Harnessing the potential of peptides as bioinsecticides to control *Drosophila suzukii*

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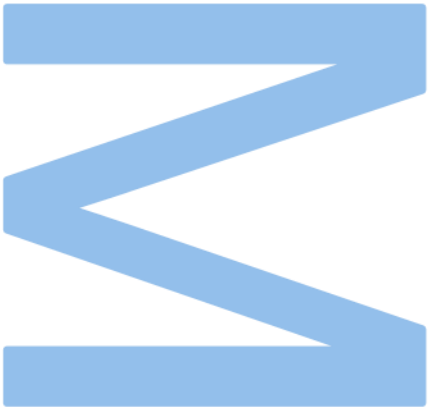
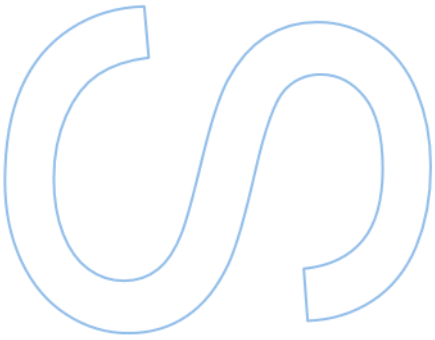
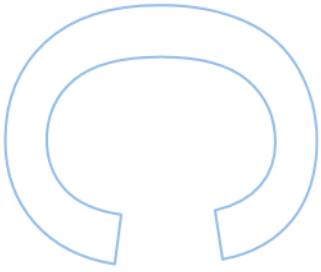
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Para o meu irmão Francisco.

Dedicated to my brother Francisco.

Sworn Statement

I, Laura Silva Ferreira Regalado, enrolled in the Master Degree in Applications of Biotechnology and Synthetic Biology at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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Laura Silva Ferreira Regalado

September 30th, 2022

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Resumo

Drosophila suzukii, também conhecida como drosófila da asa manchada, é uma das pragas de insetos mais importantes e ameaçadoras, encontrando-se em todas as principais regiões produtoras de fruta a nível mundial. Sendo polífago, este inseto alimenta-se em vários pequenos frutos, tais como mirtilo, framboesa, amora e morango como hospedeiros preferidos. As fêmeas têm a capacidade de pôr ovos em frutos maduros e não danificados, por disporem de um ovipositor fortemente esclerosado com a capacidade de perfurar a pele fina destes frutos. Os ovos eclodem e as larvas desenvolvem-se no interior do fruto, percorrendo-o e tornando-o não comerciável e inconsumível.

Atualmente, o controlo da drosófila da asa manchada baseia-se em práticas culturais e na aplicação de inseticidas de largo espetro, principalmente espinosinas, organofosforados, piretróides e nenonicotinóides. Contudo, estas estratégias revelaram-se ineficientes, havendo já registos de problemas de resistência adquirida a estes compostos, bem como de toxicidade associada a insetos não-alvo e mamíferos. A crescente consciência dos impactos ambientais e sanitários destes inseticidas encorajou a procura e estudo de novas moléculas inseticidas com novos alvos moleculares.

Neste contexto, os péptidos de veneno de aranha (PVAs) surgem como potenciais novas estratégias de controlo para gerir pragas de insetos, como a *D. suzukii*. Os PVAs são moléculas de estrutura complexa, suportada por múltiplas pontes de dissulfureto, que conferem a maior atividade inseticida, assegurando ao mesmo tempo uma maior estabilidade térmica e química. A partir de uma pesquisa bibliográfica, foram selecionados dois PVAs com base na sua eficiência contra insetos e em características estruturais: J-Atracotoxin-Hv1c, um péptido com 37 resíduos de aminoácidos e 4 pontes de dissulfureto; e o péptido μ-Theraphotoxin-Hhn2b, com 33 resíduos de aminoácidos e uma estrutura complexa estabilizada por 3 ligações de dissulfureto. Estes PVAs foram sintetizados por síntese peptídica em fase sólida, usando a estratégia Fmoc, e a sua conformação natural foi conseguida através do *folding* oxidativo.

A atividade oral dos PVAs contra *D. suzukii* foi avaliada para concentrações entre 2.23 e 223 μM para diferentes tempos de exposição. Maior mortalidade foi observada para o tratamento com J-ACTX-Hv1c a 111.5 μM durante 48 h. Em contraste, o tratamento correspondente com μ-TRTX-Hhn2b levou a um aparente aumento da sobrevivência das moscas em comparação com o controlo. Este efeito também foi observado para

moscas tratadas com 2.23 µM de J-ACTX-Hv1c, o que poderá ser uma evidência de hormese. A Análise de Componentes Principais (ACP) valida uma separação clara entre controlo e tratamento com PVAs, e entre ambos os tratamentos. Estes resultados correlacionam a expressão do gene *cyp12d1-d* com o tratamento de 111.5 µM µ-TRTX-Hhn2b (48 h), que pode estar ligado à desintoxicação deste péptido. Por outro lado, de acordo com a ACP, o tratamento com 111.5 µM J-ACTX-Hv1c (48 h) está associado à expressão do gene *hsc70-4*, podendo conferir alguma tolerância a este péptido.

A possibilidade de controlar *D. suzukii* por manipulação do seu microbiana intestinal foi avaliada com a aplicação de péptidos antimicrobianos (PAMs). Dos três PAMs (3.1, CA-M e RW-BP100) testados em análise por antibiograma contra os isolados microbianos *D. suzukii*, o RW-BP100 foi escolhido para prosseguir com os ensaios *in vivo*. A administração oral de RW-BP100 a 400 µM não teve efeito na sobrevivência das moscas de *D. suzukii*. A sequenciação parcial da região de 16s rDNA das bactérias isoladas das moscas de controlo e tratamento não mostrou diferença entre os grupos, o que pode explicar a falta de atividade *in vivo* do péptido RW-BP100. Uma mistura dos péptidos RW-BP100 com µ-TRTX-Hhn2b mostrou afetar negativamente a sobrevivência de *D. suzukii*.

Em conjunto, estes resultados realçam o potencial interesse dos péptidos em controlar a praga *D. suzukii*, suscitando questões sobre o seu modo de ação, conhecimento que, por sua vez, poderá ser utilizado para desenvolver formulações melhoradas destes péptidos de acordo com alvos específicos.

Palavras-chave: drosófila da asa manchada, *Drosophila suzukii*, J-Atracotoxin-Hv1c, manipulação de microbioma, péptidos antimicrobianos, péptidos de veneno de aranha, péptidos neurotóxicos, RW-BP100, µ-Theraphotoxin-Hhn2b

Abstract

Drosophila suzukii, also known as spotted-wing drosophila (SWD), is one of the most important and threatening insect pests worldwide, found in all major fruit-producing regions. Being polyphagous, this insect can feed on various small fruits, such as blueberry, raspberry, blackberry, and strawberry as preferred hosts. Female SWD has the ability to lay eggs in undamaged ripening and ripe red fruits due to having a heavily sclerotized ovipositor with the capacity to pierce the thin skin of these fruits. The eggs hatch and the larvae develop inside the penetrated fruit, and these larvae will scour the fruit and make it unmarketable and unconsumable.

Current management of SWD is based on cultural practices and on the application of broad-spectrum insecticides, primarily spinosyns, organophosphates, pyrethroids, and neonicotinoids. However, these strategies have been proved inefficient, and there are already reports of resistance-acquired problems to these compounds, as well as associated toxicity to non-target insects and mammals. The increased awareness of the real environmental and health impacts of these insecticides has encouraged the search and study of new insecticidal molecules with novel molecular targets.

In this context, spider venom peptides (SVPs) emerge as potential new control strategies to manage insect pests, such as *D. suzukii*. SVPs are molecules of complex structure, supported by multiple disulphide bridges, which impart the highest insecticidal activity while providing increased thermal and chemical stability. From a literature search, two SVPs were selected based on their reported efficiency against insects and structural features: J-Atracotoxin-Hv1c, a 37-residue peptide with 4 disulphide bridges; and μ-Theraphotoxin-Hhn2b, a peptide with 33 amino acid residues and a complex structure stabilized by 3 disulphide bonds. These SVPs were synthetized by solid-phase peptide synthesis (SPPS) using the Fmoc strategy, and their native conformation was successfully achieved by disulphide oxidation.

The oral activity of SVPs against *D. suzukii* was assessed for concentrations ranging from 2.23 to 223 μM at different times of exposure. Higher mortality was observed for treatment with J-ACTX-Hv1c at 111.5 μM for 48 h. In contrast, the corresponding treatment with μ-TRTX-Hhn2b led to an apparent increase in the flies' survival compared to the control. This was also observed for flies treated with 2.23 μM J-ACTX-Hv1c, which may be evidence of hormesis. The Principal Component Analysis (PCA) validates a clear separation between control and treatment with SVPs, and between both treatments. These results correlate the expression of gene *cyp12d1-d* with the treatment with 111.5

µM µ-TRTX-Hhn2b (48 h), which may be linked to peptide detoxification. On the other hand, according to PCA, treatment with 111.5 µM J-ACTX-Hv1c (48 h) is associated with the expression of *hsc70-4,* which may confer some tolerance to this peptide.

The possibility of controlling *D. suzukii* by gut microbiome manipulation was evaluated with an application of antimicrobial peptides (AMPs) to disrupt it. Of the three AMPs (3.1, CA-M, and RW-BP100) tested in antibiogram analysis against *D. suzukii* microbiome isolates, RW-BP100 was selected to pursue the *in vivo* experiments. Oral administration of RW-BP100 at 400 µM had no effect on *D. suzukii* flies' survival. The partial sequencing of the 16s rDNA region of bacteria isolated from control and treatment flies showed no difference between groups, which may explain the lack of RW-BP100 *in vivo* activity. A mixture of RW-BP100 with µ-TRTX-Hhn2b showed to negatively affect *D. suzukii* survival.

Altogether, these results highlight the potential interest of peptides to control the pest *D. suzukii*, raising questions about their mode of action, which ultimately may be used to develop improved target-specific formulations.

Keywords: antimicrobial peptides, *Drosophila suzukii*, J-Atracotoxin-Hv1c, microbiome manipulation, neurotoxic peptides, RW-BP100, spider venom peptides, spotted-wing drosophila, µ-Theraphotoxin-Hhn2b

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List of Abbreviations

2-CTC	2-Chlorotrityl chloride
AA	Amino acid
ABC	ATP-binding cassette
AC	Adenylyl cyclase
ac13E	Adenylyl cyclase 13E
ACN	Acetonitrile
Akt	Protein kinase B
AMP	Antimicrobial peptide
ARGK	Arginina kinase
ATP	Adenosine triphosphate
BK_{ca}	Large conductance calcium-activated potassium channel
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
Ca_v	Voltage-gated calcium channel
cDNA	Complementary DNA
CI	Cytoplasmic incompatibility
CRE	cAMP-response element
CREB	cAMP-response element binding protein
cyp12d1-d	Cytochrome P450 12d1 proximal
DCM	Dichloromethane
dcp-1	Death caspase-1
DDT	Dichlorodiphenyltrichloroethane
DIEA	N,N-diisopropylethylamine
DMF	Dimethylformamide
DODT	2,2′-(ethylenedioxy)diethanethiol
ef1a48d	Elongation factor 1 beta
EPPO	European and Mediterranean Plant Protection Organization
F2F	Farm to Fork
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	FAO statistical database
Fmoc	9-fluorenylmethoxycarbonyl
FoxO	Forkead box O transcription factors
foxo	Forkhead box, sub-group O
gaad45α	Growth arrest and DNA damage-inducible 45

Gd-HCl	Guanidinium hydrochloride
GNA	*Galanthus nivalis* agglutin
GPCR	G protein-coupled receptor
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
HBTU	*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HPLC	High-performance liquid chromatography
hsc70-4	Heat shock protein 70 cognate 4
HSP	Heat shock protein
hsp83	Heat shock protein 83
ICK	Inhibitory cystine knot
IIT	Incompatible insect technique
InR	Insulin-like receptor
IPM	Integrated pest management
K_v	Voltage-gated potassium channel
LC-MS	Liquid chromatograpgy – mass spectrometry
LPS	Lipopolysaccharides
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
MAPK	Mitogen-activated protein kinase
mpk2	p38a MAP kinase
MRS	de Man, Rogosa and Sharpe
Na_v	Voltage-gated sodium channel
NMR	Nuclear magnetic resonance
OD_{600}	Optical density at 600 nm
P450	Cytochrome p450
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RT	Room temperature
RT-qPCR	Real time - quantitative PCR
S6K	Ribosomal protein S6 kinase
SIT	Sterile insect technique
sod2	Superoxide dismutase 2
SPPS	Solid-phase peptide synthesis
SS-rich	Disulphide-rich
SVP	Spider venom peptide

SWD	Spotted-wing drosophila
TBP	TATA binding protein
^{t}Bu	Tert-butyl
TFA	Trifluoroacetic acid
th	Death-associated inhibitor of apoptosis 1
TIS	Triisopropylsilane
tor	Target of rapamycin
TRP	Tryptophan
TYR	Tyrosine
UV-Vis	Ultraviolet-visible
vg	Vestigial
VGSC	Voltage-gated sodium channel

1. Introduction

1.1. The worldwide problem of pests

Pest control is a problem that dates to the beginning of agriculture, with the first reports of crop control emerging in 1500 b.c..[1] The Food and Agriculture Organization of the United Nations (FAO) defines pest as "*any species, strain or biotype of plant, animal or pathogenic agent injurious to plants and plant products*"[2], and estimates that pests' impact account for an annual loss of 20 to 40% of global production.[3] These losses slow down the efforts to produce enough food to sustainably feed a growing world population, which is expected to grow from 7 to 9.7 billion (American System) by 2050, according to the United Nations Department of Economic and Social Affairs.[4] On another hand, the loss associated with pests in the agrifood industry pressures producers to increase the use of agrochemicals, with risks to the environment, to the food chain, and ultimately to consumers' health.

