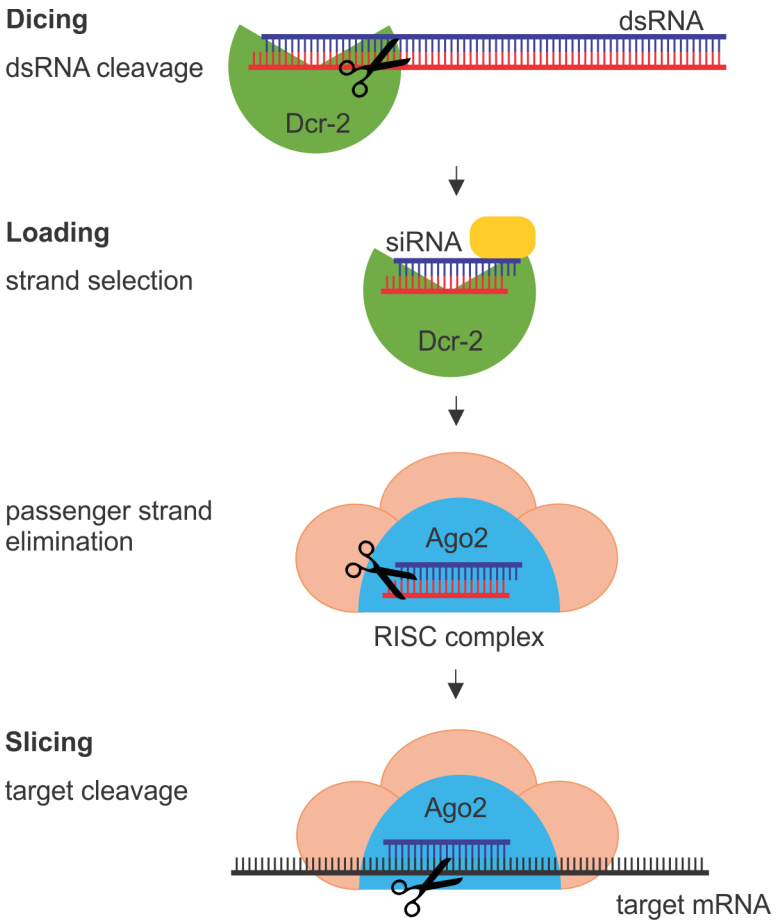


Figure 1.4: The siRNA pathway in insects. Double stranded RNA precursors are processed by Dicer-2 (Drc-2) to generate 21 nt siRNAs duplexes. Dcr-2 helps load the siRNA duplexes into the RNAi silencing complex (RISC), siRNA duplexes are unwound, and the passenger strand is destroyed. RISC searches target mRNA with the help of the guide strand and the mRNA is destroyed by Argonaute 2 (Ago2), the catalytic domain of the RISC complex. Redrawn after Mongelli and Saleh 2016; Schuster et al. 2019.

In the second step or loading, Dcr-2 helps load the siRNA duplexes into a protein complex known as the RNAi silencing complex (RISC), preventing siRNAs from diffusing freely in the cytoplasm after their production (Siomi and Siomi 2009; Vodovar and Saleh 2012). RISC is a ribonucleoproteic complex that contains several components including its catalytic domain Argonaute-2 (Ago2) (Siomi and Siomi 2009; Vodovar and Saleh 2012). Once the siRNA duplex is loaded into RISC, the siRNA duplexes must be unwound, and only one strand, the guide (antisense) strand, remains loaded onto Ago2 and assembles into the active RISC; the other strand, the passenger (sense) strand is degraded (Siomi and Siomi 2009).

In the third step or slicing, active RISC searches within the transcriptome of the cell and find potential target mRNA within the cell, with the help of the guide strand that determines the sequence specificity of the RNAi response (Siomi and Siomi 2009). The guide strand in the active RISC recognizes target mRNAs via Watson-Crick base pairing (Mongelli and Saleh 2016; Schuster et al. 2019). After the formation of the siRNA/target

mRNA duplex, Ago2 cleaves the target at the position 10 with respect to the 5' end of the siRNA (Meister 2013; Schuster et al. 2019). After cleavage of target mRNA, slicing products are rapidly degraded by cellular ribonucleases (Schuster et al. 2019).

1.5.2 Uptake and spreading of RNAi signal

High RNAi efficiency using external dsRNA not only depends on the RNAi machinery, but also involves cellular uptake of the dsRNA and systemic spreading of the RNAi signal between tissues (Figure 1.5) (Whangbo and Hunter 2008; Baum and Roberts 2014; Ivashuta et al. 2015; Denecke et al. 2018; Cooper et al. 2019). In insects, two different pathways have been described for dsRNA cellular uptake: one is mediated by sid-1-like transmembrane proteins and the other works via receptor-mediated clathrin-dependent endocytosis (Winston et al. 2002; Ulvila et al. 2006; Saleh et al. 2006; Cappelle et al. 2016).

SID-1-like transmembrane proteins

The transmembrane proteins SID1, SID2, SID3 and SID5 were shown to be involved in dsRNA uptake in the nematode *Caenorhabditis elegans* (Rocheleau 2012). Only SID-1 homologous genes have been found in many insects genomes (Cappelle et al. 2016a; Vélez and Fishilevich 2018). SID-1 encodes a multi-pass transmembrane protein that functions as a channel for the passive transport of dsRNA among cells (Winston et al. 2002; Jose et al. 2009; Shih and Hunter 2011). Although Sil seems to play a minor role in dsRNA uptake in some insects (Xu et al. 2013; Miyata et al. 2014; Yoon et al. 2016). Sil genes appear to be more closely related to ChUP-1 (previously named tag130), a protein involved in the cholesterol uptake but not in the transport of dsRNA (Tomoyasu et al. 2008; Valdes et al. 2012; Whangbo et al. 2017). As blocking cholesterol transport may decrease the clathrin-mediated endocytosis, it is possible that Sil proteins in insects mediate cholesterol rather than dsRNA uptake, explaining the divergent evidence of its participation in RNAi (Vélez and Fishilevich 2018).

Receptor-mediated clathrin-dependent endocytosis

In insects, evidence suggest that cellular uptake of dsRNA mainly occurs through receptor-mediated clathrin-dependent endocytosis (Denecke et al. 2018). According to this model, dsRNA is recognized by receptors on the plasma membrane, internalized in clathrin-coated endocytic vesicles, and then dsRNA escape the endosome to reach the core machinery in the cytoplasm (Ulvila et al. 2006; Saleh et al. 2006; Cappelle et al. 2016a; Vélez and Fishilevich 2018; Cooper et al. 2019). Two scavenger receptors, SR-CI and Eater are involved in the recognition of extracellular dsRNA and to mediate its internalization (Ulvila et al. 2006). Recently, numerous reports indicate that clathrin-dependent endocytosis together with scavenger receptors, rather than Sil genes, plays

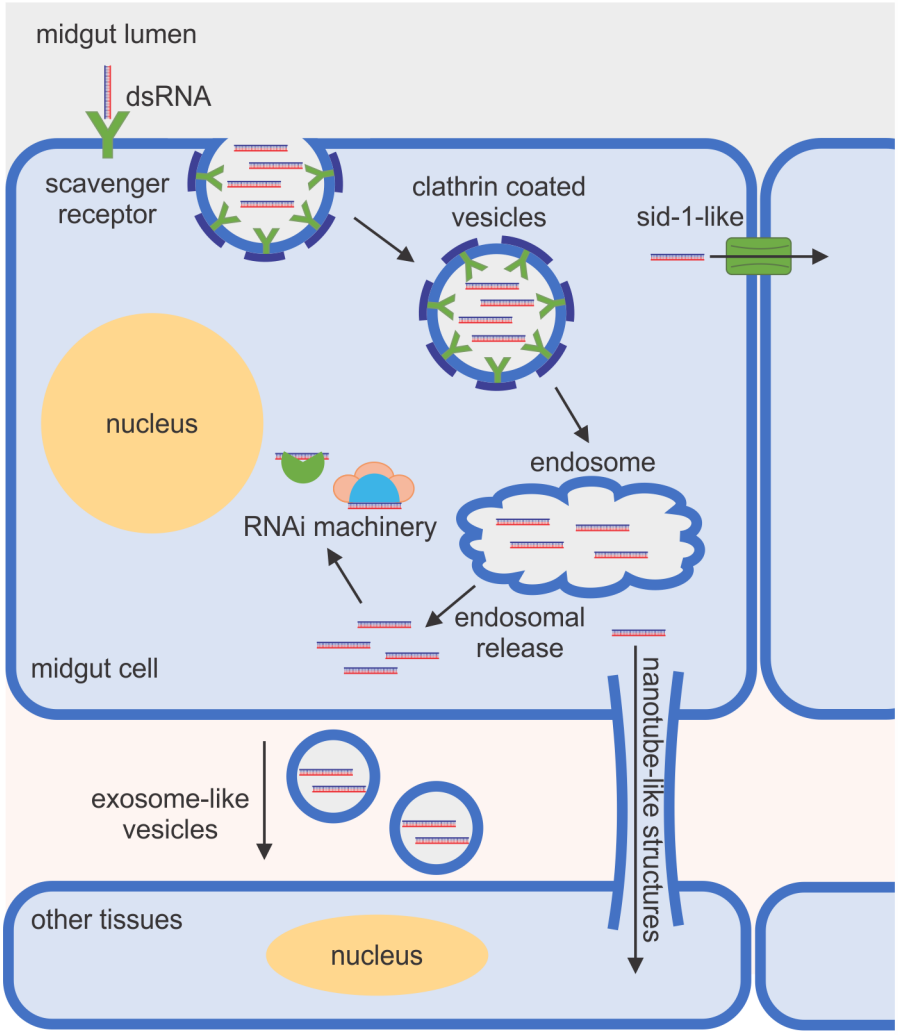


Figure 1.5: Cellular uptake of the dsRNA and systemic spreading of the RNAi signal in insects. dsRNA enters the cell via clathrin-mediated endocytosis and possibly SID-like proteins. But the specific function of SID-like proteins is unknown. First, dsRNA binds to the scavenger receptor in the plasma membrane and internalized through clathrin dependent endocytosis. Second, the clathrin coat is removed and the uncoated vesicle merges with an early endosome. Then, the dsRNA molecules must scape from endosome to the cytoplasm, where they trigger the RNAi process through the RNAi core machinery. In addition, dsRNA also can spread to other tissues, possibly through exosome-like vesicles or via nanotube-like structures. Redrawn after Fishilevich et al. 2016; Cooper et al. 2019; Vogel et al. 2019.

a predominant role in cellular uptake of dsRNA in insects (Wynant et al. 2014a; Li et al. 2015c; Xiao et al. 2015; Cappelle et al. 2016a; Yoon et al. 2016; Dong et al. 2017; Pinheiro et al. 2018). Interestingly, other non-classical endocytosis mechanisms such as micropinocytosis (Gillet et al. 2017) and phagocytosis (Rocha et al. 2011) probably are also implicated in cellular uptake of dsRNA.

Systemic spreading of the RNAi signal

Systemic spreading of the RNAi signal to distant tissues has been confirmed in some insects (Saleh et al. 2009; Wang et al. 2011; Jarosch and Moritz 2011; Bolognesi et al. 2012; Miller et al. 2012; Wynant et al. 2012; Ivashuta et al. 2015; Cappelle et al. 2016b), including Hemiptera (Wang et al. 2015). Moreover, maternal transmission of RNAi signal to the offspring has been observed in eggs laid by females fed (Khajuria et al. 2015) or injected with dsRNA (Bucher et al. 2002; Liu and Kaufman 2004; Mito et al. 2006; Pueyo et al. 2008; Piulachs et al. 2010; Nakao 2012; Yoshiyama et al. 2013; Mao et al. 2013; Lu et al. 2017), providing further evidence of the spread of RNAi effect through insect tissues. Nevertheless, the spreading mechanism(s) mediating systemic RNAi in insects remains largely unknown (Cooper et al. 2019). Possible mechanisms proposed for the transport of dsRNA between cells involve transport of siRNAs enclosed in exosome-like vesicles (Tassetto et al. 2017) or nanotube-like structures that transport dsRNA and components of the RNAi machinery between adjacent cells to mediate systemic RNAi (Karlikow et al. 2016).

In plants, nematodes and mites, exogenous dsRNAs can be amplified via RNA-dependent RNA polymerases (RdRPs) to produce endogenous dsRNAs that supplement the RNAi pathway and prolong the RNAi effect (Xie et al. 2001; Pak and Fire 2007; Grbić et al. 2011). However, RdRP homologs are not present in insect genomes (Gordon and Waterhouse 2007) and there is no evidence of secondary generated siRNAs (Li et al. 2018). Therefore, the RNAi signal that travels to distant tissues in insects probably corresponds to the dsRNA that is taken up from the environment (Li et al. 2018).

RNAi for crop protection

RNAi has the potential of multiple practical applications for crop protection, including: (i) pest management strategies against insects (Joga et al. 2016; Zhang et al. 2017; Zotti et al. 2018; Bally et al. 2018; Cagliari et al. 2019) and mites (Niu et al. 2018); (ii) resistance to plant pathogens virus, bacteria, fungi and nematodes (Auer and Frederick 2009; Rosa et al. 2018; Dalakouras et al. 2019); (iii) improvement of crop quality traits, including nutritional value, drought tolerance, elimination of allergenic and/or toxic compounds, etc (Tang et al. 2007; Auer and Frederick 2009; Habben et al. 2014; Waltz 2015b, a); (iv) treatment and prevention of diseases in beneficial insects such as honeybees and bumblebees (Hunter et al. 2010; Zotti and Smagghe 2015; Vogel et al. 2019); (v) management of pesticide-resistant insects/weeds, to investigate the role of pesticide-degrading enzymes and potentially restore pesticide activity in resistant strains (Zotti et al. 2018; Homem and Davies 2018). The sequence-dependent mode of action of RNAi allows the tailor-made design of the dsRNA for the targeted species while leaving beneficial and other NTO species unaffected (Joga et al. 2016; Bachman et al. 2016; Zotti et al. 2018). In this way, the use of RNAi for crop protection offers the potential for high

taxonomic specificity and environmental safety (Scott et al. 2013; Zhang et al. 2017; Zotti et al. 2018; Rosa et al. 2018).

Over the past decade, transgenic food crops utilizing RNAi to confer resistance to specific virus (e.g. plum-pox virus, bean golden mosaic virus), extended produce quality (e.g. apple) or nutritional enhancement (e.g. potato) have received regulatory approvals (Auer and Frederick 2009; Waltz 2015b, a; Rosa et al. 2018). Regarding insect pests, the genetically engineered corn MON 87411 has received approval for commercial planting in Canada, United States and Brazil (ISAAA 2017). This transgenic plant confers protection against the Western corn rootworm *Diabrotica virgifera virgifera* by expressing dsRNA containing a 240 nt fragment of the Sucrose non-fermenting7 (*DvSnf7*) gene, an essential vacuolar sorting protein (Head et al. 2017). After ingestion, the plant-produced dsRNA in MON 87411 is recognized by the corn rootworm's RNAi machinery, which results in a rapid decrease of DvSnf7 mRNA and protein levels leading to growth inhibition followed by mortality (Bolognesi et al. 2012; Bachman et al. 2016). This means that RNAi technology offers opportunities for control of other insect pests, including the Neotropical stink bug *E. heros*.

1.5.3 Challenges for the development of RNAi-based insecticide for the control *E. heros*

As mentioned above, RNAi technology nowadays is commercially available only for the control of Western corn rootworm in corn (Head et al. 2017; Zotti et al. 2018). Despite many studies having shown that RNAi is a feasible control strategy against a range of insect species (Joga et al. 2016; Zhang et al. 2017; Zotti et al. 2018; Bally et al. 2018; Cagliari et al. 2019), there are still many limitations that need to be addressed. The main challenge facing the successful implementation of RNAi for pest control is that many insects do no respond efficiently to externally administered dsRNA (Terenius et al. 2011; Christiaens and Smagghe 2014; Zotti and Smagghe 2015; Zhang et al. 2017; Cooper et al. 2019; Vogel et al. 2019). Consequently, in order to develop a successful RNAi-based control, first a higher effectiveness must be reached, and then the form of application and biosafety issues need to be considered (Zhang et al. 2013a, 2017)

Potential mechanisms affecting RNAi efficiency

RNAi efficiency, or the sensibility of an insect to gene suppression via treatment with dsRNA, varies greatly between different orders, but also between members of the same insect order and even within the same species (Bellés 2010; Wynant et al. 2014b; Cooper et al. 2019; Vogel et al. 2019). For instance, in RNAi sensitive insects (e.g. Western corn rootworm) gene silencing is often 90 % or higher, require only very small amounts of dsRNA and the effect can be long lasting and even hereditary (Baum et al. 2007; Rangasamy and Siegfried 2012; Bachman et al. 2013; Khajuria et al. 2015).

Conversely, recalcitrant species require large amounts of dsRNA and the gene silencing is temporary and lower than 60 % (Huvenne and Smagghe 2010; Terenius et al. 2011; Christiaens and Smagghe 2014; Bally et al. 2018). Those differences in efficiency can be the result of different biological variables including (i) instability of dsRNA prior to cellular uptake; (ii) incomplete dsRNA internalization and/or impaired systemic spreading; (iii) deficient core RNAi machinery; (iv) factors inherent to the experimental set-up (Huvenne and Smagghe 2010; Scott et al. 2013; Baum and Roberts 2014; Cooper et al. 2019; Vogel et al. 2019).

Instability of dsRNA prior cellular uptake

Instability of dsRNA in insect tissues can be attributed to the enzymatic activity of double-stranded ribonucleases (dsRNases) along with the physiological pH that could affect enzymatic activity and the stability of dsRNA (Joga et al. 2016; Singh et al. 2017; Cooper et al. 2019). Degradation of dsRNA occurs in the gut content (Arimatsu et al. 2007; Wynant et al. 2014c; Ren et al. 2014; Wang et al. 2016; Shukla et al. 2016; Singh et al. 2017; Prentice et al. 2017; Spit et al. 2017; Gillet et al. 2017; Song et al. 2017; Guan et al. 2018), as well as in the saliva of Hemiptera (Allen and Walker 2012; Christiaens et al. 2014; Lomate and Bonning 2016; Mogilicherla et al. 2018).

Incomplete dsRNA internalization and/or impaired systemic spreading

Internalization of dsRNA into the epithelial cells of the insect midgut is a critical step to generate high RNAi efficiency (Joga et al. 2016; Cooper et al. 2019). Deficient dsRNA cellular uptake through endocytosis limits RNAi efficiency in some insects (Whyard et al. 2009; Zhang et al. 2010; Taning et al. 2016). Cellular uptake, however, does not guarantee efficient RNAi response since endosomal escape remains a large obstacle (Shukla et al. 2016; Yoon et al. 2017). In addition, systemic spreading mechanisms are probably impaired by (i) nuclease activity in the hemolymph (Liu et al. 2012; Garbutt et al. 2013; Christiaens et al. 2014; Singh et al. 2017; Cao et al. 2018); and (ii) reduced uptake in certain tissues (Jarosch and Moritz 2011; Ren et al. 2014).

Deficient core RNAi machinery

Even if the dsRNA is internalized and reaches the RNAi core machinery in the cytoplasm, differences in the expression and function of the core RNAi enzymes could contribute to differences in the RNAi efficiency among insects (Singh et al. 2017; Cooper et al. 2019). Those differences are result of multiple factors, including (i) gene duplications (Tomoyasu et al. 2008; Guo et al. 2015; Yoon et al. 2016; Singh et al. 2017); (ii) poor upregulation of the core RNAi enzymes after exposure to exogenous dsRNA (Terenius et al. 2011; Kolliopoulou and Swevers 2014); (iii) overlapping between RNAi pathways (Kolliopoulou and Swevers 2014; Dowling et al. 2016; Yoon et al. 2016) and (iv) viral suppressors of RNAi that can interfere with components of the RNAi machinery or saturates the RNAi machinery (Berry et al. 2009; Swevers et al. 2016).

Factors inherent to the experimental set-up

Several important factors of the set-up of RNAi experiments such as the target selection, the length and concentration of dsRNA and the life stage of the target specie may also influence the RNAi efficiency (Huvenne and Smagghe 2010; Scott et al. 2013; Joga et al. 2016; Vogel et al. 2019). Therefore, methodological strategies such as screening of several targets (Zhang et al. 2013a), use of long dsRNA ranged from 140-500 nt (Huvenne and Smagghe 2010), optimization of the concentration (Huvenne and Smagghe 2010; Vogel et al. 2019) and use of the life stages with higher RNAi susceptibility (Araujo et al. 2006; Griebler et al. 2008; Guo et al. 2015) can result in a higher RNAi response.

And finally, to fully understand the problem of variable RNAi efficiency, intraspecies variation also need to be considered (Vogel et al. 2019). Indeed, differential susceptibility to external administration of dsRNA have been observed between insect populations of the same species (Chu et al. 2014; Sugahara et al. 2017). The cause of this intraspecific variation remains uncertain, but could be attributed to differences in genetic background of the populations, as well the presence of persistent viral infections that can reduce RNAi sensibility through viral suppressors (Swevers et al. 2016; Sugahara et al. 2017)

RNAi formulations

Clearly the obstacles of insufficient RNAi efficiency must be solved before RNAi-based strategies can be further applied in a wide range of insect species (Vogel et al. 2019). Therefore, one current challenge is the development of formulations that improve the dsRNA stability, dsRNA cellular uptake and overall RNAi response in recalcitrant species (Joga et al. 2016; Vélez and Fishilevich 2018; Vogel et al. 2019). Many different strategies have been explored to improve the efficiency of RNAi in insects, including (i) liposome coated dsRNA (Whyard et al. 2009; Cancino-Rodezno et al. 2010; Bedoya-Pérez et al. 2013; Taning et al. 2016; Lin et al. 2017); (ii) nanoparticles (Zhang et al. 2010; Mysore et al. 2013, 2014; He et al. 2013; Ramesh Kumar et al. 2016; Christiaens et al. 2018b); (iii) chemically modified siRNA (Gong et al. 2011, 2013); (iv) proteinaceous delivery systems (Gillet et al. 2017; Avila et al. 2018); and (v) the use of bacteria (Tian et al. 2009; Zhu et al. 2011; Yang and Han 2014), viruses (Kolliopoulou et al. 2017; Taning et al. 2018), microalgae (Kumar et al. 2013) and even symbiotic microorganisms (Whitten et al. 2015; Murphy et al. 2016) as vehicles for dsRNA. However, the use of nuclease inhibitors requires further investigation.

Application forms in field

In the field, RNAi can be applied using transformative and non-transformative approaches (e.g. sprayable, stem injection, root drenching, seed treatment or powder/granule products) to achieve an area-wide management strategy (Sherman et al.

2015; Joga et al. 2016; Zotti et al. 2018; Cagliari et al. 2019). Genetically modified plants expressing dsRNA are currently considered the best application form due to the junction of two factors (i) the plant can express the dsRNA in the appropriate tissue at the proper dose (Li et al. 2015a); and (ii) the growing number of in planta RNAi-based events that have been developed, reviewed, and received approval by international regulatory agencies (Sherman et al. 2015).

However, production of genetically modified plants is limited due to lack of available transformed protocols in many crops species, the instability of engineered RNA silencing traits, regulatory hurdles and the public concern of production of genetically modified crops (Wang and Jin 2017; Cai et al. 2018; Cagliari et al. 2019). Non-transformative approaches such as foliar sprays, root drenching, trunk injections confer protection against insects with reasonable duration of efficacy (Hunter et al. 2012; Li et al. 2015a; San Miguel and Scott 2016; Andrade and Hunter 2016), taking advantage of the mobility of dsRNA in the vascular system of the plant (Melnyk et al. 2011; Luo et al. 2017). However, the feasibility and applicability of these approaches relies on the development of cost-effective methods for the mass production of dsRNA, as well as the stability of dsRNA in the environment (Heidebrecht 2017; Zotti et al. 2018).

1.5.4 Biosafety

Before an RNAi-based insecticide can be commercialized, a risk assessment framework that evaluates the potential adverse effects on human health and the environment associated with this technology must be established (Romeis et al. 2013; Lundgren and Duan 2013; Ramon et al. 2014; Casacuberta et al. 2015; Alamalakala et al. 2018). As a consequence of the sequence-dependent mode of action, the spectrum of activity of insecticidal dsRNA is expected to be narrow, and thereby the unintended adverse effects are expected to be minimal (Zotti and Smagghe 2015). This is confirmed by the narrow spectrum of activity of *DvSnf7* (Bachman et al. 2013). The results from the risk assessment of *DvSnf7* demonstrate that dietary exposure to *DvSnf7* have no adverse effects on NTOs, including humans, mammals, aquatic and terrestrial vertebrates, as well as invertebrate predators, parasitoids, pollinators, soil biota (Tan et al. 2016; Bachman et al. 2016; Petrick et al. 2016). Furthermore, the abundance of arthropods present in corn agroecosystems was similar between MON 87411 and the conventional control (Ahmad et al. 2016).

However, as the potential for RNAi to cause adverse effects to NTOs is somewhat dependent on the gene, target sequence and the persistent effect (Fishilevich et al. 2016b), the ecological risk analysis need to be assessed on a case by case basis. Those risk assessments should include analysis of potential adverse effects on NTOs and assessment of the potential persistence of applied dsRNA on the environment (Romeis et al. 2013; Bachman et al. 2013).

Potential adverse effects on NTOs

The risk of RNAi-based insecticides to NTOs will depend on the potential adverse effects on those organisms and their exposure to dsRNA (Auer and Frederick 2009; Lundgren and Duan 2013). This exposure not only includes physical exposure, but also the physiological susceptibility of the NTO to dsRNA (Lundgren and Duan 2013; Zotti and Smagghe 2015). As oral ingestion is the more likely physical exposure route to NTOs (Heinemann et al. 2013; Roberts et al. 2015), physiological barriers to oral toxicity of dsRNA such as feeding behavior, diet, gut conditions and the inherent sensibility of the RNAi machinery will restrict the unwanted adverse effects (Lundgren and Duan 2013; Fishilevich et al. 2016; Bachman et al. 2016). The other probable source of exposure is through food webs with dsRNA potentially being transferred to different trophic levels, including predators and parasitoids (Roberts et al. 2015; Christiaens et al. 2018a).

In the NTOs that are sufficiently exposed to the insecticidal dsRNA, several mechanisms triggered by dsRNA were hypothesized to possibly produce unintended adverse effects (Christiaens et al. 2018a). At the first place, sequence-dependent mechanisms such as gene silencing in the NTO (Lundgren and Duan 2013; Casacuberta et al. 2015; Christiaens et al. 2018a). Additionally, the ingestion of high doses of dsRNA can affect organisms through sequence-independent mechanisms such as immune stimulation and saturation of the RNAi machinery (Lundgren and Duan 2013; Christiaens et al. 2018a).

Gene silencing

Any gene in the NTO that shares sequence homology with the siRNAs derived from the insecticidal dsRNA, could become a target of the silencing (Ramesh 2013; Lundgren and Duan 2013; Casacuberta et al. 2015; Christiaens et al. 2018a). Given the small size of siRNA, it is not surprising that off-target binding can occur in the genome of NTOs (Qiu et al. 2005; Lundgren and Duan 2013). It should be noted that not all off-target interactions will result in adverse effects, even if the expression level of a gene is reduced, significant effects on growth, development and/or survival are dependent on the function of the silenced gene (Casacuberta et al. 2015; Parker and Sander 2017). To reduce possible effects on NTOs, the application of bioinformatics can have an important role in the selection and design of the dsRNA (Ramesh 2013; Zotti and Smagghe 2015; Bachman et al. 2016).

Immune stimulation

RNAi developed evolutionary as a means of antiviral defense (Shabalina and Koonin 2008; Dowling et al. 2016), therefore, exogenous dsRNA can stimulate the immune system in both mammals and arthropods. In mammals, injection of small fragments of nanocarrier-formulated siRNA could activate the mammalian innate immune system (Robbins et al. 2009). Similarly, injection of non-specific dsRNA result in the increased expression of genes of the RNAi machinery, such as Dcr-2 and Ago2 in several insects

(Lozano et al. 2012; Garbutt and Reynolds 2012; Liu et al. 2013, 2014; Niu et al. 2016). However, it is still unclear whether such stimulation of the immune system can affect the fitness of NTOs (Lundgren and Duan 2013).

Saturation RNAi machinery

High levels of exogenous siRNAs could potentially saturate the RNAi machinery, since there is a limited number of RNAi effectors such as Dicer enzymes and RISC within the cell (Lundgren and Duan 2013). This saturation process could then temporarily inhibit the cellular use of RNAi and disturb gene regulation by endogenous miRNA (Khan et al. 2009; Jackson and Linsley 2010). However, so far this phenomenon has not been reported in invertebrate species (Christiaens et al. 2018a). Moreover, it is unknown whether this phenomenon can be induced by the concentrations of dsRNA used in pest control (Lundgren and Duan 2013; Christiaens et al. 2018a).

Environmental fate

Another possible concern relates with the fate of the insecticidal dsRNA in the environment, since the persistence and the movement of the dsRNA may impact the degree to which NTOs are exposed (Auer and Frederick 2009; Lundgren and Duan 2013; Parker and Sander 2017; Zhang et al. 2017). Recently, numerous studies indicate that dsRNA degrades rapidly in all agricultural soils, with a half-life of 15 h to 50 h (Dubelman et al. 2014; Fischer et al. 2016; Parker et al. 2019; Joaquim et al. 2019). Moreover, degradation kinetics of dsRNA degradation in soil appear to be independent of the concentration, sequence, molecular weight and structure (Fischer et al. 2016). Similarly, in aquatic environments dsRNA display a half-life of less than 3 days and no significant accumulation of dsRNA was observed in the undisturbed sediments (Albright Iii et al. 2017; Fischer et al. 2017). Overall, these results suggest that dsRNA is not anticipated to persist in environment.

Chapter 2

Imidacloprid resistance in the Neotropical brown stink bug *Euschistus heros*: selection and fitness costs

This chapter was partially redrafted from: **Castellanos NL**, Haddi K, Carvalho GA, Paulo PD, Hirose E, Guedes RNC, Smagghe G, Oliveira EE (2018) Imidacloprid resistance in the Neotropical stink bug *Euschistus heros*: Selection, metabolic mechanisms and fitness costs. Journal of Pest Science, 92: 847-860. doi: https://doi.org/10.1007/s10340-018-1048-z

2.1 Introduction

Neonicotinoids are systemic insecticides that interfere with the transmission of nerve impulses in insects by disrupting the functionality of specific insect nicotinic acetylcholine receptors (Salgado and Saar 2004; Jeschke and Nauen 2008; Oliveira et al. 2010, 2011; Salgado 2016). The use of neonicotinoids (alone or mixed with other compounds such as the pyrethroid insecticides) provides efficient control of *E. heros* infestations. However, due to the restricted number of registered compounds and their overuse, a low risk of *E. heros* control failure was recently reported in Central Brazil (Tuelher et al. 2018), indicating the likely occurrence of insecticide-resistant strains of this species (Macfadyen et al. 2014; Guedes et al. 2016, 2017). Furthermore, the bioecology of *E. heros* favors the selection of insecticide-resistant individuals, as these insects exhibit limited dispersal, keeping geographically distant populations reproductively isolated (Sosa-Gomez et al. 2004; Husch et al. 2018). Moreover, *E. heros* can produce multiple generations per soybean crop season (Sosa-Gómez and Omoto 2012) and is well adapted to survive in other crops or native plants during the soybean intercrop period (Smaniotto and Panizzi 2015), which increases their potential exposure to insecticides and the likelihood of selecting resistant individuals.

As observed for other groups of insecticides, the underlying mechanisms mitigating neonicotinoid activity are related to altered target-site insensitivity, enhanced detoxification and behavioral resistance (Nauen and Denholm 2005; Puinean et al. 2010; Seraydar and Kaufman 2015; Bass et al. 2015). Among these resistance mechanisms, although target-site insensitivity (Liu et al. 2005; Bass et al. 2011b; Zhang et al. 2015) and behavioral alterations (Seraydar and Kaufman 2015) have been reported, enhanced activity of detoxifying enzymes such as cytochrome P450 monooxygenases (Puinean et al. 2010; Yang et al. 2011b, 2013b, a, c; Bass et al. 2011a; Karatolos et al. 2011; Zhang et al. 2016b) is considered the main mechanism of neonicotinoid resistance.

Thus, this Chapter aims to evaluate the potential link between the recent reports of control failure and the occurrence of neonicotinoid-resistant *E. heros* strains. By using two *E. heros* strains with different genetic backgrounds, we evaluated the rate at which *E. heros* respond to selection for resistance to the neonicotinoid imidacloprid. We also assessed the contribution of metabolic and behavioral mechanisms of resistance and evaluated the potential adaptive fitness costs (e.g., reductions in survival and reproductive abilities) associated with imidacloprid resistance in the two laboratory-selected resistant strains.

2.2 Materials and methods

2.2.1 Insect strains and rearing

The three *E. heros* strains used in this investigation were derived from two initial colonies with different genetic backgrounds. The first is a well-known imidacloprid-susceptible strain (hereafter termed as ImiSusc) that was initially established from eggs obtained from the Semiochemical Laboratory at the Natural Resources and Biotechnology Centre of the Brazilian Agricultural Research Corporation (EMBRAPA Genetic Resources and Biotechnology; Brasília, DF, Brazil). This colony has been reared under controlled conditions (25 ± 2 ºC, 60 ± 20 % relative humidity, L:D photoperiod of 14:10 h) in an insecticide-free environment for more than 10 years. The first imidacloprid-resistant strain (hereafter termed ImiLabSel) was derived from the ImiSusc strain by subjecting early emerged (i.e., <24 h) adults to imidacloprid selection for 13 consecutive generations. We also used a second *E. heros* strain (hereafter termed as ImiGoias) as an initial background to select another imidacloprid-resistant strain (ImiRes). Adults of *E. heros* for this 2^{nd} resistance selection were originally collected in soybean fields in Santo Antonio de Goias (State of Goias, Brazil), which exhibited lower susceptibility to imidacloprid than ImiSusc (Tuelher et al. 2018). The progeny of ImiGoias were, therefore, reared under the same conditions as the other strains and were subjected to imidacloprid selection for six generations, resulting in the second imidacloprid-resistant strain, hereafter termed ImiRes.

All of the developmental stages of the *E. heros* strains were reared en masse in plastic boxes under controlled conditions and had ad libitum access to water and food. The diet provided was described elsewhere (Borges et al. 2006) and consisted of fresh green bean pods (*Phaseolus vulgaris*), soybean seeds, raw shelled peanuts (*Arachis hypogaea*) and sunflower seeds.

2.2.2 Selection for imidacloprid resistance and concentration-mortality bioassays

In each generation of selection, the insects of ImiSusc or ImiGoias were exposed to dried residues of imidacloprid using the residue-impregnated glass vial method, as detailed elsewhere (Willrich et al. 2003; Snodgrass et al. 2005; Santos et al. 2015; Tuelher et al. 2017). Briefly, the neonicotinoid insecticide imidacloprid (water-dispersible granules at 700 g active ingredient (a.i.)/kg; Bayer CropScience, São Paulo, SP, Brazil) was used to coat the inner walls of 250 mL transparent glass vials (EME Equipment, Paulicéia, SP, Brazil). Water (distilled and deionized) was used as a carrier for the commercial insecticide formulation, and 2 mL aliquots of insecticide-containing solution or water (control) were added to each glass vial. In each generation under selection,

E. heros adults (one to five days old) were exposed to dried imidacloprid residues (as described above); the number of individuals selected varied from 230 to 1532 for the ImiLabSel strain and from 645 to 1174 for the ImiRes strain. The first generation of selection for ImiRes started with 100 field-selected adults. The range of selection concentrations was 1.26 – 6.30 µg a.i./cm^2, and mortality was maintained between 40 % and 90 %. In this way, the effective population size, calculated as the 60 % of the survival parents, were maintained over 50 parents per generation to avoid inbreeding (Weber and Diggins 1990). Mortality in each selection cycle was determined after 48 h of exposure to imidacloprid. Adults surviving the insecticide exposure were transferred to an insecticide-free environment, and the populations were allowed to reproduce randomly. The rearing conditions were the same as described earlier.

The relative toxicity of imidacloprid for each stink bug strain was determined through concentration-mortality bioassays following methods previously described (Willrich et al. 2003; Snodgrass et al. 2005; Santos et al. 2015; Tuelher et al. 2017). Briefly, at least seven different insecticide concentrations (calculated as µg a.i./cm^2 of treated surface and ranging from 0.0126 to 252.0 µg a.i./cm^2, i.e., ranging from 0.003 to 60.0 times the field label rate, which corresponds to 4.2 µg a.i./cm^2) were tested in each *E. heros* strain. Seven to nine replicates (where a replicate consists of a glass vial containing 10 newly emerged adults [≤48 h]) were used for each insecticide concentration. The inner part of the top of each glass vial was coated with Teflon® PTFE (DuPont, Wilmington, DE, USA) and closed with a piece of organza and a rubber band to prevent the insects from escaping. The exposure period was 48 h, and the insects were counted as dead if they were unable to walk the length of their body when prodded with a fine hair paintbrush. Mortality data were corrected for natural mortality (i.e., mortality from the control treatment).

2.2.3 Biochemical assays

Enzyme extraction

The fat body of newly emerged adults (≤48 h old) of each E. *E. heros* strain (i.e., ImiSusc, ImiLabSel and ImiRes) was used for determining the activity of detoxifying enzymes: cytochrome P450, glutathione-S-transferases (GST) and total esterases. Cytochrome P450 activity was determined using fat bodies from five adults; the fat bodies were chilled and homogenized in 2 mL of phosphate buffer (0.1 M, pH 7.5) containing Triton X-100 (0.3 %). These procedures were performed with four groups of five insects from each strain tested. To quantify the activity of glutathione-S-transferases (GST) and total esterases, we first chilled and homogenized the fat bodies of four groups of 10 insects in 3.5 mL of phosphate buffer (0.1 M, pH 7.5) containing Triton X-100 (0.3 %) to aid in the membrane solubilization and quantification of soluble enzyme activity (e.g., GST; Hemingway 1998). Subsequently, we centrifuged the material at 10,000 g for 10 min

at 4 °C. The obtained supernatants were then used as enzymatic sources. Ultracentrifugation at 100,000 g commonly used for cytochrome P450 determinations was not employed here because this procedure and subsequent re-solubilization of the microsomal fraction exhibits low reproducibility and is not required for the heme-peroxidase bioassay used here to determined P450 activity (Brogdon et al. 1997).

Total protein content

The total protein contents of the crude extracts were determined using the Bradford method (Bradford 1976). The standard curve for protein content was generated from serial dilutions of bovine albumin (Sigma Aldrich, St Louis, MO, USA).

Cytochrome P-450 activity

The cytochrome P450 activity was determined using the heme peroxidase assay, which is an indirect measure using heme peroxidation (Brogdon et al. 1997). Heme peroxidase activity was measured using 3,3′,5,5′-tetramethylbenzidine (TMBZ) (Sigma Aldrich, St Louis, MO, USA) as the substrate. The total reaction volume per well of a 96-well microplate was 205 µl, consisting of 20 µl of enzyme solution, 60 µl of potassium phosphate buffer (1M, pH 7.2), 200 µl of TMBZ solution, and 25 µl of hydrogen peroxide (3 %). Wells with the phosphate buffer (0.1M, pH 7.5) alone were used as controls. The mixture was left for 30 minutes at room temperature. Absorbance was read at 650 nm, and the values were compared with a standard curve of absorbance for known concentrations of cytochrome C (Sigma Aldrich). The values are reported as nmol/mg protein.

Glutathione S-transferase activity

GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma Aldrich, St Louis, MO, USA) as the substrate (Habig et al. 1974). The total reaction volume was 2 ml, consisting of 1760 µL of phosphate buffer (0.1M, pH 7.5), 200 µl of enzyme solution, 20 µl of 150 mM CDNB solution, and 20 µl of 150 mM GSH solution (Sigma Aldrich, St Louis, MO, USA). Control cells consisted of 1960 µL of phosphate buffer (0.1M, pH 7.5), 20 µl of 15 mM CDNB solution, and 20 µl of 15 mM GSH solution. GST activity was determined by the change in absorbance as measured continuously every 30 s for 90 s at 340 nm using a spectrophotometer (UV-1800, Shimadzu, Tokyo, Japan). GST activity was reported as µmol CDNB conjugated/min/mg protein, using published extinction coefficients corrected for the path length (Habig et al. 1974).

Total esterase activity

Total esterase activity was determined using α-naphthyl acetate (Sigma Aldrich, St Louis, MO, USA) as a substrate (van Asperen 2003). The total reaction volume per well of a 96-well microplate was 210 µl, consisting of 10 µl of enzyme solution and 200 µl α-naphthyl acetate 0.3 mM solution. Wells with the phosphate buffer (0.1M, pH 7.5) alone were used as controls. After 15 min of incubation at 30 °C, 50 µl of DBLS solution was added to each well to stop the reaction. Absorbance of the naphthol–Fast Blue B complex was measured at 600 nm after 15 min incubation at room temperature. Absorbance levels were compared with a standard curve of absorbance for known concentrations of α-naphthol. The results were reported as µmol α-naphthol/min/mg protein.

2.2.4 Behavioral bioassays

Walking bioassays were monitored using an automated video tracking system equipped with a CCD camera (ViewPoint Life Sciences Inc., Montreal, Canada), following methods previously described elsewhere (Pereira et al. 2009; Corrêa et al. 2011; Haddi et al. 2015; Gonzales Correa et al. 2015). For each video-tracking trial, four adults were randomly selected from each *E. heros* strain (i.e., ImiSusc, ImiLabSel and ImiRes). The adults were distributed into four glass Petri dishes (135 × 20 mm) with filter paper in the bottom. The movement of each insect within the arena was recorded for 60 min. Distance walked and the time spent walking were also measured. Twenty to 27 replicates (i.e., a newly emerged [<48 h old] virgin female or male) were used for each strain. After each trial, the filter papers were replaced. For the mating behavior bioassays, 25 virgin pairs (i.e., female and male) of each *E. heros* strain (i.e., ImiSusc, ImiLabSel and ImiRes) were allowed to mate during a period of 13 h, which was digitally recorded (HDR-XR520V, Sony, Tokyo, Japan). After this mating period, the males were removed and all insects were kept in individual containers. The recorded material allowed us to determine the latency to the first mating, the number of matings, the duration of each mating and the total mating duration for each couple. The number of couples that did not mate was always less than 2 %, and their results were not used in the statistical analyses.

2.2.5 Fitness costs

Twenty to 27 mated couples per strain (i.e., ImiSusc, ImiLabSel and ImiRes) were individually monitored until death. The number of eggs laid per female, egg hatching, the percentage of females laying eggs and the survival rate of each sex were recorded daily. Insects were considered dead when they were unable to walk after being prodded with a fine hair paintbrush. Fecundity (Fd) was assessed as the average number of eggs produced per copulated female, and hatchability (Ha) was recorded as

(neonates)/(neonates + unhatched eggs). A total of 90 neonates were randomly collected from each *E. heros* strain. The survival and duration of each developmental stage were recorded. The obtained data were used to assess the survival from neonate to 3^{rd} instar (Sr1), survival from 3^{rd} to 5^{th} instar (Sr2), emergence rate (Er) and female ratio (Fr). The experiments were carried out in three replicates. The population trend index (I) and relative fitness were calculated following Liu and Han (2006). First, we estimated the number of individuals in the population of the next generation (Nt), $Nt = N0 \times Sr1 \times Sr2 \times Er \times Fr \times Cr \times Fd \times Ha$, which served to estimate the strain trend index $(I), I = Nt/N0$, where N0 is the number of individuals in the initial population. Finally, we estimated the relative fitness for each imidacloprid-resistant strain by using the following formula:

$$relative fitness = \frac{IR}{IS}$$

where IR represents the increase trend index of the resistant strain and IS represents the increase trend index of the susceptible strain (Liu and Han 2006).

2.2.6 Statistical analyses

The results concerning detoxifying enzyme activity, fitness costs, and mating and walking behavior were subjected to univariate analysis of variance (ANOVA), or a Kruskal-Wallis (H) one-way ANOVA by ranks when the assumptions of normality and homoscedasticity were not satisfied. 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). The insects that remained alive at the end of the bioassay were censored for the analyses. Overall similarity among the survival and median survival times (LT_{50} values) was tested using the χ^2 log-rank test, and pairwise comparisons among the curves were conducted using the Holm-Sidak test ($P < 0.05$). When appropriate, regression analysis was performed using the curve-fitting procedure of SigmaPlot 12.0. Regression analyses were performed to detect trends in fecundity and fertility parameters that resulted from each treatment through time. The regression model was chosen based on parsimony, low standard errors, and steep increases in R^2 with model complexity. The regression models for each treatment were considered different from each other if the confidence limits of their parameters did not overlap.

2.3 Results

2.3.1 Selection for imidacloprid resistance

The mortality values of E. heros adults from two strains with different genetic backgrounds (i.e., ImiSusc and ImiGoias) to increasing concentrations of imidacloprid are

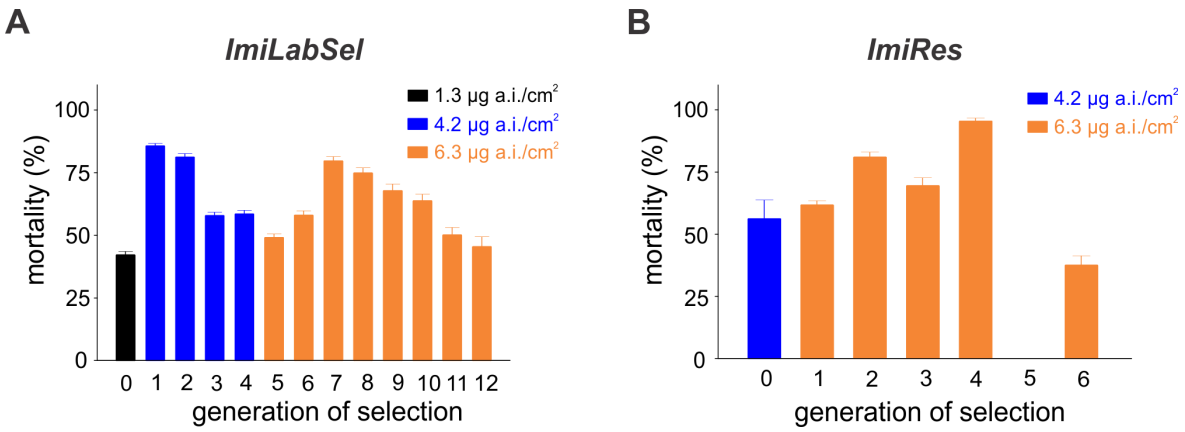


Figure 2.1: Response to selection for imidacloprid in two strains of the Neotropical brown stink bug *Euschistus heros*. Mortality of *E. heros* individuals derived from a well-known imidacloprid-susceptible strain (***A***, ImiLabSel) and from another strain (ImiGoias) that was originally field collected (***B***, ImiRes). Data are means ± standard error (SE). The assay was conducted with 10-53 repetitions, each consisting of 10 adults.

shown in Figure 2.1. The ImiLabSel strain was obtained after 13 consecutive generations, while ImiRes was obtained after selecting from the ImiGoias strain for six generations. As ImiSusc is a very well-known imidacloprid-susceptible strain, it was necessary to start the selection procedure with the application of 1.26 µg a.i./cm^2 (the equivalent of its estimated LC_{50}) (Figure 2.1*A*). The progeny of these survivors was subjected to the field rate (i.e., 4.2 µg a.i./cm^2), and after 4 generations of selection the mortality reduced from 85.5 ± 1.1 % to 58.3 ± 1.6 %.