The emergence of new pests and the evolution of existing ones are being magnified by climate change and trade globalization, which hinder and effect pest management. For example, the increasing temperatures in geographical regions with usually colder climates, has a severe impact on the geographical distribution of insect pests, as numerous pests are becoming established across new regions, facilitated by international commodity trading.[5] Besides, this increase of temperature has also direct effects at the level of reproduction, survival, population dynamics, and their relationships with other species (e.g., hosts) and natural enemies.[6] Since insects are poikilothermic animals, whose body temperature depends on environmental temperature, these changes will have a significant effect on their behavior. Figure 1 shows a prediction for *D. suzukii* distribution under the representative concentration pathways (RCP) 4.5 and 8.5 (two distinct greenhouse gas concentration scenarios), indicating expansions of suitable area predominantly in the Northern Hemisphere due to ongoing climate change.[7]

Invasive insect pests have proven to be a severe problem as they culminate in drastic production and economic losses for producers, estimated at US$70 billion (in the American System) worldwide per year.[3] Although this value is considered to be underestimated due to a lack of studies compiling service estimates, it comprises costs linked with prevention (e.g., quarantine measures, ecological research, specialist training, and vaccination), damage associated to reduced abundance and range of native species, and response or follow-up to insect pests, namely damage repair, education and research, and control measures.[8] One of the insect pests that have been causing

severe production losses worldwide is *Drosophila suzukii*, a dramatic pest for small fruits and grapes in general. It is estimated that in the United States alone this pest comprises an economic loss of US$500 million annually.[9] Its impact in the agrifood industry in Portugal is yet to be quantified, but is extremely relevant to producers of red fruits and grapes (mostly in the North and Centre of Portugal). According to the Tridge,[10] a global trade platform on the agrifood industry, and to FAO statistical database (FAOSTAT),[11] Portugal ranks in the top producers of some small fruits (like raspberries and blueberries), and several leading regions in these fruits' production (e.g., Resende) are infested with this pest.

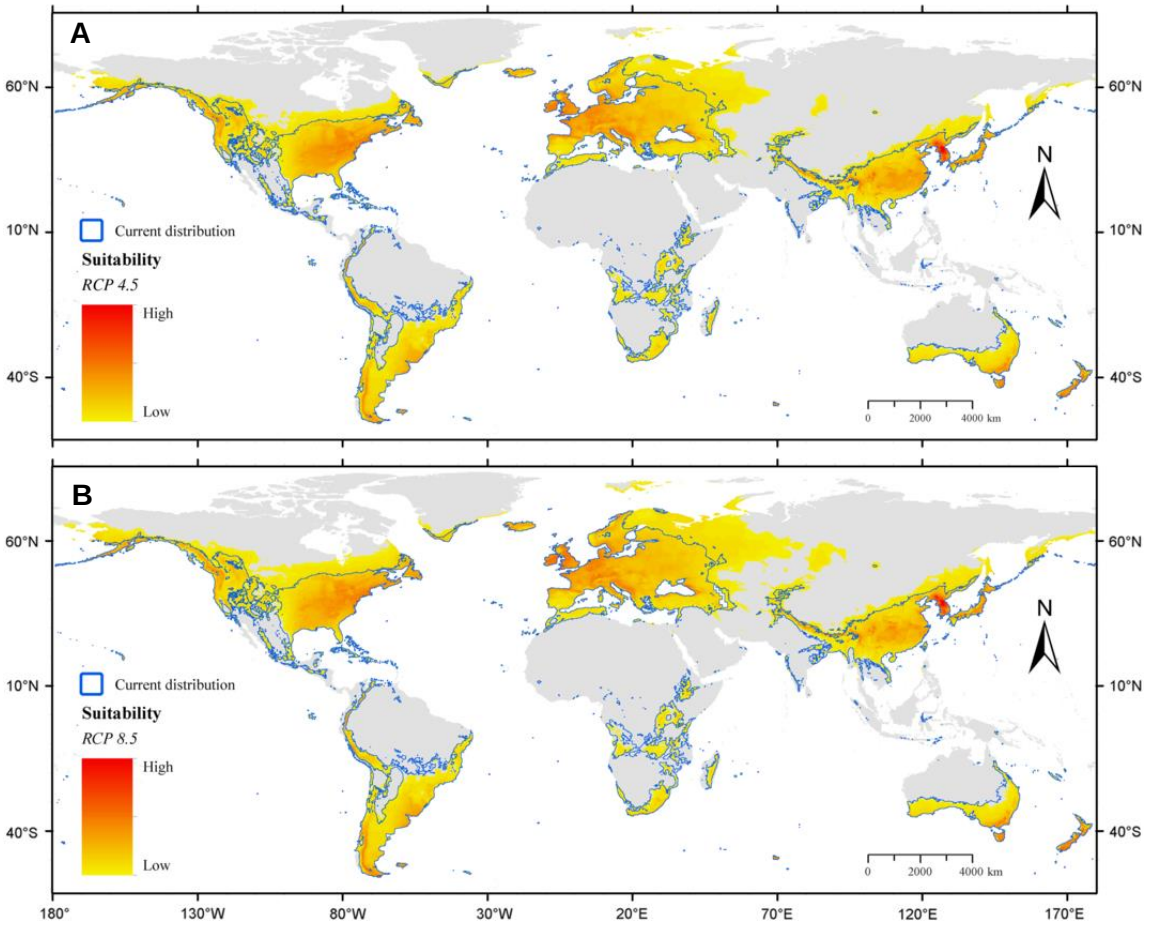


Figure 1 - Predicted habitat suitability for *Drosophila suzukii* in 2050 under A. low (RCP 4.5) and B. high (RCP 8.5) emission scenarios (image from Reyes and Lira-Noriega[7]).

1.2. Drosophila suzukii

Drosophila suzukii (Diptera: Drosophilidae), also known as spotted-wing drosophila (SWD), is one of the most relevant invasive pest species in the world.[12] SWD is a polyphagous species, as it feeds on several different small fruits, such as blueberry (*Vaccinium* spp.), raspberry (*Rubus* spp.), blackberry (*Rubus* spp.) and strawberry

(*Fragaria* spp.) as preferred hosts, and also fruits from the genera *Actinidia*, *Vitis* and *Ficus*, among others, although SWD is not considered a significant pest of these crops.[1, 12, 13] The fruit host choice is related to several factors, such as organic volatile compounds (VOCs) emitted by the fruit during the ripening process, and naturally occurring yeasts.[14, 15] In fact, there is evidence that *D. suzukii* is attracted to olfactory cues produced by food-associated microorganisms, like acetic acid, ethanol, ethyl acetate and acetoin.[16] In addition to olfactory signals, attraction is also mediated by visual traits like leaf color and reflectance, and size and shape of the fruit.[17]

Unlike other species of the same family, like *D. melanogaster*, SWD females have the singular ability to lay eggs in undamaged soft-skinned fruits, as they have a more developed ovipositor.[12] This organ consists of a strong and black saw of 30-36 teeth by which *D. suzukii* females are distinguishable from other *Drosophila* species (Figure 2).[13] The eggs develop into larvae inside the penetrated fruit, which will feed on the fruits' pulp, destroying it, and ultimately making it unconsumable and unmarketable (Figure 3).[13] In addition, the wounds created by oviposition in the fruit open the door to invasion by other insects, as well as bacterial and fungal infections, further increasing production losses, since these diseases can infect entire orchards if not controlled.[12, 19]

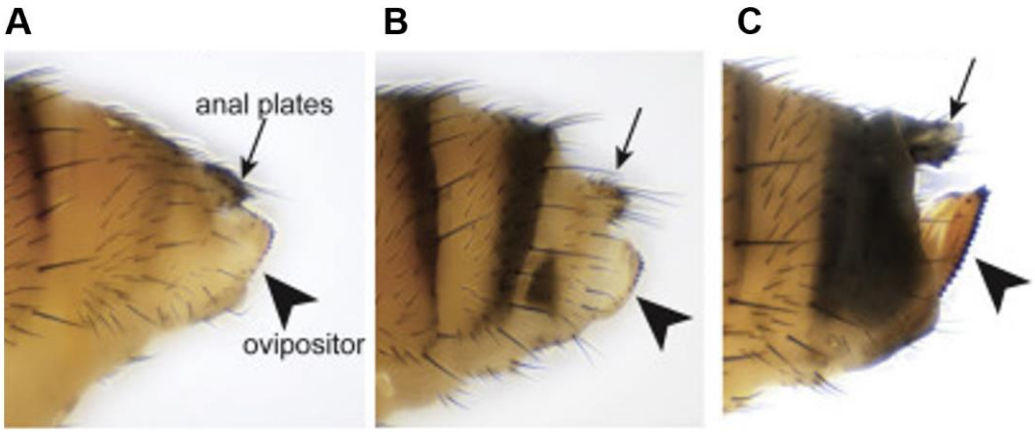


Figure 2 - Physiognomic differences between the ovipositors of A. *Drosophila melanogaster*, B. *Drosophila biarmipes*, and C. *Drosophila suzukii* (image from Green *et al.*[18]).

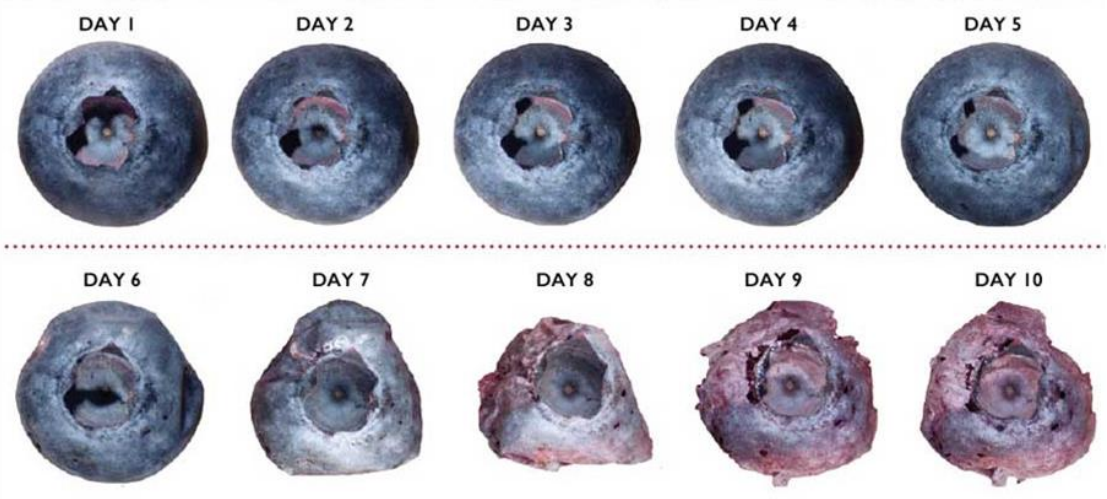


Figure 3 - Infestation of blueberries by *D. suzukii* larvae leads to the destruction, and loss of market value, of the fruits (image from Weinstock[20]).

SWD was first described in 1931 in Japan,[13] and in 2008 was observed in the USA, Spain and Italy, quickly disseminating throughout Europe in the last years (Figure 4), being currently part of the European and Mediterranean Plant Protection Organization (EPPO) A2 list as a quarantine pest.[21, 22] The ability of this species to establish itself in a wide geographic region is related to its tolerance to various climatic conditions.[23] This species benefits from the capacity of developing into an overwintering morphotype for several months beneath leaf litter and can also survive in the egg, larval and adult stages inside the fruit or transport containers.[24] In fact, *D. suzukii* can adapt to seasonal changes through some physiological and morphological adjustments.[25] In response to colder temperatures, the winter adults enter a state of reproductive dormancy, as females have



Figure 4 - Global distribution of *Drosophila suzukii*.[22] Created with mapinseconds.com.

undeveloped ovaries and males decrease sperm production.[26] Morphologically, the adults exhibit larger wings, allowing them to fly faster, develop a darker pigmentation, and have a greater cold tolerance. Most morphological changes are irreversible, but the ability to reproduce can be developed when the climatic conditions become favourable for this purpose.[23, 25]

1.2.1. Life cycle and phenotypic characteristics

During its life cycle, SWD goes through a complete process of metamorphosis, in which females can lay up to 600 eggs and around 40 per day.[12] After laying, the eggs take 12-72 h to hatch. Larvae go through three developmental stages, namely 1st, 2nd, and 3rd larval instar, characterized by a size increase, in a process that can take 5-7 days until puparium formation.[27] The metamorphic process is complete in 4 to 15 days after the beginning of the pupa stage (Figure 5). The rate of SWD development and reproduction is directly dependent on temperature and humidity. The whole metamorphic process can be completed in one to two weeks if the optimal conditions of 22-28°C and 80-90% humidity are ensured.[27, 28]

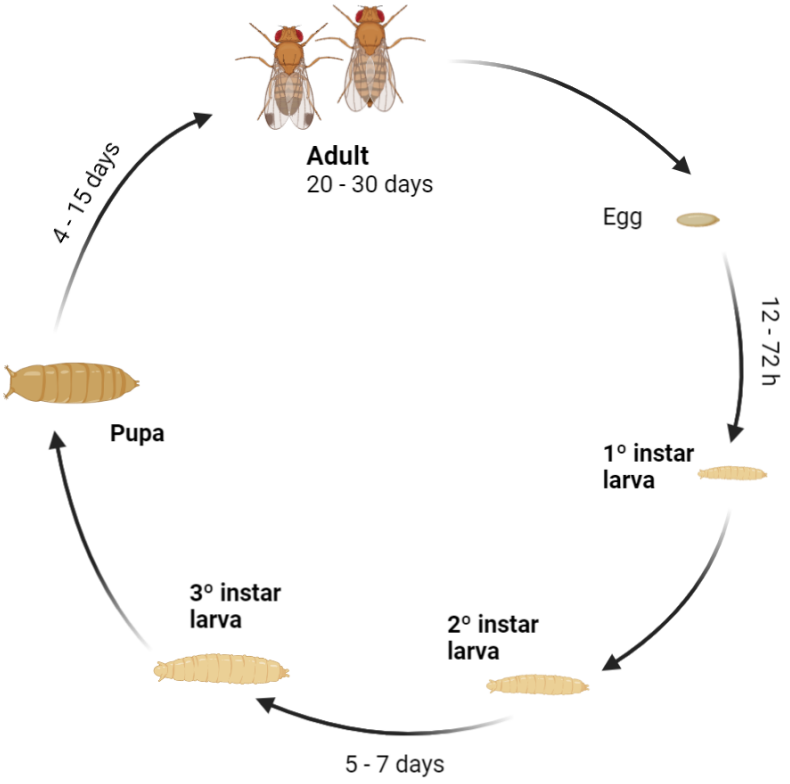


Figure 5 - *Drosophila suzukii* life cycle stages.[27] Created in BioRender.com.

The immature stages of SWD development occur inside the fruit, after oviposition beneath the fruit's skin. The pupa stage can be observed both inside or outside the fruit, depending on the infestation rate; the higher the rate, the greater the proportion of pupae

outside the fruit, as the larvae drop out of the fruit to the ground before reaching the next developmental stage, and tend to burrow the soil to ensure higher protection. As it develops inside the fruit, the immature stages are protected from the most diverse conditions, such as dehydration, rain, wind, and also to the application of insecticides. In the adult phase, the fly is more susceptible and unprotected.[27]

As *D. suzukii* is a sexually dimorphic species, some phenotypic differences between males and females allow to distinguish them, being the main one the presence of a dark spot at the top of each wing in males (Figure 6A); the position of the spot also allows to distinguish *D. suzukii* males from other *Drosophila* species.[13] Using a microscope, it is possible to observe the male sex combs on the first and second tarsomere of male's forelegs (Figure 6B), being displayed in one row only and oriented in parallel to the length of the tarsus; sex combs are not present in females. As described above, females have a strongly sclerotized ovipositor (Figure 6C). In terms of size, females are larger than males (2.5 to 4.0 mm compared to 2.0 to 3.5 mm, respectively).[13]

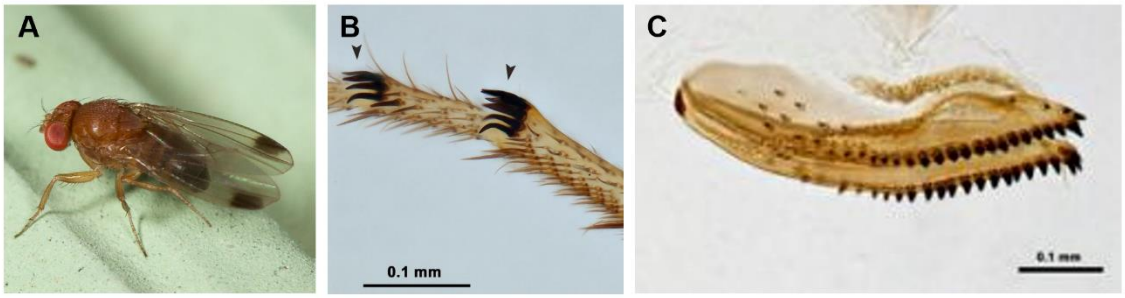


Figure 6 - *D. suzukii* physiognomy characteristics: A. spotted-wing male; B. female ovipositor; C. sex combs on male's forelegs (source: EPPO, 2022).

1.3. Insect pest control methods

1.3.1. Conventional control methods

Since the 1940s, insect pests have been controlled mostly with chemical insecticides such as dichlorodiphenyltrichloroethane (DDT), because of their fast action, low production cost, and high effectiveness.[29] It was only decades after implementation that the problems associated with its use became apparent, i.e. limitations related to species specificity, leading to non-target effects and disequilibrium of ecosystems, toxicity in vertebrate species, and development of resistant insects able to survive even with heavy doses of the chemicals.[29, 30] In reaction to this, DDT and other agrochemicals were banned from agriculture practices in many countries in the 1970s, and in the alternative, compounds such as carbamates, organophosphates, pyrethroids, synthetic growth

regulators, and metabolic disrupters were developed and applied.[31] Currently, the control of SWD is based on insecticides, physical barriers and cultural practices, which will be explored in the following subsections.[32]

1.3.1.1. Chemical control

SWD management primarily relies on broad-spectrum insecticides, such as spinosyns, organophosphates, pyrethroids, and neonicotinoids.[24] Due to *D. suzukii*'s short lifespan and short reproductive time (3-16 generations per year), high fertilization rate, and a high number of offspring, the emergence of insecticide resistance is easily developed and observed in a relatively short time window, requiring multiple insecticide applications and increasing the residues in the fruit.[32, 33] In 2018, a decrease in SWD susceptibility to spinosyn was observed in Watsonville, California. When comparing *D. suzukii* flies from a commercial caneberry crop with ones from an untreated site, the first showed to be 4.3-7.7 times less susceptible to the spinosyn.[34] The indiscriminate use of these chemicals over the years led to the development of resistant strains.[32, 33, 35]

Besides the risk of insecticide resistance, there is also an ineffectiveness of the insecticides currently homologated for use against SWD because they only target the adult stage of the insect (fly phase), failing to reach the larvae inside the fruit.[32, 33] On the other hand, side effects of toxicity to non-target insects, like natural enemies, and mammals were already reported.[35] Additionally, insecticide application usually coincides with fruit harvest, as SWD has a preference for ripe fruit.[19, 33] This makes insecticide application largely restricted since the ultimate purpose of the fruit is for consumption.[33]

Currently, there are several classes of insecticides, considering their natural versus synthetic nature and chemical structure (Figure 7). The chemical structure of an insecticide generally defines its target site and its mode of action at that target site. Most prevalent subclasses of the synthetic insecticides that prevail in the market include organochlorines, organophosphates, carbamates, and pyrethroids, being some of them (like DDT) forbidden, and most facing increased restrictions in the EU.[36]

Insecticides may also be classified according to their cellular targets (Table 1), which is a highly important classification to elucidate on pests' resistance acquisition. Insecticides

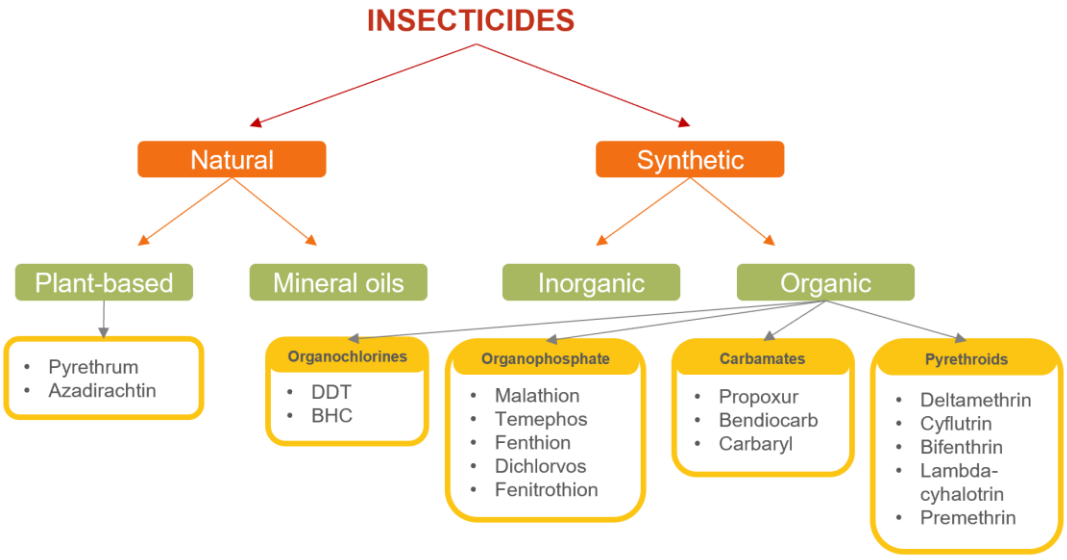


Figure 7 - Main classes of current insecticides based on natural/ synthetic nature and chemical structure (retrieved from Hassaan and Nemr[36]).

may have different targets, including the nervous system, the endocrine system, the exoskeleton building (disruption of cuticular wax adsorption), the energy production (mitochondria), or water balance (reviewed by Suiter and Scharf[37]). However, most commercially available insecticides affect the nervous system and are classified into several subclasses, for example, the Acetylcholinesterase (ACHE) disruptors (inhibitors or stimulators), Gaba-receptor blockers, Glutamate receptors modulators, and Sodium channel modulators/ blockers.

Table 1 - Chemical class of insecticides, their mode of action, target site, and primary route on entrance (retrieved from Suiter and Scharf[37]).

Mode of action, target site, and primary route of entry of various insecticide classes used in urban and structural pest management.

Chemical Class / Group	Mode of Action	Target Site	Primary Use/Route of Entry
Insecticides that Target the Insect Nervous System			
Pyrethrins / Pyrethroids	Sodium Channel Modulation	Axon of Nerve	Contact
Oxadiazines	Sodium Channel Blockage	Axon of Nerve	Oral
Semicarbazones	Sodium Channel Blockage	Axon of Nerve	Contact & Oral
OPs / Carbamates	Acetylcholinesterase Inhibition	Nerve Synapse	Contact
Neonicotinoids	Acetylcholine Receptor Stimulation	Nerve Post-synapse	Contact & Oral
Spinosyns	Acetylcholine Receptor Stimulation	Nerve Post-synapse	Oral
Phenylpyrazoles	GABA Receptor Blockage	Nerve Post-synapse	Contact & Oral
Avermectins	Glutamate Receptor Stimulation	Nerve Post-synapse	Oral
Insecticides that Do Not Target the Insect Nervous System			
Diamides	Muscle Stimulation	Muscular Calcium Channel	Oral
Juvenile Hormone Analogs	Mimic Juvenile Hormone Action	JH Degradative Enzymes / Receptor	Contact & Oral
Chitin Synthesis Inhibitors	Block Chitin Formation	Exoskeleton	Oral
Amidinohydrazones	Inhibit Energy Production	Mitochondria within Cells	Oral
Pyrroles	Inhibit Energy Production	Mitochondria within Cells	Contact
Fumigant (sulfuryl fluoride)	Inhibit Energy Production	Citric Acid / Glycolysis Cycles in Cells	Inhalation
Borates	Non-Specific Metabolic Disruption	Cells	Oral
Dehydrating Dusts	Adsorption of Cuticular Wax Layer	Exoskeleton	Contact

1.3.1.2. Cultural control

The cultural control of pests relies on practices such as pruning, mulching, irrigation, and periodic harvesting.[19, 24, 32] The efficiency of these practices is based on the negative effect that abiotic factors, such as high temperatures and low humidity, can have on *D. suzukii*. Both oviposition and survival are threatened under severe climate conditions.[19, 38, 39]

Pruning is a routine procedure consisting of the removal of parts of the plant that are not essential for growth or reproduction, which aims to increase plant health and consequently fruit quality.[19] Although this technique does not directly affect the target of *D. suzukii*, which is the fruit, it contributes to pest control since it will alter the abiotic conditions of the yield, to which this species is sensitive.[19, 39] In the field, adults seek for high humidity areas and milder temperatures to escape from the unfavourable weather conditions. Thus, pruning can be a technique that is favourable to both crop productivity and SWD control.[39]

Similar to pruning, mulching is a control technique that promotes increasing fruit yield and quality and can affect insect survival.[40] Mulches consist of covering the ground around the plants with a synthetic material to modify the microclimate. Retaining the water and nutrients, this technique protects the roots from desiccation and induces earlier ripening.[19, 40] With covered soil, it is also easier to remove the dropped fruit from the ground, which could be used by *D. suzukii* for reproduction and feeding. Cultural techniques that manipulate the soil surface can also hinder the survival of SWD since third instar larvae usually exit the fruit and fall to the ground to pupate. When facing modified soil, *D. suzukii* larvae and pupae are more prone to predation and to more extreme environmental conditions.[38] One limitation of this practice is that it should desirably be thought first to the establishment of the crop, since mulching may be hard to set up once plantations are established.[19, 41]

Irrigation methods may also be used to reduce the SWD population in crops. Changing the amount of irrigation can modify temperature and relative humidity.[19] For example, drip irrigation lowers the relative humidity compared to sprinkler irrigated crops, leading to higher mortality of larvae and lower emergence of adults from pupae, which dissect under more extreme conditions.[12] However, different insect species are susceptible to different environmental conditions, so decreased irrigation may lead to increased populations of other insect pests.[12, 42]

While SWD prefers undamaged fruits, it can also infest other hosts, such as fruit wastes and decaying fruit present in the soil.[19] As a way to control this, periodic harvesting is implemented. Leach *et al*.[43] showed that increasing harvesting frequency to every one or two days can lead to a 60% reduction of *D. suzukii* in raspberries. The same study highlighted the importance of good sanitation practices, namely bagging infested waste berries after the harvest process, which can be used as hosts for SWD oviposition.[43]

Another cultural method is the physical exclusion with fine mesh netting, which can be implemented on structures around the crops.[19] These nets have already proved to reduce *D. suzukii* infestation in raspberries and a three-weak delayed detection on fruit infestation without affecting the size and quality of the fruits, and the need for insecticide applications decreased.[44] Their efficacy heavily depends on the timing of the installation, which should be done before *D. suzukii* colonises and becomes active in the crop. Other drawbacks of this approach may be a reduced abundance of natural *D. suzukii* predators and parasitoids, insufficient crop pollination, and also plastic waste.[19, 45]

Although these techniques may seem simple, they mean a high burden for producers, due to all the necessary labour involved and associated costs.

1.3.2. Green strategies to control *Drosophila suzukii*

Due to the lack of economically and environmentally sustainable methods to control *D. suzukii*, as well as the inefficiency of those currently applied, there is an urgent need for new methods to control this pest.

The Green Deal, created in 2019 by the European Commission, is defined as a roadmap of key policies to guide Europe through a climate agenda to make this continent the first climate-neutral by 2050, thus improving human health and life quality, while taking care of nature.[46]

As part of the Green Deal, several strategies were designed, namely the Farm to Fork (F2F), presented in May 2020, which aims to rethink the entire agri-food production chain to increase sustainability, thus accelerating the transition to a sustainable food system with a neutral or positive environmental impact, hindering the biodiversity loss, and ensuring global access to sufficient and safe food (Figure 8).[47, 48] Some of the approaches taken to achieve these goals include acting in such a way that by 2030 the use of pesticides is reduced to 50%, and 25% of the European agricultural area is converted to organic practices, among other measures.[48] Furthermore, FAO has estimated that 20 to 40% of agricultural production may be affected annually as a

consequence of pests. Since there is a limitation of space, preventing the extension in space of agricultural production, the increase will have to be accompanied by an increase in resistance to diseases.[49]



Figure 8 - Farm to Fork strategy (source: European Commission, 2022).