In the next 8 consecutive generations of selections, the insects were subjected to a higher concentration (i.e., 6.3 µg a.i./cm^2), resulting in the ImiLabSel strain, which exhibited a mortality level of only 45.3 ± 4.1 %. ImiRes was obtained from individuals that were field collected (ImiGoias) and already exhibited a high resistance level before the start of selection (F0), such that the application of 4.2 µg a.i./cm^2 killed only 56.0 ± 7.7 % (Figure 2.1*B*). The progeny of these survivors was subjected to higher imidacloprid concentrations (i.e., 6.3 µg a.i./cm^2), and after 6 generations of selection, the mortality reached only 37.4 ± 3.9 %.

The probit model satisfactorily described the concentration-mortality data (goodness-of-fit tests exhibited low χ^2 -values [<6.1] and high *P*-values [>0.05]), allowing the estimation of the desired toxicological endpoints (i.e., LC_{50}) and the resistance ratios. The ImiLabSel (LC_{50} = 14.5 [7.3 - 36.8] µg a.i./cm^2, $\chi^2_{(1,5)}$ = 3.0, *P* = 0.43) strain was 11.6 [10.3 – 22.5]-fold more resistant to imidacloprid than the ImiSusc strain was (LC_{50} = 1.2 [0.9 – 1.9] µg a.i./cm^2, $\chi^2_{(1,5)}$ = 1.7, *P* = 0.71) (Fig. 2), while ImiRes exhibited an LC_{50} of 16.8 [12.5 – 23.9] µg a.i./cm2 ($\chi^2_{(1,5)}$ = 6.1, *P* = 0.10) and 13.5 [8.4 – 37.6]-fold resistance to imidacloprid compared with the ImiSusc strain (Figure 2.2).

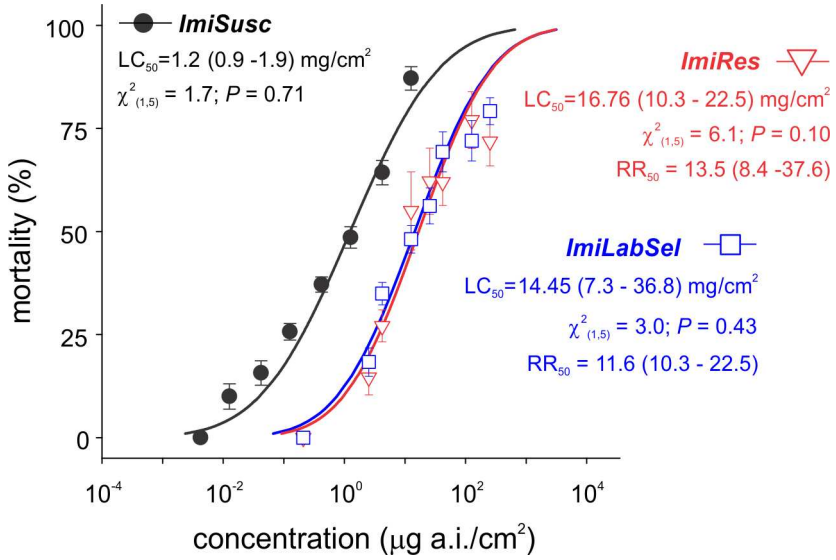


Figure 2.2: Toxicity of imidacloprid to three strains of the Neotropical brown stink bug *Euschistus heros*. Lethal concentration (LC) values were estimated based in concentration-mortality bioassays using probit analysis. The resistance ratio (RR) is expressed as the LC_{50} of imidacloprid in the resistant strains divided by the LC_{50} in the susceptible stain (ImiSusc). The symbols show the averaged mortality, and the vertical bars represent the standard error (SE). All concentrations were repeated seven to nine times, each consisting of 10 adults

2.3.2 Enhanced detoxification in imidacloprid-resistant strains

Although the activity levels of glutathione-S-transferase ($F_{2,9}$ = 0.55, *P* = 0.60) and total esterases ($F_{2,8}$ = 0.18, *P* = 0.84) were not significantly different among strains (Figure 2.3*A,B*), the activity of cytochrome P450 enzymes was significantly increased ($F_{2,6}$ = 17.0, *P* = 0.003) in both ImiLabSel (72.3 %) and ImiRes (40.5 %) compared with ImiSusc (Figure 2.3*C*).

2.3.3 Behavioral activity, fecundity and fertility

The Krukal Wallis ANOVA indicated that imidacloprid resistance had no effect on walking duration (males: *H* = 1.15, *df* = 2, *P* = 0.56; females: *H* = 1.75, *df* = 2, *P* = 0.42) or distance walked (males: *H*= 1.46, *df* = 2, *P* = 0.48; females: *H* = 2.04, *df* = 2, *P* = 0.436) compared with the results recorded for ImiSusc (Figure 2.4). A similar absence of effects was recorded for the mating results (number of matings: *H* = 1.79, *df* = 2, *P* = 0.41; mating duration: *H* = 0.01, *df* = 2, *P* = 0.99) (Figure 2.5).

However, imidacloprid resistance reduced the survivorship of nymphs and the longevity of adults of both resistant strains (Figure 2.6). The longevity of the males varied significantly (log-rank test, χ^2 =12.8,*df* = 2, *P* = 0.002) among the susceptible and resistant strains (Figure 2.6*A*). The males of the ImiSusc strain lived longer (LT_{50} = 82.0 [78.5 – 85.5] days) than the males of the ImiLabSel strain (LT_{50} = 58.0 [52.4 – 63.6] days) or the ImiRes strain (LT_{50} = 57.0 [54.4 – 59.6] days). However, when we compared the longevity of females, no significant differences (log-rank test, χ^2 = 3.8,

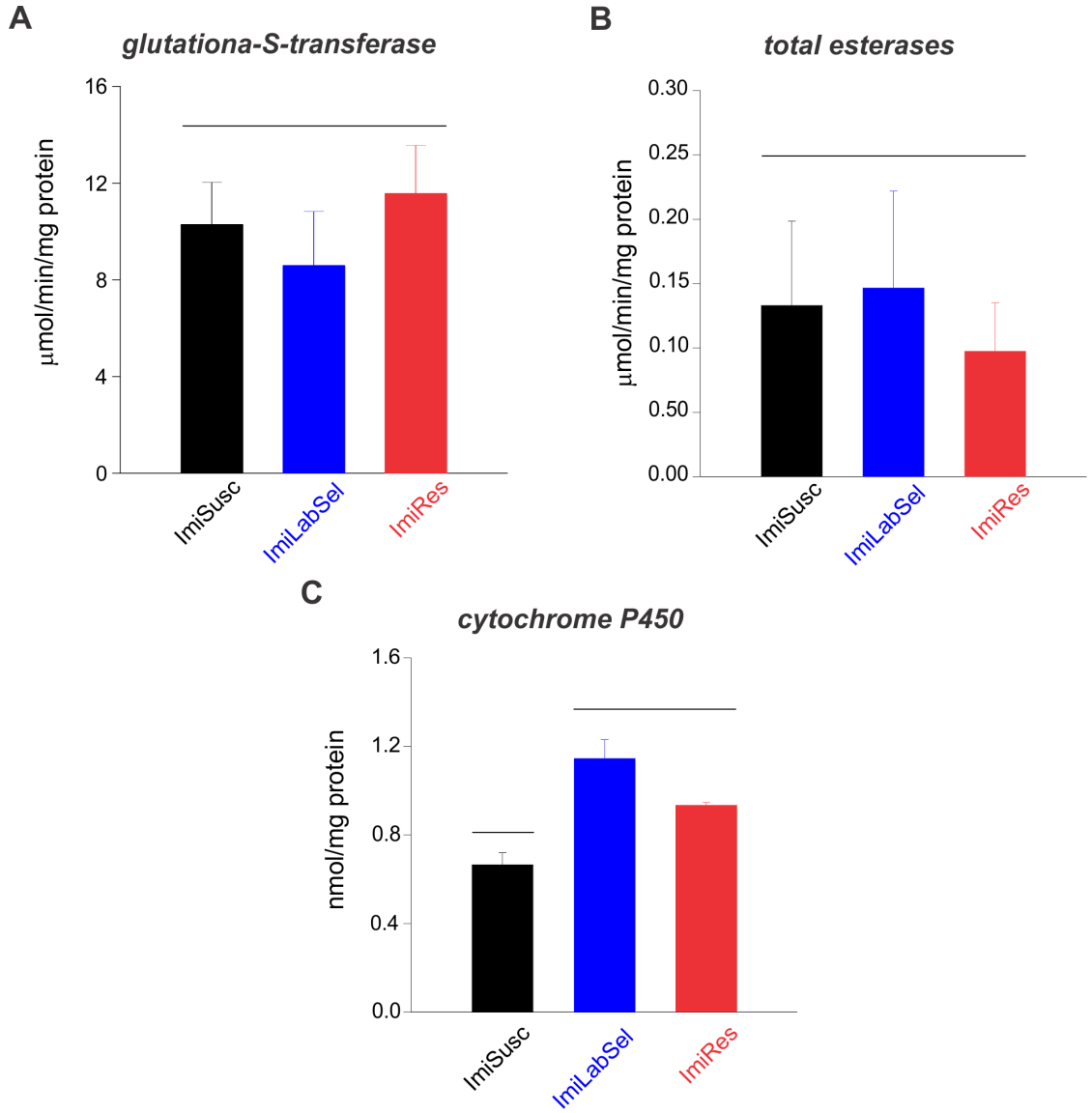


Figure 2.3: Activity of glutathione-S-transferases (***A***), total esterases (***B***) and cytochrome P450 (***C***) in one imidacloprid-susceptible (ImiSusc) and two imidacloprid-resistant strains (ImiLabSel and ImiRes) of the Neotropical brown stink bug *Euschistus heros*. The results represent the mean of three independent samples (± standard error, SE). Each sample contains 10 (A-B) or 5 (C) pooled fat bodies. (***A-C***) Means grouped under the same horizontal line are not significantly different by Tukey's HSD test ($P < 0.05$).

df = 2, *P* = 0.15) were observed between the ImiSusc strain (LT_{50} = 52.0 [39.7 – 64.3] days) and the two resistant strains (ImiLabSel: LT_{50} = 39.0 [22.6 – 55.4] days; ImiRes: LT_{50} = 55.0 [39.0 – 71.0] days). The nymph survivorship of ImiSusc (49.5 ± 4.7 %) was significantly higher than that of ImiLabSel (27.9 ± 2.2 %) or ImiRes (40.9 ± 2.8 %) (Figure 2.6*B*).

Both daily and cumulative fecundity were significantly affected in the resistant strains (Figure 2.7*A, B*). The daily fecundity results fit well with the three-parameter log-normal model with a bell-shaped curve skewed to the left and a long tail to the right (Figure 2.7*A*). The ImiSusc strain exhibited the highest peak of the daily fecundity, while the Im-

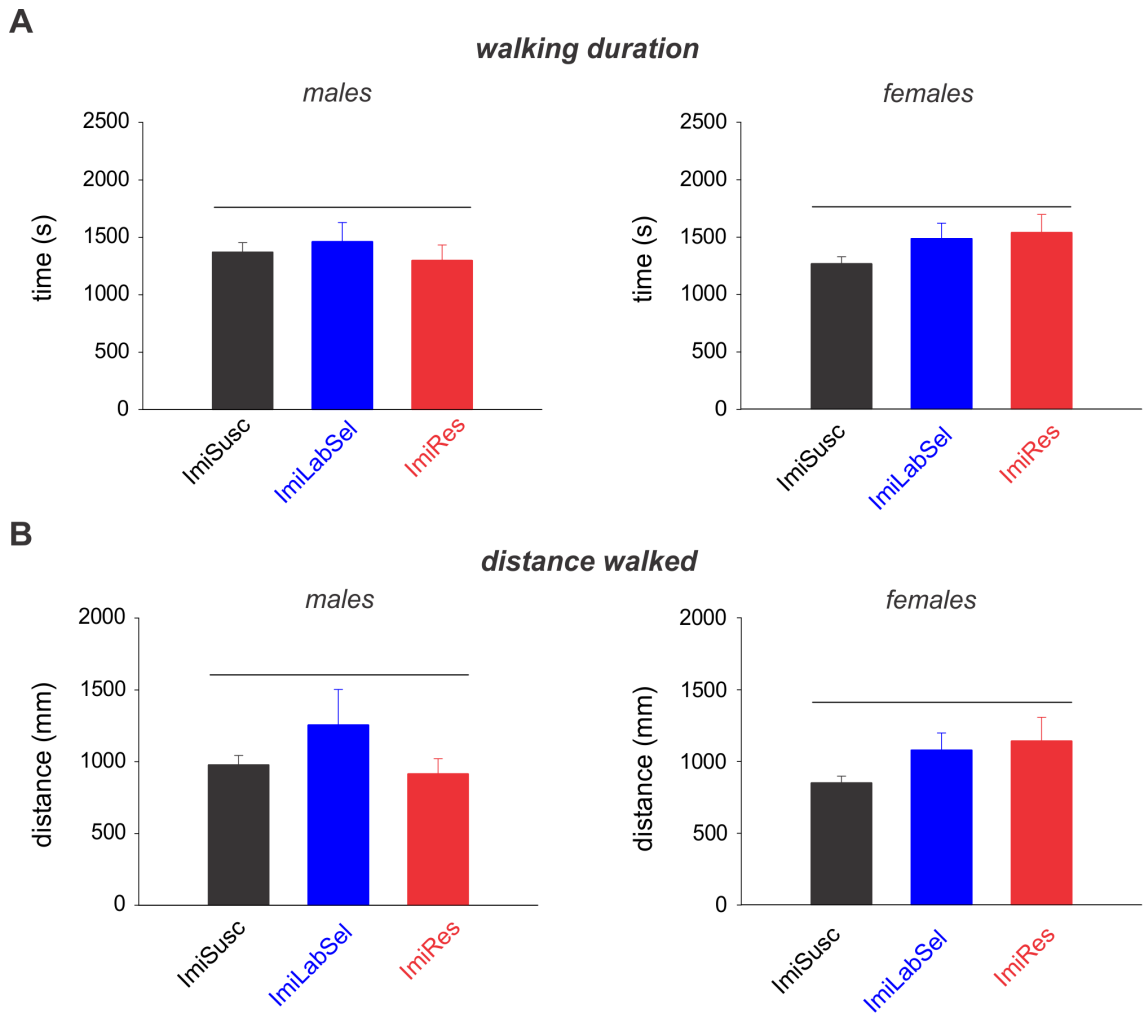


Figure 2.4: Walking duration (***A***) and distance walked (***B***) in one imidacloprid-susceptible strain (ImiSusc) and two imidacloprid-resistant strains (ImiLabSel and ImiRes) of the Neotropical brown stink bug *Euschistus heros*. (***A-B***) The left panels refer to data collected from *E. heros* males, while the right panels present data collected from *E. heros* females. Bars represent the mean observed data (n=20-22), and the ones grouped under the same horizontal line are not significantly different (post hoc Kruskal–Wallis test, $P < 0.05$).

iLabSel strain showed the lowest peak (Appendix 2.1). Furthermore, the ImiSusc strain exhibited higher levels of daily fecundity than either resistant strain, reflecting higher cumulative fecundity following logistic growth, where the rate of increase (c) decreases as the population increases towards a maximum value (a). The ImiSusc strain exhibited a higher maximum value as well as a smaller rate of decrease than either of the imidacloprid-resistant strains (Figure 2.7*B*; Appendix 2.2). Furthermore, the cumulative number of eggs obtained for the ImiRes strain was significantly higher than the number recorded in the ImiLabSel strain.

Daily and cumulative fertility (Figure 2.7*C,D*) exhibited trends similar to the daily fecundity, which were higher for the ImiSusc strain, with the ImiLabSel strain exhibiting the greatest reproductive impairment. The ImiSusc strain exhibited the highest peak of daily fertility, followed by the ImiRes (Figure 2.7*C*, Appendix 2.1). For the cumulative num-

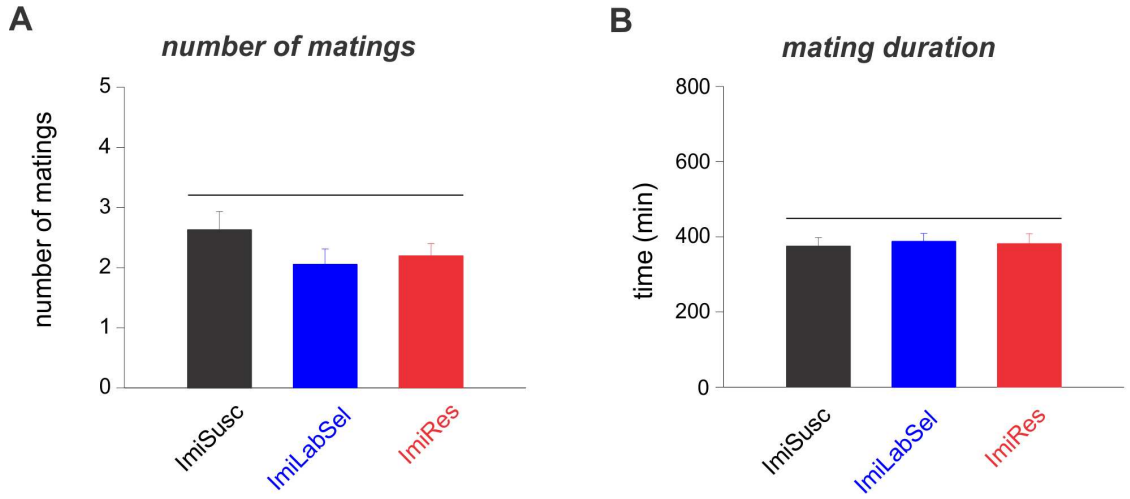


Figure 2.5: Number of matings (***A***) and mating duration (***B***) in one imidacloprid-susceptible strain (ImiSusc) and two imidacloprid-resistant strains (ImiLabSel and ImiRes) of the Neotropical brown stink bug *Euschistus heros*. (***A-B***) Bars represent the mean observed data (n=20-27), and the ones grouped under the same horizontal line are not significantly different (post hoc Kruskal–Wallis test, $P < 0.05$).

ber of hatched nymphs, the highest maximum value was observed in the ImiSusc strain while the lowest maximum value was recorded for the ImiLabSel (Figure 2.7*D*, Appendix 2.2). Furthermore, significant differences ($F_{2,60}$ = 7.3, P = 0.001) were recorded for the egg incubation time in the three E. heros strains. While the egg incubation time for the ImiSusc (7.1 ± 0.06 days) and ImiRes (7.2 ± 0.10 days) strains were not significantly different, eggs from the ImiLabSel strain took longer (7.5 ± 0.09 days) to hatch.

2.3.4 Fitness costs in resistant strains

As shown in the Table 2.1, the survival of the earlier nymphal phases (i.e., neonate to third instar), as well as the fecundity and fertility (i.e., hatchability) rates, were all significantly reduced in the imidacloprid-resistant strain ImiLabSel, but not in ImiRes, when

Table 2.1: Adaptive fitness of one imidacloprid-susceptible (ImiSusc) and two imidacloprid-resistant strains (ImiLabSel and ImiRes) of the Neotropical brown stink bugs, *Euschistus heros*.

Parameter	ImiSusc	ImiRes	ImiLabSel
Neonate number	92	92	92
Survival rate from 1^{st} to 3^{rd} instar	0.673±0.022 a	0.597±0.009 ab	0.488±0.033 b
Survival rate from 3^{rd} to 5^{th} instar	0.741±0.058 a	0.690±0.039 a	0.587±0.045 a
Emergence rate	0.867±0.012 a	0.896±0.020 a	0.966±0.033 a
Female ratio	0.671±0.067 a	0.466±0.058 a	0.675±0.050 a
Copulation rate	0.929±0.035 a	0.958±0.041 a	0.952±0.047 a
Fecundity	276.666±31.044 a	223.428±18.471 ab	145.100±25.227 b
Hatchability	0.299±0.042 a	0.192±0.026 ab	0.141±0.026 b
N, predicted number of offspring	2060.2	654.6	336.3
I, population trend index	22.4	7.1	3.7
Relative fitness	1.000	0.317	0.163

Values followed with the same letter in the raw do not differ significantly according to a Tukey's HSD test ($P < 0.05$)

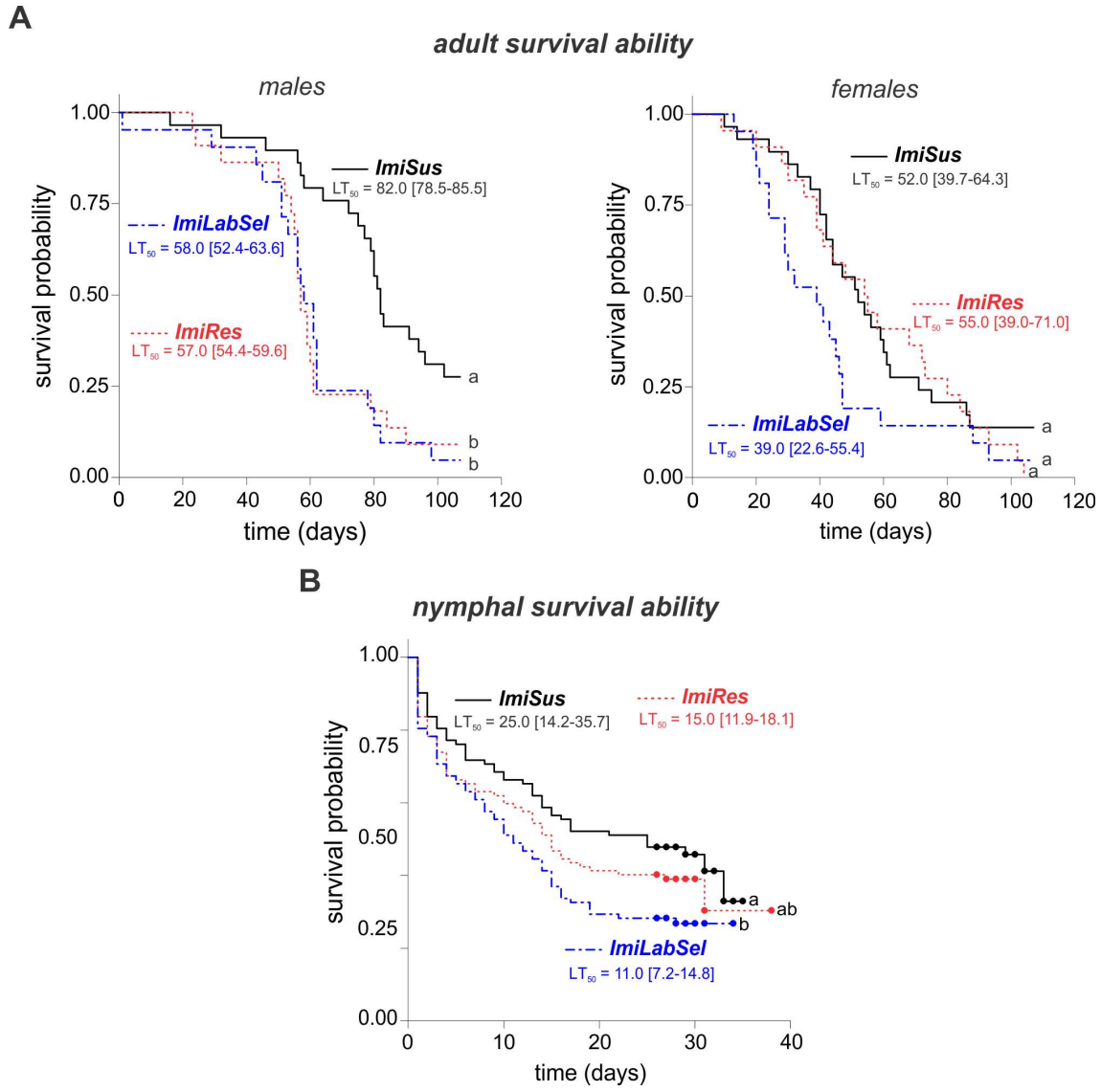


Figure 2.6: Survival curves in one imidacloprid-susceptible strain (ImiSusc) and two imidacloprid-resistant strains (ImiLabSel and ImiRes) of the Neotropical brown stink bug *Euschistus heros*.(***A***) Longevity of adult males (left panel) and females (right panel) (n=20-27). (***B***). Nymphal survival curves (n=92). The symbols at the end of each curve indicate the censored data. (***A-B***) The curves label with the same letter are not significantly different according to a Holm-Sidak test ($P > 0.05$).

compared with the ImiSusc strain. All the other fitness parameters (i.e., the survival ability of older nymph phases (i.e., third to fifth instar), emergence rates, sex [female] ratio and copulation rates) were not significantly different among strains (Table 2.1). Severe reductions of relative fitness were observed for both imidacloprid-resistant strains, where the ImiRes exhibited only one-third (0.317) and the ImiLabSel only one-sixth (0.163) of the relative fitness recorded for the ImiSusc strain. Furthermore, as demonstrated by the population trend index values (Table 2.1), the ImiSusc could breed 22.4 times in a generation, while reduced breeding abilities were observed for the ImiRes (7.1) and ImiLabSel (3.7) strains.

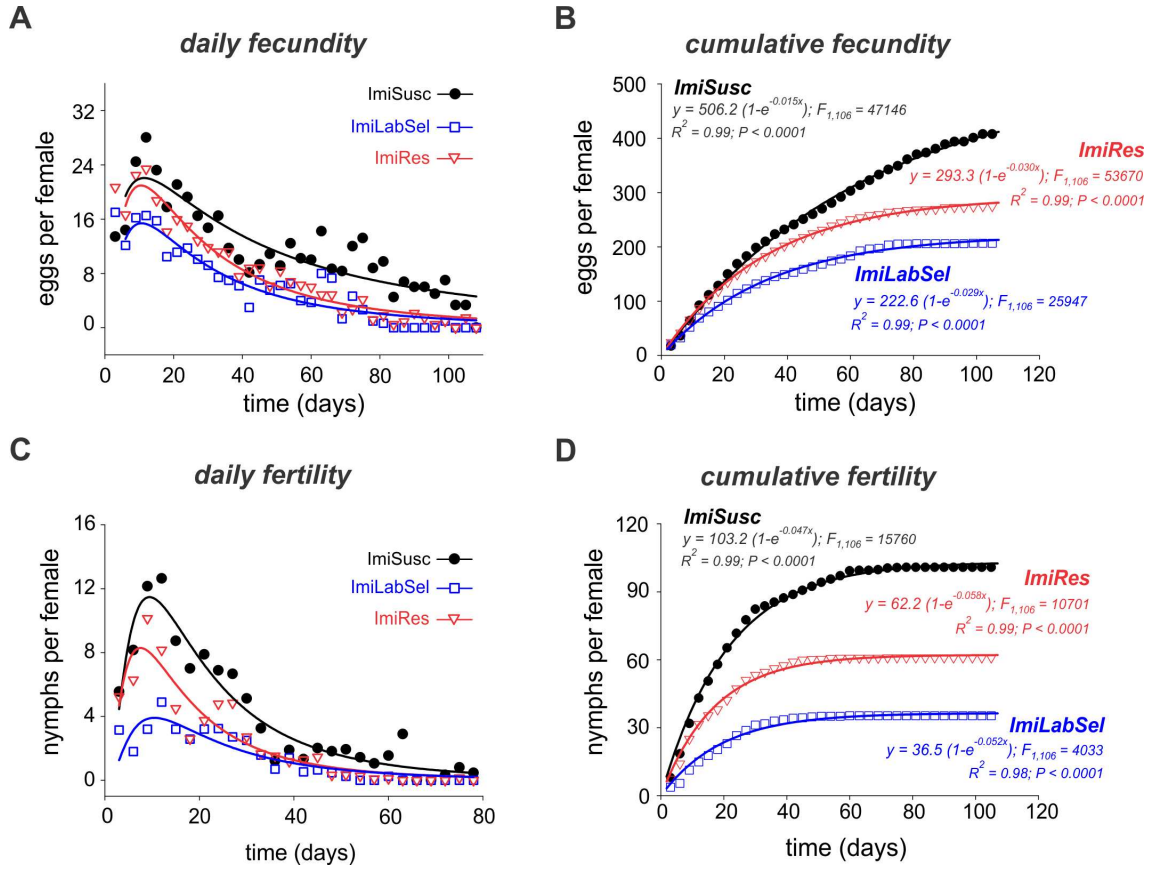


Figure 2.7: Daily (***A***) and cumulative fecundity (***B***), and daily (***C***) and cumulative fertility (***D***) of one imidacloprid-susceptible strain (ImiSusc) and two imidacloprid-resistant strains (ImiLabSel and ImiRes) of the Neotropical brown stink bug *Euschistus heros*. The symbols represent the mean observed data (n=20-27), and the lines represent the predicted trend of the best models selected.

2.4 Discussion

The sustainability of the neonicotinoids as a management tool against insect pests relies on the absence or scarcity of neonicotinoid-resistant populations, among other things. In this Chapter, we described for the first time that *E. heros* respond to selection for resistance to the neonicotinoid imidacloprid. Furthermore, by demonstrating that a field-derived *E. heros* strain (ImiRes) required only six generations of selection to achieve significant resistance levels (i.e., resistance ratio of 13.5 [8.4 – 37.6]), our findings indicate that preventive management practices are urgently required to delay the occurrence of imidacloprid-resistant strains and consequent field control failures. The data also suggest that detoxification mechanisms, particularly enhanced cytochrome P450 activity, might play relevant role in imidacloprid resistance in *E. heros*. Furthermore, the expression of resistance in *E. heros* was associated with fitness costs, reflected by reduced male longevity and reduced female fecundity and fertility.

Despite the recently described unintended effects of insecticides on *E. heros* (Santos et al. 2015, 2016; Haddi et al. 2016; Tuelher et al. 2017), the use of neonicoti-

noid insecticides (alone or mixed with pyrethroids) has been providing effective control of this soybean insect pest (Sosa-Gómez and Silva 2010; Hegeto et al. 2015; Tuelher et al. 2018). However, recent initiatives of spatial surveys reported a low risk of *E. heros* control failure with imidacloprid in Central Brazil, which also extended to the pyrethroid insecticide beta-cyfluthrin (Guedes 2017, Tuelher et al. 2018), and raised the concern of potential selection for imidacloprid resistance in this pest species. Thus, the rapid selection response obtained in the ImiRes strain is likely due, at least partially, to the genetic background of the field-collected individuals from which this strain was selected for imidacloprid resistance. These field-collected insects may have already faced selection for insecticide resistance, exhibiting a higher genetic variability and/or higher frequency of resistant alleles within the population (Roush and McKenzie 1987; Ffrench-Constant 2013). The reported occurrence of Brazilian *E. heros* populations resistant to organophosphate insecticides (Sosa-Gomez et al. 2001; Sosa-Gómez and Silva 2010) and of populations exhibiting cross-resistance between organophosphate and imidacloprid in *B. tabaci* lends credence to this hypothesis (Naveen et al. 2017).

Imidacloprid toxicity is determined by this compound mode of action on existing insect nicotinic receptor (nAChR) types that may prevail in different insect species (Salgado and Saar 2004; Jeschke and Nauen 2008; Oliveira et al. 2010, 2011; Salgado 2016). As a result, some non-hemipteran insects exhibited faster responses for imidacloprid selection than the stink bug *E. heros*. This seems to be the case for the housefly (*Musca domestica*), which exhibits a 138-fold resistance to imidacloprid at 14 generations of selection (Abbas et al. 2015). Furthermore, the presence of two high-affinity imidacloprid binding sites has been reported for hemipteran insects, while other insects apparently have only one high-affinity binding site (Lind et al. 1998; Tomizawa and Casida 2005).

Enhanced detoxification activity has been reported as the major mechanism involved in the imidacloprid resistance (Nauen and Denholm 2005; Bass et al. 2015). In the present investigation, cytochrome P450 activity was enhanced in both resistant strains (72 % in ImiLabSel and 40 % in ImiRes) when compared with the imidacloprid-susceptible (ImiSusc) strain, suggesting a relevant role of these enzymes for the imidacloprid resistance in *E. heros*. Indeed, cytochrome P450 enzymes are capable of detoxifying imidacloprid, and more than 20 P450 genes from the families CYP4, CYP6 and CYP9 have been associated with imidacloprid resistance in insects (Bass et al. 2011a, 2015; Ding et al. 2013; Højland et al. 2014; Zhu and Luttrell 2015; Zhang et al. 2016a; Clements et al. 2016, 2017). So far, the capacity to break down imidacloprid has been confirmed only in three insect P450s, CYP6G1 (*D. melanogaster*), CYP6CM1vQ (*B. tabaci*) and CYP6AY1 (*Nilaparvata lugens*) (Daborn et al. 2001; Karunker et al. 2009; Ding et al. 2013). Furthermore, our findings also revealed that total esterases and glutathione-S-transferases do not seem relevant to the imidacloprid resistance in *E. heros*, as their activity levels in the two imidacloprid-resistant strains were not significantly different from the levels in the susceptible strain.

Although behavioral disturbances (e.g., reduced response to the aphid alarm pheromone and greater susceptibility to parasitoid attack) have been reported in other imidacloprid-resistant insects (Foster et al. 2003b, a, 2007; Guedes et al. 2016), no significant effects were observed on walking or mating among individuals of either imidacloprid-resistant strain. However, significant reproductive and survival costs were recorded in both ImiRes and ImiLabSel strains, indicating a physiological trade-off between detoxification and reproduction, which leads to fitness costs associated with imidacloprid resistance. Although insecticide-resistant insect strains do not necessarily exhibit associated fitness costs (and even fitness advantages were reported in some cases) (Berticat et al. 2002; Guedes et al. 2006; Hardstone et al. 2009; Rinkevich et al. 2013; Bacca et al. 2017), enhanced detoxification is expected to be costly in insecticide-free environments, generally requiring resource and energy allocation that would otherwise be used for survival and reproduction (Roush and McKenzie 2003; Foster et al. 2007; Kliot and Ghanim 2012).

Here, both imidacloprid-resistant strains showed reduced adult survival and reduced population growth. However, the adaptive costs were elevated for the ImiLabSel individuals, resulting in a population growth rate approximating one-sixth (0.163) of that recorded for imidacloprid-susceptible strain. Such results indicate that although the ImiRes population growth was severely reduced (i.e., to one-third [0.317] that of ImiSusc), this field-derived resistant strain performed twice as well as the similarly resistant individuals of ImiLabSel. This difference between the fitness of the field (ImiRes) vs. laboratory-derived strains (ImiLabSel) could be the result of the reduced genetic variability in the ImiLabSel. Since selection in the field acts on large population sizes and a potentially limitless source of rare mutations, whereas selection of a few individuals in the laboratory can lead only to an accumulation of a number of traits of minor effect with higher fitness cost (Roush and McKenzie 1987; Ffrench-Constant 2013). This discrepancy between laboratory and field resistance illustrates the importance of monitoring insecticide resistance in field, as well as characterize the mechanisms, heritance and cross resistance patterns (Roush and McKenzie 1987; Ffrench-Constant 2014).In addition, the high fitness cost associated with the insecticide resistance reinforces the usefulness of adopting preventive management practices, such as using alternative control methods and rotating use of insecticides with different modes of action, which are urgently needed to delay the occurrence of imidacloprid resistance and associated control failure in the field levels. This temporary suppression of imidacloprid use has been a successful initiative as an insect pest management tool (Wang et al. 2009; Abbas et al. 2015; Khan et al. 2015; Yang et al. 2016).

Thus, although imidacloprid resistance in field conditions is still in the initial stages (Tuelher et al. 2018), the present investigation, by demonstrating that *E. heros* readily responded to imidacloprid selection, reinforces the concern with this phenomenon among stink bugs (particularly *E. heros*). As the use of imidacloprid is rather frequent

not only against stink bugs, but also against whiteflies in Brazilian soybean fields, with few alternative insecticides and low dispersal and survival of the former pest species on common intercrops in the region, selection for imidacloprid resistance is sustained. A sound resistance management practice for this scenario is the rotation of imidacloprid with insecticides of other modes of action and detoxification, as it would allow erosion of imidacloprid resistance due to its associated fitness cost. Further experiments are necessary, however, to ascertain of the imidacloprid resistance mechanisms involved (e.g., target-site insensitivity, identification of the individual P450 enzymes), as well as the ability of resistant individuals to compete with susceptible ones (either susceptible conspecifics or heterospecifics) under field conditions, which will highlight how quickly imidacloprid resistance can spread in soybean-growing regions.

Chapter 3

The fitness and economic benefits of rearing the parasitoid *Telenomus podisi* under fluctuating temperature regime

This chapter was partially redrafted from:

Castellanos NL,Bueno AF, Haddi K, Silveira EC, Rodrigues HS, Hirose E, Smagghe G, Oliveira EE (2019) The fitness and economic benefits of rearing the parasitoid *Telenomus podisi* under fluctuating temperature regime. Neotropical Entomology, accepted. Fourth-place award in the Biological Control Symposium (SICONBIOL)-Londrina, Brazil-2019.

3.1 Introduction

Thermal conditions have a strong influence on physiological and adaptive traits (e.g., immune function, survival and behavioral abilities) of poikilothermic organisms such as insects (Colinet et al. 2015; Furlong and Zalucki 2017; Abram et al. 2017). As terrestrial insects normally have to face field conditions with daily thermal fluctuations and periods of extremely high or low temperatures (Bannerman and Roitberg 2014; Colinet et al. 2015), it would be natural to expect that insect production facilities mass-rear the biological control agents using fluctuating temperatures. However, the vast majority of mass-rearing insect production facilities neglect such information and use constant conditions (e.g., optimal temperatures) aiming to maximize the rearing productivity and achieve the required levels demanded for augmentative biological control programs (Parra 2010; Chidawanyika and Terblanche 2011; Sørensen et al. 2012).

Instead of aiming for increasing numbers of reared individuals, biological control programs would rather benefit, in terms of quality, efficiency and costs, from developing rearing protocols that enhance field performance of the released individuals (Sørensen et al. 2012). Rearing insects on constant optimal temperature could impair the biological performance of the mass-reared insects when they are exposed to field conditions, negatively impacting the efficacy of biological control programs (Chidawanyika and Terblanche 2011; Sørensen et al. 2012; Terblanche 2014; Chidawanyika et al. 2017). Since multiple generations of rearing in a stable temperature is predicted to reduce the levels of plasticity, due to unnecessary cost connected with the maintenance of the plastic machinery in a non-changing environment (Reed et al. 2003; Lande 2009; Manenti et al. 2015). Furthermore, it has been established that small changes in thermal conditions during laboratory mass-rearing can optimize the field performance of released insects for biological control without impairing the production outputs required for inundative or inoculative releases (Thomson et al. 2001; Kristensen et al. 2008; Chidawanyika and Terblanche 2011).

One approach to improve performance under field conditions of beneficial insects (e.g., parasitoids and predatory insects) is thermal adaptation (Angilletta et al. 2002; Pörtner et al. 2006; Overgaard and Sørensen 2008). Thermal adaptation in insects involves a combination of behavioral, physiological, and cellular responses that can be elicited over a range of time-scales (Angilletta et al. 2002; Wernegreen 2012; Terblanche 2014). Within the lifetime of an individual, the thermal performance can be altered through acclimation or rapid hardening (Angilletta et al. 2002; Fischer et al. 2011; Terblanche 2014). In insects, rapid, within generation, acclimation to heat or cold temperatures has been shown in laboratory conditions to increase survival and reproductive potentials, improving their locomotion and parasitism (or predation) capacities (Thomson et al. 2001; Levie et al. 2009; Chidawanyika and Terblanche 2011; Sørensen et al. 2013; Findsen et al. 2013). Furthermore, acclimation under fluctuating rather than constant

environments has been shown to increase thermal tolerance in several insect species (Sarup and Loeschcke 2010; Terblanche et al. 2010; Bozinovic et al. 2011; Fischer et al. 2011; Overgaard et al. 2011; Basson et al. 2012; Chidawanyika et al. 2017), including parasitoids (Cônsoli and Parra 1995a; Torres et al. 2002; Bahar et al. 2013; Delava et al. 2016). On the scale of multiple generations, rearing under fluctuating temperature regimes probably will favor higher levels of adaptation plasticity, and hence better performance under a wide range of environmental conditions (Ragland and Kingsolver 2008; Kingsolver et al. 2009; Manenti et al. 2014, 2015; Colinet et al. 2015; Chevin and Hoffmann 2017). Therefore, long-term use of fluctuating thermal regimes may help to optimize rearing protocols in order to enhance field performance of laboratory-reared natural enemies.

Interestingly, little is known about the potential of using fluctuating temperatures for the mass rearing of the parasitoid *T. podisi*. Thus, in this Chapter, we investigated whether rearing *T. podisi* under fluctuating temperatures (i.e., 30 ± 2 ºC during 12h and 20 ± 2 ºC during the other 12h) for consecutive generations would result in fitness (including performance levels at non-extreme constant temperature challenges) and economic (estimated production cost) benefits when compared with parasitoids reared under the constant temperature of 25 ± 2 ºC, which is currently used by most of the insect rearing facilities. Both thermal regimes investigated are situated around the optimal temperature (25 °C) but have different levels of variability. This design allowed us to test variability without exceeding the tolerance limits of the T. podisi (Torres et al. 1997). In addition, these temperatures were chosen because their amplitudes approximately represent the daily thermal variability during the soybean growing season in Brazil (Dubreuil et al. 2012; Natal and Farias 2017).

3.2 Materials and methods

3.2.1 Laboratory rearing of *T. podisi* and *E. heros*

The parasitoid wasps *T. podisi* were obtained from the Embrapa Soybean Laboratory, where a colony of this insect has been reared in *E. heros* egg masses for five years under controlled environmental conditions (i.e., 25 ± 2 °C, relative humidity 60 ± 10 % and photoperiod of 12 L: 12 h D) according to Peres and Corrêa-Ferreira (2004). New field insects have been introduced on a yearly basis to maintain insect quality. Stink bug eggs were glued onto a cardboard card (5 cm × 8 cm) and introduced into plastic cages (8.5 cm high and 7 cm in diameter sealed with plastic film) together with the eggs previously parasitized by *T. podisi*. *E. heros* eggs were previously exposed to UV light for 30 min. Small drops of *Apis mellifera*-produced honey were placed inside these cages to feed the adults as soon as they emerged. The cages were then closed, and the eggs allowed to be parasitized for 24 h. Adults that emerged from these eggs were used for

trials or colony maintenance.

E. heros eggs were obtained from a mass-reared colony Embrapa Rice and Beans. This colony was reared as described in 2.2.1.

3.2.2 Temperature rearing trial

To evaluate the effects of fluctuating and constant temperature conditions on life history traits and thermal performance of *T. podisi*, two colonies were established using laboratory natural selection. To this end, groups with approximately one thousand newly-emerged adult *T. podisi* (mixed sexes) were submitted to two different rearing temperature conditions (i.e., colonies). Both conditions shared the same mean temperature (25 ºC) but differed with respect to the intra-day temperature variation. The constant temperature (control) was 25 ± 2°C (relative humidity of 60 ± 10 % and 12 L:12 D photoperiod). The fluctuating temperature conditions had two daily 12h phases, with maximum and minimum temperatures of 30°C and 20°C respectively (i.e., 30 ± 2°C during photophase and 20 ± 2°C at scotophase, relative humidity of 60 ± 10 % and 12 L: 12 h D photoperiod). Temperatures were chosen to represent the range of seasonal temperature variation observed in Brazilian agroecosystems during soybean growing season and in order to have the same daily average of the constant temperature commonly used in parasitoid rearing facilities. Constant and fluctuating temperature conditions were performed simultaneously in incubators (Eletrolab, model 202/4, SP, Brazil) capable of controlling the two constant thermal conditions. A simple step-function transferred from one temperature to another. After 24 h of thermal acclimation to each regime, *E. heros* fresh eggs glued to pieces of cardboard were exposed to the colonies and parasitism was allowed for 24h. After this period, the parasitized eggs were removed, transferred to plastic cages and held at the same temperature regime of their parental during their complete development. The adults emerging from those eggs were considered the first generation (G1) and were used for the phenotypic assays and production of the subsequent generation. Both colonies were reared continuously under the temperature rearing conditions and were maintained separately during the four generations of the experiment.

3.2.3 Effects of temperature rearing conditions on *T. podisi* life-history traits

Developmental time and fitness

To evaluate whether the constant and fluctuating regimes caused alterations on *T. podisi* development, we measured the egg-adult period in each regime. Ten mated females (up to 48h, with no previous parasitism experience) from both temperature rearing conditions were placed into a Duran plastic tube (7 cm high ×1 cm of diameter) with a

honey droplet to feed the parasitoids and sealed with plastic film. Each female was exposed to approximately 30 UV-treated *E. heros* eggs (≤ 24 h old), which were glued with non-toxic glue onto cardboard cards (1 cm × 4 cm). Each card was previously labeled with the respective treatments. Then, these cards were exposed to parasitism for 24 h. After this period, the cards with 30 glued eggs were transferred to a new Duran plastic tube where they remained until adult emergence. Both females and cards with parasitized eggs were maintained under the same conditions of the parental female (constant or fluctuating regime). To determine the egg-to-adult period, we made daily observations of adult emergence, without making the differentiation between the sexes. For the analysis, offspring average developmental time was calculated for each female. The females that did not produce adult offspring were removed from the analysis. These bioassays were carried out with females from the G0, G1, G2 and G3 generations in order to evaluate the duration of the immature stages at generations G1, G2, G3 and G4.

To evaluate the effects of fluctuating temperature rearing on fitness, we measured the female longevity, life-span parasitism, fecundity, fertility, emergence rate and progeny sex ratio. Ten to twenty mated females from both temperature rearing conditions were individualized and fed with honey droplets. Each female was provided daily with approximately 20 UV-treated *E. heros* eggs (≤ 24 h old) that were glued with non-toxic glue onto cardboard cards (1 cm × 4 cm). These eggs were replaced daily until the parasitoid death. Parasitism was allowed for 24 h, then the cards with glued eggs were removed and transferred to plastic bags (4 x 23 cm) where they remained under the same conditions of the parental female (constant or fluctuating regime). For each female in the respective date, the following parameters were recorded: number of parasitized eggs (egg darkening); sex (antennal dimorphism) and number of emerging parasitoids. Females that did not produce adult offspring were removed from the analysis. Parasitism life-span was assessed as the average number of days that the female produced viable offspring (Dalvi et al. 2014). Cumulative or life-time fecundity was calculated as the mean of parasitized eggs by female, and daily fecundity as the average parasitized eggs by female per day (Botto et al. 2004; Dalvi et al. 2014). Similarly, cumulative fertility was assessed as the average of the adult offspring by female, and daily fertility as mean of adult offspring by female per day (Botto et al. 2004). Emergence rate or adult emergence was recorded as (adult offspring)/(parasitized eggs) (Silva et al. 2018a). Sex ratio was assessed as (female offspring)/(adult offspring) (Silva et al. 2018a). Additionally, the mean of the female offspring by female was consider the cumulative female offspring or net reproductive rate, and the average of the cumulative female offspring per female a day was considered the daily female offspring (Orr and Boethel 1990; Huey and Berrigan 2001). These fitness bioassays were carried out with females from the G3 and G4 generations.