1.3.2.1. Biological control

Biological control is one of the most sustainable approaches being studied and developed to control the pest *D. suzukii*. These approaches result from the observation of community structures and dynamics in nature, and exploiting the beneficial populations towards controlling pests and pathogens.[50] One example is the study of natural predators of insect pests that are native to each country. Moreover, indigenous or exotic parasitoids are also under study, as the first can often adapt to the emergence of an exotic species, although the latter are preferred as they have historically shown a simultaneous co-adaptation to the emergence of exotic pests, proving to be a more efficient approach.[50]

Biological-based approaches increasingly include the exploitation of predators, which occur naturally in SWD-infested fields. Currently, many insect species that feed on *D. suzukii* have been identified, such as earwigs, damsel bugs, hunting and web-building spiders, and ants.[50, 51] These species are identified by DNA analysis of *D. suzukii* in the gut of predators, and also in field and laboratory tests. Similar to parasitoids, predators also have the limitation of feeding mostly on the larval and pupal stages, failing to reach the adult stage.[50] A 2019 study using *Forficula auricularia*, the European earwig, showed a 45% decrease in the emergence of SWD adults in a laboratory study in a growth

chamber. However, this reduction was only due to predation of developing SWD, since, across all trials, the average percentage of SWD recovery was 82.37%, highlighting the limitation of earwigs in predating adult flies.[52] In 2021, a study in the north of Portugal carried out in four blueberry fields and one blackberry field showed a wide variety of *D. suzukii* predators and identified new taxonomic groups as natural predators of this pest, namely Opiliones and Hemerobiidae, as well as a new spider family - the Uloboridae. Regarding the natural abundance of the species in the studied fields, the study highlights green lacewings, ants, and spiders as the main candidates for a predator-based control.[53]

Simultaneously, the use of parasitoids is currently promising for controlling *D. suzukii.* Two of the most common *D. suzukii* natural parasitoids in North America and Europe are *Trichopria drosophilae* Perkins (Diapriidae) and *Pachycrepoideus vindemmiae* Rondani (Pteromalidae), which typically act as parasites of pupae.[50, 54] In fact, no parasitoids have yet been identified for the egg and adult stages, with parasitism only occurring in the larval and pupal stages.[50] Females of these insects use *D. suzukii* larvae or pupae for oviposition to serve as a medium for the development of the parasitoid egg, as the wasp larva eats and kills the *D. suzukii* fly.[33, 54]

Another biological control strategy is the sterile insect technique (SIT), which is based on releasing a large number of infertile males in an infested area. Infertility is promoted by ionizing radiation, causing lethal mutations in the sperm.[12, 55, 56] These males will then compete against wild-type males for copulation with females, resulting in zygotes that die during early embryogenesis, thus decreasing the number of offspring.[12, 33] This technique is a species-specific method, having no impact on non-target species, and can be combined with other biological control methods.[57] For example, the incompatible insect technique (IIT) may be combined with SIT as a complementary tool for *D. suzukii* management. The IIT is based on cytoplasmic incompatibility (CI), a mechanism induced by symbiotic bacteria such as *Wolbachia* (a maternally transmitted bacteria). *Wolbachia* can manipulate paternal nuclear material, leading to sperm-egg incompatibility when males breed with females infected with different *Wolbachia* strains.[12, 56] Both SIT and IIT require the repeated release of a high quantity of sterile males, but contrary to SIT, IIT requires that female and male separation is well defined to assure that only Wolbachia-infected males are released, since releasing females that are also infected may lead to the population being replaced by a carrier of Wolbachia infection and not have the desired CI effect.[55]

These are green, cheap, and relatively easy to apply approaches, but can face some limitations. For example, most spiders breed only once a year and are adapted to periods of starvation.[58] Additionally, in the case of web-building spiders, the webs are static, which further limits their purpose.[50] Parasitoids on the other hand are very effective but are often prohibited, as their use is heavily regulated, given the decimating effect they can have.[59] It is important to keep in mind that, once present in an ecosystem, the pest has its role, so the goal should be to accomplish a balance of levels that do not cause substantial economic losses while being aware that the extinction of a species may lead to the appearance of other undesirable ones.[60]

1.3.2.2. Peptides as toxins targeting the pest

Animal venoms are a rich source of toxins and bioactive peptides, produced by animals such as venomous spiders, cone snails, fish, wasps, scorpions, and snakes, among others.[61] From those, spider venom peptides (SVPs) are disulphide-rich (SS-rich) peptides produced by the venom glands of several spider species,[62] and these toxins have an important role in the defence against predators and prey, allowing them to quickly paralyze or kill even animals larger than the spider itself.[63, 64] The major molecular groups present in spider venom are salts and small organic compounds, linear cytolytic peptides, enzymes (such as collagenase, hyaluronidase, proteases, and phospholipase A2), large presynaptic neurotoxins, and SS-rich peptides.[62, 64] SS-rich peptides are the major component of spider venom in most species, with only a few not suiting this criterion, namely widow spiders (*Latrodectus* spp.) that contain a large proportion of large presynaptic neurotoxins, and the hobo *Tegenaria agrestis*, in which 50% of the venom is composed of sulphated nucleosides.[64]

SVPs typically have molecular weights between 3 and 9 kDa, and are structurally very complex molecules with a double or triple-stranded, antiparallel β-sheet, stabilized by ≥3 disulphide bridges, which create a compact motif known as inhibitory cystine knot (ICK).[62, 63] This conserved ICK provides exceptional chemical and thermal stability, as well as optimal resistance to proteases,[63] a very important feature when designing a bioactive compound with insecticidal activity, since insects have their own proteases.[65]

A study developed by Herzig and King[66] evaluated the stability of a disulphide-rich peptide, comparing the peptide in its native conformation (i.e., with ICK motif) with the linear one. This study highlighted the enormous stability that ICK motifs confer to the molecules in different environments, namely acidic media as extreme as pH of 1, high temperatures up to 75 ºC, as well as chemical stability to proteases, and natural environments like insect haemolymph and human serum. This study also evaluated the

peptide's stability upon mixture with some organic solvents.[66] In all scenarios, the native peptide proved to be much more stable, which gives confidence in the application of these compounds for formulations such as insecticides or therapeutic compounds, as examples.[66]

In fact, in recent years SVPs have won special attention as a tool for insect pest control.[67] Several studies have been developed to explore animal venom peptides' bioactivity alone or in conjunction with adjuvants and carrier molecules (Table 2). Their mode of action, including receptors and signalling cascades, is also being compared with that of conventional insecticides.[68] Figure 9 is a schematic representation of insect cholinergic and glutamatergic synapses showing the primary molecular targets of the major classes of chemical insecticides (red boxes) versus those of spider-venom components (green boxes).

To the best of the authors' knowledge, to date there is only one study with SVPs in SWD, tested by topical and residual exposure.[69] That work also evaluated the efficacy of three adjuvants, which significantly increased the mortality of *D. suzukii* (>90%) compared to

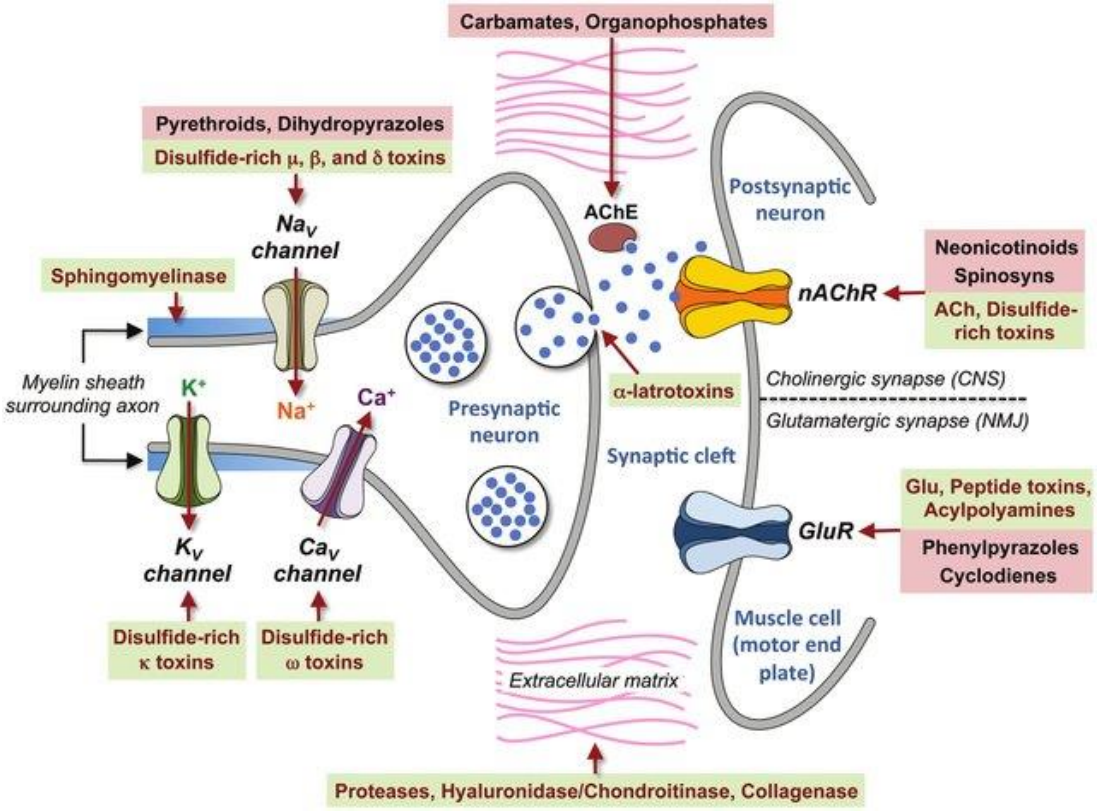


Figure 9 - Schematic comparative receptors of main conventional insecticides (red boxes) and several spider venoms, including peptides. Abbreviations: AChE - acetylcholinesterase, Ca_V - voltage-gated calcium channel, CNS - central nervous system, Glu – glutamate, GluR - ionotrophic glutamate receptor, K_V - voltage-gated potassium channel; nAChR - nicotinic acetylcholine receptor, Na_V - voltage-gated sodium channel, NMJ - neuromuscular junction (adapted by Ikonomopoulou and King[68] from the original of King and Hardy[64]).

SVP alone (10.8%). However, this mortality was linked to the action of the adjuvants rather than a synergistic effect between them.[69]

Table 2 - Review of the bioactivity of some animal venom peptides in insects.

Peptide	Sequence / Source	Target	Action	Ref.
Ae1a	GVDKEGCRYLLGACTIDDDCCLHLGCNKKYGHCGWDGTF-NH2 *Augacephalus ezendami* (African spider)	*Lucilia cuprina* (sheep blowflies)	Inhibition of insect Na_V channels PD_{50} (1h) = 963 pmol/g	[70]
		D. melanogaster	PD_{50} (3h) = 285 pmol/g PD_{50} (24h) = 1.9 nmol/g LD_{50} (24h) = 4.3 nmol/g	
ω-ACTX-Hv1a	SPTCIPSGQPCPYNENCCSQSCTFKENENGNTVKRCD *Hadronyche versuta* (Australian funnel-web spider)	*D. melanogaster*	Low concentrations can quickly breach the BBB[a] of *Drosophila*	[71]
		A. pisum	Ca_V channel antagonist LC_{50} (48h) = 1.01 mg/mL	[72]
		M. persicae	LC_{50} = 2.34 mg/mL	
Hv1a+Silwet L-77	Silwet L-77 Polyalkyleneoxide-modifed heptamethyltrisiloxane (99.5%)	*D. suzukii*	Topical application in blueberries 98.3% dead (24h)	[69]
Hv1a+LI700	LI-700 Phosphatidylcholine, methyl acetic acid and alkyl polyoxyethylene ether (80.0%)	*D. suzukii*	98.3% dead (24h)	
Hv1a+Leaf Life	Leaf Life Widespread Polyether-polymethylsiloxane-copolymer, polyether (100%)	*D. suzukii*	91.6% dead (24h)	

Hv1a-GNA	Snowdrop lectin (*Galanthus nivalis* agglutin)	*Mamestra brassicae* (cabbage moth)	Feeding with 0.2% Hv1a-GNA led to 85% mortality after 10 days	[73]
U13-MYRTX-Mri1a	DKPGQAKKIGLFDQIDKAAAAFMKLFE *Manica rubida* (ant)	*Lucilia caesar* (blowflies)	PD_{50} (1h) = 2.9 nmol/g Reversible effects after 24 h	[74]
ω-Plectotoxin-Pt1a	*ADCSATGDTCDHTKKCCDDCYTCRCGTPWGANCRCDYYKARCDT** *Plectreurys tristis* (spider)	*D. melanogaster*	Blocks presynaptic Ca_V channel currents in *Drosophila* nerve terminals	[75]
δ/ω-Plectotoxin-Pt1a	IGECAGWNDNCDKRSCCDQCHQCRCKFGSNCRCTGTKPS*C *Plectreurys tristis* (spider)	*D. melanogaster*	Enhances synaptic transmission by increasing nerve terminal excitability	[75]
ω-Hexatoxin-Hv2a	LLACLFGNGRCSSNRDCCELTPVCKRGSCVSSGPGLVGGILGGIL *Hadronyche versuta* (Australian funnel-web spider)	Honeybees	The blocker of insect VGSCs 10 000-fold preference for insect calcium channels as opposed to vertebrates	[76]
J-ACTX-Hv1c	AICTGADRPCAACCPCCPGTSCKAESNGVSYCRKDEP *Hadronyche versuta* (Australian funnel-web spider)	House flies	LD_{50} = 320 pmol/g in house flies	[77, 78]
ω-Theraphotoxin-Hh2a / Huwentoxin-V	NH2-ECRWYLGGC-SQDGDCCKHLQCHSNYEWCVWDGTFS-COOH *Haplopelma schmidti* (Chinese tarantula)	Locusts Cockroach	ED_{50} (locust) = 16 µg/g 100 µg/g – insects died	[79]
ND-Neb-colloostatin	SIVPLGLPVPIGPIVVGPR *Neobellieria bullata* (gray flesh fly)	Mealworm beetle	Inhibits ovarian development 4.15 µg NDs + 0.85 µg Neb-collooostatin	[80]