Thermal performance

To evaluate the *T. podisi* performance at different rearing temperature conditions, we measured the effects of constant temperatures (i.e., 20 °C, 25 °C, 30 °C) in development and fitness traits of the parasitoids that were reared either at constant (i.e., 25°C) or fluctuating (i.e., 30 ± 2°C during photophase and 20 ± 2°C at scotophase) thermal conditions. Ten to twenty mated females from constant or fluctuating temperature rearing conditions were transferred to environments kept at constant temperatures of 20ºC, 25ºC and 30ºC. The same methodology described above was used to assess the influence of temperature on the developmental rate and fitness traits. Both females and cards with parasitized eggs were maintained at 20ºC, 25ºC and 30ºC until the adults' emergence.

The bioassays to determine the development rate were carried out using the immature stages from the generations G1, G2, G3 and G4, using the eggs parasitized by females from the previous generations. While the bioassays to determine the fitness were carried out with females from the generations G1, G3 and G4.

3.2.4 Data analysis

A cost analysis of parasite wasp production under constant and fluctuating temperature rearing conditions was carried out as described previously by Mendes et al. (2005), including the fixed and variable production cost. Briefly, the fixed cost corresponds to all the resources that have a duration higher than the production cycle (inputs and equipment), while, the variable cost includes the inputs used for the parasitoid rearing and the labor work (Mendes et al. 2005). The fixed production cost was calculated as the depreciation sum and the alternative cost (Mendes et al. 2005). The depreciation was estimated as [new resource value – residual value]/useful life, where the residual value was assessed as 10 % of the new equipment value (Mendes et al. 2005). The alternative cost estimates the return of the capital invested and therefore it was calculated considering the monthly savings interest rates in Brazil in 2019 (Mendes et al. 2005). Similarly, the total variable cost was calculated as the sum of the variable operation cost and the alternative variable cost, where the alternative variable cost was estimated as [variable operational cost x savings interest rate]/2 (Mendes et al. 2005). For the variable cost analysis, we standardized the ingredients quantities and labor work hours required to obtain 5,000 *T. podisi* wasps per day. We based our estimations on: (i) the daily fecundity and emergence rate on the first day of the parasitoids under both regimes (i.e., constant and fluctuating temperatures) observed in this study at the fourth generation; and (ii) *E. heros* egg production under natural diet reported by Hayashida et al. (2018). The monthly average spot prices of plant ingredients and *A. mellifera*-produced honey were consulted in the Central Supply Company of soybean-producing states (CEASA 2019) and the National Supply Company (CONAB 2019b). The aver-

age price for our cost estimations was based on the year 2018. Costs of other inputs and equipment were based on online sales from January 2019 and May 2019. The labor hour price was based on the average salary of an industrial laboratory technician (CBO 3011-05), including contributions to the FGTS (Brazilian Service Assurance Fund) and INSS (Brazilian National Social Security Institute), and extra payments for holidays and the 13th salary (which is an obligation in the Brazilian work laws). The cost utilities (water, electricity, etc.) were not included in the calculations of the variable cost. The calculations in Brazilian real (i.e., BR$) were converted into US$ dollar by the foreign exchange rate in January 2019.

Bioassays results that measure the effects of two rearing temperature regimes (constant and fluctuating) on life-history traits were subjected to univariate analyses of variance (ANOVA), or a Kruskal-Wallis one-way ANOVA on ranks when the assumptions of normality and homoscedasticity were not satisfied. In the case of female longevity, survival analyses for females of the two temperature regimes were performed using Kaplan-Meier estimators as described in 2.2.6. Regression analyses were performed to detect trends in fecundity, fertility and female offspring that resulted from each treatment through time using the curve-fitting procedure of SigmaPlot 12.0. The regression model was chosen based on parsimony, low standard errors, and steep increases in R2 with model complexity. The regression models for each treatment were considered different from each other if the confidence limits of their parameters did not overlap.

The thermal performance was tested by a full factorial two-way analysis of variance (ANOVA) with rearing conditions (constant or fluctuating temperatures) and tested temperatures (20°C, 25 °C, 30 °C) as fixed effects. All assumptions were met before running the ANOVA. When the analyses showed differences between treatments, Tukey's post hoc comparisons were run to specify which treatment was different from the others ($P <$ 0.05).

3.3 Results

3.3.1 Developmental time and fitness

Fluctuating rearing thermal conditions had effects on several life-history traits of *T. podisi* (Figure 3.1, Appendix 3.2). For instance, developmental time was shorter under fluctuating temperature than at constant temperature in all generations evaluated (Figure 3.1A, Appendix 3.1).At fourth generation, fluctuating thermal regimes shorten parasitoid development time by 0.9 ± 0.16 days (Figure 3.1*A*; G4: $F_{1,16}$ = 16.8, $P < 0.0001$). Larger differences were observed at other generations with a 2.9 ± 0.14 days reduction (Appendix 3.2*A*; G1: H = 12.6, df=1, $P < 0.0001$. G2: $F_{1,16}$ = 79.9, $P < 0.0001$. G3: $F_{1,13}$ = 27.7, $P < 0.0001$). Female longevity also varied among the rearing temperature regimes at fourth generation (Figure 3.1*B*), in which the females reared under fluctuat-

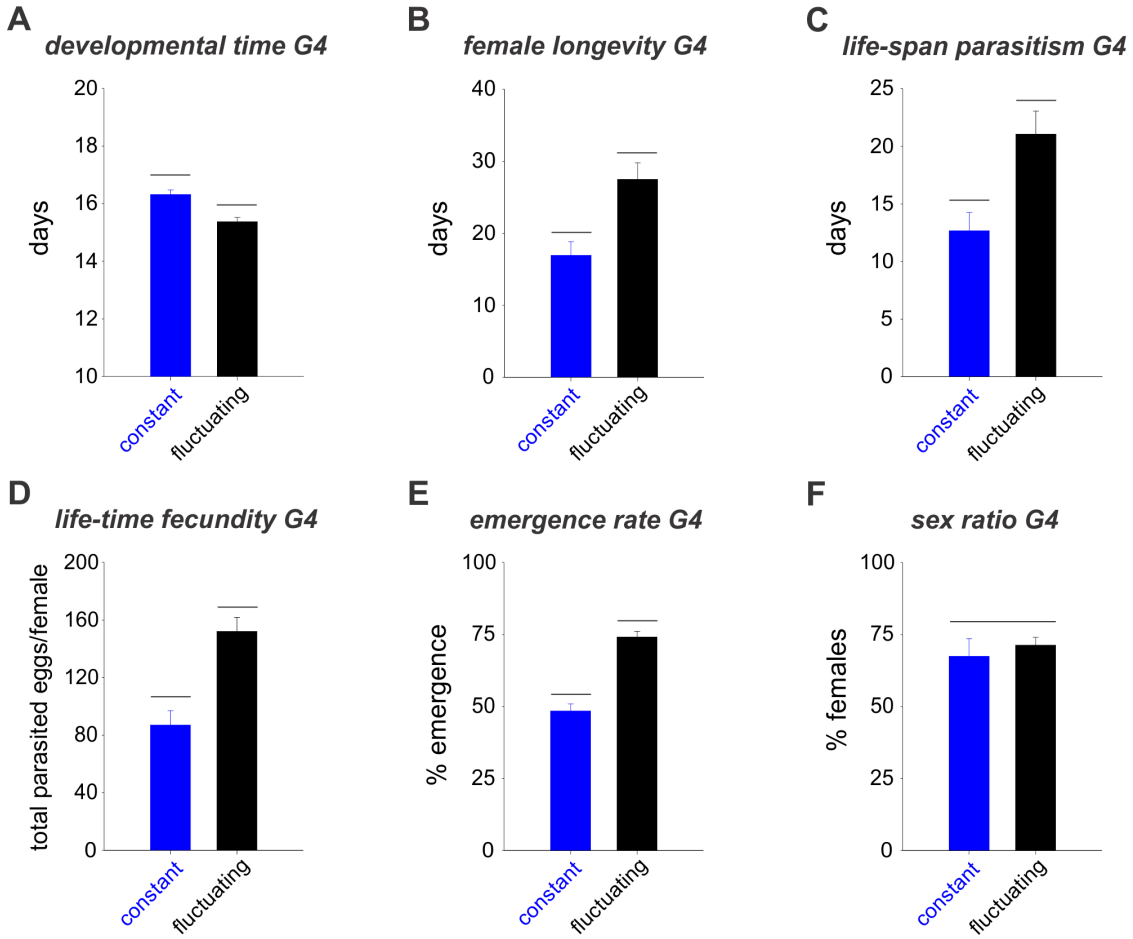


Figure 3.1: Effects of rearing temperature conditions [fluctuating (30 ± 2 °C during light phase and 20 ± 2 °C at dark phase) and constant (25ºC)] on life history traits of *Telenomus podisi* at the fourth generation. Developmental time (***A***), female longevity (***B***), life-span parasitism (***C***), life-time fecundity (***D***), emergence rate (***E***) and sex ratio (***F***). Bars represent the mean (± standard error) observed data (n=18-19) and the ones grouped with the same horizontal line are not statistically different at the 5 % significance level according to a Tukey's HSD test.

ing temperatures survived 10.5 ± 2.34 days longer than the ones reared under constant regime (χ^2 = 12.3, *df* = 1, *P* < 0.0001). Although at the third generation, the longevity did not differ between rearing conditions (Appendix 3.2*B*; χ^2 = 3.2, *df* = 1, *P* = 0.0730). Regarding the life-span parasitism or the reproductive periods, females reared under fluctuating temperatures parasitized eggs for longer time, for 8.4 ± 2.01 days more at fourth generation (Figure 3.1*C*; $F_{1,36}$ = 10.5, *P* = 0.0030) and 6.6 ± 1.03 days more at third generation (Appendix 3.2*C*; $F_{1,17}$ = 8. 7, *P* = 0.0101). As expected, the life-time fecundity was higher in females reared under fluctuating temperature, resulting in an increase of 74.6 ± 11.39 % at generation four (Figure 3.1*D*; $F_{1,35}$ =21.0, *P* < 0.0001), and even at generation three (Appendix 3.2*D*; $F_{1,17}$ = 37.6, *P* < 0.0001). At constant temperature, the emergence rate was significantly lower than for the fluctuating temperature at fourth generation, with a mean of 48.4 ± 2.53 % for constant temperature in comparison with 74.1 ± 1.98 % for fluctuating temperature (Figure 3.1*E*; $F_{1,36}$ = 64.90, *P* < 0.0001). Similarly, there were differences in the adult emergence between the thermal rearing

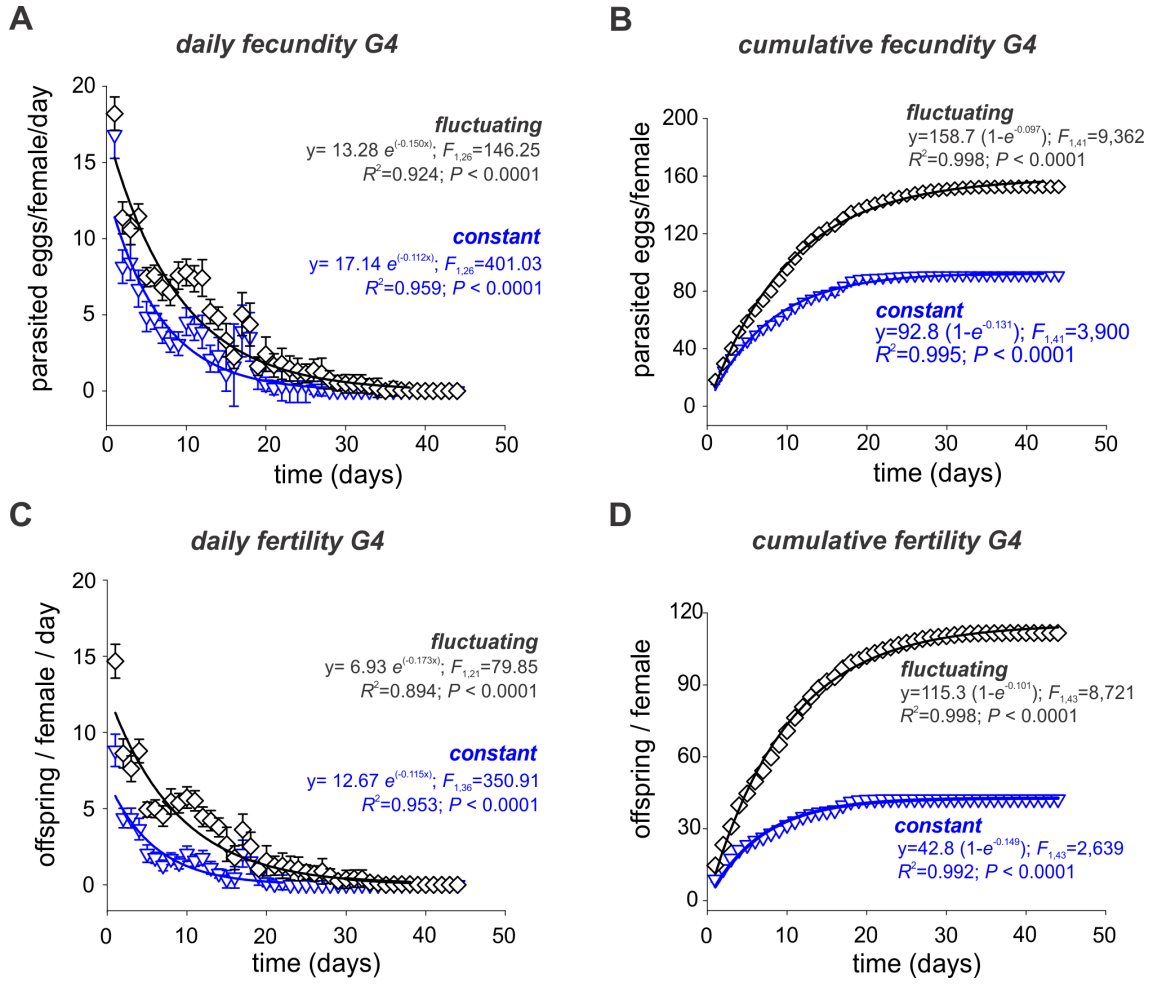


Figure 3.2: Effect of rearing temperature conditions [fluctuating (30 ± 2 °C during light phase and 20 ± 2 °C at dark phase) and constant (25ºC)] on fecundity and fertility of *Telenomus podisi* at generation four. Daily and cumulative fecundities (***A, B***) and fertilities (***C, D***). Symbols represent the mean (± standard error) observed data (n=18-19) while the lines represent the best fits.

conditions at third generation (Appendix 3.2*E*; $F_{1,17}$ = 15.0, *P* = 0.0012). Conversely, the fluctuating regime had no effects on the offspring sex ratio (% females) when compared with the constant regime at fourth generation (Figure 3.1*F*; *H* =0.001, *df* = 1, *P* = 0.9713) and third generation (Appendix 3.2*F*; G2: *H* = 0.05, *df* = 1, *P* = 0.8745).

Both daily and cumulative fecundity were also affected after four generations of rearing under the fluctuating regime (Figure 3.2*A, B*). The daily fecundity results fit well with the two-parameter exponential decay model, as it showed a higher parasitism on the experiment first day and decrease with the time (Figure 3.2*A*). The average number of eggs parasitized during the first 24 h was similar between both thermal rearing conditions, but they differed in the decay rate, fluctuating temperature rearing condition exhibited the smallest decay rate (Appendix 3.2). Regarding the cumulative fecundity, the exponential rise to maximum model was suitable because it describes a logistic growth, where the increase rate of the population decreases as the population growths towards a maximum value (Figure 3.2*B*). Consequently, the fluctuating temperature rearing condition showed the highest maximum value as well as the smallest decrease rate in compari-

son with the constant temperature rearing condition (Appendix 3.3). A similar trend was observed for daily and cumulative fertility (Figure 3.2*C, D*), since life-time fertility was higher in the fluctuating temperature rearing conditions (by 157.4 ± 21.98 %; F1,36 = 38.7, P < 0.0001) as a result of the higher fecundity and higher emergence rate. With respect to the daily fertility, the number of offspring that emerged from the parasitized eggs of the experiment on day one was higher for the fluctuating temperature rearing condition and the higher fertility rates also held for longer (Figure 3.2*C*, Appendix 3.2). For the cumulative fertility, the highest maximum value was observed in the fluctuating regime together with the highest decrease rate (Figure 3.2*D*, Appendix 3.3).

Further analysis of the survival curves at G4 showed that a median survival time (LT_{50}) of 30.0 (25.78 - 34.22) days for females reared under fluctuating regime in contrast with 17.0 (13.88 - 20.68) days for constant regime (Figure 3.3*A*; χ^2 = 12.3, *df* = 1, *P* <

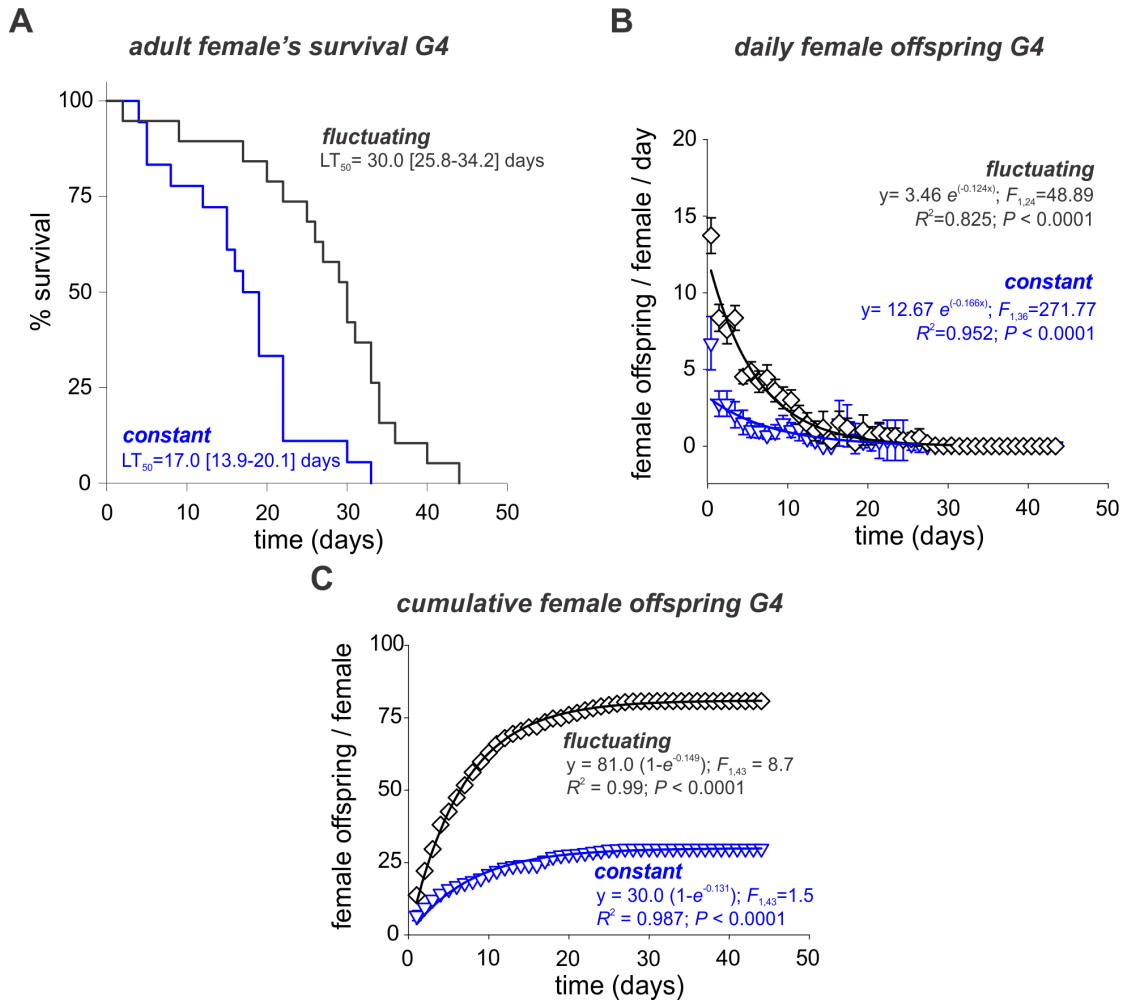


Figure 3.3: Effects of rearing temperature conditions [fluctuating (30 ± 2 °C during light phase and 20 ± 2 °C at dark phase) and constant (25ºC)] on life history traits relevant for mass-rearing of *Telenomus podisi* at generation four. Adult female's survival curve (***A***) and daily and cumulative female offspring (***B, C***). The survival curves were significantly different by Holm-Sidak's test (χ^2 = 12.3, *df*=1, *P* < 0.001). For the panels containing the female offspring (***B, C***), symbols represent the mean (± standard error) observed data (n=18-19) while the lines represent the best fits.

0.0001). Moreover, at the time the mean life-span parasitism was reached, 84.2 % of the females were alive for the fluctuating temperature rearing condition, compared to 72.2 % for the constant regime. Regarding female offspring produced per female, rearing under the fluctuating temperature conditions in generation four led to an increase of 173.7 ± 22.11 % in comparison to the constant regime ($F_{1,31}$ = 31.4, *P* < 0.0001). Regarding the daily production of female offspring, the highest maximum value was almost four times higher for the fluctuating temperature rearing condition, despite a similar decrease rate between both regimes (Figure 3.3*B*, Appendix 3.2). For the cumulative female offspring, the fluctuating temperature rearing condition showed the highest maximum value, but a slightly higher decrease rate in comparison with the constant temperature rearing condition (Figure 3.3*C*, Appendix 3.3).

3.3.2 Thermal performance

A two-way ANOVA revealed that at G4 the constant test temperatures had a strong effect for most of the life-history traits (Table 3.1, Figure 3.4). under different thermal conditions (constant or fluctuating temperatures) did not affect the duration off egg-adult development (Table 3.1, Figure 3.4*A*). Regarding the fitness, at G4 tested temperature rearing conditions had an effect on parasitism life-span (Table 3.1, Figure 3.4*C*), life-time fecundity (Table 3.1, Figure 3.4*D*) and female offspring (Table 3.1, Figure 3.4*F*), but not in the emergence rate (Table 3.1, Figure 3.4*E*). Although both test temperature and temperature rearing conditions affected life-history traits, no interaction between these two terms was found in any trait evaluated (Table 3.1). Moreover, the thermal performance of reproductive parameters showed an improvement along generations, at G1 the rearing conditions had no evident effect on reproductive performance, but at G3 it had an effect on the life-time fecundity and female offspring (Appendix 3.4, Appendix 3.6). Parasitic wasps that developed under fluctuating regime performed better at 25°C than the ones under constant regime at G4, considering the higher female longevity (Figure 3.4*B*), and higher female offspring (Figure 3.4*F*). A similar trend was observed in the

Table 3.1: Results for the effect of rearing under fluctuating [30ºC during 12 hours (day) and 20ºC during 12 hours (night)] or constant (25ºC) temperature regimes on different life-history traits at generation four from full factorial model ANOVA.

Life-history traits	**Rearing temperature condition (constant or fluctuating)**		**Test constant temperature (20°C, 25°C or 30°C)**		**Temperature rearing conditions X Test constant temperature)**	
	F	*P*	F	*P*	F	*P*
Developmental time	0.00865 $_{(1,55)}$	0.926	1815.371 $_{(2,55)}$	<0.001	2.433 $_{(2,55)}$	0.099
Life-span parasitism	4.512 $_{(1,75)}$	**0.037***	13.069 $_{(2,75)}$	<0.001	1.577 $_{(2,75)}$	0.214
Life-time fecundity	4.767 $_{(1,73)}$	**0.032***	12.208 $_{(2,73)}$	<0.001	0.294 $_{(2,73)}$	0.746
% emergence	0.750 $_{(1,74)}$	0.389	47.365 $_{(2,74)}$	<0.001	0.360 $_{(2,74)}$	0.699
Female offspring	5.744 $_{(1,65)}$	**0.020***	14.697 $_{(2,65)}$	<0.001	0.921 $_{(2,65)}$	0.404

Degrees of freedom are given within parentheses. The table shows the F-ratio (F) with associated probability (*P*) for all traits. Asterisks indicate significant differences (P < 0.05)

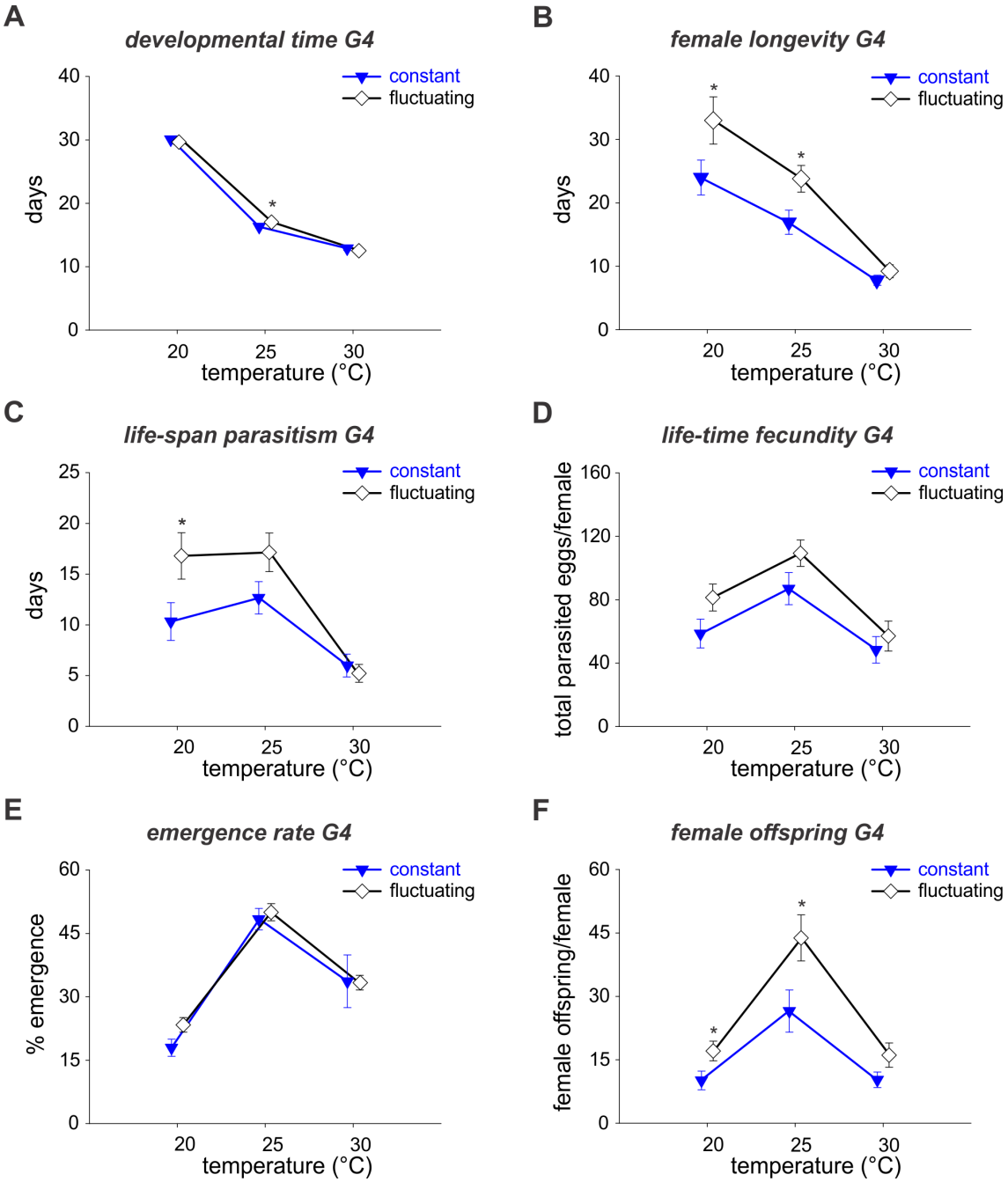


Figure 3.4: Effects of rearing temperature conditions [fluctuating (30 ± 2 °C during light phase and 20 ± 2 °C at dark phase) and constant (25ºC)] on *Telenomus podisi* thermal performance at generation four. Means of life-history traits (± standard error) of parasitic wasps from different rearing regimes when transferred to different constant test temperatures (20°C, 25°C, 30°C) (n=9-20). Developmental time (***A***), female longevity (***B***), life-span parasitism (***C***), life-time fecundity (***D***), emergence rate (***E***) and female offspring (***F***). Asterisks indicate significant differences ($P < 0.05$) between constant and fluctuating regime according to a Tukey's HSD test.

previous generations, wasps that developed under fluctuating regime and transferred to constant 25°C in their adult life also have higher female longevity (at G1; Appendix 3.6*B*), life-span parasitism (at G1; Appendix 3.5C), life-time fecundity (at G3; Appendix 3.6*D*) and female offspring (at G1 and G3; Appendix 3.6*F*) that the wasps reared under constant 25°C over the entire life-cycle. At 20°C, the reproductive performance

only moderately improved after four generations of rearing under fluctuating regime, as exemplified by higher female longevity (Figure 3.4*B*), longer life-span parasitism (Figure 3.4*C*) and higher female offspring (Figure 3.4*F*). By contrast, fluctuating regime had no effects on the performance at 30°C in any of the generations evaluated (Figure 3.4, Appendix 3.6).

3.3.3 Cost analysis

The production cost of *T. podisi* under the different thermal regimes was calculated as the sum of the total fixed cost (Table 3.2) and the variable cost (Table 3.3). The total cost of producing 5,000 *T. podisi* wasps per day was estimated to be US$ 16.57 for the parasitoids reared under constant temperature, compared with the cost of US$ 12.67 under fluctuating temperature rearing condition, which represents a decrease of 23.49 % (Table 3.3). The monthly fixed production cost of *T. podisi* was estimated to be US$ 77.14 under both rearing conditions (Table 3.2). The estimated variable cost indicates

Table 3.2: Detailed fixed rearing cost of *Telenomus podisi* wasps using *Euschistus heros* as the host insect

Resources	**Value (US$)**	**Useful live (months)**	**Residual value**[a]	**Depreciation**[b]	**Alternative cost**[c]	**Monthly fixed cost**[d]
Building 12 m^2	3219.91		240	13.42	11.96	25.38
Conditioned air	580.23	120	58.02	4.35	2.16	6.51
BOD chamber with alternating temperatures	1870.29	120	187.03	14.03	6.95	20.98
Refrigerator	404.73	120	40.47	3.04	1.50	4.54
Precision balance	189.30	120	18.93	1.42	0.70	2.12
Stereomicroscope	387.72	120	38.77	2.91	1.44	4.35
Thermo-hygrometer (2 units)	68.92	120		0.57	0.26	0.83
Exhaust fan	43.19	120		0.36	0.16	0.52
Shelf	28.62	120		0.24	0.11	0.34
Table	101.99	120		0.85	0.38	1.23
Chair	27.00	60		0.45	0.10	0.55
Tweezers	21.06	48		0.44	0.08	0.52
Scissors	13.99	48		0.29	0.05	0.34
Soldering iron	6.73	24		0.28	0.02	0.31
Plastic pots for parasitoid rearing (20 units)	43.19	24		1.80	0.16	1.96
Plastic pots for host rearing (50 units)	96.93	24		4.04	0.36	4.40
Pen, Pencils, Paper and Crepe tape (100 m)	16.14	12		1.35	0.06	1.41
Aluminium foil (100 m)	9.99	12		0.83	0.04	0.87
Plastic foil (600 m)	11.34	12		0.94	0.04	0.99
Organza veil	3.74	12		0.31	0.01	0.33
Total				50.66	26.49	77.14

[a] Value of the equipment after the useful life, estimated as 10 % of the final value
[b] Depreciation = [Value of the new resource – Residual Value] / Useful life
[c] Alternative cost = Value of the new resource x monthly savings interest rates
[d] Monthly fixed cost= ∑ Depreciation + ∑ Alternative cost

Table 3.3: Cost comparison between constant and fluctuating regime for the production of 5,000 *Telenomus podisi* wasps per day using *Euschistus heros* as the host insect

	Constant regime	**Fluctuant regime**
Host eggs required		
Maintenance of host colony	1000	1000
Maintenance of parasitoid colony	1400	500
Production parasitoid wasps for biological control	9600	6200
Total	12000	7700
Time required (h)		
Maintenance of host colony	2.61	1.69
Maintenance of parasitoid colony	0.50	0.50
Total	3.11	2.19
Detailed variable cost (US$)		
Materials for maintenance of host colony		
Green bean pod	0.3680	0.2381
Peanut seeds	0.0617	0.0399
Sunflower seeds	0.0151	0.0098
Soybean seeds	0.0166	0.0107
Materials for maintenance of parasitoid colony		
Honey	0.0016	0.0016
Non-toxic glue	0.0032	0.0032
Cardboard paper	0.0016	0.0016
Labor cost	12.5901	8.8610
Variable operational cost[a]	13.0579	9.1660
Total alternative variable cost[b]	0.0008	0.0005
Total variable cost[c]	13.0587	9.1666
Estimated production cost of 5,000 T. podisi wasps per day (US$)		
Fixed cost production per day[d]	3.5066	3.5066
Average total cost[e]	16.5653	12.6732

[a] Variable operational cost = ∑ Materials for maintenance of host and parasitoid colonies + Labor cost
[b] Total alternative variable cost = variable operational cost x daily savings interest rates / 2
[c] Total variable cost = Variable operational cost + Total alternative variable cost
[d] Fixed production cost per day = Monthly fixed cost of production / number working days per month
[e] Total cost = Total variable cost + Fixed production cost per day

that the differences between the rearing conditions were mainly caused by the amount of *E. heros* eggs required for the maintenance of the insect colonies and production of the parasitic wasps for biological control. Based on the daily fecundity and the emergence rate of the parasitoids on the first day at G4, 12,000 *E. heros* eggs were required for constant temperature, in comparison with 7,700 for fluctuating temperatures (Table 3.3). This was reflected in the higher time and materials spent on the maintenance of *E. heros* colony for the constant regime (Table 3.3).

3.4 Discussion

In this Chapter, we demonstrated how life story traits of a mass-rearing population of *T. podisi* can be affected by constant or fluctuating thermal rearing conditions. Our results showed that thermal fluctuating regime was more suitable for mass-rearing *T.*

podisi as it resulted not only in parasitoids with better fitness but also reduced the costs (by 23.5 %) for producing these insects when compared to parasitoids produced at constant thermal regime. Furthermore, we also demonstrated that parasitoids that originated from the fluctuating thermal regime can cope better with the constant exposure to 25 °C temperature and do not suffer any detrimental effects on the fitness to tolerate constant exposures to 20 °C or 30°C temperatures.

The effects of constant temperatures on the life history traits of *T. podisi* are well established (Yeargan 1980, 1982; Orr and Boethel 1990; Torres et al. 1997; Silva et al. 2018a). However, only the investigations described in Yeargan (1980) dealt with the potential effects of fluctuating temperatures on the developmental time of this parasitoid. As previously described for parasitoids (Yeargan 1980; Cônsoli and Parra 1995b; Butler and Trumble 2010; Bahar et al. 2013; Delava et al. 2016), *T. podisi* development time was significantly shorter for individuals reared under fluctuating thermal regime (Yeargan 1980). A possible explanation for faster growth at fluctuating temperatures might be related to an increased food intake and assimilation during daytime, owing to favorably high temperatures, but decreased metabolic losses during the cooler night-time (Karl and Fischer 2008; Fischer et al. 2011; Carrington et al. 2013; Kingsolver and Woods 2015).

It has been well established that parasitoid longevity, fecundity and flight activity are closely linked to the amount of energy reserves (Colinet et al. 2007; Berger et al. 2008; Abram et al. 2016) and unaffected by fluctuating temperatures (Foray et al. 2014). Intriguingly, in the present investigation, the female longevity was significantly higher for individuals' mass-reared under fluctuating thermal regime. Such increased longevity can be related with less oxidative damages under fluctuating temperatures (Lalouette et al. 2011; Foucreau et al. 2016), which is consistent with the role of oxidative damage as an underlying mechanism of ageing (Colinet et al. 2015). Alternatively, such female longevity increases may be related with the duration of thermal experiment (i.e., four generations), since the longer the period that individuals are reared under fluctuating thermal conditions, the higher will be the abilities of their progeny to tolerate temperature fluctuations in natural environments (Chidawanyika et al. 2017). Probably, such effects derive from transmissible transgenerational traits that favor the performance and plasticity under fluctuating regimes (Manenti et al. 2014, 2015; Sgrò et al. 2015; Chidawanyika et al. 2017). For example, organisms that live in highly variable thermal environments frequently induce heat shock proteins (HSPs) within the range of body temperatures normally experience in field (Dahlhoff and Rank 2007; Tomanek 2010). Experimental evidence support epigenetic transgenerational inheritance of HSPs induction after intense heat shock in some invertebrates, including Drosophila, Artemia and marine mollusks (Garbuz et al. 2003; Norouzitallab et al. 2014; Fellous et al. 2015). This transmission of the phenotypic traits is normally associated with DNA methylation and histone modifications that can persist for one to five generations (Tetievsky and Horowitz

2010; Norouzitallab et al. 2014; Sgrò et al. 2015). Although maternal intergenerational effects exist, it is not clear if they can be triggered by temperature variations that remain within the permissive thermal range. Additional studies including the expression levels of HSPs under fluctuating temperature regimes, as well as experiments to precisely characterize the role of epigenomic modifications in HSPs gene regulation and their contribution to plastic transgenerational effects are required to shed light to this issue.

Regarding the fitness, *T. podisi* reared under fluctuating regime parasitized more eggs (up to 74.6 %), exhibited higher (up to 25.7 %) parasitism viability and reached 80 % cumulative parasitism faster. This represents an estimate of the parasitoid efficiency in field (Dalvi et al. 2014) compared to the individuals reared under constant thermal regime. The increased reproductive output under fluctuating thermal regimes have been demonstrated in several insects, but the increased fitness is often accompanied by shortened longevity (Siddiqui et al. 1973; Torres et al. 2002; Mironidis and Savopoulou-Soultani 2008; Siddiqui and Barlow 2015). Decreased longevity in response to reproductive effort has been repeatedly observed (reviewed De Loof 2011), particularly if resources are limited in the adult life, as in the case of parasitoids, that are unable to synthesize lipids during adult stage (Visser et al. 2010). Although body size was not measured in this study, a possible explanation for the increased longevity may be related with a larger body size in the parasitoids reared under fluctuating regime. As a generally accepted guideline, increased fitness-related traits are often related with larger body size, and the parasitoid size itself depends on the developmental temperature (Colinet et al. 2007; Abram et al. 2016). At constant temperatures, development time and body size are negatively correlated with temperature during development (Angilletta and Dunham 2003; Sibly and Atkinson 2006). However, fluctuating temperatures have been reported to decrease, or have no effect on the body size depending on the amplitude and time of the variation (Kingsolver et al. 2009; Terblanche et al. 2010). Other possible explanations for these findings may be related to improved ovarian development (Verdú et al. 2010; Zhao et al. 2016), or an increase in egg maturation (Moiroux et al. 2018) associated with increased food intake and assimilation during development. Alternatively, environmental heterogeneity has been shown to be an important factor in maintenance of genetic variation for fitness (Reed et al. 2003). Further studies that evaluate body size, ovary development, and even expression level profiles using transcriptome would help identify genes involved in the adaptation to fluctuating temperatures. In any case, the increase in reproduction and longevity observed in the present investigation suggest that *T. podisi* might be better equipped (e.g., machinery plasticity to monitor the environment or in term of genetic makeup) to adapt to thermal fluctuations that occur in natural environments (Boher et al. 2016; Bozinovic et al. 2016; Dey et al. 2016; Kubrak et al. 2017) and, in other words, a constant 25°C may be not the optimal temperature for *T. podisi*.

Despite the fact that fluctuating temperatures can increase the synthesis of HSPs, which normally help the organisms to cope with thermal stress (Tomanek 2010; Folguera et al. 2011), rearing *T. podisi* for four consecutive generations did not increase these parasitoids' performance to tolerate constant high temperature (30°C) challenge. The lack of adaptation to the high temperature derived from the fact that four generations of rearing under fluctuating regime might be not enough to favor the plasticity required to increase such environmental tolerance (Cooper et al. 2012b, a; Manenti et al. 2014, 2015; Chevin and Hoffmann 2017). However, it worked well for the tolerance to low temperature (i.e., 20°C), as the same four consecutive generations of rearing under fluctuating regimes resulted in individuals with better performance in the survival, parasitism lifespan and female offspring. These results seem to support the hypothesis that lower thermal limits are less constrained that upper limits (Bozinovic et al. 2014), especially taking into account that the lower threshold temperature for *T. podisi* was estimated at 11.1°C (Torres et al. 1997).

Interestingly, after only one generation, the exposure to a fluctuating regime during development increased the survival, life-time fecundity and female offspring of the strains that faced constant 25°C, suggesting again that fluctuating environments are more adequate for *T. podisi* development. These findings have implications for the success of augmentative releases of parasitoids in Neotropical soybean fields. For instance, Brazilian soybean production is concentrated in two main regions (i.e., the South and the Center-West regions), where the ranges of monthly average temperatures during soybean growing season are 19.2-29.6°C (mean temperature 23.6°C in the Brazilian South region) and 21.8-32.0°C (mean temperature 27.1°C in the Brazilian Center-West region) (Dubreuil et al. 2012; Natal and Farias 2017; CONAB 2019a). Consequently, *T. podisi* reared under fluctuating temperatures (e.g., diurnal temperature fluctuations of 10°C around 25°C) may have higher efficiency when released in the field.

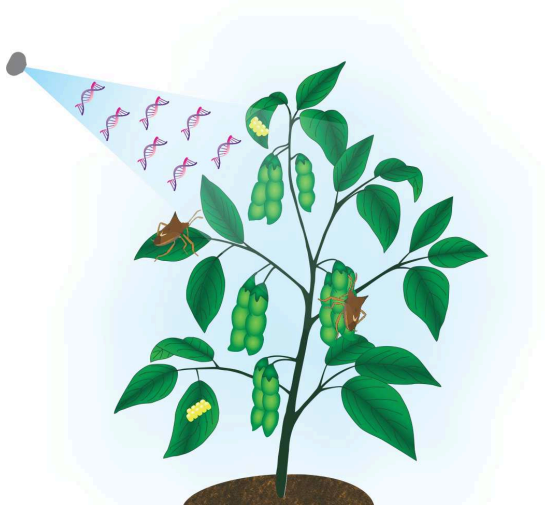
The cost estimations for producing 5,000 parasitoid wasps [i.e., which is the amount recommended per hectare in soybean fields (Corrêa-Ferreira and Moscardi 1995; Silva et al. 2008; Bueno et al. 2011a), decreased from US$ 16.57 under constant regime to US$ 12.67 under fluctuating regime. Fluctuating regime was more cost-effective than the constant regime even in the case that the climatic chamber was replaced by air conditioning. With this replacement, the price of rearing the parasitoids under constant regime can be reduced to US$ 15.91, still 20,33 % higher in comparison with the fluctuating regime (data not shown). Moreover, as our fluctuating temperatures can resemble the exterior conditions, savings on costs for electricity could be probably achieved when compared to a standard growth room equipped at constant temperature regimes. This is especially significant since large numbers of individuals are needed to achieve measurable levels of population control, and financial expenditure is typically high when carried out in area-wide basis as large farmlands of soybean in Brazil (Terblanche 2014). Furthermore, as the economic aspects normally are key factors for adoption of any pest

management by farmers (Bernardo et al. 2008; Colinet and Boivin 2011; Yang et al. 2011a; Queiroz et al. 2017), such reduction of rearing costs may facilitate the adoption of *T. podisi* as a tool for controlling insect pests. If we consider that most of the production costs relies on the high labor cost associated to the maintenance of the parasitoid hosts (i.e., *E. heros*), the development of alternative artificial diets for *T. podisi* could further reduce the production costs of these parasitoid wasps (Fortes et al. 2006; Mendoza et al. 2016; Hayashida et al. 2018). Furthermore, this reduction of the labor cost would facilitate the production on a large scale required to meet the market demand for extensive land area cultivated with soybean in Brazil (Chapter 1).

Collectively, the findings described in this Chapter indicate that parasitoids that faced thermal fluctuating regime during their mass-rearing production can better survive the thermal temperature challenges associated to transport and field releases. Further studies aiming to investigate the impact of long-term fluctuating thermal challenge in behavioral (e.g. host location and mating success), genetical (e.g. plasticity, diversity and selection responses) and physiological (e.g., heat shock proteins and detoxificative enzymes) responses will contribute to better understand whether such fitness and economic advantages will retain over time, which would facilitate the adoption of this more environmental friendly insect pest control tool.

Chapter 4

Liposome encapsulation and EDTA formulation of dsRNA targeting essential genes increases oral RNAi-caused mortality in the Neotropical stink bug *Euschistus heros*



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Castellanos NL, Sharma R, Smagghe G, Oliveira EE, 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 Management Science, 75: 537-548. doi:10.1002/ps.5167

4.1 Introduction

Double-stranded RNA (dsRNA)-mediated gene silencing, commonly referred as RNA interference (RNAi), is becoming a widely-used tool to knockdown sequence-specific target genes in insects and it also has a great potential to combat insect pests (Price and Gatehouse 2008; Huvenne and Smagghe 2010; Gu and Knipple 2013; Joga et al. 2016). Because there are no reports of insecticidal proteins such as Bt toxins with activity against pentatomid stink bugs (Cunha et al. 2012; Schünemann et al. 2014), RNAi offers a potential novel approach to control this insect pest complex (Chougule and Bonning 2012; Fishilevich et al. 2016a). To induce an RNAi response in the insect, dsRNA can be delivered into the body through different methods: ingestion, soaking and microinjection (Yu et al. 2013; Scott et al. 2013; Christiaens and Smagghe 2014). Previous experiments have shown that RNAi is effective against *E. heros* via injection of chromatin-remodeling ATPases dsRNAs (Fishilevich et al. 2016a) and against the brown marmorated stink bug *Halyomorpha halys* via injection (Bansal et al. 2016; Lu et al. 2017) and ingestion of dsRNA (Ghosh et al. 2017; Mogilicherla et al. 2018), demonstrating the existence and functionality of the RNAi machinery in pentatomid bugs.

For a role in crop protection, oral uptake of dsRNA and subsequent efficient uptake in the gut will have to be evaluated (Christiaens and Smagghe 2014). Supplying dsRNA in artificial diet resulted in the knockdown of targeted genes in different species of Hemiptera including the small brown planthopper (*Laodelphax striatellus*) (Wan et al. 2014), the brown planthopper (*N. lugens*) (Chen et al. 2010; Li et al. 2011; He et al. 2011; Ge et al. 2013), the corn planthopper (*Peregrinus maidis*) (Yao et al. 2013), the white-backed planthopper (*Sogatella furcifera*) (Yang et al. 2014; Wan et al. 2014), the kissing bug (*Rhodnius prolixus*) (Araujo et al. 2006), the pea aphid (*Acyrthosiphon pisum*) (Shakesby et al. 2009; Whyard et al. 2009; Mao and Zeng 2012), the wheat aphid (*Sitobion avenae*) (Zhang et al. 2013b; Deng and Zhao 2014), the potato/tomato psyllid (*Bactericera cockerelli*) (Wuriyanghan et al. 2011) and the tobacco whitefly (*B. tabaci*) (Upadhyay et al. 2011). However, the oral delivery in some hemipteran pests, such as the tarnished plant bug, *Lygus lineolaris*, the pea aphid, *A. pisum* and the green stink bug *N. viridula*, has been confounded by high ribonuclease activity in the saliva that degrades the dsRNA (Allen and Walker 2012; Christiaens et al. 2014; Lomate and Bonning 2016). Such degradation of the nucleic acids leads to an impaired RNAi response in an organism unless dsRNA is protected(Zhang et al. 2010). To enhance the stability of dsRNA and enhance their cellular uptake, transfection reagents have been used to encapsulate dsRNA in cationic liposome complexes that slow down the degradation and increase the effectiveness of RNAi silence in fruit flies (Whyard et al. 2009; Taning et al. 2016), mosquitoes (Cancino-Rodezno et al. 2010; Bedoya-Pérez et al. 2013) and in German cockroach (Lin et al. 2017). Another alternative for the dsRNA delivery is the use of the chelating agent ethylenediaminetetraacetic acid (EDTA) that can act as a protein inhibitor of the nucleases present in the saliva, as recent studies have reported

that EDTA may inhibit nuclease digestion of DNA in blood samples (Barra et al. 2015).