μ-TRTX-Hhn2b	ECKGFGKSCVPGKNECCSGYACNSRDKWCKVLL *Selenocosmia hainana* (spider)	*D. melanogaster*	Pore blocker - block insects' channels with high affinity	[81]
LqhαIT	VRDAYIAKNYNCVYECFRDAYCNELCTKNGASSGYCQWAGKY GNACWCYALPDN VPIRVPGKCHRK *Leiurus quinquestriatus hebraeus* (scorpion)	*Sarcophaga falculata* (flesh flies)	PD50 = 16 pmol/g (injection) Target insect Na_V channels	[82]
BoiTx1	GVPINVKCRGSRDCLDPCKKAGMRFGKCINSKCHCTP *Buthus occitanus israelis* (scorpion)	*Drosophila*	Inhibited currents through *Drosophila Shaker* channels with an IC50 = 3.5 ± 0.5 nM, with significantly lower effects on mammalian orthologs	[83]
Ba2	IFECVFSCDIKKEGKPCKPKGEKKCTGGWRCKIKLCLKI *Brachypelma albiceps* (spider)	*Acheta domestica* Crickets	Injected intrathoracically LD50 (15min) = 9.2 μg/g	[84]
SF1	KECMTDGTVCYIHNHNDCCGSCLCSNGPIARPWEMMVGNCM CGPKA *Segestria florentina* (spider)	*Heliothis virescens* larva	LD50 = 1000-2000 pmol/g	[86]

[a] Blood-brain barrier
[b] Schneider 2 cells

J-Atracotoxin-Hv1c (J-ACTX-Hv1c) is a Janus-faced atracotoxin, whose name derives from the marked asymmetrical distribution of charged residues, as one face presents almost all charged residues, while the other lacks ionizable side chains.[78, 87] This peptide, discovered and characterized in 2000 by Glenn F. King's group, was isolated from the venom of the Australian web-tunnel spider *Hadronyche versuta.* J-ACTX-Hv1c has 37 amino acids (AA) and 4 disulphide bridges (I-VI, II-VII, III-IV, V-VIII), and comprises the rare feature of having a vicinal disulphide bridge, which is defined as a bridge formed between contiguous cysteines (Figure 10A, C).[87] This feature has been shown to have importance in the function of the molecule, rather than being only a structural characteristic, because when Cys13 and Cys14 residues (responsible for this secondary structure element) were replaced by Ser residues, there was no change in the tertiary structure of the protein, but there was an almost total loss of the insecticidal activity of this mutant when injected into crickets at concentrations up to 60 fold higher than the native peptide.[87]

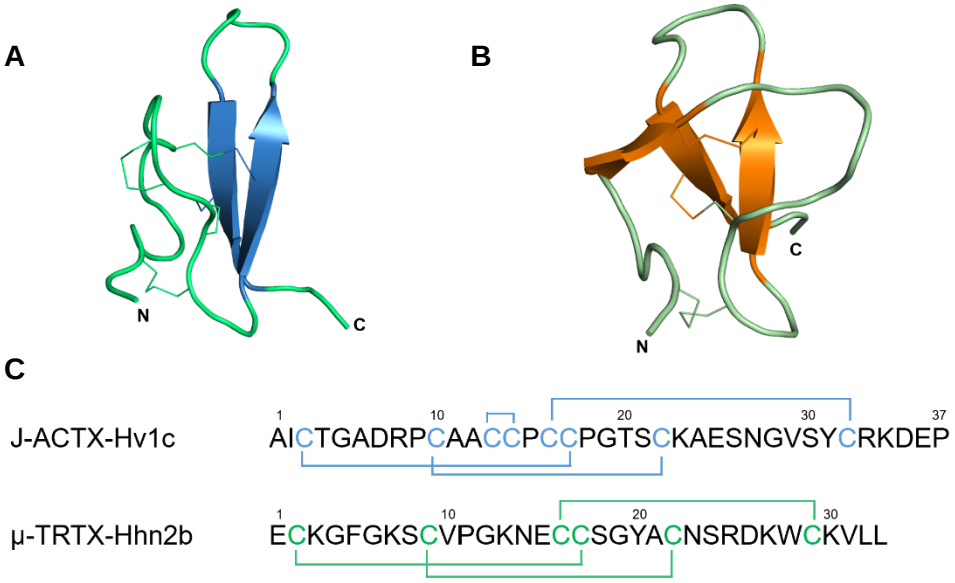


Figure 10 - Three-dimensional structure of A. J-ACTX-Hv1c and B. μ-TRTX-Hhn2b showing the β-sheets and highlighting the disulphide bridges (created with PyMOL Molecular Graphics System). C. amino acid sequence and disulphide connectivity of peptides J-ACTX-Hv1c and μ-TRTX-Hhn2b. The N- and C-terminal are labelled. Standard one-letter amino acid abbreviations are used.[81, 87]

J-ACTX-Hv1c has already proved to be efficient against insects of the dipteran order, which include flies and mosquitos, which medically represents an important order as these insects are responsible for transmitting several infectious diseases, namely malaria, dengue fever, and leishmaniasis, to name a few.[88] In 2002, Maggio and King[78] determined that the LD_{50} for J-ACTX-Hv1c is 320 ± 20 pmol/g when administered by dorsal thoracic injections in house flies (*M. domestica*). In another work, the same group reported lethal activity against *Drosophila melanogaster*, a fruit fly from the same family

as *D. suzukii*, but they couldn't determine the concentrations in which J-ACTX-Hv1c displayed this activity.[88] Furthermore, a toxin of the J-ACTX family (ω-ACTX-Hv2a) showed to be inactive in vertebrate neuromuscular junction preparations and when applied by subcutaneous injection in newborn BALB/c mice, an inbred model mostly used in immunology and infectious diseases research, at doses of up to 800 pmol/g.[76] This toxin selectively targets the insect's large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels with high affinity, displaying an IC_{50} of 2 nM, without acting on Na_V and Ca_V and other K_V channels.[77]

μ-Theraphotoxin-Hhn2b (μ-TRTX-Hhn2b; formerly, hainantoxin I or HNTX-I)[67] is a peptide toxin derived from the venom of the Chinese bird spider *Selenocosmia hainana*, and comprises 33 AA residues and a disulphide linkage of I-IV, II-V, III-VI (Figure 10B, C).[81] This toxin blocks insects' voltage-gated sodium channels (VGSCs) with high affinity. A study showed that μ-TRTX-Hhn2b displays a 15-fold selectivity for the *Drosophila* para (DmNaV1) channel when compared with rat NaV1.2, with no effect on rat NaV1.1 and NaV1.4–1.8 channels (Li *et al.*[81], as interpreted by Windley *et al.*[67]), thus potentially safer for mammals than for insects.

1.3.2.3. Microbiome manipulation as a novel approach to control *D. suzukii*

1.3.2.3.1. The *D. suzukii* microbiome

The *D. suzukii* microbiome is the community of microbiomes that inhabit the insect surface, organs, haemolymph, and principally the gut, where the widest diversity is found.[89, 90] The gut microbiome generally consists of bacteria from the families Acetobacteraceae, Enterobacteriaceae, and Lactobacillales. However, its constitution changes with time and exposure to different environments and food sources.[91] The symbiotic relations established between the insect and the microbiome modulate and condition the insect's behaviour and survival.[92] In fact, microorganisms are acquired through different factors: the first contact starts at the egg stage when the female inoculates microorganisms-colonised faeces on top of the egg so that the hatching larvae can feed on them; coprophagy and ingestion of microorganisms-rich food also help modulate the microbiome of the fly. Bing *et al*.[93] showed that laboratory-reared *D. suzukii* presents a significantly less varied microbiome than wild-type flies. Under reach yeast-carbohydrate diets, laboratory flies are predominantly colonised by lactic acid bacteria, such as *Enterococcus*, *Lactobacillus*, and other species from the family Enterococcaceae.[93] Besides bacteria, the microbiome also includes fungi, protozoa and

viruses.[94] Another study with gnotobiotic individuals showed that some of the identified bacteria or yeast species promoted larval development.[95]

Recently, *D. suzukii* microbiome manipulation has become of particular interest as a way to control this pest, given the fundamental role of the microbiome in regulating several physiological processes essential for the proper development and survival of the fly, namely as digestion of dietary substrates to essential micronutrient supply, but also shaping insect behaviour.[90, 96] For instance, a study carried out with *D. melanogaster*, a species closely related to *D. suzukii*, showed a diet-induced mating preference after exposure of only one generation to different growth media, and linked this preference to the commensal bacteria *Lactobacillus plantarum*.[97]

Microbiome-based insect management approaches rely on the symbiont elimination or replacement, genetic modification of symbionts, and microbial semiochemicals.[98] Manipulating the microbiome may thus be a powerful tool to control insect pests. An example is the maternally transmitted symbiont *Wolbachia*, which causes cytoplasmic incompatibility, and in some insect host species it feminizes genetic males into functional females.[99] Targeting in *D. suzukii* gut candidate microbial symbionts like the *Acetobacter thailandicus* (that was shown in *D. melanogaster* to accelerate larval maturation, and boost host fecundity and fertility)[100] may be an important tool to control this pest.

1.3.2.3.2. Antimicrobial peptides targeting the gut microbiome

Antimicrobial peptides (AMPs) may be explored as an innovative strategy to manipulate the gut microbiome of *D. suzukii* leading to its feebleness and eventual death. AMPs are molecules comprising about 10 to 60 AA residues, with each peptide having its unique structural characteristics, which influence their biochemical properties, such as the isoelectric point and ionization behaviour.[101] Depending on their composition, these peptides can be anionic, neutral, or cationic. Cationic AMPs are the most abundant ones and have a net charge ranging from +2 to +9 at neutral pH.[101-103]

Another key feature of AMPs is their hydrophobicity, since it is proportional to the extent of the AMP being capable of entering into the membrane lipid bilayer, and is defined as the percentage of hydrophobic residues, i.e., aliphatic and aromatic AAs (e.g. valine, leucine, isoleucine, and tryptophan), in the peptide sequence.[103] However, this percentage, which should be around 30-50%, must be regulated to avoid causing toxicity in mammalian cells and not compromise their selectivity.[104]

It is widely recognized that one of the AMP main mechanisms of action involves the disruption or permeabilization of the cell membrane, triggered by electrostatic interactions between positive charges of the cationic AMP and the negatively charged cell wall constituents, such as lipopolysaccharides (LPS) on Gram-negative bacteria and teichoic and teichuronic acids on Gram-positive bacteria.[105, 106] This interaction leads to a competitive exchange of divalent cations (e.g. Mg^{2+} and Ca^{2+}) linked to the negatively charged membrane of Gram-negative bacteria, which will promote destabilization and give access to AMPs for both outer and inner membranes.[101, 102] In fact, the outer leaflet of the lipid bilayer has a high composition of negatively charged phospholipid head groups, in contrast to the animal and plant cell membranes, which have no charge on the outer membrane leaflet.[107] These characteristics make AMPs more suitable for bacterial cells than for animal/ plant cells (Figure 11), thus reducing the possibility of unwanted toxic effects.[104] Given the structural differences dividing these cell types, the peptide-bacteria interaction is stronger, mediated by hydrophobic and electrostatic interactions, whereas the interaction with the membrane of multicellular animals and plants is only hydrophobic, as they lack negative charges in the external leaflet, thus decreasing the strength of the interaction.[104]

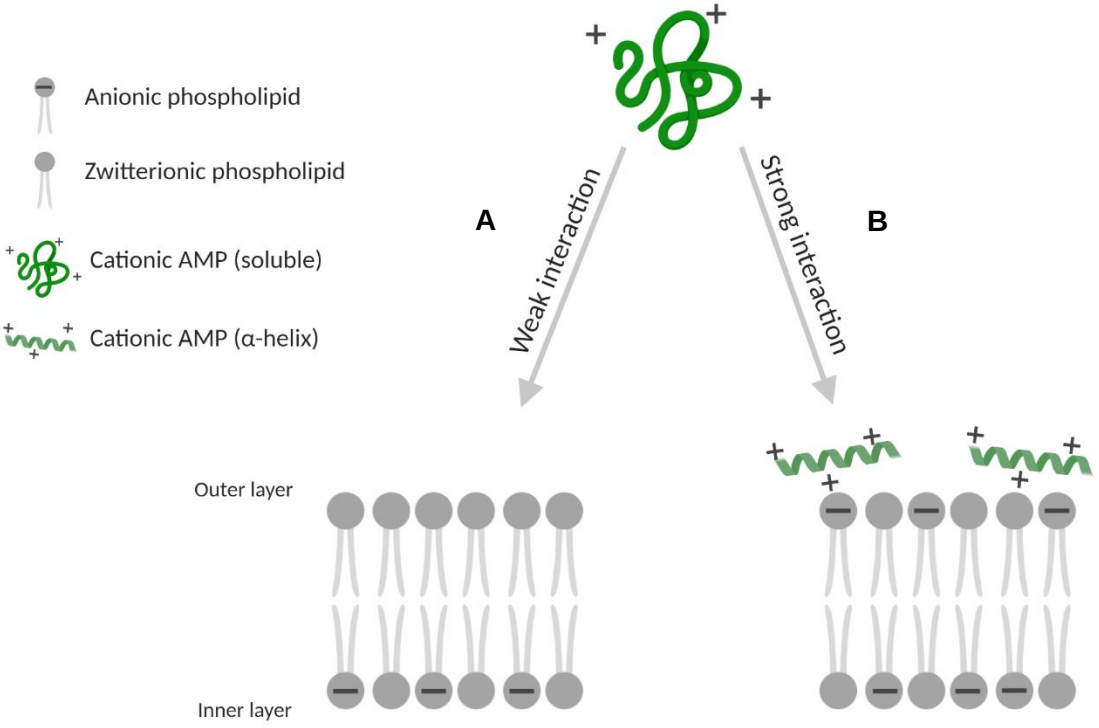


Figure 11 - The specificity interaction between a cationic peptide with A. an animal/ plant cell membrane or B. a bacterial cell membrane. The details in the composition of the membranes were hidden to simplify the interpretation of the scheme. Created in BioRender.com (inspired in Kumar *et al*.[104]).

There are some models, namely transmembrane pore and non-pore models, which are currently being proposed to explain how AMPs interact with the cell membranes.[102, 104, 108] In the *barrel-stave model*, after binding to the membrane, AMPs are inserted perpendicularly into the lipid bilayer and form transmembrane pore-like structures through side peptide-peptide interactions. Here, peptide amphipathic structure is crucial as the hydrophobic regions interact with the membrane lipids, while the hydrophilic portion forms the lumen of the channels. In the *toroidal model*, the transmembrane pore is partly formed by peptides and by the phospholipid head groups, leading to disruption of the hydrophobic and hydrophilic arrangement of the lipid bilayer. Ultimately, pore-forming models can lead to membrane depolarization and cell death.[104] The *carpet model*, a non-pore forming method, describes that instead of a transmembrane pore formation, AMPs bind parallel to the lipid bilayer and, when reaching a concentration threshold, can form a "carpet" that covers the membrane surface, which eventually loses its structure due to unfavorable interactions, leading to the disruption of the membrane through a detergent-like effect that promotes the formation of micelles or pores. [104, 106, 108] Ultimately, these mechanisms may be linked to leakage of intracellular metabolites and cell lysis.

Although the interaction between peptide and membrane is initially mediated by electrostatic forces, it is the AMP's biophysical properties, *i.e.* size, amphipathicity, and secondary structure, that will influence its mode of action.[102] Although the majority of AMPs act in a membranolytic way since they hamper the membrane architecture either by forming pores and/or increasing the permeability, AMPs can also act on targets beyond the membrane structure, via diminishing the transport across the membrane, or inhibiting the synthesis of extracellular biopolymers or even targeting intracellular constituents.[108, 109] Cell-penetrating peptides can cross the lipid bilayer without disrupting the cell membrane, as the pores are transient after disintegration, and act on intracellular targets.[110] Once in the cytoplasm, AMPs bind and inactive multiple targets in important cellular and metabolic pathways, such as the inhibition of DNA and RNA synthesis, enzymatic activity, and cell wall synthesis.[111-114] If acting in higher concentrations, these intracellular actions can be combined with the action of disrupting the membrane.[115]

1.3.3. Integrated pest management

Previous data provide evidence that different approaches should be studied and developed to be applied alone or in integrated pest management (IPM). IPM is an approach that takes advantage of the current information on pests' life cycles and their role in the environment.[29] It considers the various pest control approaches, and creates

synergies by integrating biological, chemical and cultural controls to design the best plan that protects crops.[116] IPM is thus an ecologically based approach that evaluates the possible interactions between the different control tactics, preventing the dominance of one pest control method over another, to ensure the maximum benefit from the resources of an ecosystem while keeping to a minimum the competition between the organisms involved.[60] In other words, the control approach chosen should have an effect as unobtrusive as possible, to maintain the balance of the ecosystem in the study.[29]

1.3.4. Gene expression in response to toxic compounds

How the peptides from venoms or AMPs may influence the different cell pathways of the insect are yet unknown. Some studies search the pathways already explored for xenobiotics, namely responses involving the Cytochrome P450 pathway, insulin, and target of rapamycin (TOR) signalling pathway, apoptotic pathways, or defence molecules like the heat shock proteins.[117-122]

1.3.4.1. Drosophila Cytochrome P450 pathway

G protein-coupled receptors (GPCRs) are membrane receptors that respond to the binding of numerous types of molecules, such as ions, vitamins, hormones, proteins, neurotransmitters, odorants, and intermediary metabolites.[120, 123] Once inside the cells, these molecules can be expelled without harming the organism through the activity of ATP-binding cassette (ABC) transporters, or they can activate signalling pathways of GPCRs. These pathways include a cascade of phosphorylations through mitogen-activated protein kinase (MAPK), as well as the activation of adenylyl cyclase (AC). This activation leads to the conversation of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), a second messenger involved in intracellular signal transduction, which ultimately binds to the cAMP-response element binding protein (CREB) that in turn binds to the cAMP response element (CRE).[124] This pathway ultimately leads to the activation of the promoter regulating the expression of cytochromes P450 (P450) and glutathione S-transferase (GST).[123]

P450 genes are a large family of genes associated with numerous biological processes, namely detoxification of xenobiotics, such as insecticides, drugs, and plant toxins, and associated with the metabolism of endogenous compounds like hormones and fatty acids.[120] Many P450 genes were found in all sequenced genomes of insects.[125] The presence of various P450s is believed to be an evolutionary response to the need for the detoxification of toxic compounds that insects face in the environment.[125] Furthermore, P450s have been associated with the metabolization of synthetic insecticides, and their

basal and/or up-regulation is associated with the development of insect resistance to these compounds and adaptation to phytochemcials.[120, 126] This resistance is linked to increased metabolic detoxification and accelerated excretion of the xenobiotics.[126] Nevertheless, to the best of the author's knowledge is still unclear the regulatory pathway of P450 related to insecticide detoxification. Up to now, numerous studies have identified P450s involved in insect resistance, many belonging to the CYP6 family.[126, 127] In a study performed by Daborn *et al.*,[125] the authors promoted the expression of P450s in transgenic *Drosophila melanogaster* to assess the susceptibility to different insecticides. The response to insecticides differed according to which P450 was being over-expressed. An increase in survival of flies exposed to nitenpyram and diazinon was detected when the *cyp6g2* gene was over-expressed, whereas the expression of *cyp12d1* led to a decrease in susceptibility to the insecticides DDT and dicyclanil.[125]

1.3.4.2. Heat shock proteins

Heat shock proteins (HSPs) are a family of proteins found in all organisms and are produced in coping with stressful conditions, namely temperature, environmental changes, chemicals and oxidative stress.[119, 128] The expression of genes coding for HSPs has already been associated with cellular protection in response to insecticides and other xenobiotic stressors.[122] When an extracellular signal of stress reaches the cell, some HSPs are expressed to overcome possible harmful effects of this stress and protect cellular components. HSPs with approximately 70 kDa represent one of the major HSPs families, as they are very conserved over species evolution.[129] A study led by Gupta *et al.*[130] evaluated the different responses to insecticides of tissues expressing and not expressing the *hsp70* gene. In the absence of expression, accessory glands of *D. melanogaster* males showed tissue damage in response to insecticides dichlorvos and chlorpyrifos, suggesting that *hsp70* is involved in the cellular response to these chemicals in *D. melanogaster*, and may be used as a cellular hazard biomarker in response to environmental chemicals.[130] In addition, modulated expression of HSPs has been associated with a longer lifespan in insects.[128, 131]

1.3.4.3. Insulin-like receptor/TOR signalling pathway

In *Drosophila*, the insulin-like receptor (InR) pathway regulates the size of cells, organs, and the whole organism in response to nutrient uptake. This pathway intersects with TOR signalling, which interprets growth factors, oxygenation, hormones, and nutrition levels, ultimately leading to changes in cell growth, proliferation, and control of egg development.[132] Under favourable conditions, the InR signalling causes the Akt (also known as protein kinase B, PBK) to phosphorylate the Forkead box O (FoxO)

transcription factors, thus inhibiting their transcriptional functions.[133] This results in no expression of genes involved in the response to oxidative stress, metabolism, cell-cycle control, and DNA repair, while the *vg* gene, encoding for vitellogenin (yolk protein), is being expressed.[133] On the contrary, under adverse conditions, FoxO inhibition will cease, leading to the expression of *gaad45α*, superoxide dismutase, and ribosomal protein S6 kinase (S6K), activating stress response mechanisms, whereas *vg* expression is suppressed, thus decreasing fecundity.[118, 133] Concomitantly, Akt inhibits the TSC1/2 complex (tuberous sclerosis complex genes 1 and 2), which act together to inhibit TOR, coupled to the activation of S6K, ribosomal biogenesis by *tif-IA*, and inhibition of the translation initiation factor 4E-binding protein (4E-BP) encoded by *thor*, thus suppressing translation initiation and growth.[134]

1.3.4.3. Apoptotic stimuli pathway

The mechanism of apoptosis, defined as programmed cell death, is essential for maintaining the homeostasis of an organism. This process depends on the balance between inhibitory and apoptosis-promoting caspases.[135] Caspases are major characters in most apoptotic cell deaths.[136] In *Drosophila*, seven caspases are identified, of which three are initiator caspases [Dronc (also called Nc), Dredd, and Strica], and four classified as effectors (DrICE, Dcp-1, Decay, and Damm).[136, 137] Death-associated inhibitor of apoptosis 1 (Diap1, encoded by gene *th*) is essential to prevent undesired caspase activation (such as the caspase-9 ortholog Dronc) and ubiquitous apoptosis.[138] In response to different apoptotic stimuli (e.g., DNA damage, unfolded proteins, ROS, and cellular stress), Reaper-family proteins (encoded by genes *reaper*, *hid* and *grim*) are recruited to mitochondria, increasing its ability to promote Diap1 degradation.[138, 139] This degradation of Diap1 lead to the activation of the initiation caspase Dronc, which ultimately promotes the activation of effector apoptosis caspases DrICE and Dcp-1.[138]

1.4. Synthesis of disulphide-rich peptides

1.4.1. Solid-phase peptide synthesis

Peptide synthesis can be performed in liquid or solid phase (SPPS), the former being currently used (mostly in industrial contexts) to obtain large amounts of peptides of moderate size.[101] On the contrary, SPPS in its current, highly optimized formulation, allows to obtain long and complex peptides in either manual or automated modes.[101] In both cases, chemical peptide synthesis proceeds in the C- to N-terminal direction to avoid epimerization, in contrast to *in vivo* ribosomal peptide biosynthesis.[140] The

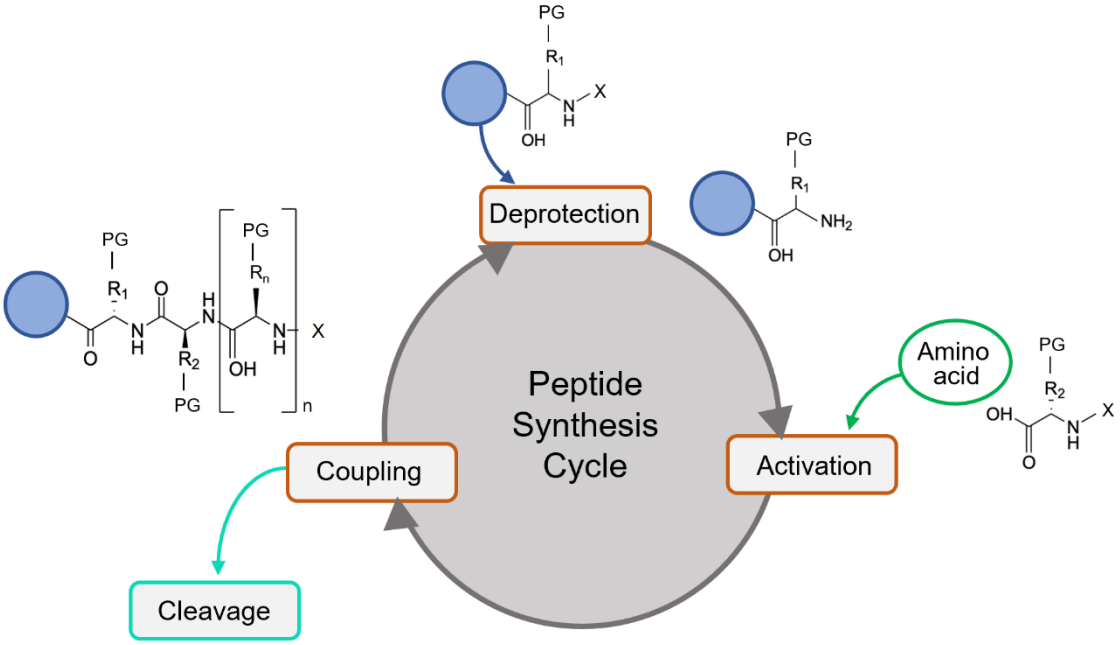


Figure 12 - Principal steps of SPPS: 1. the C-terminal amino acid (AA) is coupled to the resin (blue circle); 2. removal of α-amino protecting groups with a strong base, such as piperidine (deprotection); 3. Activation of the AA with a coupling reagent (e.g. HBTU); 4. Coupling of the following AA; 5. Cleavage of the permanent protecting groups and the resin at the end of the complete chain assembly. PG: permanent protecting groups; X = Fmoc or Boc (side-chain protecting group); $R_{1,2,n}$ = amino acids side-chain (inspired in the scheme from Julian Pampel[141]).

formation of the peptide chain takes place by the successive coupling of the AAs in correct order, with a covalent peptide bond being in each case.[142] In SPPS this is accomplished on a solid matrix (or resin), which serves as a support for the peptide chain being assembled. This solid support is an insoluble polymer that maintains its physical stability during the various steps (stirring, filtering, occasional or systemic microwave heating) throughout the synthesis procedure.[143] The synthesis starts by coupling the first (C-terminal) AA in N-protected form to the resin. Before the coupling of the second AA, the first one must be deprotected, i.e., the N-terminal protecting group must be removed (Figure 12).[144] The use of protecting groups is mandatory to prevent unwanted reactions, and there are different protection schemes to block reactive groups (at N-terminal and AA side chains), the most widely used being the 9-fluorenylmethoxycarbonyl/tert-butyl (Fmoc/^{t}Bu) orthogonal protection scheme.[144] This method relays on the principle that the different protecting groups (^{t}Bu, Pbf, Boc, Trt as the side chain protecting groups; and Fmoc for the N-terminal end) have very distinct acid/base labilities, which allows using a strong base (mainly secondary amines, such as piperidines) to deprotect the temporary protecting groups (Fmoc) before each coupling step while keeping the permanent ones attached, as ^{t}Bu, Pbf, Boc or Trt groups are easily removed with a strong acid such as trifluoroacetic acid (TFA).[145, 146] The Fmoc/^{t}Bu approach gained popularity over the earlier Boc/Benzyl strategy because it avoided the use of the anhydrous hydrogen fluoride

(corrosive and dangerous) for cleavage and final deprotection.[147] Implementation of the Fmoc/^{t}Bu approach has "democratised" peptide synthesis by making it accessible to many laboratories.[146]

As already mentioned, in chemical peptide synthesis, coupling (chain elongation) is achieved by reaction between the carboxyl group of one AA and the amino group of the next one. To accomplish this reaction, the carboxyl must be converted into a reacting species [e.g. by activation with *N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU)].[144, 148, 149] Chain assembly is thus accomplished by repeated deprotection and coupling cycles until the target sequence is obtained (Figure 12).[144]

After coupling the entire peptide sequence, the product obtained represents the peptide in its linear and reduced conformation.[150] For cysteine-rich peptides, it is necessary to promote *in vitro* its natural structure (i.e., the disulphide bonds).