The purpose of this Chapter was to assess possibilities of RNAi as a tool for pest control, using injection and oral delivery of dsRNA in *E. heros*. First, we conducted a screening of different target genes encoding proteins that are essential for growth and development by microinjection into the hemolymph of *E. heros* nymphs. Furthermore, feeding trials were conducted using different formulations of dsRNA: naked dsRNA, EDTA and the transfection reagent lipofectamine 2000. Additionally, we attempted to evaluate dsRNA stability in the saliva for possible degradation upon oral delivery.

4.2 Materials and methods

4.2.1 Candidate genes ortholog identification

Lethal candidate genes were selected according to the database of essential genes in *D. melanogaster* (http://tubic.tju.edu.cn/deg/), large scale screens for target genes in coleopterans (Baum et al. 2007; Ulrich et al. 2015), published RNAi research in *E. heros* (Fishilevich et al. 2016a) and the target gene list used and published previously (Prentice et al. 2015).Candidate genes were identified with tBLASTn searches in *E. heros* pooled transcriptome using the sequences of red flour beetle (*Tribolium castaneum*) (Table 4.1).

E. heros total RNA was isolated from second instar nymphs using a RNeasy kit (Qiagen, Hilden, Germany). The cDNA was reverse transcribed from 500 ng of total RNA

Table 4.1: Description of candidate genes for RNAi studies in *Euschistus heros*.

Gene symbol	Tribolium homolog	E value	Identity	Locus description	Source
Eh_cact	TC002003	3E-53	41 %	NF-kappa-B inhibitor cactus-like	Ulrich et al. 2015
Eh_Spr54k	TC002574	5E-103	83 %	signal recognition particle 54 kDa protein	Ulrich et al. 2015
Eh_pp1α96a	TC015321	1E-94	49 %	serine/threonine-protein phosphatase 2A catalytic subunit beta isoform	Ulrich et al. 2015
Eh_inr-a	TC008263	4E-44	43 %	kinesin-like protein KIF17	Ulrich et al. 2015
Eh_Rpn7	TC006375	4E-65	40 %	26S proteasome non-ATPase regulatory subunit 6	Ulrich et al. 2015
Eh_gw	TC006679	1E-35	49 %	protein Gawky	Ulrich et al. 2015
Eh_βcop	TC013867	2E-32	52 %	coatomer subunit beta	Baum et al. 2007
Eh_vATPase A	TC008354	0	92 %	V-type proton ATPase catalytic subunit A	Baum et al. 2007
Eh_ATPsynβ	TC015322	2E-171	79 %	ATP synthase subunit beta, mitochondrial	Prentice et al. 2015
Eh_syb	TC015183	2E-28	69 %	synaptobrevin-1-like	Prentice et al. 2015
Eh_γcop	TC011806	3E-56	53 %	coatomer subunit gamma	Prentice et al. 2015
Eh_Taf-1	TC002507	2E-34	58 %	transcription initiation factor TFIID subunit 1	Prentice et al. 2015
Eh_Prosα2	TC004868	1E-141	81 %	proteasome subunit alpha type-2	Prentice et al. 2015
Eh_RpS13	TC004990	1E-90	91 %	40S ribosomal protein S13	Prentice et al. 2015
Eh_act-2				Muscle actin	Fishilevich et al. 2016a

template and oligo dT primer using SuperScript III First-Strand synthesis (Invitrogen, Merelbeke, Belgium). Primers were designed using the web application E-RNAi-Version 3.2 and T7 promoter sequence were placed at 5' ends of both forward and reverse primer, to enable dsRNA transcription (Table 4.2). The DNA templates were amplified using Taq DNA Polymerase (Invitrogen) using 500 ng of cDNA as a template. For the negative control, a green fluorescent protein (GPF) was amplified from a plasmid containing a GFP insert (Genbank ID: NC_011521.1). The DNA templates were purified using Wizard SV gel and PCR clean up system (Promega, Madison, WI). The dsRNAs were synthesized using 2 µg of PCR product as template with the MEGAscript RNAi kit (Ambion, Austin, TX). Nuclease-free water was used for dsRNA elution. The dsRNA was quantified on a NanoDrop ND-1000 s (Nanodrop Technologies, Wilmington, DE) at 260 nm and analyzed by gel electrophoresis to determine purity. The dsRNAs were diluted to 500 ng/µl in nuclease free water.

Table 4.2: Primers used for dsRNA synthesis. Only gene-specific parts of the primer are listed. These are preceded by the T7 adaptor TAATACGACTCACTATAGGG for dsRNA synthesis.

Gene symbol	Forward primer	Reverse primer	Amplicon size
Eh_cact	CAACGTTTCAGATCAAGCGA	TAGGGTAGGTGGCTCAGGAA	397
Eh_Spr54k	TTCACCATGCGGGATATGTA	TCATTTTCGCCATTTGTTGA	418
Eh_pp1α96a	TGCCATTGACAGCTCTCGTA	CGTGTGGTTCTCCTCTCCTC	441
Eh_inr-a	GTTGGCATAGCGAAGGGTAG	TTTAATGAGCAAGGGATGGG	433
Eh_Rpn7	GCTGTTTTCGCCCTGTTTAG	TGCCAGAGCGACTAGGAAAT	417
Eh_gw	TGCAACTCAGCCAGGTTCTA	ATCTCCTGCCCACAAAGAAG	391
Eh_βcop	CCTCGAGTTGTTGGCCTATG	AGCTGTTTTCGGCTGCTTAG	366
Eh_vATPase A	TGCCTGCTGACAGTGGTTAC	TGAGTAGCTTGGCGATTTCA	444
Eh_ATPsynβ	GGGTACCATGCAGGAAAGAA	TCAAGGGGTACAAGTTTGCC	438
Eh_syb	AGCAACTTTGAAGGCAACCA	TTTTTCAACCACATGTTCCG	343
Eh_γcop	GCAGCACCAAAGTTGCTTTT	TGTTCAGAAGTTCCGATCCC	436
Eh_Taf-1	AATCAGCCTGGTCGTGTTTT	GTGGGAGGAGTCGTAGGGTT	425
Eh_Prosα2	CAACATTTAGCCCCTCTGGA	TTTCCAAGCAAAGTATGCCC	446
Eh_RpS13	ATCATGGGTCGTATGCACG	CCGCCCTTGGTTTTGTAGTA	404
Eh_act-2	GATGACCCAGATCATGTTTGAGAC	CAAGATTCCATACCCAAGAAGGAAG	462
GFP	TACGGCGTGCAGTGCT	TGATCGCGCTTCTCG	455

4.2.2 dsRNA injection

Nanoinjection of *E. heros* was performed using a nanoinjector (FemtoJet, Eppendorf, Hamburg, Germany), equipped with an injection needle prepared with glass capillary tubes. Bioassays were carried out with 0-1 day-old *E. heros* second-instar nymphs. Cages having a large number of first-instar nymphs were selected and second-instar nymphs removed. The cages were kept in isolation for the emergence of new second-instar nymphs. Insects were anesthetized with diethyl ether for 2 min and immobilized in an agarose plate at 1.5 %. Each nymph was injected with 54.75 nl of 500 ng/µl dsRNA solution (i.e., 27.4 ng dsRNA in 1-1.5 mg insect) as previously used by (Fishilevich et al. 2016a) to induce significant mortality in *E. heros* second instar nymphs. The injection site was the ventral metathoracic region near the hind coxa. dsRNA targeting GFP was

used as a negative control. 25 nymphs were injected per treatment. After injections, stink bugs were rested for 2 h before being moved to green bean slices of 5x5 mm in petri dishes. Nymphs were evaluated phenotypically every day for 14 days. To measure mortality due to expression silencing, we compared the survival among injected individuals using 23-25 nymphs (25 total minus the dead insects at 24 h post-injection). The injection bioassay was repeated two times, each with independent sets of insects. Cumulative mortality was compared using Kaplan-Meier survival as described in 2.2.6.

For the molecular analysis, each nymph was injected with 52.3 nl of 500 ng/µl dsRNA solution for the target genes *Eh_vATPase A* and *Eh_act-2*. dsRNA targeting GFP was used as negative control. 16 nymphs were injected per treatment. *E. heros* nymphs were collected at 48 and 72 h post-injection. At each time point, 8 nymphs of each treatment (divided into two biological samples of 4 individuals each) were collected and total RNA was extracted from the whole insect body.

4.2.3 Feeding of dsRNA in an artificial diet

For the feeding assays, dsRNA was mixed with a diet, provided by Bayer AG, adapted from a liquid meridic diet for rearing of *Lygus hesperus* (modified after (Debolt 2015)). The effectiveness of different formulations of dsRNA: naked dsRNA, the transfection reagent lipofectamine 2000 (Invitrogen) and the protein inhibitor EDTA were tested. Besides being a magnesium chelator that may inhibit the activity of nucleases, EDTA is produced on a huge scale, is cheap and is already used as an adjuvant of pesticides with an acceptable safety profile (U.S. EPA 2004). Bioassays were carried out with 0-1 day old *E. heros* second instar nymphs. To feed the stink bugs, Parafilm™ (Bemis NA, Neenah, WI) was folded with a soldering iron to form small sachet pockets. The process of sachets making was carried out aseptically using UV-sterilized Parafilm to minimize the possibilities of degradation in test samples and prevent microbial contamination. The exposure to the amended diet was 6 days, the sachet was replaced on day three. After the exposure period, the artificial diet was substituted with green bean slices of 5x5 mm, plant matter was changed daily. Nymphs were evaluated phenotypically every day for 14 days, and weights were taken on day 4, 7 and 14 after the beginning of the feeding assay. Survival was analyzed using Kaplan-Meier survival curves and the log-rank test.

For naked dsRNA, nymphs were fed ad libitum on a sachet containing 40 µl of artificial diet amended with dsRNA. The final concentration of dsRNA in the diet was 320 ng/µL, using *Eh_vATPase A* and *Eh_act-2* as target genes and GFP as control. A total of 20 nymphs were used per treatment in each replicate, and two replicates were performed. The concentration was chosen based on the concentration of the synthetized dsRNA (3 µg/µl) and the dilution of the artificial diet that allowed the normal development of *E. heros* nymphs.

For all assays where lipofectamine 2000 was used, the mixture with dsRNA was prepared as follows: 10.7 µl of dsRNA (3 µg/µl) was mixed with 4.3 µl of nuclease free water and 1 µl of lipofectamine 2000. The mixture was incubated at room temperature for 5 min and then mixed with the artificial diet. Sachets containing 30 µl of artificial diet amended with freshly prepared lipoplex solution (dsRNA with liposome) were used to the feeding assays. The final concentration of dsRNA in the diet was 300 ng/µL, using *Eh_vATPase A* and *Eh_act-2* as target genes and GFP as control. A total of 20 nymphs were used per treatment in each replicate, and two replicates per treatment were performed.

For the EDTA assays, we used a concentration of 3 % w/v suggested by the EPA for agricultural products. Nymphs were fed ad libitum on a sachet 30 µl of artificial diet amended with the mixture of dsRNA and EDTA. The final concentration of dsRNA in the diet was 300 ng/µL, using *Eh_vATPase A* and *Eh_act-2* as target genes and GFP as control. Another control group without dsRNA treatment was fed with the same amount of EDTA solution. A total of 20 nymphs was used per treatment in each replicate, and two replicates per treatment were performed.

For expression analysis of feeding of dsRNA, bioassays were carried out with 0-1 day old E. heros fourth-instar nymphs. Naked dsRNA, and mixtures of dsRNA with lipofectamine 2000 and EDTA 3 % w/v were mixed with the artificial diet. Sachets containing 30 µl of the diet amended with dsRNA were made aseptically using UV-sterilized Parafilm. The exposure time were 24, 72 and 120 h using *Eh_vATPase A* and *Eh_act-2* as target genes and GFP as control. The final concentration of dsRNA in the diet was 300 ng/µL for naked dsRNA, lipofectamine 2000 and EDTA. Total RNA was extracted from the guts after continuous feeding with dsRNA, each treatment contained 4 biological samples of 3 pooled guts. For dissection, larvae were first chilled on ice, the posterior and anterior ends were removed, and entire guts were excised.

4.2.4 *Ex vivo* saliva degradation assays

To collect watery saliva from *E. heros*, adult insects were chilled on ice for about 5 min, then placed ventral side up and observed with a dissecting microscope. As the bugs returned to room temperature, the watery saliva was secreted from the tip of the beak (Peiffer and Felton 2014). This saliva was collected with a microloader pipet tip (Eppendorf). After collection, the saliva was expelled into a 1.5 ml tube and stored in ice until enough saliva was collected. For the digestion assay, 20 µl of a 200 ng/µL-dsRNA solution was incubated in 2 µL of RNase-free water, 2 µL of saliva or 2 µL of saliva diluted at 1/10. dsGFP and ds*Eh_act-2* were used as dsRNA for degradation assays. To test the ability of EDTA to inhibit the enzymatic activity of nucleases in the saliva of *E. heros*, 1 µl of 10 mM EDTA was added to the mixture before the incubation. All digestions were performed at 25° C. Aliquots of 5 µL were collected after 10, 30, 60 and 120 min, added a same volume of EDTA (10 mM) to stop the enzymatic reaction and run on a 1.5 %

agarose gel.

To evaluate the ability of lipofectamine 2000 to protect dsRNA against degradation by nucleases present in the insect saliva a similar experiment was made. 20 µl of dsRNA of 200 ng/µL (with lipofectamine 2000, same ratio as before) was incubated in 2 µL of RNase-free water, 2 µL of saliva or 2 µL of saliva diluted at 1/10. Aliquots of 5 µL were collected after 10, 30, 60 and 120 min. To stop the enzymatic reaction and disassemble the dsRNA complexes, 7.5 µL of SDS (1 % w/v) was added before the samples were run on a 1.5 % agarose gel.

4.2.5 Real-time quantitative PCR

The RNeasy Mini Kit (Qiagen) was used for RNA extraction following the manufacturer's instructions. The total RNA was purified with DNase treatment and removal reagent using a turbo DNA-free kit (Invitrogen) following the suppliers' recommendations. After DNase I treatment (Ambion), RNA was quantified using a NanoDrop ND-1000 (Nanodrop Technologies) and verified by 1.5 % agarose gel electrophoresis. The cDNA was reverse transcribed from 500 ng of total RNA template and oligo dT primer using SuperScript III First-Strand synthesis (Invitrogen).

E. heros qRT-PCR specific primers were designed using Primer3 Plus free-software (Table 4.3). The reference genes *Eh_ARL2*, *Eh_ARP8* and *Eh_UB4A* exhibit the most stable expression following the dsRNA treatment in the stink bug *H. halys*, making these genes appropriate for qRT-PCR data normalization for gene silencing analysis (Bansal et al. 2016).

The qRT-PCR reactions were performed in the CFX 96TM real-time system (Bio-Rad, Hercules, CA) with SYBR green dye as the fluorescence reporter for each elongation cycle. 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 temperature range from 60 to 95°C. The reaction included 10 µl of GoTaq qPCR Master Mix for Dye-Based Detection (Promega), 0.5-1 µl of 10 µM forward primer (Invitrogen), 0.5-1 µl of 10 µM of reverse primer (Invitrogen), 0-1 µl of nuclease-free water and 8 µl of cDNA (dilution 1/100th), 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

Table 4.3: Description of candidate genes for RNAi studies in *Euschistus heros*.

Target gene	**Forward primer**	**Reverse primer**	**Efficiency body (%)**	**Efficiency guts (%)**	**Amplicon size**
Eh_vATPase A	TGCCTGCTGACAGTGGTTAC	CCCTCCCTCTCTGGATTACC	103	97.6	88.9
Eh_act-2	ATCACCAACTGGGACGACAT	GAGCCTCAGTCAGGAGGATG	100	94.9	94.9
Reference gene	**Forward primer**	**Reverse primer**	**Efficiency body (%)**	**Efficiency guts (%)**	**Amplicon size**
Eh_ARL2	GGTTGGCATTCTTCAGTTGG	GCGCAATCGTAACTGGTACA	101	86.7	95.2
Eh_ARP8	TGCCATTCTTGCTTGTCTTG	GGCCCTCTTTCTCGTATCAA	96	103.7	102.5
Eh_UB4A	AGCTTCATCGAGCAGGAAAA	CTCGTGAGGCGGAAACTAAC	100	93.4	92.5

60°C. The reactions were set-up in 96-well format Microseal PCR plates (Bio-Rad) in duplicates. The endogenous controls, *Eh_ARL2*, *Eh_ARP8* and *Eh_UB4A*, were used for normalization of the data. Appropriate controls, no-template control and no-reverse transcriptase control, were also included in the assay. Relative expression values of genes in biological samples were calculated using the equation ratio 2-ΔΔCt (Livak and Schmittgen 2001).

4.3 Results

4.3.1 dsRNA injection

The insecticidal activity of 15 dsRNAs targeting essential genes was studied following direct injection in second-instar larvae of *E. heros*. dsRNA injection of 27.5 ng mg^{-1} body weight (∼1.0 mg) led to significant differences in survival (log-Rank test: χ^2 = 349.358, *df* = 16, *P* < 0.001, Figure 4.1). Of the 15 candidate genes tested, the silencing of the genes *Eh_prosα2*, *Eh_Spr54k*, *Eh_act-2*, *Eh_pp1α96a*, *Eh_taf-1*, *Eh_γcop* and *Eh_rpn7* showed a mortality rate of over 95 %. After 7 days comparing both repetitions, the injection of dsRNA against the genes *Eh_βcop*, *Eh_pp1α96a*, *Eh_act-2*, *Eh_rpn7*, *Eh_syb*, *Eh_vATPase A*, *Eh_cact* and *Eh_ATPsynβ* produced more than 50 % (Figure 4.1A). These values differed from the values obtained for the dsRNA treated insects (*F* > 39.87, *P* < 0.001). At 14 days, the mortality reached 90–100 % for the silencing of the genes *Eh_prosα2*, *Eh_Spr54k*, *Eh_act-2*, *Eh_pp1α96a*, *Eh_taf-1*, *Eh_γcop*, *Eh_Rpn7*, *Eh_βcop* and *Eh_syb* (Figure 4.1B), which were significantly different from the dsGFP control (*F* > 21.3, *P* < 0.001).

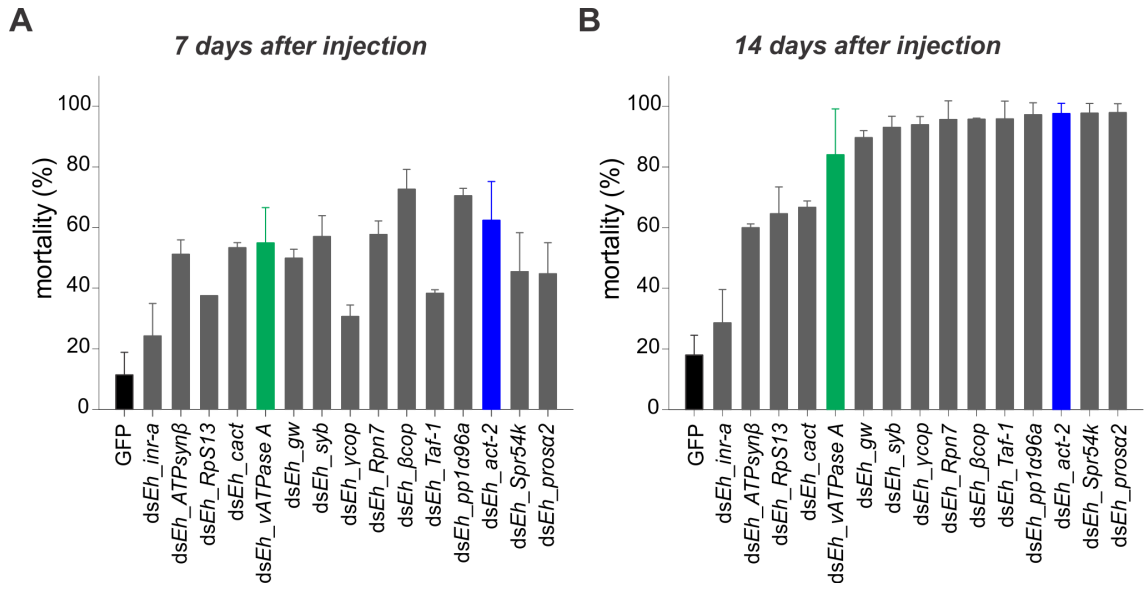


Figure 4.1: Mortality of second instar nymphs of *Euschistus heros* after injection of dsRNA. Injection with dsRNA targeting GFP was used as a control. Values represent the mean mortality percentages ± SEM from two independent replicates, each consisting of 23-25 nymphs. (***A***) 7 days post-injection, and (***B***) 14 days post-injection

The survival curves with the silencing of the genes *Eh_prosα2*, *Eh_spr54k*, *Eh_act-2*, *Eh_pp1α96a*, *Eh_taf-1*, *Eh_βcop*, *Eh_rpn7*, *Eh_γcop*, *Eh_syb*, *Eh_gw* and *Eh_vATPase A* were not significantly different from each other, but differed from the values obtained for the dsGFP-treated insects (Holm-Sidak's statistics < 23.9, $P > 0.0001$, not shown). Only the silencing of the genes *Eh_inr-a*, *Eh_ATPsynB*, *Eh_RpS13* and *Eh_cact* showed a mortality rate below 70 %. The survival rate of *Eh_inr-a* dsRNA treated insects was not significantly different for the values obtained for the dsGFP-treated insects (Holm-Sidak's statistics < 0.18, $P = 1.00$, not shown).

Figure 4.2 represents the survival curve over time for the 3 genes causing the highest mortality in the injection assays (*Eh_prosα2*, *Eh_Spr54k* and *Eh_act-2*) and *Eh_vATPase A*, a target gene which is commonly used in RNAi feeding experiments and has shown to be effective in causing mortality upon knockdown in many other insect species (Baum et al. 2007; Upadhyay et al. 2011; Wuriyanghan et al. 2011; Rangasamy and Siegfried 2012; Thakur et al. 2014; Mao et al. 2015; Christiaens et al. 2016). ds*Eh_act-2* caused early mortality, with a median survival time (LT_{50}) of 3.0 days (95 % confidential limits: 1.0 - 4.9). The silencing of genes *Eh_prosα2*, *Eh_Spr54k* and *Eh_vATPase A* exhibited intermediate survival times, 6.0 days (95 % confidential limits: 4.1-7.9) for *Eh_vATPase A*, and 7.0 days (95 % confidential limits: 5.5-8.5) for *Eh_Spr54k* and *Eh_prosα2*. Normal development was observed in the control (dsGFP), while detrimental effects were demonstrated under dsRNA treatments. These detrimental effects included lower mobility, lower weight, slower development and defects in molting. These results confirmed that *E. heros* is highly susceptible to RNAi even at low doses injected. Based on these results, we have chosen to continue our investi-

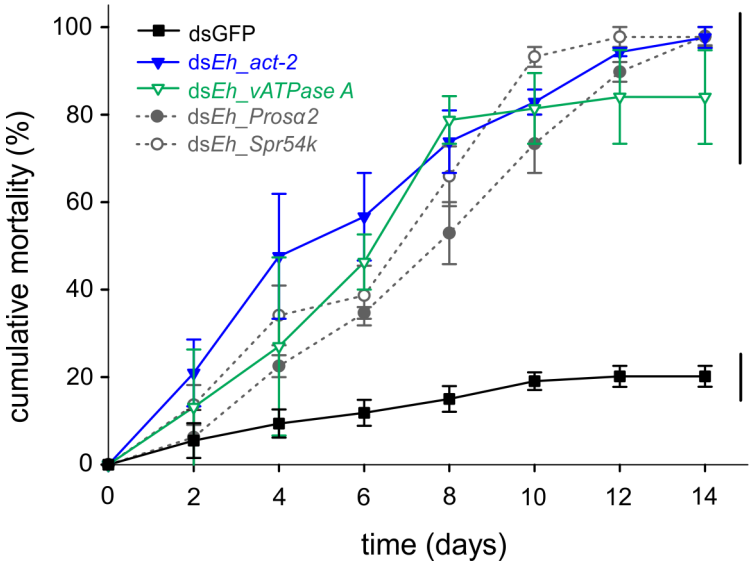


Figure 4.2: Cumulative mortality of *Euschistus heros* after injection of dsRNA targeting a selection of target genes in second instar nymphs. Injection with dsRNA targeting GFP was used as a control. The curves encompassed by the same vertical bar at the right side of the plot are not significantly different according to Holm-Sidak's test ($P > 0.05$). The assay was conducted with two replications each consisting of 23-25 nymphs.

gation with *Eh_act-2* and *Eh_vATPase A*. The former was chosen purely based on the high and rapid mortality observed after injection of the specific dsRNA, while *vATPase A* was chosen partly based on the results from the injection assay but also because it is a commonly used target gene in oral RNAi assays, often leading to a high mortality upon feeding the ds*Eh_vATPase A*.

Real-time quantitative PCR

To confirm the knockdown of the genes *Eh_vATPase A* and *Eh_act-2* after dsRNA injection, we performed a qRT-PCR analysis using cDNA of injected insects. For *Eh_act-2*, dsRNA injection reduced transcript levels by 69 % (P = 0.002) after 48 h of injection and 59 % (P = 0.032) after 72 h of injection (Figure 4.3*A*). After 48 h of injection, the *Eh_vATPase A* transcript levels were reduced by 54 %, but were not significantly different from the control (P = 0.12) (Figure 4.3*B*). 72 h after injection, dsRNA injection reduced the *Eh_act-2* transcript levels by 90 % (P = 0.019).

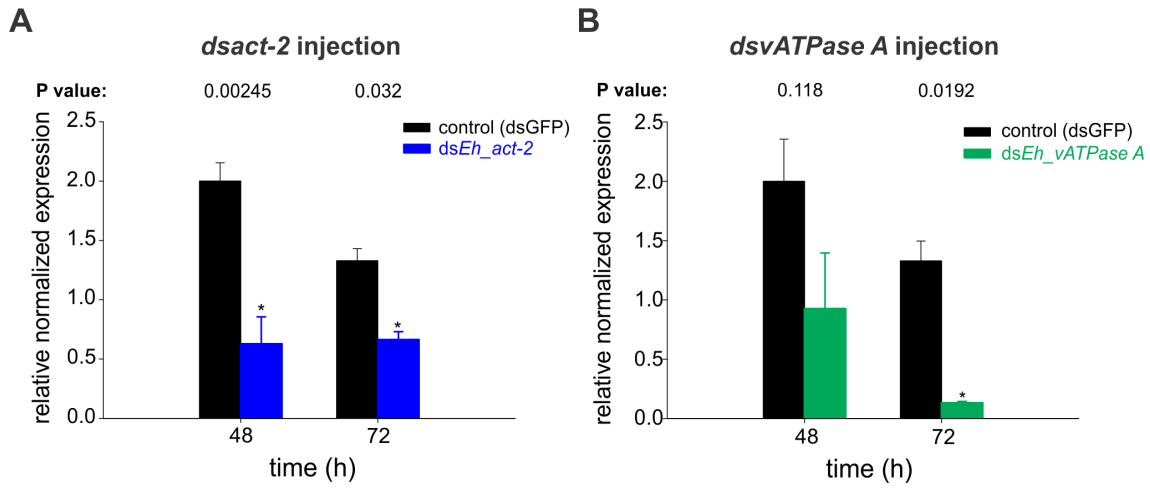


Figure 4.3: Knockdown of E*Eh_vATPase A* and *Eh_act-2* in *Euschistus heros* second instar nymphs injected with target gene specific dsRNA. Injection with dsRNA targeting GFP was used as a negative control. (***A***) *Eh_vATPase A*, and (***B***) *Eh_act-2*. As internal controls, *Eh_ARL2*, *Eh_ARP8* and *Eh_UB4A* were used. Values are based on two biological samples and expressed as mean ± SEM. Each sample contains 4 pooled insects. The p-values were calculated by unpaired t-test.

4.3.2 Feeding of dsRNA in an artificial diet

Next, dsRNA was administered via feeding by mixing with the artificial diet using different formulations of dsRNA: naked dsRNA, dsRNA mixed with liposomic transfection reagent and dsRNA mixed with the chelating agent EDTA, which is able to inhibit nuclease activity. Because the transcript silencing of the genes *Eh_vATPase A* and *Eh_act-2* was confirmed with qRT-PCR after microinjection, these genes were selected for the artificial diet assay. For the naked dsRNA test, diet containing 320 ng/µl of dsRNA resulted in 33 % mortality for ds*Eh_act-2* and 30 % for ds*Eh_vATPase A* (Fig-

ure 4.4*A*). According to the survival analysis, only the observed mortality for ds*Eh_act-2* was significantly different from the control dsGFP (Holm-Sidak's statistics = 9.485, *P* = 0.023). As a second formulation, we tested the influence of the liposomic transfection agent on dsRNA delivery. The diet containing 300 ng/µl of dsRNA combined with liposomes resulted in 45 % mortality for ds*Eh_vATPase A* and 42 % for ds*Eh_act-2* after 14 days (Figure 4.4*B*). According to the survival analysis, the observed mortality for ds*Eh_vATPase A* and ds*Eh_act-2* was significantly different from the liposome-coated dsGFP control (Holm-Sidak's statistics = 12.266, *P* = 0.007). In the last treatment, EDTA was added to the artificial diet to test the influence on dsRNA delivery. EDTA is a chelating agent that could inhibit the enzymatic activity of nucleases present in the saliva of the insect. The diet containing 300 ng/µl of dsRNA and EDTA at 3 % (w/v) resulted after 14 days in 51 % mortality for ds*Eh_vATPase A* and 22 % for ds*Eh_act-2* (Figure 4.4*C*). The observed mortality for ds*Eh_vATPase A* was significantly different from the

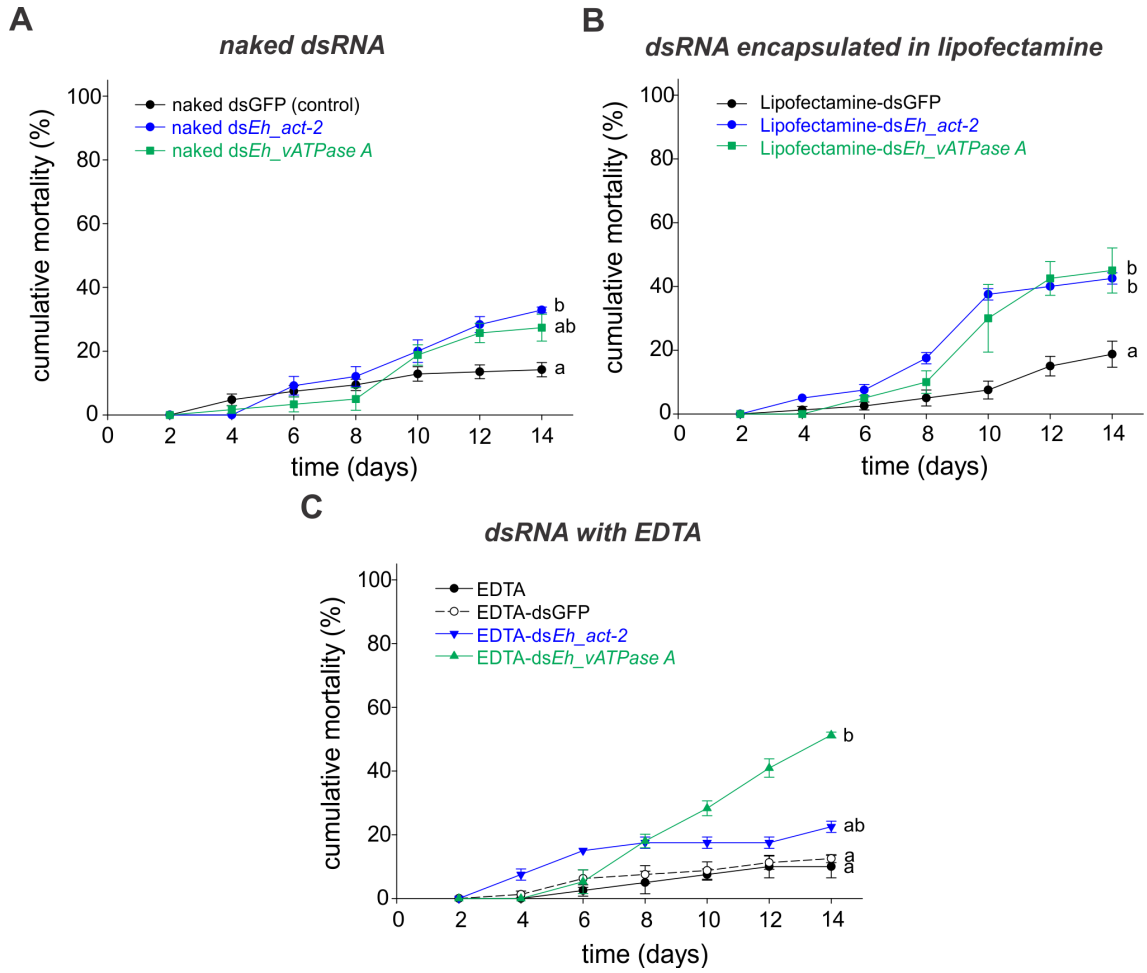


Figure 4.4: Cumulative mortality of second instar nymphs of *Euschistus heros* after feeding dsRNA with different formulations of dsRNA: (***A***) naked dsRNA, (***B***) liposome-encapsulated dsRNA, and (***C***) EDTA-formulation. Feeding with dsRNA targeting GFP was used as a negative control. The curves encompassed by the letter at the right side of the plot are not significantly different according to Holm-Sidak's test (*P* >0.05). The assay was conducted with two replications each consisting of 20 nymphs.

EDTA-dsGFP and EDTA controls (Holm-Sidak's statistics = 52.739, $P < 0.001$), while no significant mortality was shown with the target gene *Eh_vATPase A*. Significant differences in weight between the liposomic and EDTA treatments for both target genes compared to the controls were already recorded after 4 days of feeding (Appendix 4.2).

Real-time quantitative PCR

To confirm the knockdown of the *Eh_vATPase A* after dsRNA feeding with different formulations of dsRNA, we performed qRT-PCR using cDNA of dissected guts after 24, 72 and 120 h of feeding. A significant decrease in *Eh_vATPase A* and *Eh_act-2* mRNA levels was confirmed by qRT-PCR for naked dsRNA, EDTA-dsRNA and liposome-coated dsRNA (Figure 4.5). For naked dsRNA, the *Eh_vATPase A* transcript levels were reduced by 53 % after 72 h ($P = 0.0357$; Figure 4.5*A*) and *Eh_act-2* mRNA levels were reduced by 43 % after 24 h ($P = 0.0483$; Figure 4.5*B*). The feeding of ds*Eh_vATPase A* lipoplexes resulted in a small significant reduction of 36 % in the transcription ($P = 0.0483$; Figure 4.5*C*) compared with dsGFP lipoplex control. After feeding of lipoplexes with ds*Eh_act-2*, a significant reduction of *Eh_act-2* expression was observed at 24h (39 %; $P = 0.0428$; Figure 4.5*D*) and 72 h (46 %; $P = 0.0428$). A transcript depletion effect after continuous feeding on dsRNA with EDTA was observed; for *Eh_vATPase A*, transcript levels were lowered by 49 % after 24 h ($P = 0.0126$; Figure 4.5*E*) and *Eh_act-2* transcript levels by 40 % after 72 h ($P = 0.00472$; Figure 4.5*F*).

Ex vivo saliva degradation assays

An ex vivo assay with watery saliva of *E. heros* adults was conducted to evaluate whether dsRNA is degraded during extra oral digestion. The saliva was found to rapidly digest double stranded RNA because after 10 min the dsGFP and ds*Eh_act-2* were completely degraded (Figure 4.6*A,B*). To establish an effective concentration of saliva necessary for dsRNA digestion, raw saliva was diluted in concentrations of 1/10, 1/50, 1/200 and 1/1000. After 10 min at 25°C, the most concentrated sample resulted in a complete digestion of the dsRNA, a 1/50 dilution resulted in a minimal degradation of dsRNA, and the dsRNA in the less concentrated saliva samples appeared intact (data not shown). Based on these results, a 1/10 dilution was used in the further experiments. With the 1/10 dilution, an incubation of 30 min resulted in a clear smear below the band representing the intact dsGFP fragment, indicating digestion of the dsRNA. After 60 min of incubation, the dsGFP was partially degraded, and after 120 min the dsRNA was completely degraded (Figure 4.6*C*). To examine whether liposomes and EDTA could protect dsRNA from degradation, similar experiments were set up to test the integrity of dsRNA, using gel electrophoresis after incubation in saliva. The liposomes were less effective in protecting the dsRNA against saliva degradation. After 30 min of incubation with non-diluted saliva, the dsGFP and ds*Eh_act-2* were partially degraded (Figure 4.6*D,E*); and after 120 min dsGFP was almost completely degraded (Figure 4.6*D*) while

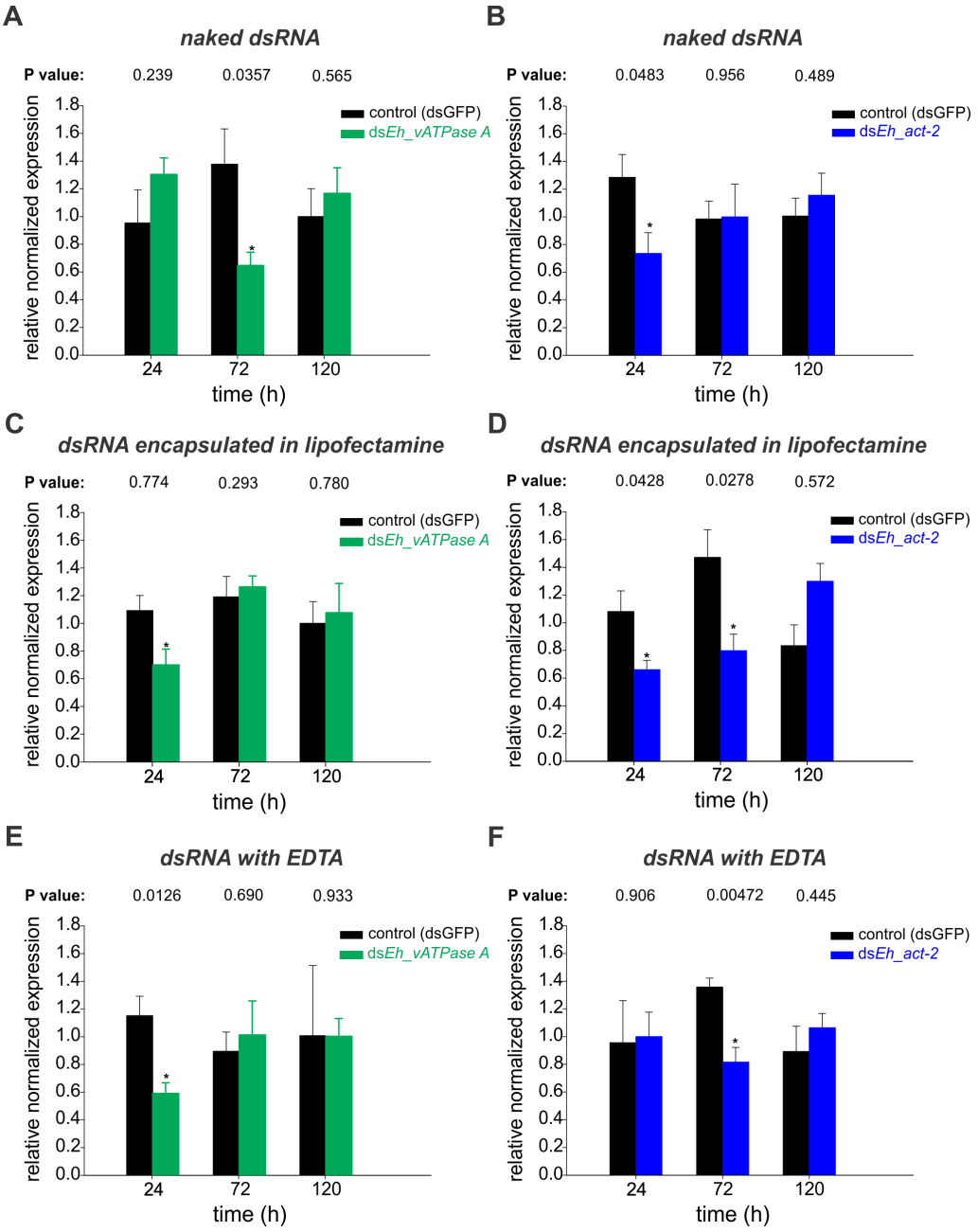


Figure 4.5: Expression of *Eh_vATPase A* and *Eh_act-2* in fourth instar nymphs of *Euschistus heros* after feeding on dsRNA using different formulations: naked dsRNA, liposome-coated dsRNA and an EDTA formulation for 24, 72 and 120 h. Feeding with dsRNA targeting GFP was used as a control (***A***) naked ds*Eh_vATPase A*, (***B***) naked ds*Eh_act-2*, (***C***) liposome-ds*Eh_vATPase A*, (***D***) liposome-ds*Eh_act-2*, (***E***) EDTA-ds*Eh_vATPase A* and (***F***) EDTA-ds*Eh_act-2*. As internal controls, *Eh_ARL2*, *Eh_ARP8* and *Eh_UB4A* were used. Values are based on four biological samples and expressed as mean ± SEM. Each sample contains 3 pooled insect guts. The p-values were calculated by unpaired t-test.

for ds*Eh_act-2* and small band was still present (Figure 4.6*E*). The EDTA protected the dsGFP and ds*Eh_act-2* longer against enzymatic degradation by the saliva with non-diluted saliva (Figure 4.6*F,G*). dsGFP and ds*Eh_act-2* bands were still present after 2 h of incubation, despite signs of ongoing degradation illustrated by the smearing on the gel (Figure 4.6*F,G*).

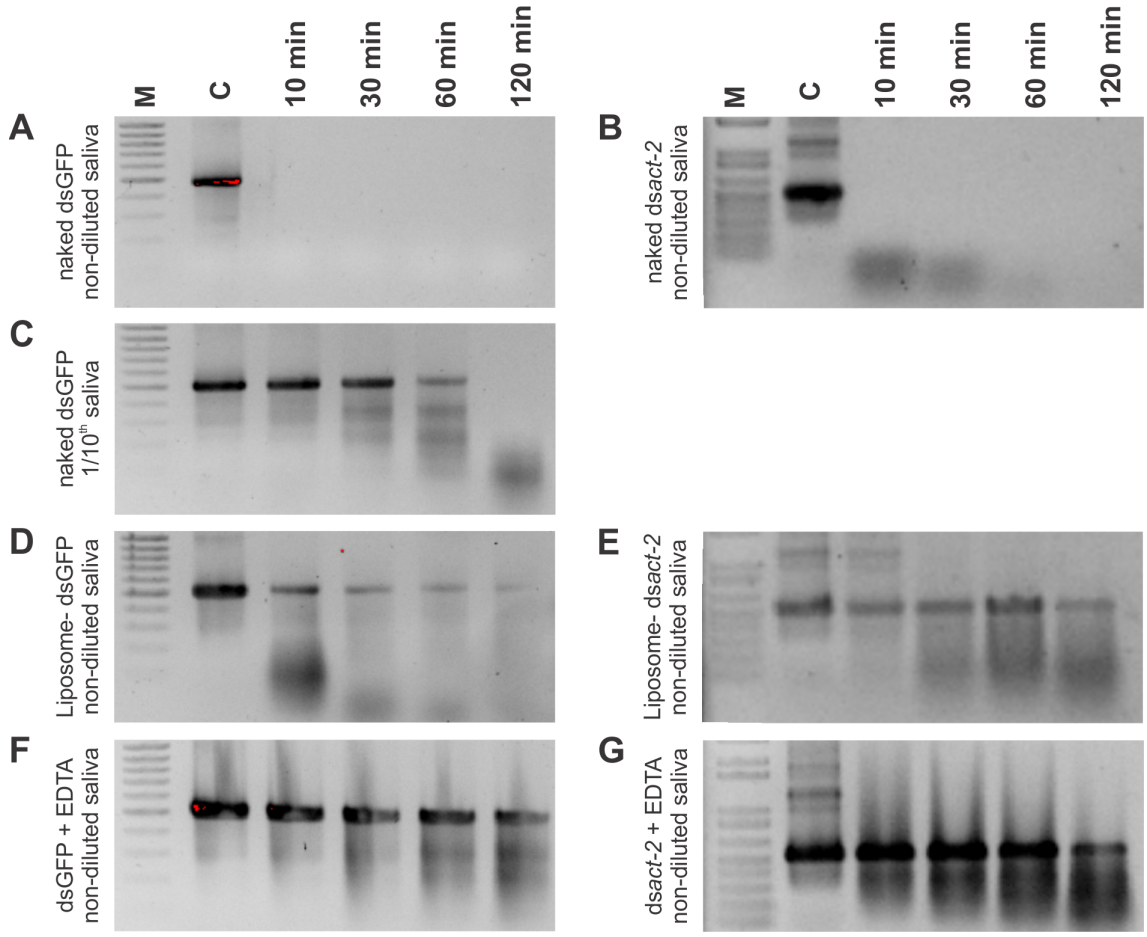


Figure 4.6: Ex vivo dsRNA degradation assay of different formulations of dsRNA: (***A***) naked dsGFP non-diluted saliva, (***B***) naked ds*Eh_act-2* non-diluted saliva, (***C***) naked dsGFP at 1/10 diluted saliva, (***D***) dsGFP with liposome, (***E***) ds*Eh_act-2* with liposome, (***F***) dsGFP with EDTA and (***G***) ds*Eh_act-2* and EDTA. Watery saliva of *Euschistus heros* were extracted and incubated with 0.9 µg of dsGFP per well for different time periods and run in 1.5 % agarose gel. M = DNA ladder, C = 0.9 µg of dsGFP at 2 h of incubation.

4.4 Discussion

In this chapter, we demonstrated that RNAi effects can be induced in *E. heros* by dsRNA microinjection and oral delivery. Recently, it was reported that *E. heros* is highly sensitive to dsRNA by injection (Fishilevich et al. 2016a). The functionality of RNAi in *E. heros* was confirmed by the screening by microinjection of 15 potential genes. The class of target genes selected here represented varied functions such as signaling pathways, intracellular transport, degradation of proteins, cell energization, pH homeostasis, transcriptional regulation, protein synthesis and muscle movement. These genes were chosen based on the expectation that their knockdown would result in a lethal phenotype (Prentice et al. 2015; Ulrich et al. 2015).

In the present investigation, microinjection experiments were chosen for the first selection round, as only small amounts of dsRNA are needed for these experiments, and because even a dose of 20 ng/g body weight of dsRNA of an essential gene (actin) led

to a higher mortality (Fishilevich et al. 2016a). A high insecticidal activity was observed, with almost a total mortality for seven of the dsRNAs, targeting the genes *Eh_prosα2*, *Eh_Spr54k*, *Eh_act-2*, *Eh_pp1α96a*, *Eh_taf-1*, *Eh_γcop* and *Eh_rpn7*, at 14 days post-injection compared with the low mortality (18 %) in the control injected with dsGFP. While the dsRNAs against four other genes, namely *Eh_inr-a*, *Eh_ATPsynB*, *Eh_RpS13* and *Eh_cact*, failed to cause mortality over 70 %. Interestingly, we observed that many of the selected genes showed an efficient silencing leading to abnormal phenotypes such as a significant reduction of the nymphal weight, reduction of mobility and molting abnormalities. We then selected two target genes to continue our investigation in *E. heros* RNAi, namely *Eh_act-2* and *Eh_vATPase A*. The former encodes a muscle actin, that is a crucial component for muscle contraction (Rosa et al. 2010) and *Eh_vATPase A* is a catalytic subunit of the vacuolar-type proton ATPase. vATPase is an enzyme complex which functions to acidify intracellular organelles through pumping protons across the plasma membranes. This enzyme is also found in the plasma membrane of many animal cell types and is involved in pH homeostasis and ion-transport (Thakur et al. 2014; Li et al. 2015b). vATPase A has revealed a high RNAi efficacy by ingestion in different insects including *Helicoverpa armigera* (Mao et al. 2015), *Diabrotica* spp. (Baum et al. 2007; Rangasamy and Siegfried 2012), *Cylas brunneus* (Christiaens et al. 2016), *Leptinotarsa decemlineata* (Baum et al. 2007), *B. tabaci* (Upadhyay et al. 2011; Thakur et al. 2014) and *B. cockerelli* (Wuriyanghan et al. 2011). In contrast, act-2 has only been silenced in *E. heros* by injection (Fishilevich et al. 2016a) and in *Homalodisaca vitripennis* cells via lipid-based transfection (Rosa et al. 2010). Finally, we also confirmed by RT-qPCR that a significant transcript reduction of the expression of *Eh_act-2* and *Eh_vATPase A* (69 % and 90 %, respectively) was obtained after dsRNA injection. The delay in the gene suppression observed for *Eh_vATPase A* is probably caused by differences in the mRNA turnover rate and the half-life of the protein (Huvenne and Smagghe 2010; Scott et al. 2013; Vogel et al. 2019). These results indicated that microinjection is a very promising technique for the study of functional genomics in *E. heros*.

As injection of dsRNA is not possible in field conditions, oral delivery and uptake of the dsRNA in the gut becomes very critical (Upadhyay et al. 2011). Therefore, to examine the potential of RNAi for crop protection against *E. heros*, we evaluated the toxicity through oral delivery of the two selected target genes. While *E. heros* displayed an RNAi response that is highly sensitive to dsRNA injection, we observed in this study that oral delivery of dsRNA is much less effective. In our feeding experiments, even with high dsRNA quantities applied, we could only detect a significant downregulation of the *Eh_vATPase A* expression in the isolated gut within 72 h (53 %) and we did not find any significant difference in the survival rates compared with that of the controls (Figure 4.4*A*). This lower response upon oral delivery of the dsRNA has been reported in *Ly. lineolaris* (Allen and Walker 2012), *Locusta migratoria* (Luo et al. 2013), *Bombyx mori* (Liu et al. 2012), *Schistocerca gregaria* (Wynant et al. 2014c), *Drosophila suzukii* (Taning et al. 2016), *C. brunneus* (Christiaens et al. 2016), *C. puncticollis* (Prentice et

al. 2017), and in *Blattella germanica* (Lin et al. 2017). Possible explanations for the large discrepancy between RNAi efficiency through microinjection and oral delivery of dsRNA could be the amount of dsRNA ingested by the insect, the frequency of feeding, degradation of dsRNA in the digestive tract, cellular uptake in the gut and systemic transport to the target tissues (Huvenne and Smagghe 2010; Yu et al. 2013; Scott et al. 2013).

This premise was further studied to observe specifically whether the saliva of *E. heros* has ribonuclease activity. Our ex vivo incubation test showed a rapid degradation of dsRNA because within 10 min the dsRNA had essentially disappeared from our gels (Figure 4.6*A*), indicating the complete digestion to monomers. This finding is in agreement with the strong degradation due to nucleases in the saliva of in *Ly. lineolaris* (Allen and Walker 2012), *A. pisum* (Christiaens et al. 2014) and *N. viridula* (Lomate and Bonning 2016). It is becoming increasingly evident that non-specific nucleases in the gut lumen could contribute to the degradation of dsRNA in Lepidoptera (Arimatsu et al. 2007; Shukla et al. 2016; Wang et al. 2016; Singh et al. 2017; Guan et al. 2018), Blattodea (Wang et al. 2016), Orthoptera (Wynant et al. 2014c; Ren et al. 2014; Spit et al. 2017; Song et al. 2017) and Coleoptera (Singh et al. 2017; Prentice et al. 2017; Gillet et al. 2017), and even in the highly oral RNAi sensitive *L. decemlineata* (Spit et al. 2017). Unlike other insects that primarily digest the food internally, hemipterans use digestive enzymes to the extra-oral digestion for liquefy the vegetal tissue before the ingestion.(Boyd 2003; Silva et al. 2012; Peiffer and Felton 2014) Then these nucleases have to be expressed in the salivary glands. Liu et al. (2012) demonstrated that a DNA/RNA non-specific alkaline nuclease is in fact present in several different tissues of *B. mori* larvae including epidermis, fat body, thoracic muscles, Malpighian tubules, brain and silk glands. A recent study in *B. tabaci* identified three homologs of this protein and demonstrated that suppression of dsRNase genes resulted in an enhanced efficacy of RNAi against 2 other insect genes, *AQP1* and *SUC1* (Luo et al. 2017). However, extra-oral degradation of dsRNA is not substantial in the whitefly, but dsRNA ingested by the whiteflies is subjected to non-specific degradation within the insect body (Luo et al. 2017), indicating that different nucleases are responsible for the degradation of dsRNA by the saliva, making in turn that future research should identify these enzymes on a biochemical and genetic level.

An alternative approach is to protect the dsRNA for its delivery, and so various delivery vectors including liposomes, polymers and nanoparticles have thus been developed in order to avoid these problems (Wu and McMillan 2009). In *D. melanogaster* and other drosophilid species, a transfection reagent has been used to enhance the uptake of encapsulated dsRNA into the target cell, due to its efficient interaction with cell membranes and nucleic acids (Whyard et al. 2009; Wu and McMillan 2009; Taning et al. 2016). Recently, Lin et al. (2017) demonstrated that liposomes can be a protective vehicle of dsRNA against the degradation that takes place in the midgut juice of *B. ger-*

manica. In this study, ingestion of liposome-encapsulated *Eh_vATPase A* increased the mortality 1.6-fold compared with naked *Eh_vATPase A* (45.0 % and 27.4 % mortality, respectively), and a significant but incomplete gene suppression in the isolated gut tissues was observed after 24 h after feeding (35 %). For *Eh_act-2*, the liposome was able to increase mortality 1.3-fold compared to naked dsRNA and resulted in an incomplete gene suppression of 39 % after 24 h and 46 % after 72 h of feeding. These observations could be linked to an improved or more rapid cellular uptake and a partial protective activity against degradation by the saliva (Figure 4.5C). Our qPCR analysis showed a prolonged silencing at the *Eh_act-2* transcript level for liposome-dsRNA compared to the naked dsRNA. However, we could not observe a similarly improved transcript reduction for the *vATPase* liposome-dsRNA. In general, our observations at the transcript level do not clearly support the increased effect of this formulation at the phenotypical level. One possible explanation is that the limited number of timepoints analyzed resulted in a hidden difference in silencing duration, which we were not able to detect. While these results make it difficult to explain the phenotypical differences seen between naked dsRNA and the liposome-formulated molecule, it does prove that gene silencing occurs, which can explain the observed mortality and link it to an RNAi effect. Additionally, previous studies showed the restrictive depleting effect in the midgut by dsRNA ingestion might not pass through the gut cells into the hemocoel to cause a strong silencing effect in other tissues of the German cockroach (Lin et al. 2017).

This study also showed that the degradation of dsRNA in the saliva was inhibited *in vitro* in the presence of EDTA, a chelating agent for divalent cations (Ca^{+2}, Mg^{+2} and Mn^{+2}). These results provide evidence that a metal-dependent enzyme is responsible for degradation of dsRNA in the saliva, which was also previously reported to be the case in the degradation in the hemolymph plasma of *Manduca sexta* (Garbutt et al. 2013) and in the midgut of *C. puncticollis* (Prentice et al. 2017) , suggesting that we could use EDTA as dsRNA protective vector for our feeding assays. The ingestion of ds*Eh_vATPase A* amended with EDTA caused a significant mortality (51 %), but the formulation did not appear to affect the mortality upon *Eh_act-2* feeding. However, a significant weight decrease was observed for both target genes using EDTA as a formulation (Appendix 4.2). The expression level of *Eh_vATPase A* decreased 49 % within 24 h when feeding EDTA-dsRNA, while gene silencing with naked dsRNA was only significantly reduced after the third day. In contrast however, for *Eh_act-2* we observed gene silencing later when EDTA was added to the dsRNA compared to naked dsRNA. Similar to the results found for liposome-formulation, the effect at the transcript level appeared to be difficult to reconcile with the significantly increased mortality for these formulations. In this study, the goal of the transcript analysis was to confirm that gene silencing occurred but further investigations into these dynamics, for example by evaluating samples taken at many more time points, or by looking at multiple tissues rather than the gut only, could perhaps help us explain these observations. As the insects lived for several days while consuming the dsRNAs, this observation suggested that the mortality

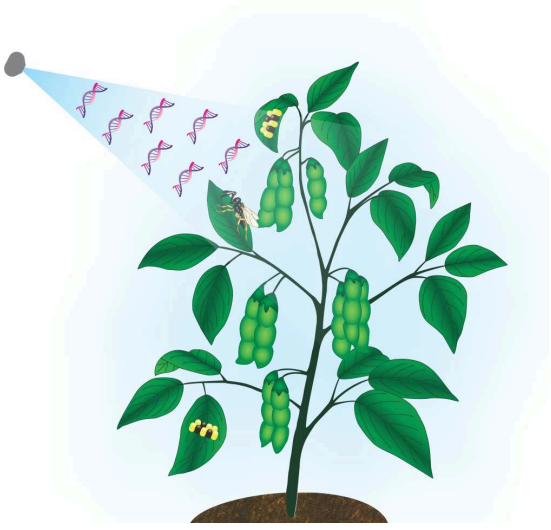
was a consequence of latent effects of the dsRNA, reducing gene function sufficiently to disrupt normal gut cell function, thereby leading to death of the growing insect (Whyard et al. 2009). In future studies, it will be of interest to examine whether the ingestion of dsRNA with these formulations leads to a systemic RNAi response by evaluating gene suppression in different tissues or using target genes that are not present in the midgut.

In our feeding experiments with EDTA-dsvATPase A, we detected a significant but transient RNAi silencing at high concentrations (300 ng/µl), with transcript levels in silenced individuals returning to levels comparable with those in control individuals after 3 days. This transient silencing might be due to the lack of dsRNA at the latter stages of insect development with the dilution of dsRNA through processing or cellular replication (Sijen et al. 2001). These results are consistent with the reports in other hemipterans that also required higher concentration of dsRNA, such as *A. pisum* (Shakesby et al. 2009; Mao and Zeng 2012), *B. cockerelli* (Wuriyanghan et al. 2011), *N. lugens* (Chen et al. 2010; He et al. 2011) and *R. prolixus* (Araujo et al. 2006). Despite the reports that in some hemipterans low concentrations as 3.4-50 ng/µl could evoke RNAi responses (Whyard et al. 2009; Upadhyay et al. 2011; Li et al. 2011; Yao et al. 2013; Zhang et al. 2013b; Deng and Zhao 2014), our data were comparable to gene silencing levels observed in many other insects that have been fed dsRNA, where the extent of RNAi-induced silencing typically ranged between 40 % and 60 % (Zhou et al. 2008; Zhang et al. 2010; Li et al. 2011; Wan et al. 2014; Deng and Zhao 2014; Lin et al. 2017). It should be remarked that if high dsRNA concentrations are required for RNAi effects in target insects, this might compromise the practical application of RNAi approaches for insect control (Wuriyanghan et al. 2011). Several studies have demonstrated that increasing the concentration beyond an optimal does not always improve the efficiency of dsRNA (Shakesby et al. 2009; Wan et al. 2014), suggesting that lower concentrations could generate the same silencing effects. Therefore, further studies are needed to find the optimal concentrations to evoke efficient RNAi in *E. heros*.

To summarize, the results presented in this Chapter reveal an RNAi effect in *E. heros* through injection and oral delivery, although less sensitive than the latter. We have identified several target genes for which the injection of low doses of dsRNA lead to effective silencing and high mortality. The rapid dsRNA degradation that occurred during the extra-oral digestion impaired the RNAi response in the feeding assays. Feeding of lipofectamine-encapsulated dsRNA increased the mortality and protected the dsRNA against saliva degradation. Furthermore, this study tests for the first time the potential to use of EDTA to prolong the stability of dsRNA and to induce stronger RNAi effects. However, EDTA could only effectively increase RNAi-caused mortality for one of our target genes, despite showing clear capabilities to protect the dsRNA targeting the other target gene in the *E. heros* midgut environment. Further research will have to elucidate the exact reasons for this observation.

Chapter 5

Risk assessment of RNAi to the egg parasitoid *Telenomus podisi*



5.1 Introduction

Insects are ubiquitous components of agricultural landscapes (Romeis et al. 2013). Some insects are considered important pests and reduce productivity (Zhang et al. 2007). Beneficial insects such as natural enemies, pollinators and decomposers also inhabit cultivated areas and provide valuable ecosystem services to agriculture (Zhang et al. 2007; Kremen and Miles 2012; Bommarco et al. 2013; Schellhorn et al. 2014; Duru et al. 2015). In particular, biological control by natural enemies is a key ecosystem service and an underlying pillar of IPM (Cock et al. 2010; van Lenteren 2012; Naranjo et al. 2015; van Lenteren et al. 2018), with an estimated value of $4.5 billion per year in the United States alone (Losey and Vaughan 2006). Consequently, natural enemies and the ecosystem services they provide should be protected from the pest control measures, to ensure the sustainability of agricultural production (Romeis et al. 2013; Lescourret et al. 2015). Accordingly, pesticides are subject to an environmental risk assessment before being released for commercialization (Romeis et al. 2008, 2013; Sanvido et al. 2012; Boivin and Poulsen 2017; Schiemann et al. 2019). By ensuring that new technologies will not have significant adverse effects on natural enemies regulatory agencies indirectly ensure that these technologies fit well into IPM programs (Matten et al. 2008).

In this perspective, the use of RNAi offers potential to control insect pests with high taxonomic selectivity and environmental safety (Scott et al. 2013; Joga et al. 2016; Zhang et al. 2017; Zotti et al. 2018). Nevertheless, as with any pest control technology, one of the major concerns regarding the environmental risk of RNAi is the potential adverse effects on NTOs. Potential hazards of RNAi include gene silencing, immune stimulation and saturation of the RNAi machinery (Lundgren and Duan 2013; Casacuberta et al. 2015; Christiaens et al. 2018a). It is widely recognized that different barriers will restrict the unwanted adverse effects on NTOs, including behavior, diet, gut conditions and the inherent sensibility of the RNAi machinery (Lundgren and Duan 2013; Fishilevich et al. 2016; Bachman et al. 2016). However, knowledge gaps in the genomics and physiologies of exposed NTOs currently affect our ability to predict the risks of RNAi-based insecticides to NTOs and agroecosystems as a whole (Lundgren and Duan 2013; Zotti and Smagghe 2015). For instance, the potential utility of bioinformatics as a prediction tool for risk assessment is limited by the lack of clear rules on sequence specificity in the siRNA pathway in insects (Casacuberta et al. 2015; Sherman et al. 2015).

Given these limitations, assessments of whether RNAi-based insecticides pose potential ecological risks to NTOs should include both lethal and sublethal measurement endpoints (i.e., growth, development, reproduction, gene expression) (Lundgren and Duan 2013; Bachman et al. 2013; Ramon et al. 2014; Roberts et al. 2015). In this regard, some works have been done to evaluate both lethal and sublethal effects in insect pollinators (Bachman et al. 2013, 2016; Tan et al. 2016; Vélez et al. 2016), predators (Bachman et al. 2013, 2016; Haller et al. 2019) and soil decomposers (Pan et al. 2016;

Bachman et al. 2016). Surprisingly, despite the ecological and economical importance of parasitoids, to date, only mortality has been the measurable endpoint to assess the potential risks of RNAi on parasitic wasps (Bachman et al. 2013, 2016). Furthermore, the hypothetical pathway of harm indicates that these organisms can be exposed to insecticidal dsRNA: (i) directly via consumption of pollen and nectar in the adult life; or (ii) indirectly during the development inside the herbivore host (Bachman et al. 2013, 2016; Roberts et al. 2015; Rusch et al. 2017). Therefore, evaluation of the potential sublethal effects of non-specific dsRNA in parasitoids is fundamental to ensure that the pest suppression provided by these insects is preserved (Romeis et al. 2008; Sanvido et al. 2012).

In the previous chapter, the expression level of *Tp_vATPase A* and *Eh_act-2* was significantly reduced after *E. heros* nymphs were fed an artificial diet containing dsRNA targeting those genes (Chapter 4). In this chapter, we examine the risk hypothesis that *E. heros* active *Tp_vATPase A* and *Eh_act-2* dsRNA has no adverse impact on its parasitoid *T. podisi*. More importantly, we examined the link between sequence similarity, potential off-target gene silencing and parasitism capacity of this egg parasitoid in order to increase our understanding of the sequence homology requirements to trigger silencing in insects. The worst-case scenario was established, which involves exposure in the laboratory to a maximum hazard dose with purified active ingredients in artificial diets (US EPA 2007; EFSA 2010). To test this risk hypothesis under the worst-case scenario, we (i) determined the susceptibility of *T. podisi* adults to dietary RNAi after ingestion of specific dsRNA; (ii) assessed the impact of ingestion of high concentration of *E. heros* active dsRNAs on life history traits of *T. podisi* adults; (iii) conducted a bioinformatic analysis to detect shared sequence identity between *Tp_vATPase A* and *Eh_act-2* and the published *T. podisi* transcriptome (Farias et al. 2015) for possible off-target genes; (iv) investigated the expression levels of core RNAi genes and predicted off-target genes in response to non-specific dsRNA; (v) characterized the exposure routes to *T. podisi* larvae; and (vi) evaluated the development of *T. podisi* larvae on eggs previously exposed to dsRNA.

5.2 Materials and methods

5.2.1 RNAi-related genes and phylogenetic analysis of siRNA core genes

A list of RNAi-related genes employed by Swevers et al. (2013) was selected, covering the RNAi core machinery, auxiliary factors, nucleases, antiviral RNAi and dsRNA uptake. Homologous sequences from *D. melanoganster* and *B. mori* corresponding to these genes were used as a query to search the transcriptome from *T. podisi* for the presence of RNAi-related genes using the tBLASTn tool at NCBI BLAST Service (http://www.ncbi.nlm.nih.gov/).

A phylogenetic tree of Dicer enzymes and Argonaute proteins were constructed using the sequences of *T. podisi* and 10 insects from different orders. The selected species were: *T. castaneum* (Coleoptera), *B. mori* (Lepidoptera), *A. pisum* (Hemiptera), *H. halys* (Hemiptera), *Bombus terrestris* (Hymenoptera), *A. mellifera* (Hymenoptera), *Nasonia vitripennis* (Hymenoptera), *Acromyrmex echinatior* (Hymenoptera), D. melanogaster (Diptera) and *L. migratoria* (Orthoptera). For protein alignment and phylogenetic tree construction, only the hit with the lowest E-value was chosen. Amino acid sequence alignments and phylogenetic analyses were performed with the ClustalW program, integrated in the Molecular Evolutionary Genetics Analysis software (MEGA 7.0.26). Phylogenetic trees and P-Distances were constructed using the Maximum Likelihood method with MEGA 7.0.26 software. Bootstrapping was used to estimate the reliability of phylogenetic reconstructions (1000 replicates).

5.2.2 dsRNA design and synthesis

Tp_vATPase A and *Tp_act-2* genes identified with tBLASTn searches in *T. podisi* pooled transcriptome using the sequences of *T. castaneum* (Table 5.1). These target genes were selected based on their essential functions and their insecticidal activity in the stink bug host *E. heros* (Chapter 4). In addition, homology between *E. heros* and *T. podisi* genes was investigated through phylogenetic trees using the Maximum Likelihood method with MEGA 6.06 software (Appendix 5.2). vATPase A and act-2 sequences from six insect species were used in the analysis, including *T. castaneum*, *B. mori*, *A. pisum*, *A. mellifera*, *N. vitripennis* and *D. melanogaster*. Primers were designed within the same region targeted by *E. heros* active *Eh_vATPase A* and *Eh_act-2* dsRNA using the web application E-RNAi-Version 3.2 (Table 5.2, Appendix 5.3). For the evaluation of the non-target effects and the exposure routes, *E. heros* active *Eh_vATPase A*, *Eh_act-2* and *Eh_ATPsynβ* dsRNA previously designed in Chapter 4 were used. DNA fragments were amplified by PCR using cDNA of *T. podisi* adults or second instar *E. heros* nymphs as templates. For the negative control, a GPF fragment was amplified from a plasmid containing a GFP insert. The dsRNAs was synthetized using MEGAscript RNAi kit as described in 4.2.3.

Table 5.1: Description of candidate genes for RNAi studies in *Telenomus podisi*.

Gene symbol	Tribolium homolog	E value	Identity	NCBI accession *T. podisi*	Locus description
Tp_vATPase A	EFA02908	3.00E-138	84.51	GBEU01005926.1	V-type proton ATPase catalytic subunit A
Tp_act-2	XP_966495.1	3.00E-153	56.10	GBEU01006906.1	Muscle actin

Table 5.2: Primers used for dsRNA synthesis. Only gene-specific parts of the primer are listed. These are preceded by the T7 adaptor TAATACGACTCACTATAGGG for dsRNA synthesis.

Gene symbol	Forward primer	Reverse primer	Amplicon size
Eh_vATPase A	TGCCTGCTGACAGTGGTTAC	TGAGTAGCTTGGCGATTTCA	444
Eh_act-2	GATGACCCAGATCATGTTTGAGAC	CAAGATTCCATACCCAAGAAGGAAG	462
Eh_ATPsynβ	GGGTACCATGCAGGAAAGAA	TCAAGGGGTACAAGTTTGCC	438
Tp_vATPase A	AATTTGGCAACCATTGGAAA	GGTGGCATGATCATTCTGTG	494
Tp_act-2	ACCAGCCTGTGGTAATCGAC	CCCCGGCATCTAAAACTACA	458
GFP	TACGGCGTGCAGTGCT	TGATCGCGCTTCTCG	455

5.2.3 Adult feeding assays

On-target effects: Feeding assays using dsRNA targeting *T. podisi* genes

Assays were initiated with newly emerged *T. podisi* adults that were ≤24 h from first observed emergence. Assays were carried out in Duran plastic tubes (17 mm diameter × 100 mm tall). In each tube 30 adults were fed 20 µl of sucrose 50 % (w/v) mixed with dsRNA using *Tp_vATPase A* and *Tp_act-2* as target genes. The concentration of the dsRNA was 1000 ng/µL. dsGFP served as negative control, and the experiment was performed in triplicate. Diet treatments were administered over a period of 4 days and renewed after 48 h. Following the initial 4-day exposure period, diet of the parasitoid wasp was replaced by 50 % sucrose without dsRNA. Parasitic wasps were evaluated phenotypically every day for 14 days. The *T. podisi* adult mortality was recorded and compared using Kaplan-Meier survival curves as described in 2.2.6.

For the evaluation of the life history traits, an additional batch of 30 mated females was treated with the sucrose solution containing ds*Tp_vATPase A*, ds*Tp_act-2* and ds-GFP. After the 4-day exposure period, 10-15 treated females were individualized into a Duran plastic tube with a droplet of honey to feed the parasitoid and the tube was sealed with plastic film according to the procedure of Silva et al. (2018a). Parasitoids were provided daily with approximately 20 eggs of *E. heros* (≤ 24 h old) as described in 3.2.3. The life-span parasitism, the life-time fecundity, emergence rate, the development time and sex ratio of the offspring was measured.

Non-target effects: Feeding assays using dsRNA targeting *E. heros* genes

Newly emerged *T. podisi* adults were exposed to *E. heros* dsRNA incorporated into a sucrose solution 50 % (w/v). Each dietary exposure treatment consisted of 30 adult wasps housed together in a Duran plastic tube and replicated three times. Wasps in the test group were fed 20 µl of sucrose 50 % (w/v) mixed with dsRNA using *Eh_vATPase A* and *Eh_act-2* at the concentration of 1000 ng/µL. As negative control, wasps were fed with the same quantity of dsGFP or RNA-free water. As a positive control for the dietary exposure, the neonicotinoid insecticide imidacloprid, was incorporated into the sucrose solution for a toxicity assay. Imidacloprid was used as a positive control to test whether parasitic wasps were feeding on the diet due to its high acute lethal activity toward *T.*

podisi (Turchen et al. 2016b). The final concentration of imidacloprid in the diet was 271.2 ng/ml, 1 % of the maximum recommended label rates as recommended by the manufacturers. Diet treatments was administered over a period of 4 days and renewed after 48 h. Following the initial 4-day period, the parasitoid wasps were fed only with 50 % sucrose without dsRNA and observed for an additional 10 days. The *T. podisi* female mortality was recorded. For the evaluation of the life history traits, and additional batch of 30 mated females were treated with the sucrose solution containing ds*Eh_vATPase A*, ds*Eh_act-2*, dsGFP and water. The evaluation of the life-history traits was performed as described above.

Feeding assays for expression analysis

For expression analysis, parasitic wasps were fed with sucrose solution 50 % (w/v) mixed with *Tp_vATPase A*, *Tp_act-2*, *Eh_vATPase A*, *Eh_act-2*, dsGFP at the concentration of 1000 ng/µL. Each dietary exposure treatment consisted of 30 adult wasps housed together in a Duran plastic tube and replicated three times. The diet was renewed after 48h. Samples of treated adults were collected at different time points depending of the purpose. Adults treated with dsRNA targeting *T. podisi* genes and dsGFP were collected at 24, 48, 72 and 96 h to test the susceptibility of *T. podisi* to dietary RNAi. To quantify the expression levels of predicted off-target genes, wasps treated with *E. heros* active dsRNAs and dsGFP were collected after 96 h. Adults treated with dsGFP or sucrose solution were collected after 12, 24, and 96 h to evaluate the immune activation of core RNAi enzymes by random dsRNAs. After the collection, total RNA was extracted from whole bodies. For each treatment, 3 biological samples of 25 pooled adult wasps were prepared.

dsRNA stability assay

To determine the stability of dsGFP and ds*Tp_vATPase A* incorporated into the sucrose solution in the presence *T. podisi*, after 24 h and 48 h of the feeding assay the sucrose solution was collected and subsequently analyzed on a 1.5 % agarose gel.

5.2.4 Off-target gene search

Potential off-target genes were predicted based on sequence complementarity regions using a Python script (Figure 5.1). First, the *Eh_vATPase A* and *Eh_act-2* dsRNA sequences were diced into 21 nt substrings of an siRNA cocktail. Second, an exhaustive off-target BLAST search in the transcriptome of *T. podisi* was performed for all individual siRNAs. Every siRNA sequence that has at least 15/21 nucleotide sequence identity with the predicted target gene was recognized as a hit. At this way, even if two consecutive siRNAs had similarities with the same region of the predicted off-target gene, both were considered different hits. All the hits with 17-21 nt length, with a maximum of two mis-

matches and/or one gap were selected for our further analysis. The total number of hits were counted individually for every off-target gene candidate and sorted in descending order.

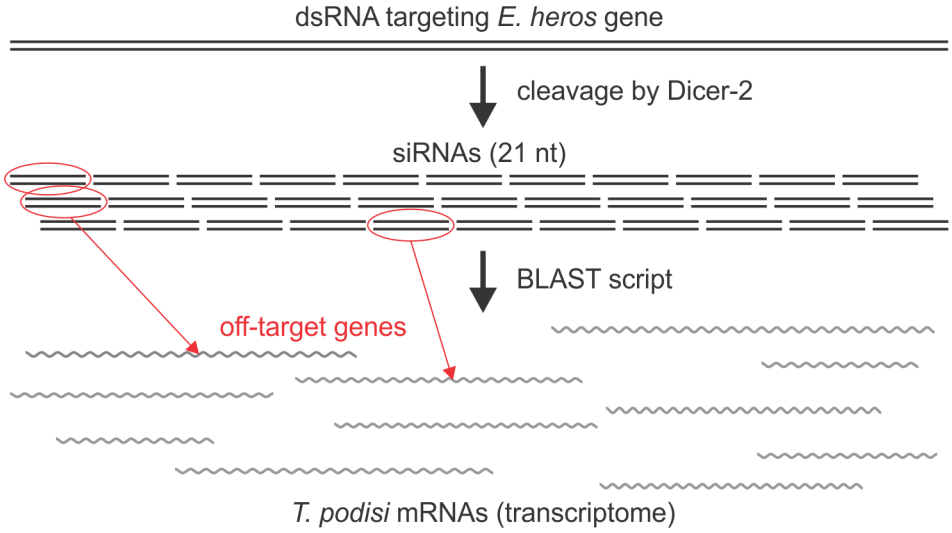


Figure 5.1: Schematic representation of the python script used for prediction of off-target genes. *E. heros* active dsRNA sequences were diced into 21 nt creating a pool of all possible siRNA. Each sequence within the siRNA pool was compared for sequence identity to all possible siRNA sequences in the transcriptome of *T. podisi*

5.2.5 Exposure routes *T. podisi* larvae: transfer dsRNA to eggs of *E. heros*

Egg soaking assays

In order to evaluate if the *E. heros* egg shell can absorb dsRNA, we evaluated the presence of dsGFP in the embryos after simulating the spraying of dsGFP in the field using Tween 40 (Sigma-Aldrich) as surfactant. To that end, 80 eggs of 120 h old were soaked in 50 µl solution of 300 ng/µl dsGFP and 300 ppm of Tween 40 for 4 h. The age of the egg was chosen based on the facility do dissect the embryo at that stage. An additional batch of 40 eggs were soaked in 300 ppm of Tween 40 under the same conditions and was used as negative control. After the 4 h, the eggs were washed 3 times with 100 µl nuclease free water, placed in a petri dish covered by filter paper and allowed to dry for 15 minutes at room temperature. The eggs were equally divided into groups of 20 eggs. For each treatment, the RNA was extracted from the eggs of one of those groups without dissection (with the shell). For the remaining groups of 20 eggs, the embryos were dissected and collected in an Eppendorf tube (without the shell) and stored in ice until the RNA extraction. For the dissection of the embryo, we cut the cuticle of the egg through the opercular rim, then the embryo was removed carefully with the forceps to avoid the contact with the dsRNA present in the egg shell.

In addition, the expression levels of *Eh_vATPase A* and *Eh_ATPsynβ* were measure

by qRT-PCR after soaking the eggs in dsRNA solution. 60 eggs of 24 h old were soaked in 50 µl solution of 300 ng/µl dsRNA and 300 ppm of Tween 40 for four hours, using d*Eh_vATPase A* and *Eh_ATPsynβ* as target genes and dsGFP as control. Total RNA was extracted from the eggs after 72h of the beginning of the soaking experiment. For each treatment, 3 biological samples of 20 eggs were prepared.

Injection assays

To evaluate parental transference of dsRNA to *E. heros* eggs, virgin sexually mature *E. heros* females (10-12 days old) were injected with dsRNA using a nanoinjector (FemtoJet, Eppendorf). Briefly, insects were anesthetized in ice and each female was injected with approximately 150 nl of a 1000 ng/µl dsRNA solution (150 ng dsRNA). The injection site was the ventral metathoracic region near the hind coxa. Thirty females were injected with dsRNA targeting GFP were used for detection of dsRNA in the egg. Injected females were reared individually with untreated males and allowed to lay eggs. Eggs laid were recorded every day for 14 days. 60 eggs were collected at days 1, 2, 5 and 10 after injection. As a negative control, eggs laid by untreated females were used. After 120 h of oviposition, eggs were divided in groups of 20 eggs and total RNA was extracted from the complete eggs of one group and the embryos without the shell of the other two groups.

For the expression analysis, *E. heros* females were injected with *Eh_vATPase A* as target gene and dsGFP as negative control. 30 females were injected per treatment. At least 90 eggs per treatment were collected at days 1, 3, 5 and 7 after injection. Total RNA was extracted from complete eggs after 48 h hours of oviposition. For each treatment, 3 biological samples of 30 eggs were prepared.

Detection of dsGFP

The cDNA was reverse transcribed from 500 ng of total RNA template and random hexamer primers (Invitrogen) using SuperScript IV First-Strand synthesis (Invitrogen) and a 10 min 75 °C denaturing step was added directly preceding the reverse transcription reaction in order to denature the secondary structure of the dsRNA allowing the reverse transcription of dsRNA. As a positive control, 150 ng of dsGFP was used as template for cDNA synthesis.

For the detection of dsGFP, a PCR was carried out using 1 µl of cDNA as a template, GFP specific primers (2.5 pmols of forward CACATGAAGCAGCACGACTT and reverse primer TGCTCAGGTAGTGGTTGTCG, amplicon size 379 nt) and Taq DNA Polymerase (Invitrogen). The amplification conditions were 2 min at 94°C followed by 5 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. Subsequently, 30 additional cycles were performed with a constant annealing temperature of 65°C. The reaction was completed with a final elongation step at 72°C

for 3 min. As an additional positive control, 0.64 ng of PGEM-GFP plasmid was used as a template for amplification. Amplified fragments were analyzed by gel electrophoresis.

5.2.6 *T. podisi* larvae bioassays

While feeding assays focus on the impact of dietary RNAi in the adult life, this experiment was intended to investigate the impact of dsRNA on the development of *T. podisi* using eggs laid by females injected with dsRNA. dsGFP was used as negative control, ds*Tp_vATPase A* was used to evaluate the effects of specific dsRNA in the development, while ds*Eh_vATPase A* was used to evaluated non-target effects. 30 females were injected per treatment. Egg masses were collected at days 1, 2 and 3 after injection. In total, 480 eggs were collected per treatment. After the collection, *E. heros* eggs (≤ 24 h old) were separated in groups of 20 eggs and glued with non-toxic glue onto cardboard cards (1 cm × 4 cm). Each group of 20 eggs were offered to a single *T. podisi* mated female (up to 48h) and placed inside a Duran plastic tube. This procedure was repeated with 24 females per treatment, for a total of 480 eggs. Parasitism was allowed for 24 h. After this period, the eggs were transferred to a new Duran plastic tube where they remained until the adult emergency. The parameters assessed were: developmental time, emergence rate, and sex ratio.

5.2.7 Real-time quantitative PCR

qRT-PCR was performed as described in 4.2.6 to analyze the expression levels of (i) target genes to test the functionality of RNAi machinery in *T. podisi*; (ii) off-target genes to test non-target effects of *E. heros* active dsRNAs in *T. podisi*; (iii) core RNAi effectors in *T. podisi*, such as Dcr-2 and Ago2 to test immune activation by random dsRNAs; and (iv) target genes to evaluate the transfer of dsRNA to *E. heros* eggs. qRT-PCR specific primers were designed using Primer3 Plus free-software (Table 5.3).

For the feeding experiments in *T. podisi*, the stability of four candidate reference genes (*Tp_RPL3*, *Tp_EF1A*, *Tp_RPL27* and *Tp_RPII3*) was evaluated after feeding of dsRNA using geNorm tool integrated in the qBase+ software. Genes with the lowest stability values (M) were selected, and coefficient of variation (CV) was used to determine the optimum number of reference genes (Hellemans et al., 2008; Vandesompele, 2002).The reference genes *Tp_EF1A* and *Tp_RPL27* exhibit the most stable expression following the dietary treatment with dsRNA (Appendix 5.4), making these genes appropriate for qRT-PCR data normalization for gene silencing analysis. For the experiments in *E. heros* eggs, the reference genes *Eh_ARL2*, *Eh_ARP8* and *Eh_UB4A* were used due to their stable expression after dsRNA feeding and injection (Chapter 4). Differences in the expression levels were calculated by an unpaired t-test ($P < 0.05$) and performed in qBase+ software.

Table 5.3: Primers used for qRT-PCR assay and primer efficacy results for *T. podisi* adults or *E. heros* eggs.

	Forward primer	Reverse primer	Amplicon size	Efficiency (%)
Target genes feeding assays *T. podisi*				
Tp_vATPase A	GCACCCGCAGGAAATTATAC	ACGGACTGGCCAAACTTGTA	99	89.4
Tp_act-2	CGTTACCCGATGGTAGCACT	TGGATGCCTTCGTATTCCTC	103	93.0
Predicted off-target genes ds*Eh_vATPase A* in *T. podisi*				
Tp_vATPase A-like	CAACATGCCTGTAGCTGCTC	AGTGGAATCGGCCATCATAG	106	98.0
Tp_unknown1	GGCTTGTCGTACGATTCCTC	TCGATCCGACCTCCATAGTT	100	97.0
Tp_GDF8-like	GCGAGGTCGTTTCAAGTTTC	TCCGTCTCAAGTCTCAACCA	100	95.3
Tp_HARBI1-like	TTGCGCTGATTGTTCGTATC	TGCGTCGTTGATACATTCGT	103	105.5
Tp_ G5I_03042	AGCGCTCGAAATTTCCTGTA	CAGACATTCTTGCAGGCGTA	94	95.9
Tp_ GPN1-like	AAGCGTTTCGGTTATGATGG	GACAAGGCTGGAAAGGAACA	102	93.7
Tp_ ZNF502-like	GTTGGACTGGTTCCTGGTGT	CTCCACCCCTCTCTTCACAG	100	100.6
Tp_SPTB	GCAGTCGCAGTAAATCACCA	CATCAAACGCATCGTCACTC	99	103.0
Predicted off-target genes ds *Eh_act-2* in *T. podisi*				
Tp_act-3-like	GCGATATTGCGTTTGGATCT	GTTCCGCCGTAGTTGTGAAC	97	95.2
Tp_ SPTBN4-like	AATTTTGCAGGACCTGAACG	GTGCGATTGTAACCATGCTG	105	103.7
Tp¬_ BAZ2B-like	TATGCAAACGTTCCACAGGA	GTTCTCGCGGTTCTCAATGT	99	100.5
Tp¬_ SLC38A10-like	CCAAAGAATCCGACACACAA	CGACACCATACCAAATGCAA	99	105.0
Tp_LYRM7-like	ACTGACAGCGACAACGAGTG	ATCGTGGGCAAAAAGTGAAC	97	97.4
Tp_unknown2	TTTTGATTCGTCCCAGGTGT	GGAAGAGACCAACCAACGAA	105	97.2
Core RNAi genes *T. podisi*				
Tp_Dcr-2	TGACGAACTCCCTCTGCTCT	CAGTGAGACACGGTTTGCAT	102	112.0
Tp_Ago-2	CTGACCCTGGCGTAAAAGAA	GGCTGAAGTCGAATGGTGAT	100	97.6
Reference genes *T. podisi*				
Tp_RPL3	TAAAGGCCTTCCCAAAGGAT	CTGTCAGCTTCACGCACAAT	100	100.2
Tp_EF1A	GTCGTGGCATCTGGAGTTTT	TGTCCAATGACGACGATGTT	100	98.0
Tp_RPL27	CGAGTTGGCATAAGGTGGTT	GATACCCACGAGCAGTCCAC	111	93.3
Tp_RPII3	AATTGGGCATCACCAGATGT	AAGCACAAACGGACCATACC	93	99.7
Target genes exposure routes eggs *E. heros*				
Eh_vATPase A	TGCCTGCTGACAGTGGTTAC	CCCTCCCTCTCTGGATTACC	103	103.1
Eh_ATPsyn β	CCAGCATCTTGGTGAAAACA	TGATGGGAAATCCTGTGTCA	98	90.6
Reference genes *E. heros*				
Eh_ARL2	GGTTGGCATTCTTCAGTTGG	GCGCAATCGTAACTGGTACA	101	87.2
Eh_ARP8	TGCCATTCTTGCTTGTCTTG	GGCCCTCTTTCTCGTATCAA	96	95.0
Eh_UB4A	AGCTTCATCGAGCAGGAAAA	CTCGTGAGGCGGAAACTAAC	100	91.7

5.3 Results

5.3.1 On-target effects on the NTO

Core RNAi genes in *T. podisi*

As a first step to test the susceptibility of *T. podisi* adults to dietary RNAi, the *T. podisi* transcriptome was screened for the presence of the most important genes related to the RNAi machinery (Table 5.4). All sequences representing the core proteins of the three RNAi pathways found in insects siRNA, miRNA and piRNA were identified, except R2D2, the cofactor of Dcr-2 in the siRNA pathway. Phylogenetic analysis support the identity of the Dicer enzymes and Argonaute proteins found in the *T. podisi* transcriptome (Figure 5.2).

Feeding assays using dsRNA targeting *T. podisi* genes

To test the susceptibility to dietary RNAi, *T. podisi* adults were fed with sucrose solution containing dsRNA targeting *Tp_vATPase A* and *Tp_act-2* at the concentration of 1000 ng/µl. The dsRNA treatments significantly affect the survival of *T. podisi*, feeding of ds*Tp_vATPase A* and ds*Tp_act-2* resulted in 76.4 ± 9.9 % and 76.7 ± 8.8 % mortality respectively (Figure 5.3*A*). According to the survival analysis, the observed mortality for ds*Tp_vATPase A* and ds*Tp_act-2* was significantly different from the dsGFP control (Holm-Sidak's statistics χ^2 = 95.12, *df*=2, *P* <0.001). Relative to the negative control (dsGFP), the life-span parasitism was significantly reduced by 5.5 days (corresponding to 37.9 %) for ds*Tp_vATPase A* and by 5.0 days (corresponding to 34.5 %) for ds*Tp_act-2* (Figure 5.3*B*; *H* = 17.21, *df*=2, *P* < 0.0001). Consequently, life-time fecundity or parasitism was reduced by 57.1 % for ds*Tp_vATPase A* and by 49.0 % (Figure 5.3*C*; *H* = 21.22, *df*=2, *P* < 0.0001). The emergence rate (Figure 5.3*D*; $F_{2,29}$ = 0.94, *P* = 0.402) and the developmental time (Figure 5.3*E*; F2,44 = 1.50, *P* = 0.236) of the offspring of females treated with *T. podisi* specific dsRNAs were unaffected. However, the offspring sex ratio (% females) was significantly higher in the females treated with ds*Tp_act-2* (Figure 5.3*F*; *H* = 9.818, *df*=2, *P* = 0.007).

Table 5.4: RNAi-related genes identified in the transcriptome of *T. podisi* and their Accession IDs.