1.4.2. *In vitro* oxidative folding

The oxidation of peptide thiol groups can be mediated by specialized enzymes, low molecular weight disulphide reagents, or by other electron-acceptor reagents.[151] The simplest approach consists of completely deprotecting the linear polythiol peptide and promoting a redox environment (e.g. with thiol disulphide oxidoreduction buffers, mimicking the physiognomic environment) [152, 153] that will facilitate the achievement of the thermodynamically more stable conformation.[151, 154]

The formation of disulphide bonds by an oxidation reaction involves the exchange of two electrons, where oxidizing equivalents are transferred from a disulphide reagent to cysteine thiolates (Figure 13).[151] For a peptide with more than two cysteines, there are multiple possible oxidation products to be formed. For instance, a peptide with six cysteines can adopt fifteen different arrangements depending on the different disulphide bonds created.[151] However, naturally-occurring peptides tend to adopt unique, thermodynamically determined foldings, as opposed to alternative, non-native disulphide pairings. In aiming to reproduce native peptide structure from synthetic precursors, it is crucial to ensure that only intramolecular bonds are formed during the oxidative folding process. To this end, highly dilute peptide solutions avoiding non-productive disulphide pairings are used.[151, 154] Also, nondenaturing chaotropic agents, like guanidinium hydrochloride (Gd·HCl), are commonly used to prevent the peptide to fall out of the

solution and aggregate, as the salt interacts with the peptides, decreasing the electrostatic energy between peptide molecules, thus increasing their solubility.[155]

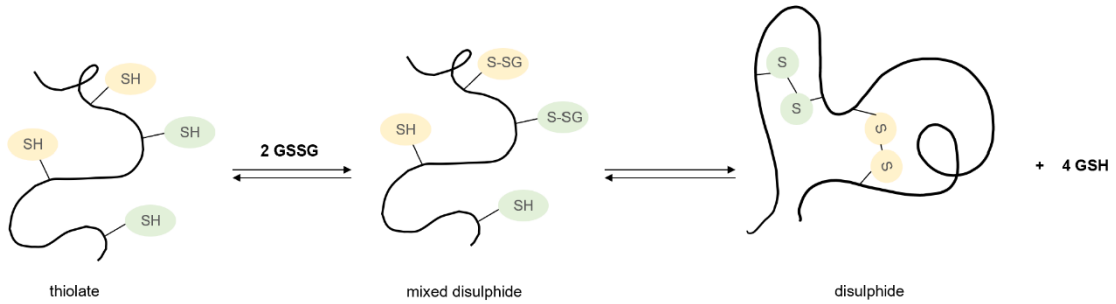


Figure 13 - Schematic representation of the mechanism by which disulphide bonds are formed in an *in vitro* redox environment (inspired in the scheme from Bulaj[151]).

Besides the described method, other chemical approaches could be implemented. One consists of selecting a set of protecting groups with different stabilities and removal conditions for the various cysteine groups.[156] This approach can afford higher yields, fewer by-products and shorter reaction times when compared with the oxidation method.[157] However, it also presents some weaknesses, such as being chemically more demanding as more cysteine groups are involved; the orthogonality of protecting groups is compromised for the synthesis of peptides with multiple disulphides; and some thiol protecting groups are labile under basic conditions and therefore incompatible with the Fmoc/^{t}Bu strategy.[156, 157] Moreover, this approach can suffer from intermolecular dimerization and oligomerization side reactions, although this can be avoided with a high dilution of the peptide.[156]

Overall, SPPS is a method that offers numerous advantages over genetic engineering or protein recombination, namely the possibility of incorporating several modifications. From C- (e.g. amide, acid, ester) and N-terminal (e.g. formylation, acetylation, fatty acid addition) modifications, to fluorescent labelling (to study cell processes, and binding and localization studies), and also incorporating unnatural AAs (to enhance stability and activity), it is possible to explore the creativity and implement an endless number of modifications to the molecules of interest.[101]

1.5. Objectives and Hypotheses

The increasing challenge of meeting the demand for food, posed by current world population growth, has been challenged by several factors, from new paradigms caused by climate change, and principally by food pests. Altogether with the current urgent need of developing more sustainable agriculture, new green control measures to reduce the impact and dissemination of insect pests, such as *D. suzukii*, must be explored.

Thus, going in line with the challenge proposed both by the Sustainable Development Goals of the United Nations, and the European Green Deal, with special incidence with the Farm to Fork strategy, to end hunger and foster more sustainable agriculture (e.g., rethink and reduce the use of chemical applications in agriculture practices),[158] this work aimed to develop new methods to control *D. suzukii*, a major pest responsible for large production and economic losses worldwide.

To achieve this main goal, five specific objectives were defined:

1. Chemically synthesize two complex multi-disulphide SVPs. For achieving this objective, after being selected in the literature, it was hypothesized that the synthesis of complex SS-rich peptides could be achieved by SPPS and their expected stable conformation confirmed;
2. Establish an evaluation method for the insecticidal activity through oral absorption of peptides on *D. suzukii* and evaluate the potential insecticidal activity of both SVPs against *D. suzukii* by oral administration. For achieving this objective, pre-selected doses and best administration methods were selected on literature and adapted in the laboratory, until their efficacy and reproducibility were validated;
3. Assess the activity of AMPs and their conjugates with SVPs as a way to control *D. suzukii* by weakening its microbiome. For this, and benefiting from the author's previous experience in synthesising and testing AMPs, some AMPs already showing antimicrobial effects were selected, and also supplied by oral administration;
4. Evaluate the biological response of key cell pathways of *D. suzukii* to the SVPs. For that, and based on literature for other toxic compounds, we designed the hypothesis that the SVPs will trigger cell responses of pathways that also respond to xenobiotics in *D. suzukii*, namely the cytochrome P450 pathway, the increase of HSPs, target the TOR pathway, and eventually induce apoptosis. This hypothesis will be tested by quantifying the expression of genes associated with resistance and response to xenobiotics.

To achieve these goals, throughout this study SPPS of complex multi-disulphide SVPs and subsequent *in vitro* oxidative folding were performed, as well as antibiogram assays, 16s rDNA sequencing, survival analysis and gene expression evaluation through RT-qPCR were employed. Obtained data can contribute to establishing a method to obtain complex multi-disulphide peptides, and to elucidating the potential of natural peptides as a green control measure for *D. suzukii* in particular, whilst highlighting their promising role in insect pest management in the agro-food industry.

2. Material and Methods

2.1 Peptide synthesis

2.1.1. Chemicals

Fmoc-protected amino acids, HBTU, 2-chlorotrityl chloride (2-CTC) resin, H-L-Pro-2-chlorotrityl resin and Fmoc-L-Leu-Wang resin were acquired from Irish Biotech GmbH (Marktredwitz, Germany). HPLC-grade acetonitrile (ACN) and peptide synthesis-grade dimethylformamide (DMF), dichloromethane (DCM), *N,N*-diisopropylethylamine (DIEA), and TFA were purchased from Carlo Erba-SDS (Sabadell, Spain). Unless otherwise indicated, all other chemicals were purchased from Sigma-Aldrich (Madrid, Spain).

2.1.2. Peptide synthesis

Two SVPs (Figure 10) were synthesized at the Proteomics and Protein Chemistry group facilities at Pompeu Fabra University, Barcelona Biomedical Research Park. Peptides J-ACTX-Hv1c – AICTGADRPCAACCPCCPGTSCKAESNGVSYCRKDEP - and μ-TRTX-Hhn2b - ECKGFGKSCVPGKNECCSGYACNSRDKWCKVLL - were assembled on 2-CTC resin on a Prelude (Gyros Protein Technologies, Tucson, AZ) instrument running Fmoc SPPS protocols. Resin amount was determined by dividing the desired peptide amount (0.1 mmol) by resin functionalization (0.6 mmol/g). Before coupling the first AA, the resin was preconditioned in DCM (3 × 1 min + 5 min) and DMF (3 × 1 min). The C-terminal amino acid was coupled manually to the resin in 8-fold molar excess before automated synthesis, using a mixture of the N^{α}-Fmoc protected AA, suspended in a small volume of DMF, and the base (DIEA, 16 mmol). Automated double coupling of amino acids was performed with an 8-fold molar excess of Fmoc-L-AA/HBTU in the presence of 16-fold molar excess of DIEA in DMF as solvent. Fmoc removal was done with piperidine/DMF (20:80 v/v) followed by DMF washes.

2.1.3. Cleavage and extraction

Once the target sequences were assembled, full deprotection of protecting groups on the side chains and cleavage from the resin was done with TFA/H_2O/DODT/TIS (94:2.5:2.5:1 v/v) at RT, for 2h under constant shaking (1 mL of cleavage cocktail/100 mg resin).

Crude peptide was isolated from the TFA solution by precipitation with chilled diethyl ether and centrifugation at 4700 rpm for 10 min at 4 ºC, while other impurities (largely

the derivates of side-chain protecting groups) remained in the supernatant. The peptide pellet was taken up with water and lyophilized.

2.1.4. Peptide purification

Peptide purification was done by preparative scale RP-HPLC, on a Shimadzu (Kyoto, Japan) instrument equipped with an SPD-20A UV/Vis detector and a LC-20AP pump, on a reverse-phase C18 column (250 × 21.2 mm, 5 µm pore size, Phenomenex®), using a 15-50% linear gradient of solvent B into A (A: 0.1% TFA in water; B: 0.1% TFA in ACN), at 20 mL/min flow rate, with detection at 220 nm. Fractions considered to be of adequate purity by analytical HPLC were pooled and lyophilized. Peptide identity was confirmed by mass spectrometry (LC-MS) and MALDI-TOF analysis.

2.1.5. Oxidative folding

For the oxidative folding, the peptides previously purified by HPLC were diluted to 10^{-5} M in water, and 0.1 M ammonium acetate (NH_4OAc) containing 1M Gd·HCl was added. The pH was adjusted to 7.8-8.0 and the oxidation was allowed to run for 20-24 h under an anaerobic environment (N_2 bubbling), in the presence of reduced (GSH) and oxidized glutathione (GSSG) (1:100:10 molar ratio of peptide/GSH/GSSG) to promote disulphide oxidation and shuffling. The oxidation process was monitored by HPLC (15-50% and 5-50% linear gradient of solvent B into A over 15 min, at a 1 mL/min flow rate for J-ACTX-Hv1c and µ-TRTX-Hhn2b, respectively) and LC-MS (gradient: 5-50% of solvent B into A over 15 min, at a 1 mL/min flow rate). To quench oxidation when the process was judged to be completed, acetic acid was added until pH of approximately 4.

2.1.6. Solid-phase extraction

To remove folding buffer components and extract the peptides after oxidation, the oxidation mixtures were applied through a peristaltic pump model 313S (Watson Marlow) to a Strata® reverse-phase C18-E cartridge (Phenomenex®). The cartridge was first washed with 2 mL of 0.1% TFA in ACN and then with 2 mL of 0.1% TFA in water. The peptides were eluted from the cartridge with a flow of 4 mL/min, the column was washed with 2 mL of 0.1% TFA in water and then the peptides were eluted with ACN/H_2O with 0.1% TFA (49.5:49.5:1 v/v) and lyophilized.

The lyophilized peptides were then dissolved in Milli-Q water and purified by RP-HPLC as described in section 2.1.4. Peptide purification final products were stored at -20 ºC until further use.

2.1.7. Peptide quantification

Stock solutions of peptides were prepared in sterile Milli-Q water at 1 mg/mL for quantification using a NanoDrop™ One UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For J-ACTX-Hv1c, quantification method 31 was used, assuming an extinction coefficient ε_{205} of 31 mL mg^{-1} cm^{-1}, often used for peptides without tryptophan (Trp) and tyrosine (Tyr) residues.[159] Peptide μ-TRTX-Hhn2b was quantified by the Scopes method, which uses an Abs_{280}/Abs_{205} ratio to correct the contribution of Trp and Tyr side-chain absorbance.[159]

2.1.8. MALDI-TOF analysis

The molecular weight of both peptides in their linear conformation and after oxidative folding was confirmed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectra analysis, performed at the facilities of the University of Barcelona.

Spectra were acquired in a 4800 Proteomics Analyzer (AB Sciex, Darmstadt, Germany) operated in positive ion mode at an acceleration voltage of 20 kV, 80% grid voltage, 1.227 ns delay time, and 2.19 kV detector voltage. Equal volumes of peptide (1 mg/mL) and matrix solution (α-cyano-4-hydroxy-cinnamic acid, 15 mg/mL in 50% MeCN in H_2O) were mixed on the MALDI plate and air-dried. Spectra were recorded in reflector TOF mode in the 1000 – 4000 m/z range by accumulating 30 subspectra at a fixed laser intensity of 4900.