Protein		Organism	Accession	E value	Identity (%)	NCBI accession *T. podisi*
miRNA core genes						
Dicer-1	RNase III, conversion of pre-miRNA to miRNA	*D. melanogaster*	AAF56056	2.00E-152	65.71	GBEU01005709.1
Ago1	Argonaute, catalytic subunit of RISC	*D. melanogaster*	NP_725341.1	0	91.85	GBEU01006610.1
Drosha	RNase III, cleavage of pri-miRNA to pre-miRNA	*D. melanogaster*	AAF59169	0	70.04	GBEU01024687.1
Pasha	dsRNA-binding, co-factor of Drosha	*D. melanogaster*	AGB96528	0	54.74	GBEU01025694.1
Loquacious	dsRNA-binding, co-factor of Dicer-1	*D. melanogaster*	AAF53296	1.00E-33	50.6	GBEU01005007.1
siRNA core genes						
Dicer-2	RNaseIII, processing of long dsRNA into siRNAs	*D. melanogaster*	AAF57830	4.00E-64	33.56	GBEU01012739.1
Ago2	Argonaute, catalytic subunit of RISC	*D. melanogaster*	NP_648775	3.00E-126	35.19	GBEU01014776.1
R2D2	dsRNA-binding, co-factor of Dicer-2	*D. melanogaster*	Not found			
piRNA core genes						
Piwi	Argonaute (PIWI subfamily), catalytic subunit of RISC	*D. melanogaster*	AAF53043	1.00E-165	37.03	GBEU01040097.1
Aubergine	Argonaute (PIWI subfamily), catalytic subunit of RISC	*D. melanogaster*	AGA18945	0	43.55	GBEU01018389.1
Ago3	Argonaute (PIWI subfamily), catalytic subunit of RISC	*D. melanogaster*	ABO27430	0	38.31	GBEU01037523.1
DsRNA uptake						
HPS4	Hermansky-Pudlak Syndrome 4 protein regulation of association of late endosomes with GW bodies (dsRNA soaking)	*D. melanogaster*	ACJ13135	7.00E-63	28.01	GBEU01018923.1
FBX011	F-box motif, Beta-helix motif regulation of association of late endosomes with GW bodies (dsRNA soaking)	*D. melanogaster*	AAQ23623	0	83.58	GBEU01019864.1
SR-C	Scavenger receptor type C, uptake of dsRNA in Drosophila S2 cells	*D. melanogaster*	AAW79537	4.00E-21	26.15	GBEU01037287.1
Sid-1	Homolog of putative dsRNA transporter in C. elegans	*Bo. mori*	BAF95807	1.00E-117	53.94	GBEU01026812.1
Auxiliary factors (RISC)						
Translin	Complex of RNA-binding protein & endonuclease, stimulates miRNA and siRNA pathway	*D. melanogaster*	AAF58784	2.00E-82	54.71	GBEU01026724.1
Trax-B		*D. melanogaster*	ABE01846	1.00E-52	40.34	GBEU01014864.1
Hen-1	Mediates 2′-O-methylation of piRNAs and siRNAs	*D. melanogaster*	AAF58575	2.00E-34	35.14	GBEU01021387.1
Gawky	Component of RNA-processing GW-bodies, interacts with Argonaute proteins	*D. melanogaster*	AAX52511	9.00E-69	39.05	GBEU01007113.1
FXMR	Fragile-X-related protein, component of RISC complex in S2 cells, RNA binding RGG and KH motifs	*D. melanogaster*	AFH06335	9.00E-160	59.6	GBEU01012609.1

Protein		Organism	Accession	E value	Identity (%)	NCBI accession *T. podisi*
Auxiliary factors (RISC)						
Belle	DEAD-box RNA helicase, interacts with RISC and small RNAs	*D. melanogaster*	AGB95761	5.00E-124	47.56	GBEU01023973.1
p68 RNA helicase	DEAD-box RNA helicase, required for RNAi	*D. melanogaster*	AAG22212	0	67.05	GBEU01015593.1
Armitage	ATP-dependent helicase distinct from the DEA(H/D) box proteins required for RISC maturation	*D. melanogaster*	AFH04274	0	32.39	GBEU01013339.1
Staufen	Tubulin-binding & dsRNA-binding domain transport of mRNA	*D. melanogaster*	AAF57753	1.00E-51	39.22	GBEU01003494.1
Maelstrom	piRNA pathway domain weak similarity to HMG box, Mael domain with weak homology with the DnaQ-H 3′-to-5′ exonuclease	*D. melanogaster*	AAF51851	5.00E-20	25.32	GBEU01009551.1
PRMT5	Protein methyltransferase; methylates Piwi proteins at conserved Arg-residues	*D. melanogaster*	AAM68510	1.00E-34	47.85	GBEU01029180.1
Clp-1	RNA kinase, phosphorylation of siRNAs	*D. melanogaster*	Q7K284	0	61.87	GBEU01015491.1
Antiviral RNAi						
Ars2	Regulator of siRNA- and miRNA-mediated silencing, suppressor of RNA virus infection	*D. melanogaster*	AAM68345	4.00E-100	51.38	GBEU01018148.1
Egghead	Seven transmembrane- domain glycosyltransferase, uptake of dsRNA, innate immunity against RNA virus (Drosophila)	*D. melanogaster*	CAB72293	7.00E-180	73.49	GBEU01047202.1
CG4572	Unknown, proteolysis/peptidolysis motifs, uptake of dsRNA, innate immunity against RNA virus (Drosophila)	*D. melanogaster*	ACU33957	2.00E-51	47.54	GBEU01001911.1
Nucleases						
3′hExo/Eri-1/Snipper	Histone mRNA metabolism, siRNA degradation, apoptosis	*D. melanogaster*	NP_726149.1	1.00E-25	30.96	GBEU01018083.1
MUT-7/Nibbler	Processing of 3′ends of miRNAs (Drosophila)	*D. melanogaster*	AET07634	6.00E-28	25.44	GBEU01036737.1
Rex1p-Rex4p	Single-strand-specific small RNA degrading nucleases; degradation of mature miRNAs (Arabidopsis)	*D. melanogaster*	NP_648689.1	9.00E-58	60.62	GBEU01012108.1
Sdn1-like		*D. melanogaster*	AAZ41783	8.00E-134	38.74	GBEU01007060.1
		D. melanogaster	AAN71264	1.00E-99	41.7	GBEU01013092.1

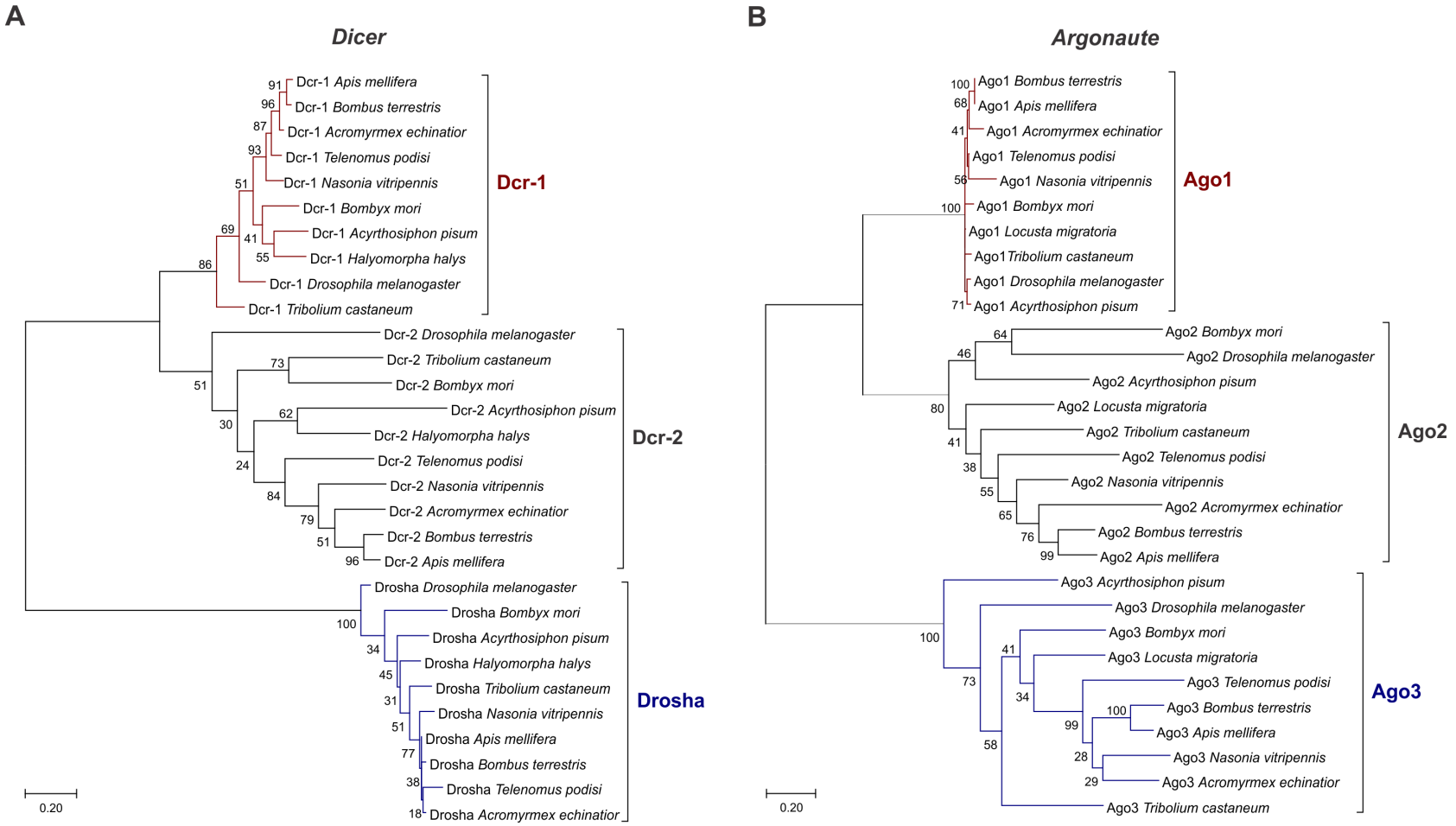


Figure 5.2: Phylogenetic trees of the Dicer enzymes (**A**), and Argonaute proteins (**B**). The phylogenetic tree was constructed using the c method with MEGA, version 7, software. The numbers associated with the branches refer to bootstrap values (confidence limits) resulting from 1000 replicate resampling.

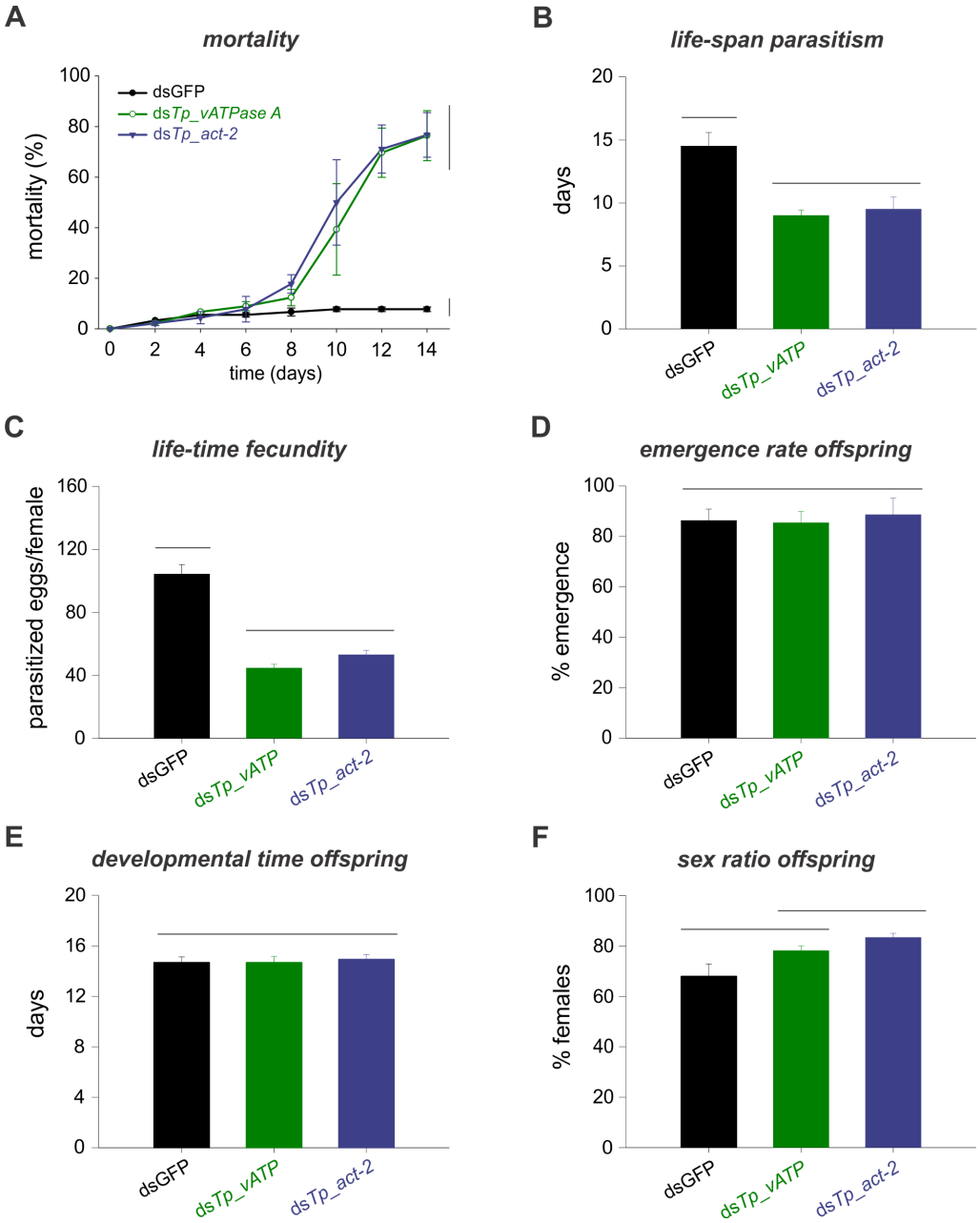


Figure 5.3: Effects of feeding *T. podisi*-specific dsRNA on the life history traits of *T. podisi* adults. Cumulative mortality (***A***), life-span parasitism (***B***), life-time fecundity (***C***), emergence rate of the offspring (***D***), developmental time of the offspring (***E***) and sex ratio of the offspring (***F***). Feeding with dsRNA targeting GFP was used as a negative control. (***A***) Values represent the mean mortality percentages ± SEM from three independent replicates, each consisting of 29-31 parasitic wasps. The curves encompassed by the vertical line at the right side of the plot are not significantly different according to Holm-Sidak's test (P >0.05). (***B-F)*** Bars represent the mean (± standard error) observed data (n=10-15) and the ones grouped with the same horizontal line are not statistically different at the 5 % significance level according to a Tukey's HSD or post hoc Kruskal–Wallis test.

Stability dsRNA in the diet

dsGFP and ds*Tp_vATPase A* remained stable in the sucrose solution after being fed by T. podisi adults for 24 h and 48 h (Figure 5.4). This indicated that the environment

and the salivary enzymes did not influence the stability of dsRNA.

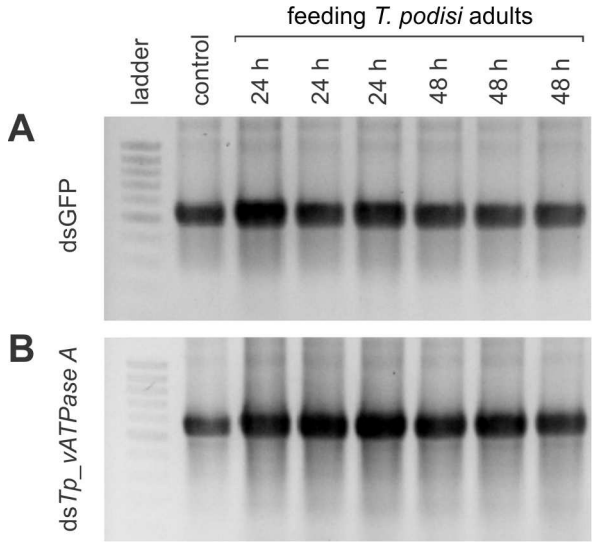


Figure 5.4: Stability of dsRNA incorporated on *T. podisi* adult diet at the concentration of 1000 ng/µl. Positive control consisted of dsRNA diluted in water at the same concentration. (***A***) stability of dsGFP, and (**B**) stability of ds*Tp_vATPase A*.

Gene expression of target *T. podisi* genes

To confirm the knockdown of the genes *Tp_vATPase A* and *Tp_act-2* after dsRNA feeding, we performed a qRT-PCR analysis using cDNA of treated wasps. For *Tp_vATPase A*, dsRNA feeding reduced transcript levels by 36.6 % (*P* = 0.007) after 72 h and 40.1 % (*P* = 0.006) after 96 h (Figure 5.5*A*). *Tp_act-2* transcript levels were lowered by 59.1 % after 72 h of continuous feeding (Figure 5.5*B*; *P* = 0.005).

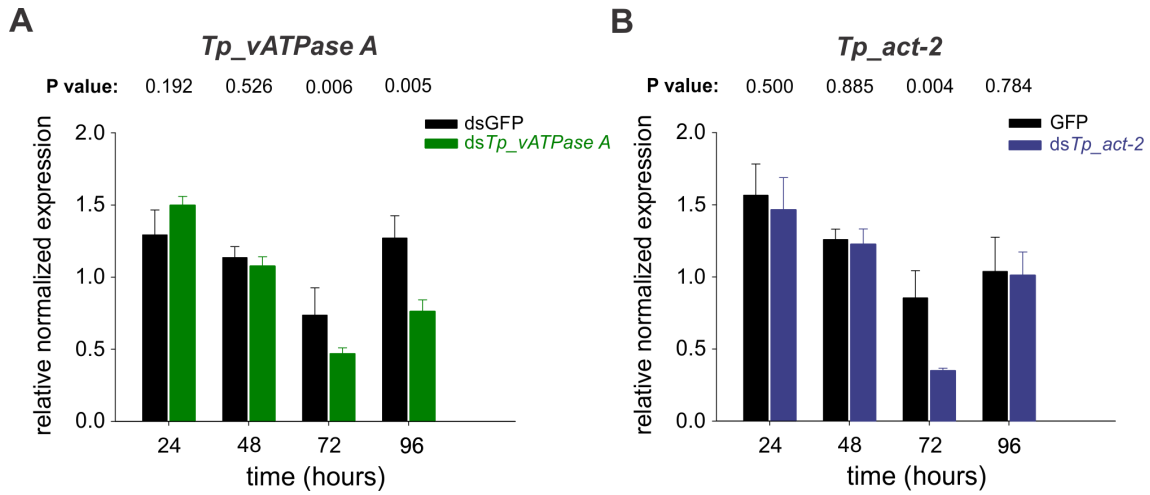


Figure 5.5: Expression levels of *Tp_vATPase A* and *Tp_act-2* in *T. podisi* adults fed with target gene specific dsRNA. (A) *Tp_vATPase A*, and (B) *Tp_act-2*. Feeding with dsRNA targeting GFP was used as a negative control. As internal controls, *Tp_EF1A* and *Tp_RPL27* were used. Values are based on three biological samples and expressed as mean ± standard error. Each sample contains 25 pooled adult wasps. The p-values were calculated by unpaired t-test in qBase+ software.

5.3.2 Non-target effects

Feeding assays using dsRNA targeting *E. heros* genes

Lethal and sublethal non-target effects were evaluated under the worst-case scenario, *T. podisi* adults were fed with *E. heros* active dsRNAs at the concentration of 1000 ng/µl. Mean survival was nearly identical between the assay control and the dsRNA treatments, with 93.1 ± 2.81 % survival for ds*Eh_vATPase A*, 91.1 ± 2.4 % for ds*Eh_act-2*, 92.2 ± 0.9 % for dsGFP and 96.6 ± 0.8 % the sucrose solution (the assay control) (Figure 5.6*A*; $P > 0.879$). Conversely, there was no survival in the positive controls with the pesticide imidacloprid by day 4 and without food by day 5, confirming the effectiveness of the dietary feeding exposure (Holm-Sidak's statistics $\chi^2 = 748.125$, $df=5$, $P <0.001$). Regarding the reproductive parameters, the life-span of parasitism (Figure 5.6*B*; $H = 3.80$, $df=3$, $P = 0.284$) and the life-time fecundity (Figure 5.6*C*; $H = 5.76$, $df=3$, $P = 0.124$) were similar across the dsRNA treatments and the assay control. Moreover, the emergence rate (Figure 5.6*D*; $F_{3,39} = 0.54$, $P = 0.659$), developmental time (Figure 5.6*E*; $F_{3,59} = 0.52$, $P = 0.669$) and the sex ratio (Figure 5.6*F*; $H = 1.93$, $df=3$, $P = 0.588$) of the offspring were not significantly different across the treatments as well.

Predicted off-target genes

We determined whether any of the possible siRNAs present in the *E. heros* active ds*Eh_vATPase A* and ds*Tp_act-2* might be complementary to the sequences found in the transcriptome of *T. podisi*. The Phyton script algorithm revealed that there is no single hit of 21 nt continuous matches in the *T. podisi* transcripts. However, for both dsRNAs evaluated, there are multiple off-target transcripts that share hits of 17-21 nt with a maximum of two mismatches and/or one gap. For ds*Eh_vATPase A*, 251 transcripts have at least one hit of 17-21 nt with one of the possible siRNAs, and the longest continuous hit contained 20 nt matches (Appendix 5.4). While for ds*Tp_act-2*, 188 transcripts were found, and the longest continuous hit contained 17 nt matches (Appendix 5.5). Surprisingly, the alignment comparison of the available orthologous target gene fragments between *E. heros*s and *T. podisi* showed only a 33.5 % identity for *vATPase A* and 52.0 % for *act-2*. In addition, there were no 17-21 nt contiguous sequence matches in the target genes between the two species.

Gene expression potential off-target genes

To further evaluate the effects of *E. heros* active dsRNAs on *T. podisi* adults, we quantified the mRNA levels of some of the predicted off-target genes using the qRT-PCR assay. We selected the predicted off-target genes that met one the following criteria: (i) transcripts with more than seven hits, (ii) transcripts with seven hits with at least one hit of 19 nt with one gap or one mismatch, (iii) transcripts with six hits with at least four hits with 19 nt with one gap or one mismatch, or (iv) transcripts with six hits with at

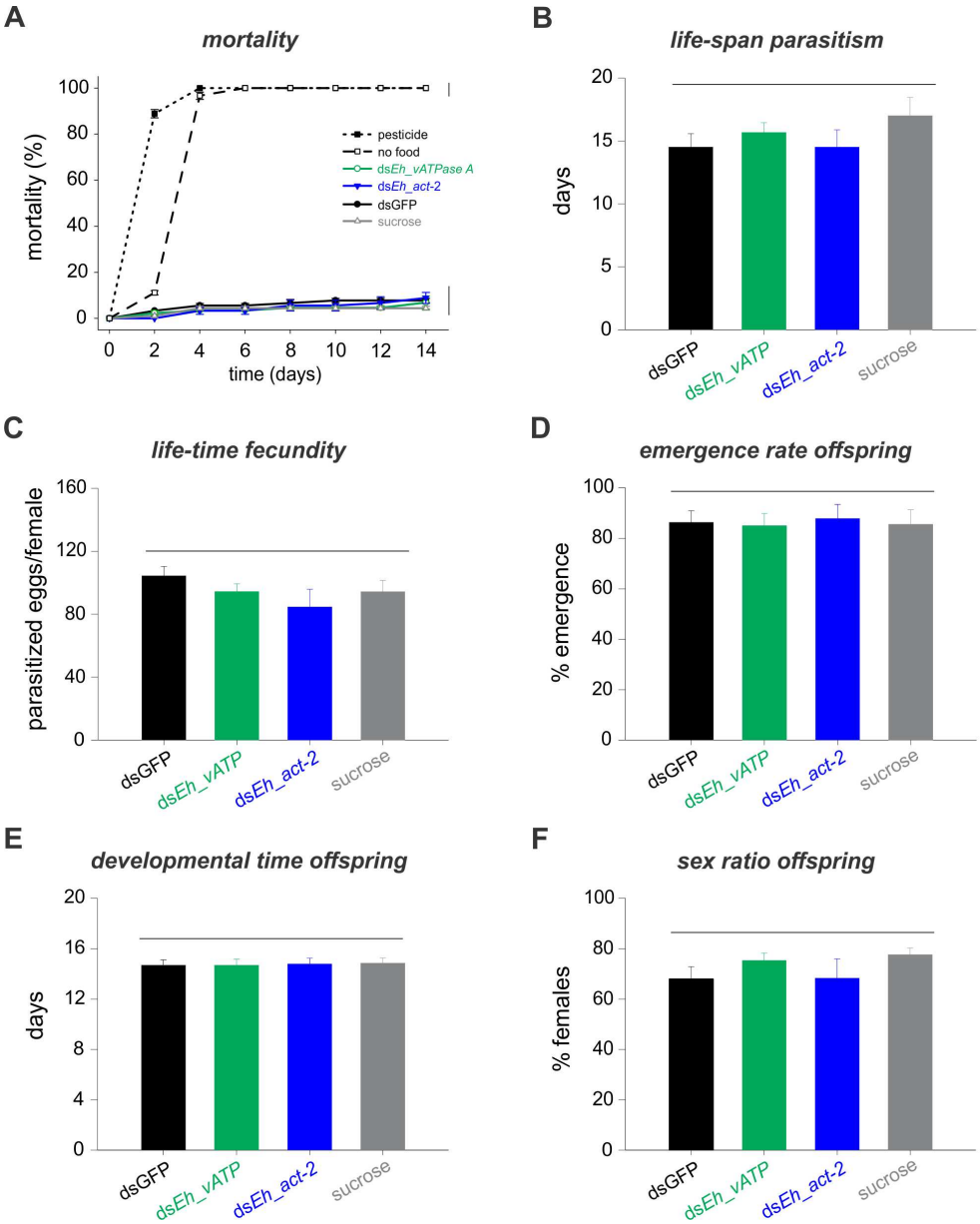


Figure 5.6: Effects of feeding *E. heros*-specific dsRNA on the life history traits of *T. podisi* adults. Cumulative mortality (***A***), life-span parasitism (***B***), life-time fecundity (***C***), emergence rate of the offspring (***D***), developmental time of the offspring (***E***) and sex ratio of the offspring (***F***). Feeding with the sucrose solution and dsGFP were used as negative controls. As positive controls, imidacloprid solution at the concentration of 271.2 ng/ml and no food were used. (***A***) Values represent the mean mortality percentages ± SEM from three independent replicates, each consisting of 29-31 parasitic wasps. The curves encompassed by the vertical line at the right side of the plot are not significantly different according to Holm-Sidak's test (P >0.05). (***B-F)*** Bars represent the mean (± standard error) observed data (n=10-15) and the ones grouped with the same horizontal line are not statistically different at the 5 % significance level according to a Tukey's HSD or post hoc Kruskal–Wallis test.

least four hits with 18 nt without gaps or mismatches.For practical reasons, the criteria of selection of the off-target genes was based on the probability to detect changes in the gene expression with a limited number of genes. Thus, we assume that genes with higher number of hits and/ or longer continuous matches have higher risks of potential

Table 5.5: Predicted off-target genes of *E. heros* active ds*Eh_vATPase A* in the transcriptome of *T. podisi* selected for quantification of gene expression

***T. podisi* NCBI**	**Number of hits**											**BLASTx top hit**	**Abbreviation**
Gap/mismatch	**0**				**1**					**>1**	**Total**		
Length of the hit	20	19	18	17	21	20	19	18	17	17-21			
GBEU01005918.1	2	2	1	3	9	9	6	3	2		37	PREDICTED: V-type proton ATPase catalytic subunit A-like [*Megachile rotundata*]	*Tp_vATPase A-like*
GBEU01005925.1	2	2	1	4	9	8	5	1	2		34		
GBEU01005924.1					3	2	2	1	9		17		
GBEU01005916.1					3	2	2	1	7		15		
GBEU01041559.1								8	2		10		*Tp_unknown1*
GBEU01021593.1						2	2	2	2		8	PREDICTED: growth/differentiation factor 8-like isoform 2 [*Bombus terrestris*]	*Tp_GDF8-like*
GBEU01021596.1						2	2	2	2		8		
GBEU01021597.1						2	2	2	2		8		
GBEU01021598.1						2	2	2	2		8		
GBEU01012239.1							3	2	2		7	PREDICTED: putative nuclease HARBI1-like [*Nasonia vitripennis*]	*Tp_HARBI1-like*
GBEU01012242.1							3	2	2		7		
GBEU01012244.1							3	2	2		7	hypothetical protein G5I_03042 [*Acromyrmex echinatior*]	*Tp_ G5I_03042*
GBEU01012245.1							3	2	2		7		
GBEU01012942.1			4	2							6	PREDICTED: GPN-loop GTPase 1-like [*Apis florea*]	*Tp_ GPN1-like*
GBEU01012943.1			4	2							6		
GBEU01012945.1			4	2							6		
GBEU01012947.1			4	2							6		
GBEU01012948.1			4	2							6		
GBEU01006921.1											6	PREDICTED: zinc finger protein 502-like [*Apis florea*]	*Tp_ ZNF502-like*
GBEU01006920.1							4	2			6		
GBEU01015354.1							4	2			6	Spectrin beta chain [*Camponotus floridanus*]	*Tp_SPTB*
GBEU01015362.1							4	1	1		6		
GBEU01015363.1							4	1	1		6		

Table 5.6: Predicted off-target genes of *E. heros* active dsds*Eh_act-2* in the transcriptome of *T. podisi* selected for quantification of gene expression

***T. podisi* NCBI**	**Number of hits**									**BLASTx top hit**	**Abbreviation**
Gap/mismatch	**0**	**1**					**<1**		**Total**		
Length of the hit	17	21	20	19	18	17	21	20			
GBEU01002114.1	3	1	4	3	1	3			15	PREDICTED: actin-3-like isoform 1 [*Nasonia vitripennis*]	*Tp_act-3-like*
GBEU01002108.1	3	1	4	3	1	2			14		
GBEU01037618.1						7	1	1	9	PREDICTED: spectrin beta chain, brain 4-like [*Megachile rotundata*]	*Tp_ SPTBN4-like*
GBEU01015664.1				3	2	2			7	PREDICTED: bromodomain adjacent to zinc finger domain protein 2B-like [*Apis florea*]	*Tp_ BAZ2B-like*
GBEU01015665.1				3	2	2			7		
GBEU01015666.1				3	2	2			7		
GBEU01024258.1				3	2	2			7	PREDICTED: putative sodium-coupled neutral amino acid transporter 10-like [*Apis mellifera*]	*Tp_ SLC38A10-like*
GBEU01024259.1				3	2	2			7		
GBEU01024260.1				3	2	2			7		
GBEU01037250.1		2	2	1	1				6	PREDICTED: LYR motif-containing protein 7-like [*Nasonia vitripennis*]	*Tp_LYRM7-like*
GBEU01037252.1		2	2	1	1				6		
GBEU01037253.1		2	2	1	1				6		
GBEU01003695.1				4	2				6		*Tp_unknown2*
GBEU01005404.1				4	2				6		

gene suppression. In this way, we evaluated the expression of eight predicted off-target genes for ds*Eh_vATPase A* (Table 5.5), and six for ds*Eh_act-2* (Table 5.6). In addition, we also quantified the expression levels of the target genes in *T. podisi* (i.e. *Tp_vATPase A* and *Tp_act-2*). Feeding ds*Eh_vATPase A* to *T. podisi* adults did not significantly affect the transcript levels of the selected genes when compared to adults fed dsGFP after four days (Figure 5.7*A*). Similarly, the expression levels of most of selected

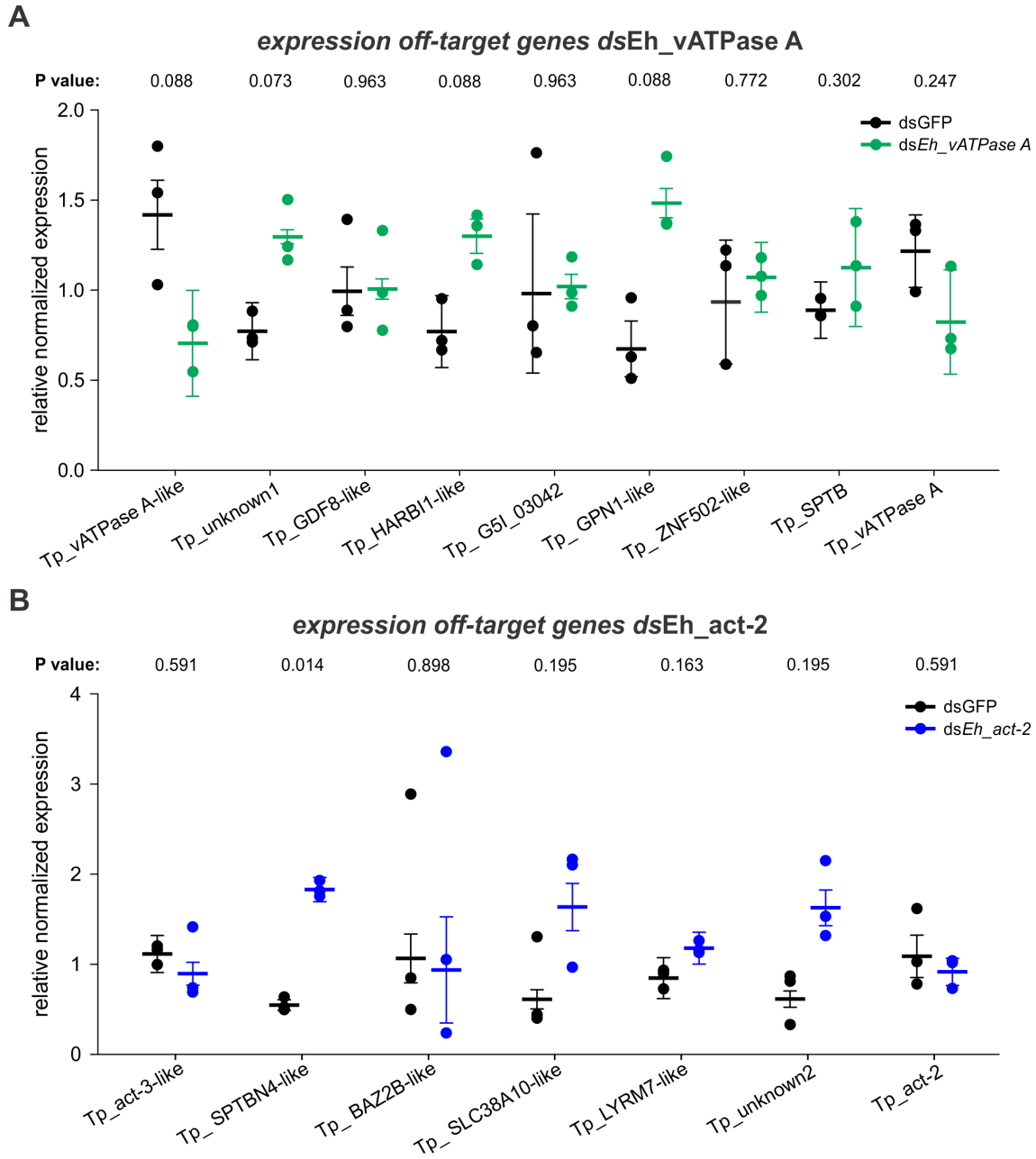


Figure 5.7: Expression levels of selected off-target and target genes in *T. podisi* adults fed with *E. heros* active dsRNA. (***A***) ds*Eh_vATPase A*, and (***B***) ds*Eh_act-2*. Feeding with dsRNA targeting GFP was used as a negative control. As internal controls, *Tp_EF1A* and *Tp_RPL27* were used. The values for the three biological replicates are shown as circles and the mean is depicted with a dash with its correspondent standard error. Each biological replicate contains 25 pooled adult wasps. The p-values were calculated by unpaired t-test in qBase+ software.

genes were not significantly different between the groups fed with ds*Eh_act-2* and dsGPF (Figure 5.7*B*). Only the transcript levels of *Tp_SPTBN4-like* significantly increased by 151.2 % (P = 0.014) after feeding of ds*Eh_act-2*.

Immune stimulation

In order to investigate the immune stimulation of core RNAi genes, the expression of *Tp_Dcr-2* and *Tp_Ago2* was determined after feeding of non-specific dsRNA. After 12 h of dsGFP feeding, *Tp_Dcr-2* transcript levels were significantly increased by 30.2 % (P = 0.037) when compared to adults fed with sucrose solution (Figure 5.8*A*). *Tp_Ago* transcript levels were significantly up-regulated by 48.1 % (P = 0.015) after 24 h of dsGFP feeding (Figure 5.8*B*).

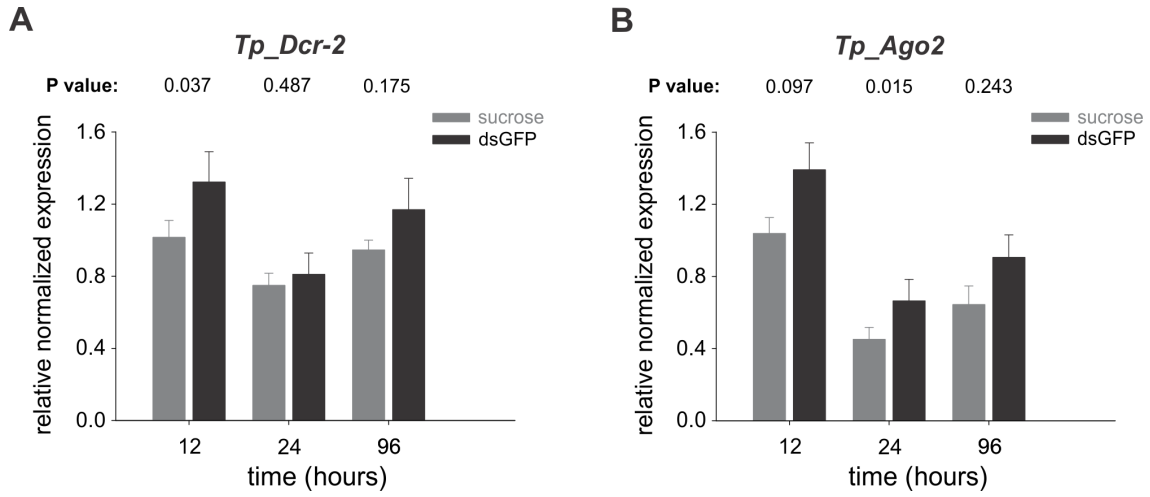


Figure 5.8: Immune stimulation of *T. podisi* adults fed with dsGFP. (***A***) *Tp_Dcr-2*, and (***B***) *Tp_Ago2*. Feeding with sucrose solution was used as a negative control. As internal controls, *Tp_EF1A* and *Tp_RPL27* were used. Values are based on three biological samples and expressed as mean ± standard error. Each sample contains 25 pooled adult wasps. The p-values were calculated by unpaired t-test in qBase+ software.

5.3.3 Effects of dsRNA on *T. podisi* larvae

Exposure routes to *T. podisi* larvae

The route of exposure to *T. podisi* larvae is the development inside stink bug eggs containing dsRNA. There are two hypothetical ways of transference of dsRNA to those eggs: (i) sprayed dsRNA penetrates the egg shell and could reach the embryo; or alternatively (ii) stink bug adults could take up the dsRNA, and if the dsRNA reaches the germline cells, the embryos/eggs of the next generation could be exposed. To test whether the egg shell can absorb dsRNA, *E. heros* eggs were soaked in dsGFP solution at the concentration of 300 ng/µl for 4 h and afterwards the dsGFP was detected by PCR amplification. dsGFP was detected in the complete eggs, and to a lesser extent in the dissected embryos that were not in direct contact with dsGFP solution (Figure 5.9*A*).

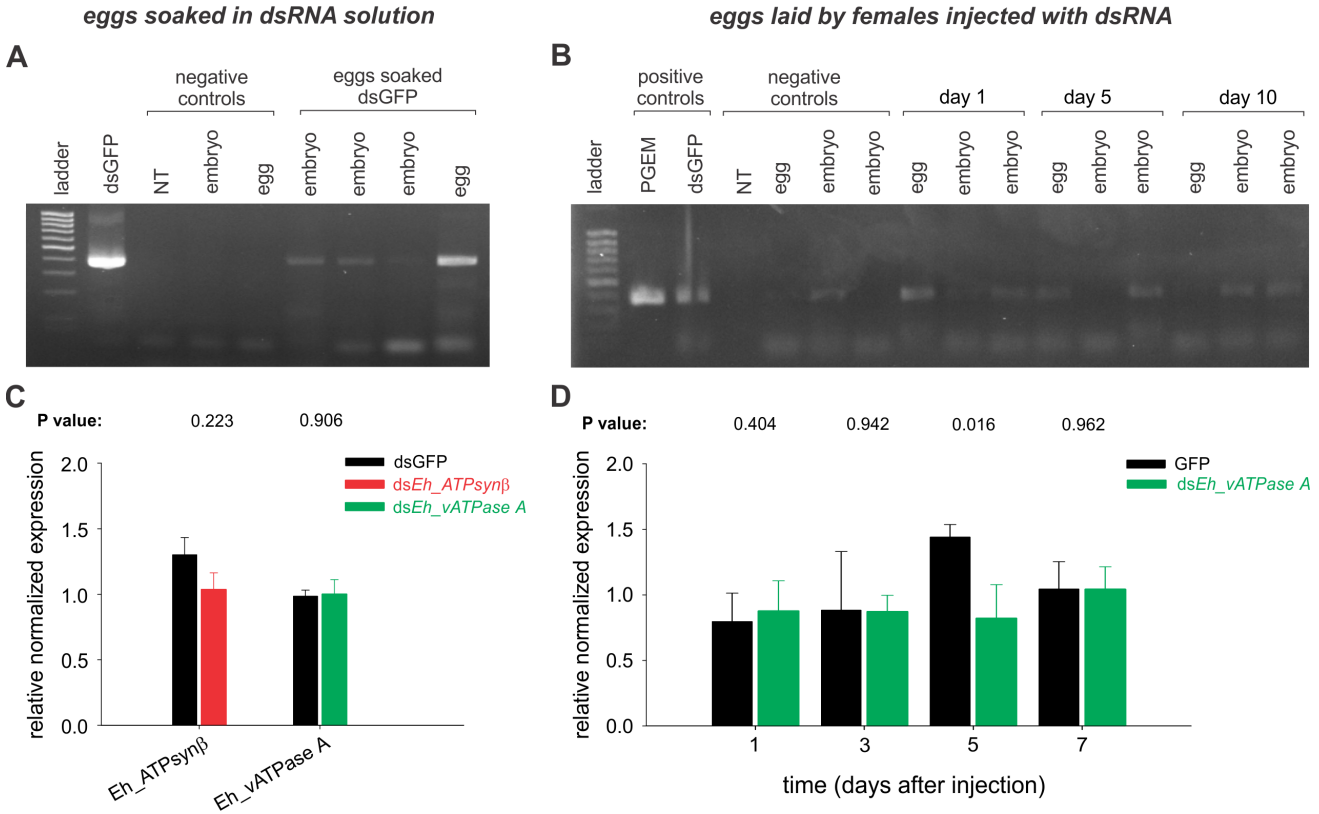


Figure 5.9: Transfer of dsRNA to *E. heros* eggs (***A, C***) *E. heros* eggs that were soaked 50 µl solution of 300 ng/µl dsRNA and 300 ppm of Tween 40 for 4 h, (***B, D***) eggs laid by females injected with 150 ng of dsRNA. (***A, B***) Detection of dsGFP in the eggs and dissected embryos, the samples were incubated at 75 °C for 10 min just before reversed transcription to denature the dsRNA. The levels of remaining dsGFP in the cDNA were then analyzed with PCR. NT=No template. (***C, D***) Expression levels of *Eh_vATPase A* and/or *Eh_ATPsynβ* in *E. heros* eggs. dsRNA targeting GFP was used as a control. As internal controls, *Eh_ARL2*, *Eh_ARP8* and *Eh_UB4A* were used. Values are based three biological samples and expressed as mean ± SEM. Each sample contains 20 pooled *E. heros*. The p-values were calculated by unpaired t-test in qBase+ software.

To further evaluate whether dsRNA can penetrate the egg shell, we performed qRT-PCR using cDNA of soaking eggs in dsRNA targeting *Eh_vATPase A* and *Eh_ATPsynβ*. The transcript levels remain similar with the control after soaking of eggs in ds*Eh_vATPase A* (*P*=0.223) and *Eh_ATPsynβ* (*P*=0.906; Figure 5.9*C*). To investigate the parental transference of dsRNA to the eggs, we evaluated the presence of dsRNA and silencing in eggs laid by females that were injected with dsRNA. dsGFP was detected in eggs and after one, five and even ten days after the injection of the females (Figure 5.9*B*). The transcript levels of *Eh_vATPase A* in the eggs were significantly knocked down by 75.6 % (*P* = 0.016) five days after the female injection (5.9*D*), suggesting that dsRNA can reach the eggs. However, no definitive conclusion could be drawn regarding the transference of dsRNA from the female to the eggs due to the lack of reliable negative controls for the detection of dsGFP.

Development of *T. podisi* larvae on eggs laid by females injected with dsRNA

To evaluate the effects of dsRNA on the development of *T. podisi* larvae, *E. heros* eggs laid by females injected with dsRNA were offered to *T. podisi* mated females. The developmental time (Figure 5.10*A*; *H* = 3.04, *df*=2, *P* = 0.219), emergence rate (Figure 5.10*B*; *H* = 2.11, *df*=2, *P* = 0.347) and the sex ratio (Figure 5.10C; *H* = 0.91, *df*=2, *P* = 0.634) were not significantly affected in the *T. podisi* larvae that developed inside eggs laid by females injected with ds*Eh_vATPase A* and ds*Tp_vATPase A*.

5.4 Discussion

In this Chapter, we assessed the potential risks of *E. heros* active dsRNA on its egg parasitoid *T. podisi*. Evaluating the potential risks involves estimating the likelihood of exposure and the potential hazardous effects on NTOs (Matten et al. 2008; Auer and Frederick 2009; Romeis et al. 2013; Welch and Lundgren 2016). Potential adverse effects on NTOs are typically evaluated using a multi-step tiered approach that begin with controlled laboratory studies under worst-case scenario exposure conditions (Romeis et al. 2008, 2013; Duan et al. 2010; Ramon et al. 2014). Therefore, we evaluated the lethal and sublethal effects of a four-day oral exposure to 1000 ng/µl of both *E. heros* and *T. podisi* specific dsRNA in adults of this parasitic wasps. Ingestion of dsRNA targeting *T. podisi* genes resulted in reduced fitness and target gene expression, demonstrating that *T. podisi* can be physiologically exposed to insecticidal dsRNA. Nevertheless, no adverse effects were observed in either the survival and parasitism capacity after feeding of *E. heros* active dsRNA. Moreover, *T. podisi* larvae developed normally in eggs previously exposed to specific and non-specific dsRNAs. These combined results suggest that despite the likelihood of exposure of *T. podisi* to dietary RNAi the hazards of *E. heros* active dsRNAs on its egg parasitoid are negligible.

In order for insecticidal dsRNAs to cause adverse effects in parasitoids, there must

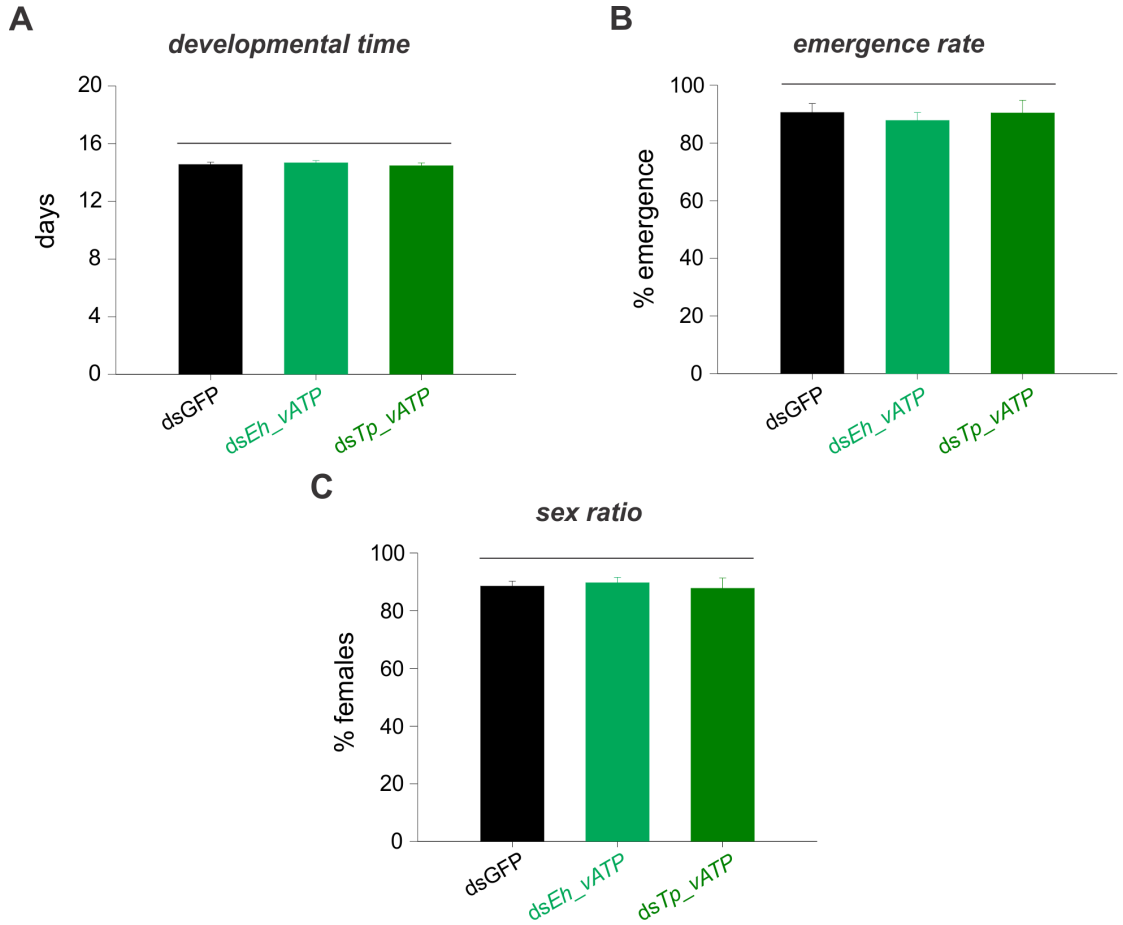


Figure 5.10: Effects of specific and non-specific dsRNA in the development of *T. podisi* larvae. Developmental time (***A***), emergence rate (***B***), sex ratio (***C***). Feeding with dsRNA targeting GFP was used as a negative control. Values represent the mean ± SEM from 24 independent replicates (n=20 eggs laid by injected females) and the ones grouped with the same horizontal line are not statistically different at the 5 % significance level according to a post hoc Kruskal–Wallis test.

be a route through which animals are exposed (Heinemann et al. 2013). This exposure involves not only the ingestion of dsRNA from nectar or pollen, but also the parasitoid should have the capacity to respond to the ingested dsRNA (Bachman et al. 2013, 2016; Roberts et al. 2015). Since insects display a wide range of sensibilities to ingested dsRNA (Zotti and Smagghe 2015; Zhang et al. 2017; Cooper et al. 2019; Vogel et al. 2019), our first step was to determine the susceptibility of *T. podisi* to dietary RNAi using dsRNA against its own genes. Feeding of ds*Tp_vATPase A* and ds*Tp_act-2* at the concentration of 1000 ng/µl led to approximately 77 % mortality after 14 days. Sublethal effects on the life-span of parasitism and life-time fecundity were statistically significant but were primary associated with the early death of the females (Teder and Knapp 2019). This was also the case for the wasps treated with ds*Tp_act-2* that have an increased proportion of female offspring. Since, *T. podisi* females, as haplodiploid parasitoids, laid most of their daughters at the beginning of the reproductive cycle (Silva et al. 2018a). The downregulation of *Tp_vATPase A* and *Tp_act-2* genes supports the lethal and sublethal effects observed were caused by feeding of specific dsRNA.

Tp_vATPase A transcript levels were reduced by 36.6 % after 72h and 41.1 % after 96 h. While *Tp_act-2* expression level decreased by 59.1 % after 72 h of continuous feeding of dsRNA. According to these data, we can infer that *T. podisi* is susceptible to dietary RNAi and therefore is an appropriate surrogate species to evaluate potential risks of RNAi to NTOs. As data generated using NTOs that are physiologically exposed to RNAi can be more consistent, of higher quality and of greater transferability (Lundgren and Duan 2013; Welch and Lundgren 2016).

Data from several studies suggest that the hymenopterans are not extremely sensitive to ingested dsRNA. Several reports have shown that feeding with high concentrations of dsRNA (more than 450 ng/µl or 500 ng per individual) is required to achieve moderate gene silencing in other hymenopterans such as the honey bee *Ap. mellifera* (Nunes and Simões 2009; Mutti et al. 2011; Chen et al. 2015; Vélez et al. 2016), the fire ant *Solenopsis invicta* (Cheng et al. 2015; Qiu and Cheng 2017), the carpenter ant *Camponotus floridanus* (Ratzka et al. 2013), the paper wasp *Polistes metricus* (Hunt et al. 2011) and the parasitoid wasp *Diachasmimorpha longicaudata* (Chen et al. 2015). Conversely, recent studies in the edible black ant *Polyrhachis vicina* have shown that concentrations as low as 15 ng/µl in the diet are enough to elicit significant silence (Zhang and Xi 2018; Xi et al. 2019). Our results and previous studies indicate that dsRNA was not degraded by salivary enzymes brought into the artificial diet after 48 h of feeding (Vélez et al. 2016) or by digestive enzymes in the crop of ant workers (Ratzka et al. 2013). Future studies are needed to explain the lower sensibility to dietary RNAi in Hymenoptera, to increase the ability to predict outcome of RNAi in other NTOs of this order (Lundgren and Duan 2013).

Feeding on high concentration of *E. heros* active dsRNA did not lead to adverse effects in the survival or parasitism capacity of *T. podisi* adults. These results are in agreement with those reported by Bachman et al. (2016, 2013) who observed that dsRNA targeting *DvSnf7* did not affect the survival of *N. vitripennis* and *Pediobius foveolatus* adult parasitic wasps. Although Bachman et al. (2016, 2013) evaluated the impact of survival using a chronic exposure for 20 days, the dsRNA concentration we used in our experiment was 200 to 1000-fold higher. However, there is no information on the expected concentration of insecticidal dsRNA to control stink bug pests, or even the application form that will be used. In the case of genetically modified plants expressing dsRNA, the expression levels of *DvSfn7* dsRNA in MON 87411 can be used as a reference. The expression levels of dsRNA in the corn plant ranges between 33.0-106 ng/g of dry weight (Bachman et al. 2016). On the other hand, the amount of dsRNA required for foliar spray application is predicted to be near 2-10 g/ha^{-1} (Zotti et al. 2018). Based on the average dry weight of soybean plants, the estimated concentration of dsRNA will be approximately 400-2000 ng/g (Hanway et al. 1984). Because parasitoid adults were fed with dsRNA at the concentration of 1000 ng/µl, the margin of exposure was at least 9433 and 500-fold higher than the estimated field exposures, respectively. The

absence of sublethal effects on the development, growth or reproduction was also reported for *DvSfn7* in 15 insect species representing different trophic levels including non-target herbivores, predators and soil decomposers (Bachman et al. 2013, 2016). No adverse effects were observed even in studies that intentionally designed *vATPase A* dsRNA based on the region with highest sequence similarity between the target *D. v. virgifera* and the non-target species *A. mellifera* (Vélez et al. 2016), the springtail *Sinella curviseta* (Pan et al. 2016) and the monarch butterfly *Danaus plexippus* (Pan et al. 2017). However, it is important to point out that the monarch butterfly larvae are not susceptible to dietary RNAi and the susceptibility of the springtail is unknown (Pan et al. 2016, 2017). In this regard, Haller et al., (2019) repeated the experimental approach using two species of ladybird beetles *Adalia bipunctata* and *Coccinella septempunctata* sensible to dietary RNAi. Feeding on *D. v. virgifera* active dsRNA slightly prolonged the developmental time for *A. bipunctata* and reduced the survival rates of *C. septempunctata* (Haller et al. 2019). The adverse effects in *C. septempunctata* can be explained by the downregulation of *vATPase A*, but that is not the case for *A. bipunctata*, thereby the off-target effects cannot be completely excluded.

Although previous studies in insects have provided evidence of non-specific downregulation of off-target genes after dsRNA exposure (Ma et al. 2006; Kulkarni et al. 2006; Jarosch and Moritz 2012), little is known about the off-target effects on NTO. Our bioinformatic analysis predicted the potential off-target binding of *E. heros* active dsRNA with several *T. podisi* transcripts, but there is no single hit with ≥21 nt continuous matches. However, in the absence of complete genome information it is possible that some significant off-target interactions may be missed (Qiu et al. 2005). The high number of predicted off-target genes in our study can be correlated with the use of long dsRNA sequences (444 and 462 bp), since off-target sequence similarity increases with the dsRNA length (Mogren and Lundgren 2017). Nevertheless, the high number of predicted off-target does not imply a higher likelihood of unintended gene silencing (Hanning et al. 2013). In fact, pooling of multiple siRNAs to the same target may help to reduce off-target silencing due to competition among the siRNAs in the pool (Jackson and Linsley 2010; Miller et al. 2012; Hannus et al. 2014). Accordingly, none of the predicted off-target genes tested experimentally were silenced in *T. podisi* fed with E. heros active dsRNA. The upregulation of *Tp_SPTBN4-like* after ds*Eh_act-2* feeding can be caused by the activation of feedback loops that try to re-establish the protein levels after the suppression of mRNA result of the RNAi response (Khan et al. 2009). However, this effect has not shown any in vivo consequence in terms of safety since did not result in detectable phenotypic changes (Casacuberta et al. 2015). Our expression analysis experiments suggest that at least 21 nt continuous matches may be required to trigger RNAi response in *T. podisi*. Although it is widely presumed that a minimum of 100 % complementary sequence length of ≥21 nt is required for efficient RNAi activity in insects (Bolognesi et al. 2012; Bachman et al. 2013, 2016; Tan et al. 2016; Fishilevich et al. 2016b; Pan et al. 2016, 2017). Some studies have shown that even a single 19-nt

hit with the off-target sequence is capable to effectively knockdown its corresponding mRNA (Kulkarni et al. 2006; Zhang et al. 2015a; Chen et al. 2015). While other studies suggest that numerous potential 21-nt hits may be required to reduce the expression level of the off-target (Poreddy et al. 2017; Haller et al. 2019). Besides the sequence similarity and the length of the dsRNA, there are further factors that may influence the likelihood of off-target silencing including the position of mismatches within the siRNA sequence, the position of the dsRNA within the off-target, the expression levels of the off-target gene, the exposure time and untranslated regions as targets of RNAi (Qiu et al. 2005; Ma et al. 2006; Alemán et al. 2007; Arvey et al. 2010; Hong et al. 2014). Hopefully, a further understanding of contribution of these factors to off-target silencing in insects, as well as the availability of genomes from representative species will increase the reliability of the bioinformatic analysis for prediction of hazards to NTOs.

To further characterize the risk of non-specific dsRNA in *T. podisi*, the levels of the core RNAi enzymes were evaluated. Since RNAi is involved in defense against RNA virus infection, dsRNAs are recognized as molecules associated with pathogens and trigger the upregulation of Dcr-2, Ago2 and other immune response genes (Hirai et al. 2004; Lozano et al. 2012; Garbutt and Reynolds 2012; Liu et al. 2013, 2014; Buchon et al. 2014; Niu et al. 2016). Feeding with non-specific dsGFP induce moderately the expression levels of Dcr-2 and Ago2 in *T. podisi*. Although the induction of defense mechanisms can be energetically costly, and may result in trade-offs with other life-history traits, such as reproduction and development (Ahmed et al. 2002; Ahmed and Hurd 2006; Freitak et al. 2007; Schwenke et al. 2016). By correlating the immune stimulation and the absence of adverse effects in the life-history traits, we can infer that the stimulation of the immune system triggered by dsRNA does not represent any risk to *T. podisi*.

Although the main emphasis of this Chapter is the evaluation of potential adverse effects under the worse-case scenario, we also assessed the effects of insecticidal dsRNA in the larvae of *T. podisi* under more realistic conditions. As the immature stages of the parasitoid are protected by the eggshell of the host (Cônsoli et al. 1998; Suh et al. 2009; Takada et al. 2009), we evaluated the possible route of transmission of dsRNA to the host egg. Our results suggest that sprayed dsRNA can penetrate the egg shell and could reach the embryo. This finding was also reported in the eggs of *Ostrinia furnalalis* after topical application of the fluorescent labeled dsRNA (Wang et al. 2011). Significant silencing was observed in eggs laid by females injected with dsRNA, suggesting that dsRNA can reach the eggs through maternal transmission. Knockdown of the gene function in the offspring after delivery of dsRNA to adult females have been also observed in multiple insect species, including the stink bug *H. halys* after injection (Lu et al. 2017), and *D. v. virgifera* after feeding (Khajuria et al. 2015). In addition, our results indicate that *T. podisi* developmental time, emergence and sex ratio were unaffected by both *E. heros* and as well as *T. podisi* dsRNA. However, these data must be interpreted

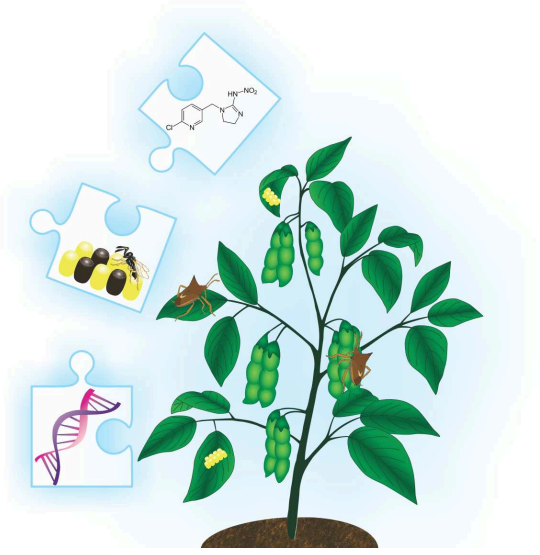
with caution because there is the possibility of false positives in the detection of dsRNA.

The lack of adverse effects in *T. podisi* adults and larvae suggest that insecticidal dsRNA can be designed to have a reduced impact on the parasitoid population dynamics, as well as on the biocontrol services they provide (Auer and Frederick 2009; Lundgren and Duan 2013; Ramon et al. 2014; Roberts et al. 2015). Laboratory tiered experiments are considered sufficiently accurate and conservative to predict potential adverse effects that might be manifested in field (Duan et al. 2010). However, different biotic and abiotic stressors absent in laboratory conditions may influence the gene expression of potential off-targets (Lundgren and Duan 2013). Genes with clear ties to phenotype and fitness tend to be highly expressed (Cutter et al. 2003). It is possible, therefore, that the potential off-target effects can be detected in laboratory assays, since highly expressed genes are more susceptible to siRNA mediated silencing (Hong et al. 2014). However, as experiments were conducted into small plastic tubes the lack of detection of possible behavioral perturbations is likely, since parasitoids may find their hosts without using long-range cues (Desneux et al. 2007). Indeed, sublethal effects of pesticides can be expressed as impairment of mobility or the ability to locate host important to guarantee optimal performance of parasitoids in the field (Desneux et al. 2007; Cloyd and Bethke 2011; Teder and Knapp 2019). It may therefore be important to add a standard behavioral test to further investigate the impact of RNAi in non-target organisms.

In summary, exposure to insecticidal dsRNA does not lead to changes in gene expression or adverse effects in the survival or parasitism capacity of the egg parasitoid *T. podisi*. The evaluation of sublethal effects in life-history traits, potential off-target silencing, and immune stimulation provided in this study contributed to the overall understanding of the potential hazards of RNAi to NTOs. Thus, this study could be used as route map for future investigations aimed to evaluate the toxicity of RNAi-based insecticides in NTOs and could serve as baseline information for the environmental risk assessment of RNAi for pest control. However, further studies evaluating the molecular mechanisms of RNAi, the susceptibility of other NTOs and possible exposure routes will improve our knowledge of the impacts of RNAi on NTO and increase the predictive power of bioinformatic analysis for the environmental risk assessment.

Chapter 6

General discussion and future perspectives for sustainable control of *E. heros*



The main challenge faced by agriculture nowadays is to minimize the environmental impact and raise productivity in order to ensure food security for a growing world population (Pretty and Bharucha 2015; Rockström et al. 2017; Struik and Kuyper 2017; Pretty et al. 2018). This challenge requires the reduction of environmentally damaging practices and the adoption of sustainable practices that do not undermine the capacity of the land to produce food in the future (Zechendorf 1999; Godfray et al. 2010; Godfray 2015; Pretty et al. 2018). For instance, while conventional insecticides prevent a significant amount of potential losses, enabling the farmers to increase yields of foods and other agricultural products (Macfadyen et al. 2014; Pretty and Bharucha 2014), the overuse of these molecules poses threats to human and ecosystem health, and their effectiveness decline as a result of resurgence and secondary pest outbreaks following the disruption of natural biological control and/or the selection for insecticide resistance (Szczepaniec and Raupp 2013; Macfadyen et al. 2014; Quarcoo et al. 2014; Guedes and Cutler 2014; Guedes et al. 2016, 2017). Thus, the over-reliance on conventional insecticidal sprays as the only tool for insect pest control, like in the case of *E. heros* in Brazilian soybean, is not sustainable (Gentz et al. 2010; Pretty and Bharucha 2014; Pretty et al. 2018).

Integrated Pest Management (IPM) is increasingly perceived as a sustainable solution to reduce pesticide use without yield penalties (Pretty and Bharucha 2014). IPM consists of a toolbox of management decisions and interventions designed to combine the use of multiple complementary tactics to control crop pest (Ehler 2006; Roubos et al. 2014; Pretty and Bharucha 2014). The aim is to reduce applications of conventional pesticides, provide economic savings for the farmer, protect both the environment and human health, and problems caused by pesticide-resistant pests (Ehler 2006; Stenberg 2017). Nevertheless, to make any IPM program successful there is a need to develop alternatives to conventional pesticides that allow an effective management of crop pests (Lamichhane et al. 2017; Pretty et al. 2018). Although biological control is widely recognized as an essential factor of IPM programs, there are still some obstacles that hinder its development and adoption (Ehler 2006; Barzman et al. 2015; Lamichhane et al. 2017; Stenberg 2017). Among these obstacles, biological control agents must meet the demands of growers in terms of quantity and effectiveness in the field under variable climatic conditions (Sørensen et al. 2012; Terblanche 2014; Lamichhane et al. 2017). In addition, molecular methods such as RNAi-based pesticides represent key opportunities to reduce pest population densities in a sustainable way, however, its use is still limited to a few transgenic crops, and only adopted in restricted parts of the world (Pretty et al. 2018; Taning et al. 2019).

For a transition towards sustainable *E. heros* control, there is a need to reduce the dependence on insecticides. To achieve this, approaches such as the adoption of IPM, improve the efficiency of natural enemies in biological control programs and development of new commercially-viable insect control technologies are required (Zalucki et al. 2009; Weddle et al. 2009; Gentz et al. 2010; Pretty and Bharucha 2015). In this PhD

dissertation, we aimed to provide relevant information for the sustainable pest control of this stink bug pest by evaluating the potential to (i) develop insecticide resistance; (ii) improve performance and productivity of mass-reared *T. podisi*; (iii) develop novel strategies for pest management based on RNAi; and (iv) combine RNAi-based control strategies and biological control with *T. podisi*. In what follows, the obtained results are situated in the context of IPM and both the short and the long-term implications for sustainable control of *E. heros* are discussed.

6.1 Evaluation of the individual strategies to control *E. heros* in the context of IPM

6.1.1 Chemical control for the control of *E. heros*

Using two laboratory selected strains with different genetic backgrounds, we showed in **Chapter 2** that *E. heros* has the potential to develop resistance to the neonicotinoid imidacloprid. Especially since the field-derived strain required only six generations of selection to achieve significant resistance levels in comparison to the 13 generations required by the laboratory-derived strain. Therefore, adoption of insecticide resistance management (IRM) is crucial to prevent the development and spread of resistance in *E. heros* in field (Puinean et al. 2010; Onstad 2014a; Nauen et al. 2019). The focus should be on preventative IRM for managing susceptibility before resistance is established in a population because curative approaches are more restrictive and have smaller chance of long-term success (Onstad 2014a). Successful IRM programs are important to regain susceptibility in the *E. heros* populations in which resistance has already arisen (Tuelher et al. 2018), thereby maintaining the efficacy of insecticides available in the market (Nauen et al. 2019). Given that new insecticides with novel mode of actions are difficult and costly to develop (Sparks 2013; Sparks and Lorsbach 2017) and the limited options of non-chemical alternatives for the control of *E. heros* (**Chapter 1**).

Imidacloprid resistance in *E. heros* was correlated with the enhanced detoxification by cytochrome P450 enzymes in both resistant strains. While the walking and mating behavior remain similar between the susceptible and resistant strains, the relative fitness of the resistant strains decreased dramatically, as result of lower fecundity, lower fertility, lower survival rate of the nymphs and shorter adult male lifespan. These results suggest the presence of a trade-off in the allocation of resources between the enhanced activity of cytochrome P450 enzymes and fitness in imidacloprid resistant strains of *E. heros* (**Chapter 2**). Consequently, IRM programs that minimize the selection for resistance, including alternations, rotations, or even mixtures of insecticides with different modes of action and detoxification may delay the resistance development (Nauen 2007; Whalon et al. 2008; Sparks and Nauen 2015; Nauen et al. 2019). For instance, the suspension of the use of imidacloprid for the control of the brown planthopper in China from 2006 has contributed to the decrease of resistance (Wang et al. 2009; Wen et al. 2009).

However, this approach has two limitations that need to be considered.

First, laboratory-selected insecticide resistance can differ from field-evolved resistance in the genetic basis, mechanism, and magnitude of resistance (Tabashnik et al. 2014). Indeed most of the strains selected in laboratory show accumulation of low level resistance mechanisms, whereas most resistant field strains show monogenic resistance (Roush and McKenzie 1987; Ffrench-Constant 2014). Therefore, monitoring of insecticide resistance and underlying mechanism involved in the field, as well as the characterization of genetic basis, heritance and cross-resistance patterns would help us to improve IRM programs (Nauen and Denholm 2005; Guedes 2017; Freeman et al. 2019). In addition, behavioral avoidance, endosymbionts, sublethal exposure, induction of detoxification enzymes also influence insecticide resistance in field, evaluating the role of these factors is also of interest for more informed decision-making (Onstad 2014a; Chanda et al. 2016; Guedes et al. 2017).

Second but more importantly, controlling *E. heros* with only a limited number of chemical classes is challenging itself, particularly when those insecticides are also used to suppress other pest species in the early season (Tuelher et al. 2018; AGROFIT 2019). The chemical options for IRM strategies include only four molecular classes of insecticides addressing three different target sites: nAChRs for neonicotinoids, voltage-gated sodium channel for pyrethroids and acetylcholinesterase for organophosphates and carbamates (Sparks and Nauen 2015; Nauen et al. 2019). In addition, due to the wide metabolism spectrum, detoxification by cytochrome P450 enzymes may potentially confer cross-resistance to insecticides independent of their target sites (Zewen et al. 2003; Yang et al. 2016). Consequently, the most advocated tactic for managing resistance is minimizing the reliance on chemical insecticides per se, thereby IRM will be more effective and more valuable when it is incorporated into IPM (Weiss et al. 2009; Sparks 2013; Onstad 2014b; Bass et al. 2015; Nauen et al. 2019). From this perspective, IPM practices such as the use of appropriate economic thresholds, recommended rates, early maturing or pest-tolerant varieties, biological control and crop rotation can contribute to delay the insecticide resistance in *E. heros* (Weiss et al. 2009; Onstad 2014b; Nauen et al. 2019).

6.1.2 Biological control of *E. heros* using *T. podisi*

As shown in **Chapter 3**, the mass rearing conditions of the parasitoid *T. podisi* can be improved using fluctuating thermal regimes. A fluctuating regime was more suitable for rearing of *T. podisi*i than the constant regime, as exemplified by shorter developmental time, increased female longevity, increased life-span of parasitism, increased fecundity and increased emergence rate. This increment in the fitness can also be translated into a lower production cost, and thereby higher chances of adoption of augmentative biological programs using this parasitoid. Although the fluctuating regime could not increase the performance at constant extreme temperatures. *T. podisi* reared under fluctuat-

ing temperatures may have higher performance when released in soybean crops, since temperature in the field fluctuates (Bannerman and Roitberg 2014; Colinet et al. 2015). These findings have significant implications for the biological control of *E. heros* using *T. podisi*. First, changes in the temperature variability during laboratory culture can result in increased productivity, or perhaps even increased performance in field (Parra 2010; Sørensen et al. 2012; Terblanche 2014; Chidawanyika et al. 2017). Second, the upper thermal limits of *T. podisi* appear to be tightly constrained, as demonstrated by the low performance at 30°C and the lack of adaptation to high temperatures after four generations of selection (Hoffmann et al. 2013; Roitberg and Mangel 2016; Furlong and Zalucki 2017). Since this temperature is frequently reached in field, the performance survival and ultimately the establishment in field of *T. podisi* can be affected in the warmer regions or during extreme heat events (Chidawanyika et al. 2017). Consequently, field releases should be accompanied by seasonal monitoring to confirm the presence and activity of the parasitoid wasps, especially after extreme thermal events (Messelink et al. 2014; Barratt et al. 2018). Alternatively, the use of fluctuating regimes more adapted to the thermal conditions in the different productive areas in Brazil could improve mass rearing and transportation protocols with the view of strengthening the efficiency of biological control programs (Thomson et al. 2001; Chidawanyika and Terblanche 2011; Terblanche 2014). Future studies evaluating the impact of different fluctuating regimes on field performance, tolerance to high temperatures and cost-effectiveness are required.

However, improving mass rearing and transportation protocols is not enough to ensure the success of biological control using *T. podisi*. First and foremost, expectations that biological control can act as a sole method for pest management in field control are generally unrealistic, and thereby integrating biological control into IPM strategies is particularly relevant for efficient pest control (Naranjo et al. 2015; Lamichhane et al. 2017; Barratt et al. 2018). Monitoring and economic thresholds are important to more accurately determine the need or timing for the inoculative releases of *T. podisi* (Collier and Van Steenwyk 2004; Mills 2009; Naranjo and Ellsworth 2009; Parra and Coelho 2019). Moreover, integration of conventional insecticides with *T. podisi* releases could also increase the effectiveness of pest suppression. Despite considerable research of defining toxicity to insecticides to *T. podisi*, little attention has been paid towards the compatibility of chemical control with biological control in field (Turchen et al. 2016a; Silva et al. 2018b; Zantedeschi et al. 2018; Stecca et al. 2018; Feltrin-Campos et al. 2018; Pazini et al. 2019). Thus, further studies in the modification of pesticide use practices, use of reduced risk pesticides and even selection of strain tolerant to pesticides would beneficiate the integration into IPM programs (Mills 2009; Roubos et al. 2014; Gurr and You 2016; Baker et al. 2020). In addition, the cultivation systems used for short-term annual crops such as soybean often prevent the establishment of viable breeding populations of natural enemy populations between the crop production systems (Bale et al. 2008; Stenberg 2017; Grab et al. 2018). To increase the efficacy of biological control, the environment can be manipulated to favor the permanence of *T.*

podisi and probably also other natural enemies in the crop (Bale et al. 2008; Gurr and You 2016; Holland et al. 2016; Perović et al. 2018). Strategies such as the application of synthetic herbivore-induce plant volatiles that attract natural enemies, introduction of semi-natural habitats, provision of floral resources may lead to a greater control and the control can be self-perpetuating over longer periods of time (Bale et al. 2008; Borges et al. 2011a; Perović et al. 2018; Kruidhof et al. 2019).

6.1.3 RNAi for the control of *E. heros*

From an IPM perspective, RNAi-based insecticides have an appeal because they are effective against the targeted pests and can be designed to exert toxicity to a very limited range of species (Joga et al. 2016; Bachman et al. 2016; Zotti et al. 2018). In **Chapter 4**, we evaluated the possibility of using RNAi technology for the control of *E. heros*. As the functionality of the RNAi machinery has been already confirmed in this stink bug (Fishilevich et al. 2016a), oral feeding assays were performed using two target genes whose effectiveness was first corroborated in the microinjection screening. DsRNA was administered by feeding to the insects with the artificial diet containing different forms of dsRNA for delivery: naked dsRNA, dsRNA with EDTA and dsRNA encapsulated in liposomes. Oral delivery of naked dsRNA resulted in lower RNAi efficiency, evidenced by incomplete and transient silencing and no clear mortality scores, probably due to dsRNA degradation the salivary secretions of *E. heros*. However, the lack of mortality with naked dsRNA should be interpreted with caution. Feeding with naked dsRNA can cause significant silencing and mortality in *H. halys* and *N. viridula*, in spite of the nuclease activity of the saliva in these pentatomid stink bugs (Lomate and Bonning, 2016; Mogilicherla et al., 2018; Sharma, 2019 Personal communication) . These conflicting results could be associated with factors inherent to the experiment set-up, including the gene selection, the life-stage and the use of artificial diet (Chapter 1). For instance, Mogilicherla et al. (2018) also found that dsATPase caused 100 % mortality by injection and less than 15 % by feeding, whereas injection and feeding of dsIAP resulted in 70 % mortality, suggesting that the gene selection is of extreme importance. Furthermore, the use of EDTA and lipid-based nanoparticles improve the mortality induced by the oral delivery of dsRNA in *E. heros*. It is not clear if the improvement was only associated with the protection from nuclease activity or whether it is related to an improved dsRNA uptake by the midgut cells. Consequently, further work is required to address the following questions (i) is the degradation in the saliva enough to impair RNAi efficiency by feeding in *E. heros*?; (ii) are there differences in the susceptibility to dsRNA between the life-stages?; (iii) is the cellular uptake and spreading of RNAi signal efficient in stink bugs? The answers to these questions may help to increase our understanding of the RNAi response in stink bugs, but still, an even greater effort is needed to translate these studies into products that can be used for the control of *E. heros* in field.

RNAi-based insecticides for the control of *E. heros* or the complex of soybean stink

bugs in the field may include the transgenic plants that express dsRNA, but sprayable products might also be formulated using dsRNA as an active ingredient (**Chapter 1**). Effectively integrating RNAi-based insecticides requires an understanding of the advantages and disadvantages of both approaches and their compatibility with principles of IPM (summarized in Table 6.1).

Table 6.1: Advantages and disadvantages of RNAi-based insecticides using transformative and non-transformative approaches in the framework of IPM.

	Transformative RNAi	**Non-transformative RNAi**
Practicality		
Ease of use	Easy, with the seed. dsRNA is expressed in the appropriate tissue at the proper dose	Require one to multiple applications. Application may be not uniform.
Efficiency	High in insects that are highly susceptible to RNAi	Formulations may increase the efficiency in recalcitrant insects.
Crops	Only for the crops with the transgenic trait	Can be used in all the crops in which the pest reach damaging levels
Cost	Associated with the transgenic trait in the seed	Associated with the cost dsRNA and formulations, applied only when required
Risks		
Persistence in the field	All season long	Low, will depend on the formulation
NTOs	Only by ingestion. Longer Exposure	By ingestion and probably by contact. Formulations may increase risk to NTOs
Resistance development	High risk	Low risk
IPM		
Compatibility principles IPM	Preventive measure, even if the pest is not present	Require monitoring of the pests, Should be applied only when the pest reach damaging levels
Compatibility with other IPM tactics (i.e. biological control)	High	High
Consumer acceptance	Low	High

Transformative RNAi

Up to now, delivery of dsRNAs through transgenic plants has been used to induce silencing in numerous hemipteran pests including aphids (Pitino et al. 2011; Mao and Zeng 2014; Xu et al. 2014; Elzinga et al. 2014), psyllids (Wuriyanghan and Falk 2013;

Hajeri et al. 2014), the cotton mealybug *Phenacoccus solenopsis* (Khan et al. 2018), the whitefly (Thakur et al. 2014; Malik et al. 2016; Ibrahim et al. 2017), the brown planthopper (Zha et al. 2011), and even the mirid bug *Apolygus lucorum* (Liu et al. 2019). Some of these studies have also demonstrated that the silencing can result in high mortality (more than 50 %; Ibrahim et al., 2017; Liu et al., 2019b; Malik et al., 2016; Thakur et al., 2014), suggesting that plant-mediated RNAi is a feasible and powerful strategy to control sucking pests. Moreover, it is promising to find that transgenic soybean can induce 72.47 % mortality in the mirid bug that have feeding strategies similar to stink bugs (Lucini and Panizzi 2018; Liu et al. 2019). Both mirids and stink bugs use their stylets to lacerate the plant cells, simultaneously secreting watery saliva to dissolve the cells and then ingest the degraded cell contents (Lucini and Panizzi 2018). However, the maximum expression of *DvSfn7* in MON 87411 maize is 5.40 x 10^{-3} µg/g of fresh weight (Bachman et al. 2016). Based on the concentration of 300 ng/µl used in our experiments (**Chapter 4**), the expression levels of dsRNA in transgenic soybean to control E. heros should be at least 8875-fold higher than in MON 87411. Expression of these amount of dsRNA by transgenic plants may be challenging, especially because the expressed long hpRNA is processed into siRNA by the plant RNAi machinery (Bally et al. 2016, 2018; Zhang et al. 2017). Transformation of chloroplast permits accumulation of much higher amounts of dsRNA which remains encapsulated in the chloroplast and thus is protected from the plant RNAi machinery (Bally et al. 2016, 2018; Zhang et al. 2017). As plastid transformation of soybean plants have been achieved, it represents an attractive alternative to ensure sufficiently high doses of intact dsRNA for effective RNAi in *E. heros* (Bally et al. 2018).

Used within the context of IPM, transgenic plants expressing insecticidal dsRNA offer several advantages: (i) ease to use, requiring only planting seeds of the transgenic plant; (ii) dsRNA is expressed in the appropriate tissue at the proper dose, even though it is expressed to the lesser extent in other tissues such as pollen (Li et al. 2015a; Bachman et al. 2016); (iii) compatibility with other IPM practices including cultural, biological and chemical controls (Kennedy 2008; Taning et al. 2019); and (iv) approval by international regulatory agencies in different countries (Sherman et al. 2015; ISAAA 2017). However, the main drawback is that the transgenic crop continuously express insecticidal dsRNA, and exerts continuous selection pressure for adaptation of the pest to the transgenic trait (Ferré et al. 2008; Kennedy 2008; Naranjo 2011). To the date, resistance to RNAi have been only documented in a laboratory selected strain of *D. v. virgifera* that possibly had impaired luminal uptake of dsRNA (Khajuria et al. 2018). But other possible resistance mechanisms to RNAi include upregulation of nucleases, inactivation of the RNAi machinery, mutations in the target gene and upregulation of other genes that can perform the similar functions (Auer and Frederick, 2009; Ramesh, 2013; Zhang et al., 2017; Zotti et al., 2018). Strategies like classical refuges, use of promoters induced by insect wounding and integration inside IPM programs may delay the emergence of insect resistance, just as is done for Bt crops (Gulbitti-Onarici et al. 2009; Baum and Roberts 2014;

Tabashnik and Carrière 2017; Pandey et al. 2019). Another drawback is that transgenic plants can only be used preventatively; they cannot be deployed mid-season to control an unanticipated insect problem (Kennedy 2008; Naranjo 2011). Other disadvantages of RNAi transformative approaches include the difficulty to generate transgenic plants for some crop species, the long regulatory process plus public concerns about genetically modified organisms (Wang and Jin 2017; Cai et al. 2018; Cagliari et al. 2019). However, in the specific case of Brazil, it should not be a problem due to large adoption of transgenic plants and approval by the National Biosafety Technical Commission (CTNBio) for release of transgenic plants expressing dsRNA (ISAAA 2017).

Alternatively, symbiotic bacteria and viruses can be engineered to generate a vector for RNAi induction through the continuous production of dsRNA into the insect host (Whitten et al. 2015; Kolliopoulou et al. 2017; Taning et al. 2018; Cagliari et al. 2019). In *R. prolixus* knockdowns were achieved by delivering the symbiotic bacteria isolated from the hindgut *Rhodococcus rhodnii* expressing dsRNA (Whitten et al. 2015). Furthermore, this method of feeding might potentially lead to horizontal transfer of the RNAi signal through symbiont-contaminated feces. Therefore, symbiont bacteria in the gastric caeca of stink bugs, that are also transferred horizontally, can be used as dsRNA delivery vehicles in *E. heros* (Hirose et al. 2006; Zucchi et al. 2012; Bistolas et al. 2014). On the other hand, *Halyomorpha halys* virus (HhV) (Picornavirales: Ilfaviridae) hast the potential to be used as silencing vector not only in *E. heros*, but also in other pentatomid species (Santos et al. 2019). However, microorganisms following genetic modification to express dsRNA will be considered as genetically modified products and will suffer the same regulatory and public acceptance obstacles as transgenic plants (Cagliari et al. 2019).

Non-transformative RNAi

On the other hand, foliar sprays and other exogenous applications (such as baits, root drenching, trunk injections, seed treatments) offer alternatives for RNAi-based pest control, with higher flexibility than the transformative approach (Zotti et al. 2018; Cai et al. 2018; Cagliari et al. 2019). Exogenous application of naked dsRNA through topical application, trunk injection or drenching of plant cuttings have been shown to work for some hemipterans, like the soybean aphid *Aphis glycines* (Zheng et al. 2019), psyllids (Hunter et al. 2012; Andrade and Hunter 2017; Killiny and Kishk 2017), the glassy-winged sharpshooter *Homalodisca vitripennis* (Hunter et al. 2012), the brown planthopper (Li et al. 2015a) and the stink bug *H. halys* (Ghosh et al. 2017; Mogilicherla et al. 2018). Non-transformative approaches can take advantage of formulations that improve dsRNA stability, dsRNA uptake and overall RNAi response in less sensitive species like *E. heros* (Joga et al. 2016; Vélez and Fishilevich 2018; Vogel et al. 2019). In addition to the liposomes and EDTA formulations tested in **Chapter 4**, other formulations to further increase the efficacy and duration of the silencing in *E. heros* remain to

be investigated. In Hemiptera for instance, the use of a peptide nanomaterial, branched amphiphilic peptide capsules (BAPCs), facilitates cellular uptake and probably protect dsRNA against degradation in the saliva in *A. pisum* (Avila et al. 2018). As demonstrated by the early mortality and gene suppression observed in aphids feeding with dsRNA associated with BAPCs compared to naked dsRNA. Different nanoparticles can be used to tested in *E. heros* including BAPCs, nanoparticles used successfully to increase RNAi efficiency in other insects and even carrier systems used in biomedical and pharmaceutical fields (Heidebrecht 2017). The list of successful examples in insects include chitosan nanoparticles (Zhang et al. 2010; Mysore et al. 2013, 2014; Ramesh Kumar et al. 2016), fluorescent cationic core-shell (He et al. 2013), and more recently guanylated polymers were designed to protect dsRNA from the alkaline conditions in the midgut of Lepidoptera (Christiaens et al. 2018b). The advantages of using nanoparticles include low-cost, low-price, biodegradability and environmental safety (Zhang et al. 2010; Joga et al. 2016). Moreover, nanoparticles might increase the stability of the topically applied dsRNA to withstand environmental conditions and provide long-term protection against the targeted pest (Mitter et al. 2017).

In the terms of IPM, advantages of non-transformative approaches compared with transgenic plants include: (i) increased the range of crops and the pest that can be targeted using RNAi technology due to use of formulations and different types of applications (Cai et al. 2018; Cagliari et al. 2019); (ii) reduced risk of resistance development since these products can be applied judiciously when established economic thresholds of pest infestation have been crossed (Huvenne and Smagghe 2010; Baum and Roberts 2014); (iii) shorter exposure of NTOs to insecticidal dsRNA (Romeis et al. 2019); and (iv) probably faster regulatory process of approval of RNAi-based products (Cai et al. 2018; Taning et al. 2019). For instance, Brazilian regulation has stipulated that dsRNA molecules released in the environment can or cannot be considered genetically modified organisms depending of origin of dsRNA (CTNBio 2018). In order to determine whether the RNAi-based insecticide would be considered a genetically modified organisms, the final product must lack of recombinant DNA/RNA and loss the recombinant capacity to insert into the target species or NTOs. Nevertheless, variation in application equipment, timing, coverage, and environmental conditions can cause variable efficacy (Ferré et al. 2008; Storer et al. 2008). Furthermore, the use of formulations designed to reduce the degradation in the environment and increase cellular uptake might increase risks to NTOs by overcoming natural barriers which could have otherwise protected the NTO (Heinemann et al. 2013; Taning et al. 2019). Lastly, it is also worth to highlight that the drop in the price of dsRNA production considerably increases the feasibility of applying RNAi-based products on large fields in a cost-effective manner (Taning et al. 2019). For instance, the cost of producing dsRNA through fermentation (using bacterial cells deficient of enzyme RNase III that degrades dsRNAs) is US$ 1.0/g with scalability to kg (Taning et al. 2019). While, a cell-free bioprocessing platform can produce dsRNA with the same bioactivity and specificity that dsRNA synthetized *in-vitro* with a projected

cost of <$0.50/g dsRNA and scalability to metric tons (Maxwell et al. 2018; Taning et al. 2019). Considering that the amount required per hectare is predicted to be 2-10 g (Zotti et al. 2018), dsRNA products can finally achieve a commercially competitive cost per application ($/ha). However, this amount may vary greatly depending on species sensibility to RNAi, systemic RNAi and efficiency of the formulation developed for delivery (Zotti et al. 2018).

6.2 Towards a sustainable the control of *E. heros*: Approaches for integrating the control strategies

The goal of IPM is to support the sustainable production crops through the integration of diverse control tactics while minimizing environmental impacts attributable to pests or pest management practices (Ehler 2006; Roubos et al. 2014; Pretty and Bharucha 2014). Adoption of IPM programs in soybean production in Brazil has the potential to: (i) minimize of evolution of insecticide resistance; (ii) enhance role of natural enemies in pest control; (iii) favor the adoption of more environmentally friendly technologies such as RNAi; (iv) reduce the cost to the farmer; and (v) reduce adverse effects of insecticides to the consumers and the environment (Roubos et al. 2014; Barzman et al. 2015; Birch et al. 2016; Stenberg 2017). While the benefits of using an IPM approach are evident, implementation of IPM can be very challenging, particularly in the case of *E. heros* due to the lack of alternative tactics for its control. It is in this regard that RNAi-based insecticides have the greatest potential to contribute significantly to the establishment of sustainable crop protection systems (Kennedy 2008; Taning et al. 2019; Romeis et al. 2019). However, as RNAi-based insecticides to control stink bugs are still in development, a gradual stepwise adaptation to IPM programs will raise the opportunity for this technology to allow sustainable soybean production in the future (Barzman et al. 2015). This process of adaptation starts with the integration of pesticide use combined with biological control in current production, followed by the introduction of new technologies like RNAi, to more radical redesigns of ecologically balanced crop production systems (Pretty and Bharucha 2015; Barzman et al. 2015; Anderson et al. 2019).

6.2.1 Short-term solutions for sustainable control of *E. heros*

The IPM concept is based on the premise that cultivated plants can tolerate certain levels of injury without economically significant yield reductions (Gu et al. 2008; Ramsden et al. 2017). In this context, adoption IPM programs based on pest population monitoring and use of insecticides only when the economic threshold is reached will increase the short-term sustainability of stink bugs control in soybean (Bueno et al. 2011b, 2013; Bortolotto et al. 2015). In Brazil, only the beat cloth is recommended as a sample method for stink bug control, and the economic thresholds are variable depending on the production system (Bueno et al. 2011b, 2013; Bortolotto et al. 2015). The economic

threshold for controlling stink bugs is 2 insects per meter for grain production and only 1 per meter for crop seed (Bueno et al. 2015). Compared with prophylactic pesticide applications, adoption of economic threshold based IPM has the potential to reduce the insecticide applications targeting *E. heros* by more than the half, without compromising the yield or seed quality (Bueno et al. 2015; Conte et al. 2019). Moreover, this reduction of insecticide applications implies also a decrease in the management cost (Conte et al. 2019). Considering that if not controlled E. heros has the potential to cause 25% (US$ 215.6/ha; Figure 6.1*A*), prophylactic use of insecticides is still profitable (US$ 89.0/ha; Figure 6.1*B*). However, the adoption of IPM programs based on economic thresholds can reduce the management cost by 46.4% (US$ 47.7/ha; Figure 6.1C) in spite of the cost of monitoring insect pests at the recommended rate (Corrêa-ferreira 2012). However, guaranteed positive results for adopting this strategy depends on the accurate and reliable monitoring of the stink bug pests, however monitoring using beat cloth can be very laborious in large scale crops such as soybean (Lefebvre et al. 2014; Parra 2014; Bueno et al. 2015). Thus, pheromone traps and precision agriculture can reduce the monitoring efforts and favor the adoption of larger soybean-IPM programs as well (Parra 2014; Godfray 2015; Pretty et al. 2018). Other measures like the prohibition of insecticides and fungicides mixtures for the control of *E. heros* will also help to reduce the preventive applications of insecticides in the early season. This scenario with reduced use of insecticides not only will reduce selection of insecticide resistant populations of stink bugs, but also allow the integration of biological control programs using *T. podisi* that are already available for the control of *E. heros* (Bortolotto et al. 2015; Koppert 2019). Nevertheless, effective IPM programs for *E. heros* that integrate chemical and biological control in soybean agroecosystem are not available to date, but selectivity could be accomplished in part through effective timing of insecticide applications. Taking the example of Arizona cotton agroecosystem, growers are encouraged to use partially selective insecticides in scenarios of large immigrating populations of *B. tabaci* (Naranjo and Ellsworth 2009). When the residual activities of the insecticides have decreased to levels that make biological control possible, biological control agents are released providing suppression that has been measured up to 12 weeks in the cotton/whitefly agroecosystem. Assuming that soybean growers will adopt similar strategies, the cost of integrating chemical and biological control will be near US$ 55.0/ha (Figure 6.1D). To achieve this, development of extension guidelines for soybean agroecosystem are required, along with extensive educational programs to support the implementation of IPM with decision support tools like monitoring and thresholds (Naranjo and Ellsworth 2009).

6.2.2 Combination of RNAi and Biological control for the control of *E. heros*

Potential risks of RNAi to *T. podisi*

To achieve more sustainable pest control, RNAi-based insecticides should work synergistically with other control tactics such as biological control (Romeis et al. 2019). Accordingly, in **Chapter 5** the potential risks of *E. heros* active dsRNA to its egg parasitoid *T. podisi* were evaluated. For insecticidal dsRNA to present risks to *T. podisi*: (i) the parasitoid has to ingest the dsRNA (physical exposure), (ii) be susceptible to ingested RNAi (physiological exposure) and (iii) dsRNA should trigger harmful unexpected phenotypic changes (hazard) (Lundgren and Duan 2013; Casacuberta et al. 2015; Roberts et al. 2015). As such, the likelihood of physical and physiological exposure and the potential hazards posed by RNAi were assessed in this study. First, parasitoid adults could be exposed through consumption of pollen or nectar containing dsRNA (Bachman et al. 2013, 2016; Roberts et al. 2015; Rusch et al. 2017). While the immature stages of *T. podisi* could be exposed through host eggs sprayed with dsRNA and probably through eggs laid by females treated with dsRNA. Second, *T. podisi* adults are sensitive to dietary RNAi when ingesting its own dsRNA, as demonstrated by reduced survival and parasitism. Gene expression analysis confirms the activity of specific dsRNA and the phenotypic results from the feeding assays. Third, no adverse effects were observed in either the survival or parasitism of adult parasitoids that were exposed to high concentration of *E. heros* active dsRNA. Moreover, the development of *T. podisi* larvae was normal inside *E. heros* eggs laid by females injected with dsRNA. Based on the lack of lethal or sublethal effects on *T. podisi*, we could already conclude that impact of *E. heros* active dsRNAs on its egg parasitoid is negligible.

However, to achieve a better understanding, potential hazards inherent to RNAi, off-target gene silencing and immune stimulation were also assessed. Despite numerous genes in the *T. podisi* transcriptome sharing one or more 15-20 nt matches with the 21 nt siRNA sequences of the *E. heros* active dsRNA, the expression levels of these potential off-target genes were not downregulated. As expected by the role of RNAi in antiviral defense (Shabalina and Koonin 2008; Dowling et al. 2016; Schuster et al. 2019), non-specific dsRNA induced the expression of core RNAi genes in *T. podisi*. Fortunately, this immune stimulation did not result in significant trade-offs with the reproduction or development of this parasitoid (Ahmed et al. 2002; Ahmed and Hurd 2006; Freitak et al. 2007; Schwenke et al. 2016). These findings provide a solid evidence base for the possibility of integrating RNAi-based insecticides and biological control using the parasitoid *T. podisi* inside IPM programs in the future. Nevertheless, due to the sequence-dependent mode of action of RNAi evaluation of the potential risks of insecticidal dsRNA should be assessed in case-by-case basis (Fishilevich et al. 2016b; Christiaens et al. 2018a; Taning et al. 2019). It is important to highlight that phylogenetically closely related species

inside the Pentatomidae family are potentially in a higher risk than *T. podisi* (Whyard et al. 2009; Lundgren and Duan 2013; Bachman et al. 2013, 2016; Roberts et al. 2015). For instance, activity of *DvSfn7* was only evident in a group of beetles within the Galerucinae subfamily of Chrysomelidae since the *Snf7* sequence diverges rapidly and the divergence of the sequence can be seen at the subfamily and tribal level (Bachman et al. 2013). The results from this study provide guidance for future RNAi risks analysis since include not only assessment of lethal and sublethal effects, but also potential hazards linked particularly with dsRNA exposure. Our results agree with the assumption that a minimum of 21 nt continuous matches are required to trigger RNAi response in insects (Bolognesi et al. 2012; Bachman et al. 2013, 2016; Tan et al. 2016; Pan et al. 2016, 2017; Fishilevich et al. 2016b). However, there is a need for a more systematic analysis that evaluate sequence specificity in the siRNA pathway to improve the prediction power of bioinformatic analysis and facilitate ecological risk assessment of NTOs in the future (see Chapter 5 for details). In addition, incorporation of foraging behavioral responses and mobility of natural enemies should improve risk assessments that extrapolate findings from laboratory assays to more realistic biological control in field conditions (Desneux et al. 2007; Cloyd and Bethke 2011; Teder and Knapp 2019). Moreover, indirect effects in the arthropod communities inside agroecosystems that could occur as a consequence of changes in the crop management or arthropod food webs (Romeis et al. 2019). As any other effective pest management practice, it is expected that RNAi-based insecticides modify the target herbivore community (Romeis et al. 2019). Therefore, future studies in the dynamic of ecological interactions associated with the host quality and availability are required to assess the impact of RNAi-based insecticides on arthropods communities (Romeis et al. 2008b; Auer and Frederick 2009).

Importance of integrating RNAi and biological control within the framework of IPM

Regardless of the effectiveness and selectivity that RNAi-based insecticides can achieve, they should represent only a tactic that must be integrated into IPM programs, rather than a stand-alone insect control measure (Kennedy 2008; Frisvold 2009; Taning et al. 2019; Anderson et al. 2019). The use of insecticidal RNAi would increase natural enemy populations and enhance the suppression of pests that they provide, even in the case that RNAi is used as the only management tool for stink bug control. Due to the lack of adverse effects of RNAi-based control in natural enemies (Bachman et al. 2013, 2016; Haller et al. 2019; **Chapter 5**) and the potential reduction or elimination of broad-spectrum insecticides targeting stink bugs. This was already illustrated by the area-wide adoption of Bt-transgenic varieties, that also have a limited spectrum of activity and that have led to substantial reductions of pesticide use (Kennedy 2008; Romeis et al. 2008b, 2019; Storer et al. 2008; Anderson et al. 2019). However, as was the case with Bt-crops overreliance on RNAi-based insecticides without appropriate IPM practices can lead to problems with secondary pests, resistance to RNAi and

the durability of the technology may fail (Kennedy 2008; Naranjo et al. 2008; Naranjo 2011; Anderson et al. 2019). Consequently, to generate more effective and sustainable results, the adoption of a well-structured IPM approach that combines multiple management strategies, monitors pests and natural enemies and uses thresholds for decisions is required (Baum and Roberts 2014; Barzman et al. 2015; Lamichhane 2017; Stenberg 2017; Anderson et al. 2019). By adopting principles of IPM, the risk of secondary pest outbreak will be reduced and the resistance development to RNAi will be delayed. Furthermore, the costs associated with management will be higher in the hypothetical scenario that RNAi is used alone (US$ 70.5/ha; Figure 6.1*E*) in comparison with the using RNAi in combination with biological control (US$ 60.7/ha; Figure 6.1*F*). Not only

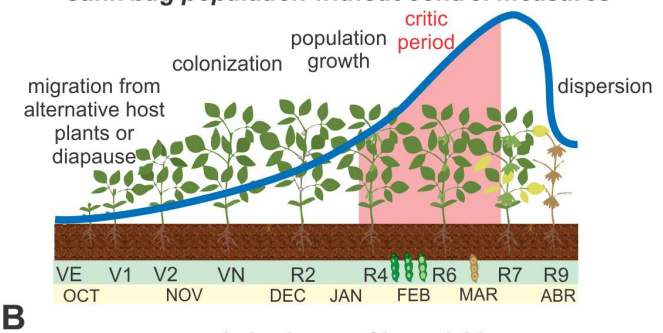
that, the favorable selectivity profile of RNAi-based insecticides will favor the establishment of inoculative releases of *T. podisi* and other biological control agents, but also will provide an environment that allows biological control by a diverse community of natural enemies (Bale et al. 2008; Gurr and You 2016; Holland et al. 2016; Perović et al. 2018; Romeis et al. 2019). Further knowledge and understanding of the RNAi technology, pest, crop, regional differences, alternative control tactics and even social context are critical for the successful integration of RNAi-based insecticides into IPM programs.

6.2.3 Future perspectives for sustainable control of *E. heros*

E. heros adaptation to effective IPM strategies and dispersion to new regions due to climate change or accidental long-distance transfer may challenge the sustainable control of this pest in the future (Onstad et al. 2003; Godfray et al. 2010; Pretty et al. 2018). IPM design, testing and practical implementation must be constantly evolving for better approaches that combine landscape design, host plant resistance, biological control, RNAi-based insecticides and other feasible tactics for pest management (Onstad et al. 2003; Godfray et al. 2010; Birch et al. 2016). These tactics could include new technologies with the potential to suppress evolving insect populations with high specificity (Lombardo et al. 2016; Douglas 2018). For instance, expression of protein toxins (e.g., lectins, protease or amylase inhibitors, chimeric toxins, etc) and genome editing using Clustered Regularly Interspaced Palindromic Repeats (CRISPR) offer the alternative to implement novel plant resistance traits against hemipterans and probably *E. heros* (Chougule and Bonning 2012; Macedo et al. 2015; Lombardo et al. 2016; Gowda et al. 2016; Douglas 2018). Genetic modification of secondary metabolite pathways or the use of plant resistance genes (R genes) offer the promise of specific toxicity or deterrence to insect pests (Douglas 2018). In addition, CRISPR-based genetic technology can be used for mass production and release of sterile males to mate with wild females that then produce inviable embryos (Scott et al. 2018; Kandul et al. 2019).

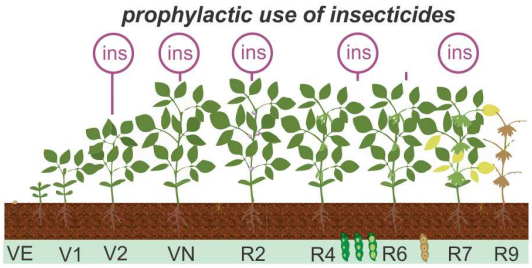
Furthermore, sustainable soybean production not only depends on the control of *E. heros*, thereby IPM programs in the future should address multiple pest species including insects/arthropods, pathogens and weeds (Kennedy 2008; Barzman et al. 2015;

A



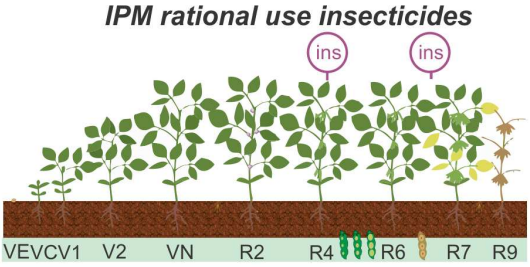
Estimated economical losses caused by *Euschistus heros* per hectare when no controlled[a]	**US$ 215.6**

B



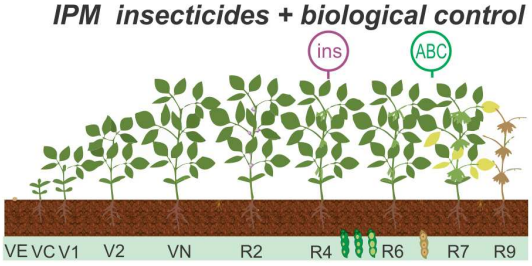
Insecticide/ha[b]	US$ 9.3
Spraying insecticide/ha[c]	US$ 8.9
Total	**US$ 89.0**

C



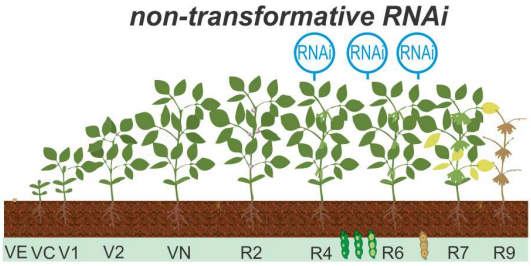
Pest monitoring/ha[d]	US$ 12.1
Insecticide/ha[b]	US$ 9.3
Spraying insecticide/ha[c]	US$ 8.9
Total	**US$ 47.7**

D



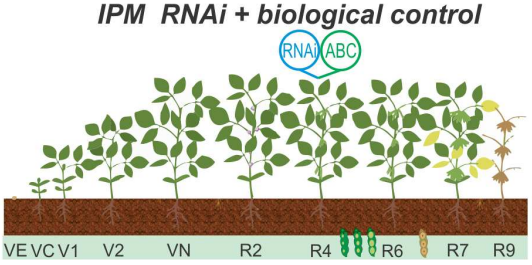
Pest monitoring/ha[d]	US$ 12.1
Insecticide/ha[b]	US$ 9.3
Spraying insecticide/ha[c]	US$ 8.9
Telenomus podisi/ha[e]	US$ 16.6
Release *Telenomus podisi*/ha[f]	US$ 8.9
Total	**US$ 55.0**

E



RNAi-based insecticide/ha[g]	US$ 15.0
Spraying RNAi/ha[h]	US$ 8.9
Total	**US$ 70.5**

F



Pest monitoring/ha[d]	US$ 12.1
RNAi-based insecticide/ha[g]	US$ 15.0
Spraying RNAi/ha[h]	US$ 8.9
Telenomus podisi/ha[e]	US$ 16.6
Release *Telenomus podisi*/ha[f]	US$ 8.9
Total	**US$ 60.7**

Figure 6.1: Scenarios for the management of Euschistus heros in Brazilian soybean crops. (***A***) stink bug population without control measures based on Saran (2008); (***B***) prophylactic use of insecticides based on Bueno et al. (2015) and Conte et al. (2019); (***C***) rational use of insecticides inside IPM programs based on Bueno et al. (2015) and Conte et al. (2019); (***D***) hypothetical scenario for the integration of chemical and biological control inside IPM programs; (***E***) hypothetical scenario use of RNAi-based insecticides as unique form of control; (***F***) hypothetical scenario for the integration of RNAi-based insecticides and biological control inside IPM programs. ^{a} Estimated yield losses caused by *E. heros* when not controlled (Oliveira et al. 2014). ^{b} Average cost of insecticides and adjuvants in Parana, Brazil (Conte et al. 2019). ^{c} Average operational cost of insecticide applications in Parana, Brazil (Conte et al. 2019). ^{d} Estimated labor cost of agricultural technician for monitoring insect pest once a week following recommended protocol (Corrêa-ferreira 2012), based on the minimum salary per hour approved by the Federal Association of agricultural Technicians (https://www.fenata.com.br/site/index.php/01-referencias). ^{e} Cost of producing 5000 parasitoid wasps per day (Chapter 3). ^{f} Assuming same price for application of insecticides. ^{g} Estimated cost of RNAi-based insecticides based on the rate of application 10g/ha (Zotti et al. 2018), cost of production of dsRNA US$ 1.0/g (Taning et al. 2019) and 33% formulation and production cost. ^{h} Assuming same price for application of insecticides.

Lamichhane et al. 2017). To reduce the pesticide applications, alternative methods for the control of whiteflies, armyworms that are not controlled by Bt toxins, the Asian soybean rust and weeds are also required. Of particular concern are sustainable and durable tactics for weed control that replace the use of herbicide tolerant transgenic plants which resulted in increased used of herbicides and resistant weeds (Heap 2014; Lucio et al. 2019; Anderson et al. 2019). Other specific solutions are needed to reduce the intensive use of land and the absence of crop rotation that also threaten the sustainability of soybean production in Brazil (Godoy et al. 2015).

To achieve desired social, economic and environmental benefits of IPM programs in the future is critically needed a joint effort among scientist, governments, label organizations, growers, grower associations, and the seed and pesticide industries (Barratt et al. 2018; Anderson et al. 2019). Collaboration and communication among stakeholder will help to promote continued research, expand implementation and highlight the value of using IPM approach for enhance the sustainability of soybean production (Birch et al. 2016; Lamichhane et al. 2017; Anderson et al. 2019).

6.3 Conclusions

Sustainable control of *E. heros* requires adoption of IPM programs that reduce the problems associated with the overuse of broad-spectrum conventional pesticides, including insecticide resistance, decreased effectiveness of biological control and risks to human health and the environment. The overall results of this PhD thesis suggest that biological control using *T. podisi* and RNAi-based insecticides are feasible control tactics that can be used in combination to enhance the durability and versatility of IPM programs in the future.

Appendices

Chapter 2

Appendix 2. 1: Summary of the non-linear regression analyses of the curves shown in Figure 2.7*A,C*

Estimated parameters*	**Strain**	**Fecundity (Figure 2.7*A*)** Value (95 % CI)	t-value	*P*	**Fertility (Figure 2.7*C*)** Value (95 % CI)	t-value	*P*
a	lmiSusc	564.9 (494.2 - 635.5) a	16.2	<0.0001	153.2 (137.7 - 168.7) a	20.4	<0.0001
	lmiLabSel	272.4 (241.4 - 303.5) c	17.9	<0.0001	58.0 (47.2 - 68.8) c	11.1	<0.0001
	lmiRes	362.1 (337.5 - 386.6) b	30.1	<0.0001	90.5 (76.9 - 104.0) b	13.8	<0.0001
b	lmiSusc	1.27 (1.00 - 1.54) a	9.6	<0.0001	0.83 (0.74 -0.93) a	17.8	<0.0001
	lmiLabSel	1.00 (0.83 - 1.18) a	11.9	<0.0001	0.83 (0.65 -1.01) a	9.5	<0.0001
	lmiRes	1.00 (0.90 - 1.10) a	20.1	<0.0001	0.86 (0.71 - 1.01) a	12.0	<0.0001
c	lmiSusc	57.4 (33.7 - 81.3) a	4.9	<0.0001	18.9 (16.5- 21.4) a	16.0	<0.0001
	lmiLabSel	29.3 (23.6 - 35.1) ab	10.4	<0.0001	21.0 (15.9 - 26.0) a	8.6	<0.0001
	lmiRes	28.5 (25.3 - 31.7) b	17.9	<0.0001	15.8 (12.7 - 18.9) a	10.4	<0.0001

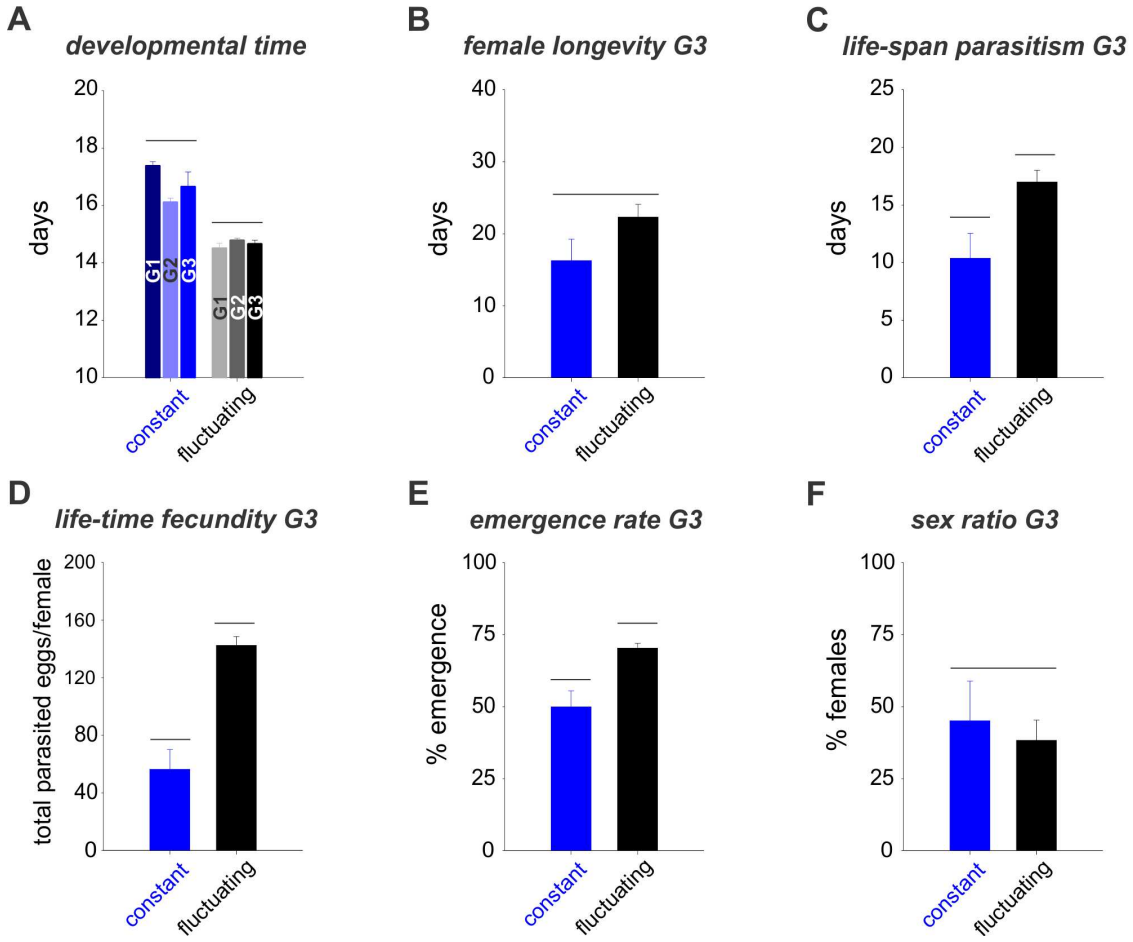
*Coefficients from the three parameters log-normal model $y = \frac{a}{x} exp[-0.5(\frac{ln\frac{x}{c}}{b})^2]$. The parameters characterize different attributes of the curves, where a is the maximum value of the dependent variable, b is the location of the peak response value on the time axis, and c is the skewness (or rate of change) of the response as a function of time. Parameter values followed by different letters in the columns were significantly different (based on non-overlapping of confidence limits).

Appendix 2. 2: Summary of the non-linear regression analyses of the curves shown in Figure2.7*B,D*

Estimated parameters*	**Strain**	**Fecundity (Figure 2.7*B*)** Value (95 % CI)	t-value	*P*	**Fertility (Figure 2.7*D*)** Value (95 % CI)	t-value	*P*
a	ImiSusc	506.2 (497.8 - 514.7) a	119.0	<0.0001	103.2 (102.5 - 103.9) a	277.6	<0.0001
	ImiLabSel	222.6 (220.5 - 224.7) c	210.0	<0.0001	36.5 (36.0 - 37.0) c	150.4	<0.0001
	ImiRes	293.3 (291.4 - 295.1) b	307.9	<0.0001	62.2 (61.8 - 62.6) b	285.7	<0.0001
c	ImiSusc	0.0157 (0.0152 - 0.0162) a	65.5	<0.0001	0.0471 (0.0459 - 0.0484) a	73.3	<0.0001
	ImiLabSel	0.0294 (0.0286 - 0.0301) b	77.6	<0.0001	0.0519 (0.0491 - 0.0546) b	37.2	<0.0001
	ImiRes	0.0300 (0.0295 - 0.0306) b	111.9	<0.0001	0.0588 (0.0570 - 0.0606) c	65.0	<0.0001

* Coefficients from the exponential rise to maximum model $y = a(1 - e^{-cx})$. The parameters characterize different attributes of the curves, where a is the maximum value of the dependent variable, and c is the skewness (or rate of change) of the response as a function of time. Parameter values followed by different letters in the columns were significantly different (based on non-overlapping of confidence limits

Chapter 3



Appendix 3. 2: Generational effects of rearing temperature conditions [fluctuating (30 ± 2 °C during light phase and 20 ± 2 °C at dark phase) and constant (25ºC)] on life history traits of *Telenomus podisi*. Developmental time (***A***), female longevity (***B***), life-span parasitism (***C***), life-time fecundity (***D***), emergence rate (***E***) and sex ratio (***F***). Bars represent the mean (± standard error) observed data and the ones grouped with the same horizontal line are not statistically different at the 5 % significance level according to a Tukey's HSD test.

Appendix 3. 2: Summary of the non-linear regression analyses of the curves shown in Figure 3.2*A,C* and Figure 3.3*B*.

Estimated parameters*	Rearing temperature conditions	Fecundity (Figure 3.2*A*)			Fertility (Figure 3.2*C*)			Daily female offspring (Figure 3.3*B*)		
		Value (95 % CI)	t-value	*P*	Value (95 % CI)	t-value	*P*	Value (95 % CI)	t-value	*P*
a	Constant	13.28 (10.22–16.35) a	8.92	<0.0001	6.93 (4.72–9.16) a	6.52	<0.0001	3.46 (2.23–4.69) a	5.82	<0.0001
	Fluctuant	17.14 (14.79–19.49) a	14.81	<0.0001	12.67 (10.81–14.52) b	13.81	<0.0001	13.53 (11.17–15.89) b	11.72	<0.0001
b	Constant	0.150 (0.124–0.177) a	11.76	<0.0001	0.173 (0.129–0.216) a	8.25	<0.0001	0.124 (0.085-0.164) a	6.46	<0.0001
	Fluctuant	0.112 (0.010–0.123) b	20.18	<0.0001	0.115 (0.102–0.127) b	18.89	<0.0001	0.166 (0.145-0.187) a	16.14	<0.0001

*Coefficients from the exponential decay model $y = a{\cdot}e^{(-bx)}$. The parameters characterize different attributes of the curves, where a is the initial value of the dependent variable, b is the skewness (or decay rate) of the response as a function of time. Parameter values followed by different letters in the columns were significantly different (based on non-overlapping of confidence limits)

Appendix 3. 3: Summary of the non-linear regression analyses of the curves shown in Figure 3.2*B,D* and Figure 3.3*C*.

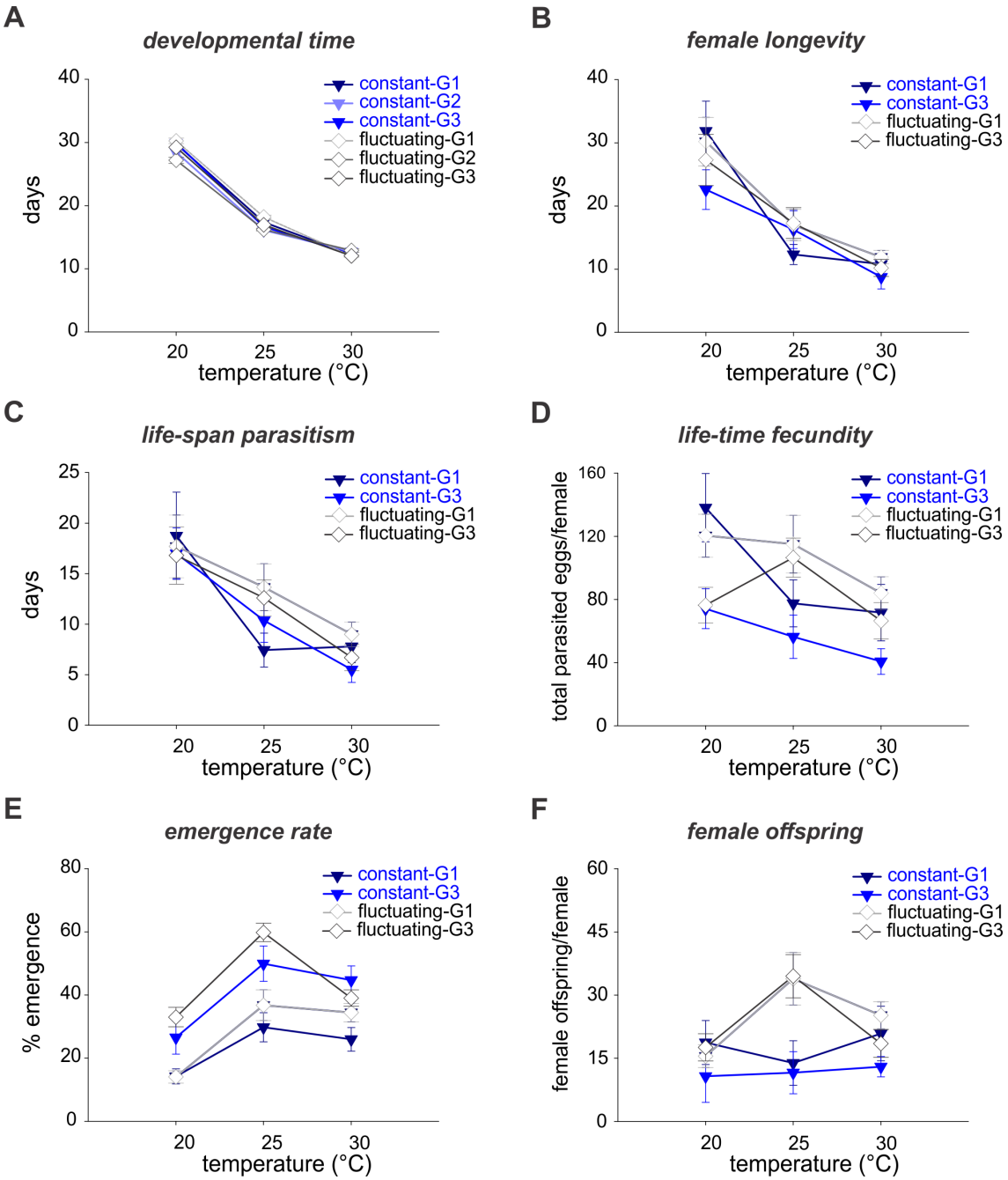
Estimated parameters*	Rearing temperature conditions	Cumulative fecundity (Figure 3.2*B*)			Cumulative fertility (Figure 3.2*D*)			Cumulative female offspring (Figure 3.3*C*)		
		Value (95 % CI)	t-value	*P*	Value (95 % CI)	t-value	*P*	Value (95 % CI)	t-value	*P*
a	Constant	158.7 (157.0–160.5) a	182.65	<0.0001	42.8 (42.3–43.3) a	169.71	<0.0001	29.96 (29.46–30.47) a	119.60	<0.0001
	Fluctuant	92.8 (91.7–93.9) b	170.82	<0.0001	115.3 (114.1–116.5) b	196.16	<0.0001	80.98 (80.64-81.33) b	476.10	<0.0001
b	Constant	0.097 (0.093–0.101) a	55.66	<0.0001	0.149 (0.141-0.157) a	37.45	<0.0001	0.132 (1.123-0.141) a	28.56	<0.0001
	Fluctuant	0.131 (0.124–0.137) b	42.37	<0.0001	0.101 (0.098–0.105) b	56.22	<0.0001	0.149 (0.146-0.152) b	104.96	<0.0001

*Coefficients from the exponential rise to maximum model $y = a(1 - e^{-bx}))$. The parameters characterize different attributes of the curves, where a is the maximum value of the dependent variable, and b is the skewness (or change rate) of the response as a function of time. Parameter values followed by different letters in the columns were significantly different (based on non-overlapping of confidence limits)

Appendix 3. 4: Results for the generational effect of rearing under fluctuating [30ºC during 12 hours (day) and 20ºC during 12 hours (night)] or constant (25ºC) temperature regimes on different life-history traits from full factorial model ANOVA.

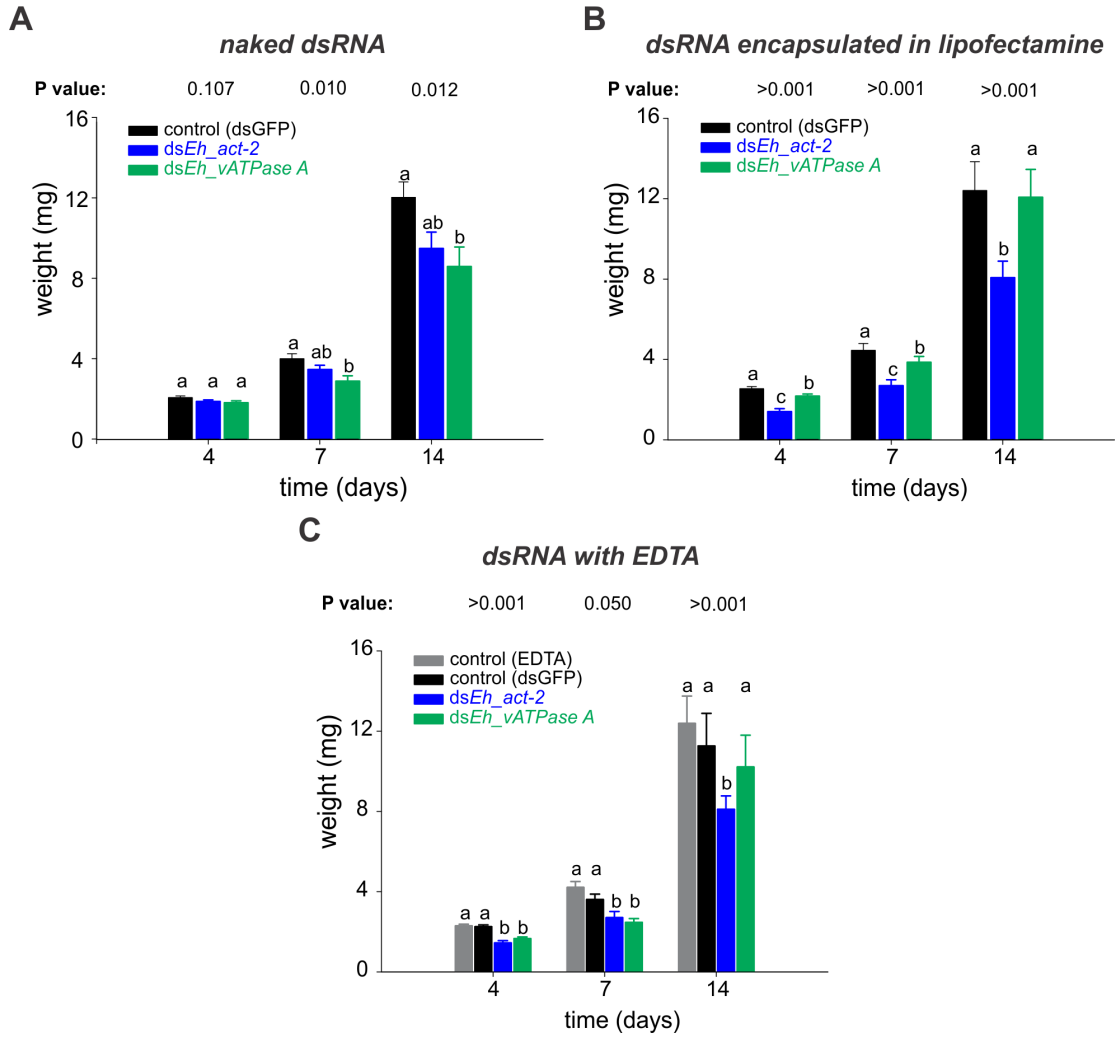
Life-history traits	**Rearing temperature condition (constant or fluctuating)**		**Test constant temperature (20°C, 25°C or 30°C)**		**Temperature rearing conditions X Test constant temperature)**	
	F	*P*	F	*P*	F	*P*
At generation G1						
Developmental time	2.163 $_{(1,52)}$	0,148	1748.946 $_{(2,52)}$	<0.001	1.745 $_{(2,52)}$	0.186
Life-span parasitism	1.095 $_{(1,55)}$	0.300	8.574 $_{(2,55)}$	<0.001	1.087 $_{(2,55)}$	0.345
Life-time fecundity	0.657 $_{(1,55)}$	0.421	5.208 $_{(2,55)}$	0.009	1.411 $_{(2,55)}$	0.253
% emergence	3.040 $_{(1,55)}$	0.087	16.018 $_{(2,55)}$	<0.001	0.828 $_{(2,55)}$	0.443
Female offspring	3.093 $_{(1,52)}$	0.085	1.137 $_{(2,52)}$	0.330	2.900 $_{(2,52)}$	0.065
At generation G3						
Developmental time	0.382 $_{(1,63)}$	0.539	1396.819 $_{(2,63)}$	<0.001	0.822 $_{(2,63)}$	0.445
Life-span parasitism	0.380 $_{(1,50)}$	0.541	12.252 $_{(2,50)}$	<0.001	0.153 $_{(2,50)}$	0.858
Life-time fecundity	6.715 $_{(1,50)}$	**0.013**	3.082 $_{(2,50)}$	0.056	1.825 $_{(2,50)}$	0.173
% emergence	1.200 $_{(1,50)}$	0.279	19.085 $_{(2,50)}$	<0.001	2.289 $_{(2,50)}$	0.113
Female offspring	19.226 $_{(1,44)}$	**0.005**	2.117 $_{(2,44)}$	0.134	2.237 $_{(2,44)}$	0.120

Degrees of freedom are given within parentheses. The table shows the F-ratio (F) with associated probability (*P*) for all traits



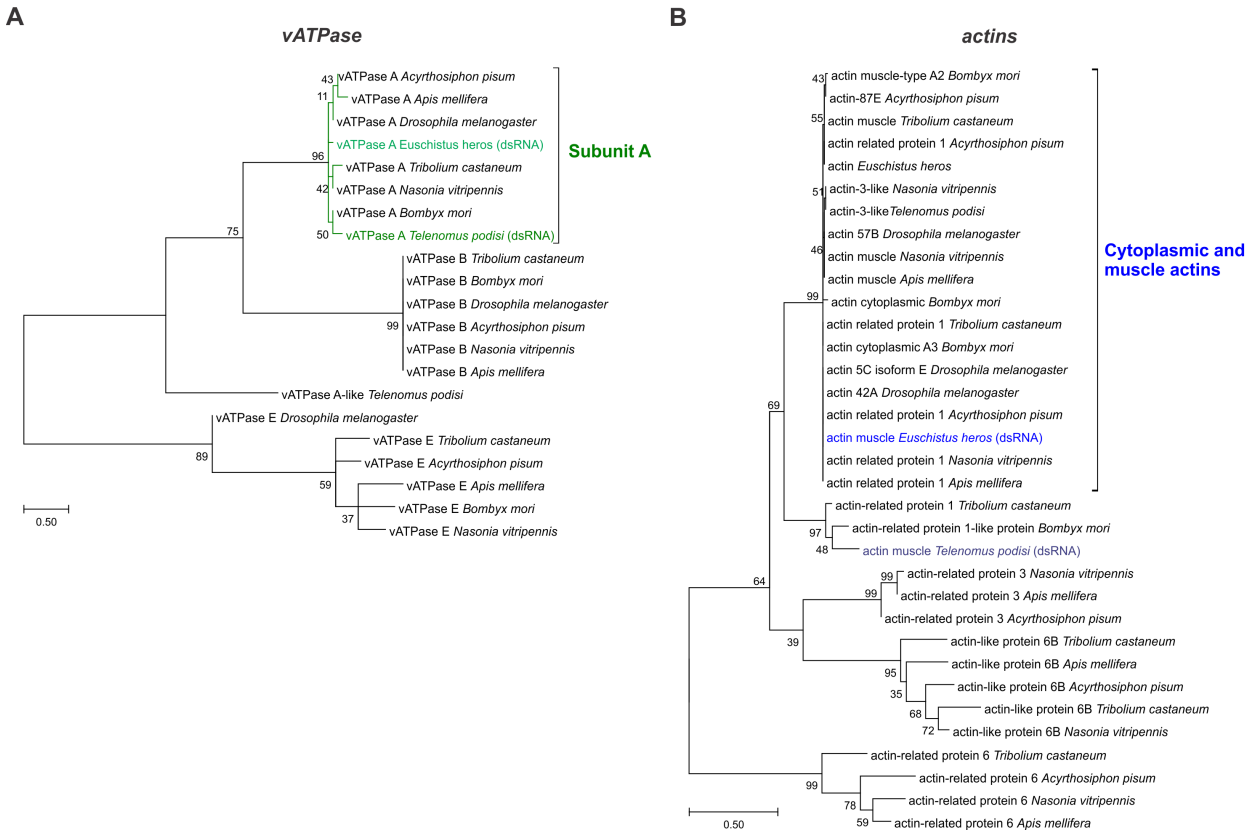
Appendix 3. 6: Generational effects of rearing temperature conditions [fluctuating (30 ± 2 °C during light phase and 20 ± 2 °C at dark phase) and constant (25ºC)] on *Telenomus podisi* thermal performance. Developmental time (***A***), female longevity (***B***), life-span parasitism (***C***), life-time fecundity (***D***), emergence rate (***E***) and female offspring (***F***).

Chapter 4

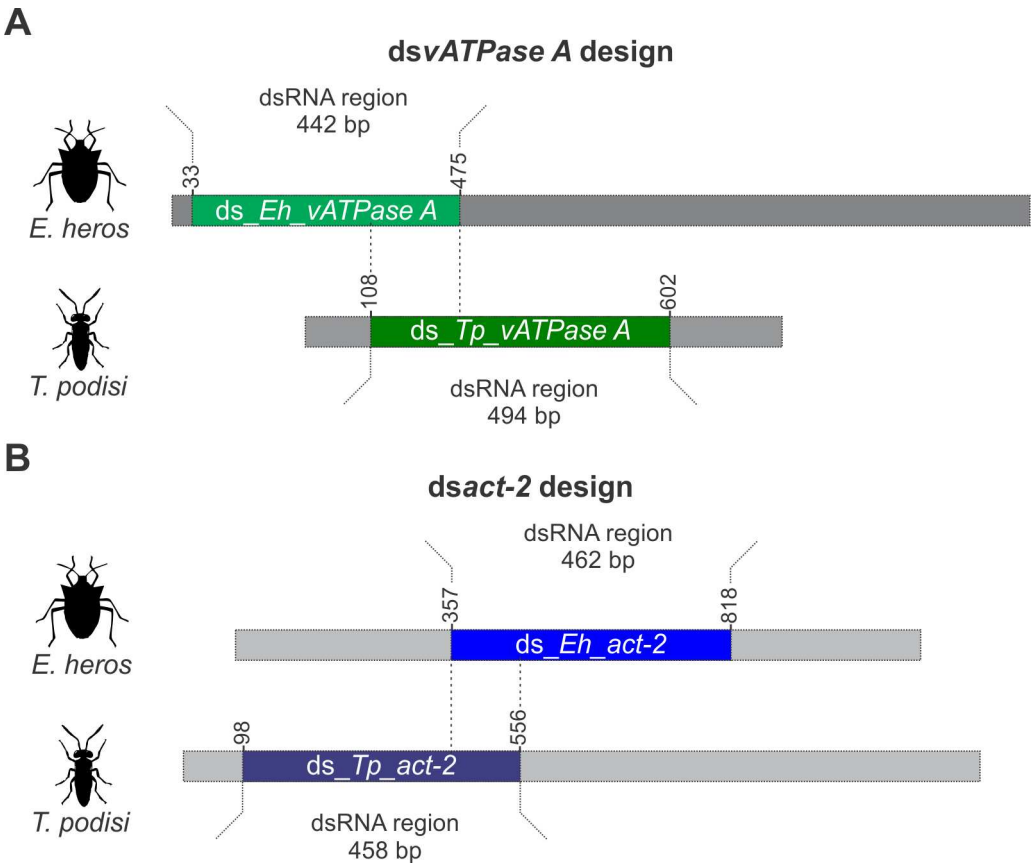


Appendix 4. 2: Weight of second instar nymphs of *Euschistus heros* after 4, 7 and 14 days of feeding experiments with dsRNA with different formulations of dsRNA: (***A***) naked dsRNA, (***B***) lipofectamine 2000, and (***C***) EDTA. Feeding with dsRNA targeting GFP was used as a control. Bars represent the mean observed data (± standard error). Bars with the same letter are not statistically different at the 5 % significance level according to a Tukey's HSD test.

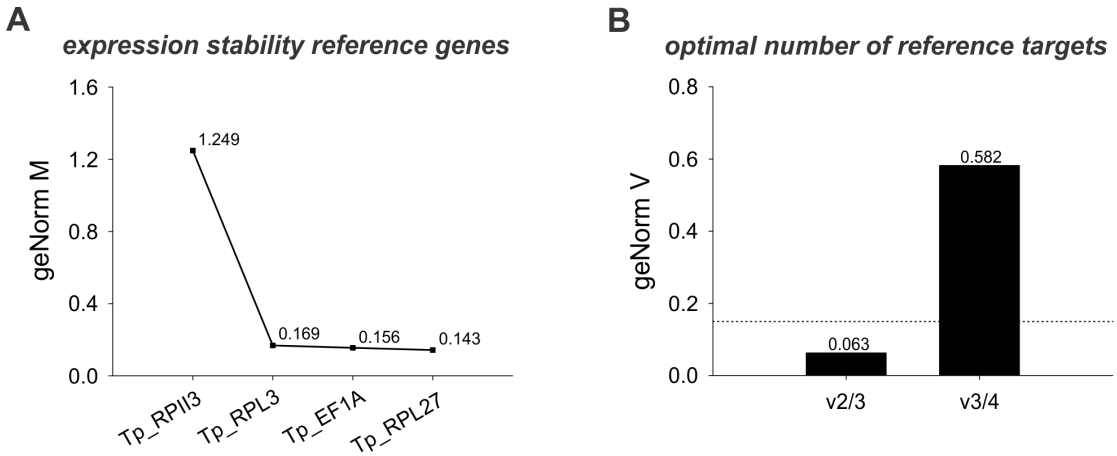
Chapter 5



Appendix 5. 2: Phylogenetic trees of the vATPase (***A***), and actin proteins (***B***). The phylogenetic trees were constructed using the Maximum Likelihood method with MEGA, version 7, software. The numbers associated with the branches refer to bootstrap values (confidence limits) resulting from 1000 replicate resampling.



Appendix 5. 3: Schematic representation of dsRNA design from the targeted insect pest *E. heros* and the non-target parasitoid *T. podisi*: (***A***) *vATPase A*, (***B***) *act-2*.



Appendix 5. 4: Ranking of candidate reference genes in *T. podisi* adults as calculated by geNorm after feeding of dsRNA. (***A***) mean expression stability (M), the least stable genes are represented on the left and the most stable on the right side of the plot; (***B***) pairwise variation (V) calculated to determine the optimal number of reference genes for normalization in *T. podisi* after feeding of dsRNA.

Appendix 5. 4: Predicted off-target genes of *E. heros* active ds*Eh_vATPase A* in the transcriptome of the non-target parasitoid *T. podisi*

***T. podisi* NCBI**	**Number of hits**																
Gap / Mismatch	**0**				**1**					**>1**							**Total**
length of hit	20	19	18	17	21	20	19	18	17	23	22	21	20	19	18	17	
GBEU01005918.1	2	2	1	3	9	9	6	3	2								37
GBEU01005925.1	2	2	1	4	9	8	5	1	2								34
GBEU01005924.1					3	2	2	1	9								17
GBEU01005916.1					3	2	2	1	7								15
GBEU01041559.1								8	2								10
GBEU01021593.1						2	2	2	2								8
GBEU01021596.1						2	2	2	2								8
GBEU01021597.1						2	2	2	2								8
GBEU01021598.1						2	2	2	2								8
GBEU01012239.1							3	2	2								7
GBEU01012242.1							3	2	2								7
GBEU01012244.1							3	2	2								7
GBEU01012245.1							3	2	2								7
GBEU01000120.1									7								7
GBEU01012942.1			4	2													6
GBEU01012943.1			4	2													6
GBEU01012945.1			4	2													6
GBEU01012947.1			4	2													6
GBEU01012948.1			4	2													6
GBEU01006921.1							4	2									6
GBEU01006920.1							4	2									6
GBEU01015354.1							4	1	1								6
GBEU01015362.1							4	1	1								6
GBEU01015363.1							4	1	1								6
GBEU01015907.1							3	2	1								6
GBEU01034300.1								5	1								6
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GBEU01017383.1								4	2								6
GBEU01016952.1								4	1		1						6
GBEU01016955.1								4	1		1						6
GBEU01016956.1								4	1		1						6
GBEU01027547.1								3	1		1	1					6
GBEU01008857.1									6								6
GBEU01008860.1									6								6
GBEU01008861.1									6								6
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GBEU01042367.1									6								6
GBEU01027055.1									5	1							6
GBEU01027053.1									5	1							6
GBEU01007172.1									4			1	1				6
GBEU01037688.1									3			2	1				6

***T. podisi* NCBI**	**Number of hits**																
Gap / Mismatch	**0**				**1**					**>1**							**Total**
length of hit	20	19	18	17	21	20	19	18	17	23	22	21	20	19	18	17	
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GBEU01011202.1							3	1	1								5
GBEU01011203.1							3	1	1								5
GBEU01011206.1							3	1	1								5
GBEU01010899.1				5													5
GBEU01021446.1				5													5
GBEU01021448.1				5													5
GBEU01024929.1				5													5
GBEU01002503.1								4	1								5
GBEU01002506.1								4	1								5
GBEU01004160.1								4	1								5
GBEU01004180.1								4	1								5
GBEU01018255.1								4	1								5
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GBEU01033122.1									5								5
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GBEU01038428.1									5								5
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GBEU01008177.1								4	1								5
GBEU01008178.1								4	1								5
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GBEU01009707.1									5								5
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GBEU01010294.1									5								5
GBEU01010295.1									5								5

***T. podisi* NCBI**	**Number of hits**																
Gap / Mismatch	**0**				**1**					**>1**							**Total**
length of hit	20	19	18	17	21	20	19	18	17	23	22	21	20	19	18	17	
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GBEU01010299.1									5								5
GBEU01010300.1									5								5
GBEU01010301.1									5								5
GBEU01010302.1									5								5
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GBEU01015604.1									5								5
GBEU01015605.1									5								5
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GBEU01023948.1									5								5
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GBEU01025257.1									5								5
GBEU01025258.1									5								5
GBEU01025261.1									5								5
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***T. podisi* NCBI**	**Number of hits**																
Gap / Mismatch	**0**				**1**					**>1**							**Total**
length of hit	20	19	18	17	21	20	19	18	17	23	22	21	20	19	18	17	
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GBEU01025441.1									5								5
GBEU01026393.1									5								5
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GBEU01026481.1									5								5
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GBEU01042568.1									4								4
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GBEU01044643.1									3								3
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GBEU01013388.1													3				3
GBEU01013390.1													3				3
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GBEU01013392.1													3				3
GBEU01013393.1													3				3
GBEU01023104.1													3				3

***T. podisi* NCBI**	**Number of hits**															
Gap / Mismatch	**0**				**1**					**>1**							**Total**
length of hit	20	19	18	17	21	20	19	18	17	23	22	21	20	19	18	17	
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GBEU01023107.1													3				3
GBEU01023108.1													3				3
GBEU01032044.1													3				3
GBEU01038768.1													3				3
GBEU01025023.1												2					2
GBEU01025025.1												2					2
GBEU01001329.1												1	1				2
GBEU01003777.1												1	1				2
GBEU01014652.1												1	1				2
GBEU01024330.1													2				2
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GBEU01025290.1													2				2
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GBEU01045897.1													2				2
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GBEU01035847.1												1					1
GBEU01035846.1												1					1
GBEU01014651.1												1					1

Appendix 5. 5: Predicted off-target genes of *E. heros* active ds*Eh_act-2* in the transcriptome of the non-target parasitoid *T. podisi*

***T. podisi* NCBI**	**Number of hits**											
gaps/mismatches	**0**	**1**					**>1**					**Total**
length of hit	17	21	20	19	18	17	21	20	19	18	17	
GBEU01002114.1	3	1	4	3	1	3						15
GBEU01002108.1	3	1	4	3	1	2						14
GBEU01037618.1						7	1	1				9
GBEU01015664.1				3	2	2						7
GBEU01015665.1				3	2	2						7
GBEU01015666.1				3	2	2						7
GBEU01024258.1				3	2	2						7
GBEU01024259.1				3	2	2						7
GBEU01024260.1				3	2	2						7
GBEU01037250.1		2	2	1	1							6
GBEU01037252.1		2	2	1	1							6
GBEU01037253.1		2	2	1	1							6
GBEU01003695.1				4	2							6
GBEU01005404.1				4	2							6
GBEU01014899.1					5	1						6
GBEU01014905.1					5	1						6
GBEU01033412.1					5	1						6
GBEU01007628.1					4	2						6
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