2.1.9. Nuclear magnetic resonance analysis

Nuclear magnetic resonance (NMR) analysis for peptides J-ACTX-Hv1c and μ-TRTX-Hhn2b were performed at the NMR facility of the University of Queensland, Brisbane, Australia. Each peptide (1 mg) was dissolved in 550 μL of 10% D_2O/90% H_2O at pH 3.2. Spectra were recorded at 298 K on a Bruker Avance III HD 600 MHz spectrometer equipped with a cryoprobe, including TOCSY (with an 80 s MLEV-17 spin lock), NOESY (mixing time of 200 ms) and natural abundance 15N HSQC (and also 13C HSQC for J-ACTX-Hv1c). Solvent suppression was achieved using excitation sculpting. Spectra were processed using Topspin 3.6 and then analyzed using CcpNMR Analysis. Chemical shifts were referenced to internal 4,4-dimethyl-4-silapentane-1-sulfonic acid.

2.2. Flies exposure to peptides

2.2.1. Biological material

2.2.1.1. *Drosophila suzukii* colony

A *D. suzukii* colony was established from a blueberry orchard (*Vaccinium* spp.) located in São Martinho de Mouros, Resende, Portugal (41°07'13.4"N 7°53'17.8"W), collected during the Summer of 2021. Eggs and larvae were obtained by collecting ripe-infested blueberries from this field/site. The colony was housed in plastic vials containing standard drosophila cornmeal diet [1.5% (w/v) agar, 4% (w/v) brewer's yeast, 8% (w/v) cornmeal, 10% (w/v) sugar, 0.08% (w/v) methyl 4-hydroxybenzoate, 0.15% (v/v) orthophosphoric acid, distilled water (dH_2O)], at 25 ºC under a 16:8 h of light:dark photoperiod, and was transferred to new vials every week.

2.2.1.2. Bacteria collection

One set of bacteria was chosen from a collection of the Integrative Biology and Biotechnology Laboratory (iB_2 Lab), which contains bacteria from the gut of adult *D. suzukii* flies and the larval stage. The bacteria species were chosen based on their relevance and prevalence in the fly's microbiome and include two *Acetobacter* sp. (FCP21F-3 and FCP21L-11), two *Tatumella* sp. (LCP21-7 and LCP21-3), one *Microbacterium* sp. (FCP21F-14), and one *Frigobacterium* sp. (MCP21F-13). Bacteria were maintained in Lysogeny broth medium (LB, Liofilchem®, Italy) with agar at 1.5%, until further use.

2.2.2. Ingestion bioassays with SVPs

2.2.2.1. Determination of spider venom peptides' concentrations

Due to a lack of work studying the potential of SVPs through oral ingestion, the peptides' concentrations were determined by resorting to the median lethal dose (LD_{50}) of J-ACTX-Hv1c when applied via injection into *Musca domestica*, i.e. 320 pmol/g[78], as a starting point. Considering that the *D. suzukii* fly has an average weight of 1.5 mg, 0.48 pmol is the equivalent amount of peptide to be ingested per fly.

To determine how much media the flies ingest on average, a set of 4 males and 4 females were left starving for 1 h before being weighted as a pool. Afterward, the flies were fed for 2 h with Formula 4-24® Instant Drosophila Medium (Carolina® medium), and then weighed again. This medium is blue stained, which allowed the ingestion to be

visualized by observing the blue coloration on the flies' abdomen after feeding. The mass of the medium ingested per fly was calculated as the difference between the weight of flies post- and pre-ingestion, divided by the number of flies, which resulted in an average value of 0.28 mg/fly. For this purpose, the excretion was assumed to be zero. Considering that the test vial has 2.6 mg of food, for this quantity 16.1 µg of the peptide is applied, which is equivalent to 2.23 µM. This was the lowest concentration tested in this study, followed by concentrations 10, 50 and 100 times higher.

2.2.2.2. SVPs incorporation within culture medium

Young (2-5 days) *D. suzukii* flies were briefly anesthetized with diethyl ether to sort males and females. Pools of 5 males and 5 females were collected in different empty vials and left in starvation for 30 min. Flies were then transferred to vials containing 0.6 g of the Formula 4-24® Instant Drosophila Medium (Carolina®), dissolved in 2 mL of µ-TRTX-Hhn2b solution with increasing concentrations (2.23, 22.3, and 223 µM) (Figure 14), or dissolved in 2 mL of sterile distilled water for the negative control.

After 2, 4, and 72 h, flies exposed to the lowest concentration (2.23 µM) were housed in vials containing Formula 4-24® free of peptide, following the aforementioned process. Flies were also exposed to µ-TRTX-Hhn2b at 22.3 and 223 µM for 72 h (Figure 14), after which were transferred to a peptide-free medium. The adults were housed in new vials as pupae appeared. For the positive control, a solution of the commercial product Spintor® (Corteva Agriscience) was incorporated in the medium to a final concentration of 5 mL/100L, corresponding to half of the concentration recommended for application in field according to manufacturer instructions. The percentage of surviving flies was monitored and daily recorded during the whole lifespan. Three replicates per combination of concentration and exposure time were used. Peptide µ-TRTX-Hhn2b was selected for protocol optimisation since we had a significantly higher quantity compared to J-ACTX-Hv1c.

2.2.2.3. SVPs soaked in a filter

As an attempt to minimize the amount of peptide used per assay, and to prevent unwanted interactions of the peptide with constituents of the Carolina® medium, that could be hindering its bioactivity, an oral ingestion assay was performed by incorporation of the peptides soaked in sterile filter paper. For this purpose, the tube-sized filter paper was embedded in 180 µL of a solution containing 0.1 M sucrose and yeast extract at 5% (1:1). 180 µL of J-ACTX-Hv1c or µ-TRTX-Hhn2b were also added, to final concentrations of 2.23, 111.5 and 223 µM. For the negative control, 180 µL of dH_20 was added. For the

positive control, a solution of the commercial product Spintor® (Corteva Agriscience) was used at a final concentration of 5 mL/100L. As previously described in section 2.2.2.2. SVPs incorporation within culture medium pools of 5 males and 5 females of *D. suzukii* flies were starved for 30 min and were then housed in the prepared vials for the appropriate assay duration (2, 4, 24, and 48h) (Figure 14), after which were transferred to peptide-free medium to follow all lifespan. The adults were housed in new vials every week, as pupae appeared. The survival percentage was monitored and daily recorded during all lifespans. For each condition, three replicates were made.

2.2.3. Biological assays with AMPs and mixture with SVPs

2.2.3.1. Preliminary assay for antibacterial activity of selected AMPs

To evaluate the bioactivity of antimicrobial peptides against a collection of bacteria relevant to the fly microbiome, a first antibiogram assay was performed. In this assay we tested the activity of three AMPs: 3.1, CA-M, and RW-BP100 (Table 3). Lyophilized peptides were solubilized in sterile distilled water, and working solutions of 200, 400 µM, and 1 mM were prepared.

Pure colonies of bacterial strains were grown overnight in LB medium at 28 ºC, 200 rpm, in an incubating orbital shaker (VWR). The optical density was measured at 600 nm (OD_{600}) in a spectrophotometer (Genesys 10uv Scanning, Thermo Scientific) and adjusted to 0.1. After that, bacterial cultures were inoculated in LB (1.5% agar) medium by spreading 1 mL of each culture, removing the excess after 1 min, and allowing it to dry for 15 min. A volume (1 µL) of each peptide dilution was loaded on previously inoculated LB agar plates. An equal volume of sterile distilled water was used as a negative control, and three antibiotics at 50 µM were tested as positive controls: chlortetracycline, ampicillin, and streptomycin sulphate. Plates were incubated at 28 ºC for 24h, after which the results were registered. AMPs were considered to perform antibacterial activity when there was no growth on the drop site of the peptide. This experiment was performed in three replicates.

Table 3 - Sequence and properties of the antimicrobial peptides (AMPs) used.

Peptide	Sequence	Net charge	MW (Da)
3.1	KKLLKWLLKLL-NH_2	5	1393.9
CA-M	KWKLFKKIGAVLKVL-NH_2	6	1769.2
RW-BP100	RRLFRRILRWL-NH_2	6	1583.0

2.2.3.2. Ingestion bioassays

After assessing through the antibiogram assay that the peptide RW-BP100 was the most efficient in inhibiting the bacteria growth, the remaining work was proceeded with this

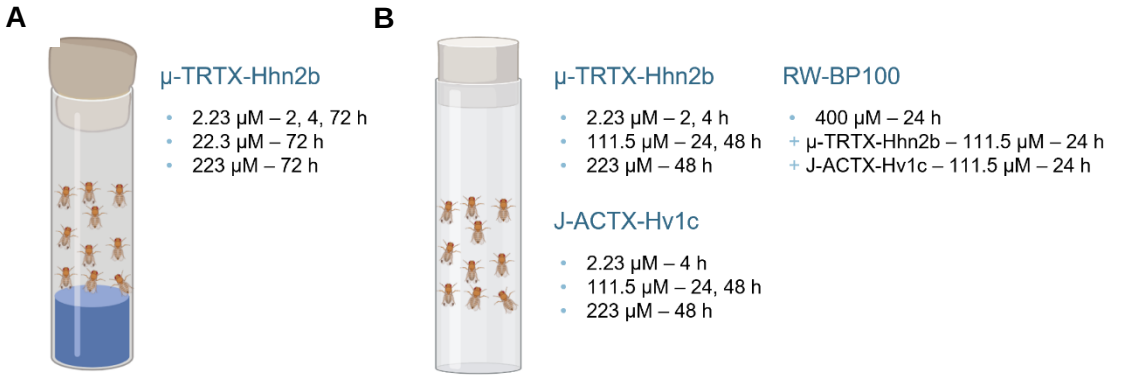


Figure 14 - Schematic overview of peptides' exposures by oral ingestion, through A. incorporation within Carolina® medium, and by B. soaking on filter paper.

AMP. To evaluate the potential effect of the peptide RW-BP100 and its mixture with SVPs on *D. suzukii* survival, oral exposures of these compounds were made following the procedure described in section 2.2.2.3. SVPs soaked in a filter. Pools of 5 males and 5 females of *D. suzukii* flies were exposed to RW-BP100 peptide at 400 μM for 24 h. Flies were also exposed to a mixture of RW-BP100 at 400 μM with both SVPs (J-ACTX-Hv1c and μ-TRTX-Hhn2b) separately at 111.5 μM for a 24 h exposure period (Figure 14). At least 3 replicates of each condition were made for this assay.

2.2.3.3. Evaluation of flies' microbiome by 16s rDNA sequencing

D. suzukii flies with 2-5 days were anesthetized with diethyl ether to allow seriation. Pools of 2 males and 2 females were separated into different empty vials and left in starvation for 30 min. After that, flies were transferred to vials containing a filter embedded in 180 μL of a solution of sucrose 0.1 M and yeast extract 5% in equal volumes (1:1) and 180 μL of RW-BP100 at 800 μM, to a final concentration of 400 μM. For the negative control, the peptide solution was substituted with 180 μL of sterile distilled water. After 24 h of exposure, flies were completely anesthetized and transferred to 2 mL Eppendorf tubes to be sterilized with 10% bleach (3 min), 70% ethanol (3 min), and washed three times with sterile dH_20 (1 min each). Flies were then transferred to 2 mL pre-filled bead mill tubes and 500 μL of PBS 1% was added, and were then homogenized using a FischerBrand™ BeadMill 24 with a 2.8 mm ceramic bead at 2.4 m/s for 60s (ThermoFisher Scientific, Massachusetts, USA), with a run velocity = 2.40 m/s for 30 s.

A volume of 100 µL of the homogenized product was spread onto plates of mannitol medium (mannitol, 25 g; peptone, 1.5 g; yeast extract, 2.5 g; agar, 1.5%; distilled water, 500 mL), Sabouraud Dextrose (Oxoid) Agar (1.5%) medium, and MRS (de Man, Rogosa and Sharpe) medium (1.5% agar). Plates were incubated at 24 ºC and results were registered after 48 h. This experiment was performed three times independently from different flies' pools.

According to morphology, size and color, colonies from the two conditions (negative control and exposed to RW-BP100) were chosen for characterization by colony PCR. For this purpose, each colony was spiked and diluted in 200 µL of sterile dH_2O, and then cell lysis was promoted at 100°C for 5 min, followed by cooling on ice to stop the reaction. Primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3')[160] and 1492R (5'-TACGGYTACCTTGTTACGACTT-3')[160] as a forward and reverse primer, namely, were employed for the amplification of the partial 16S rDNA region. PCR cycling conditions were an initial denaturation step at 94 ºC for 5 min, followed by 25 cycles of 30 s at 94 ºC, 30 s at 55 ºC, and 1 min at 72 ºC, with a final extension for 3 min at 72 ºC. PCR products were analyzed through a 1% agarose gel electrophoresis for 30 min under a constant voltage of 120 V, and amplicons of the expected size (approximately 1500 bp) were excised for DNA extraction, using the NZYGelpure Kit (NZYTech, Lisbon, Portugal) following the manufacturer's protocol. Amplicons were then sent to STAB Vida (Caparica, Portugal) for Sanger sequencing, and the samples' sequences were analyzed with Geneious Prime Software (Version 2022.1.1). The sequencing results were checked with BLASTn toll by NCBI to compare with the sequences of this database. The top blast hit was identified as the isolated microbe.

2.3. Quantitative real-time PCR

2.3.1. Total RNA isolation and cDNA synthesis

Total RNA of flies was isolated with a ready-to-use Tri-reagent (NZYol, NZYTech, Lisbon, Portugal) following the manufacturer's instructions for small quantities of tissue. A pool consisting of 1 male and 1 female fly was randomly chosen from each replicate of the following conditions: J-ACTX-Hv1c and µ-TRTX-Hhn2b at 111.5 µM for 48 h, and the corresponding negative control. Flies' tissue was homogenized in 1.5 mL RNase-free Eppendorf tubes containing 800 µL of NZYol in a FischerBrand™ BeadMill 24 with a 2.8 mm ceramic bead at 2.4 m/s for 60s (ThermoFisher Scientific, Massachusetts, USA), and then incubated for 5 min at RT. After adding 160 µL of chloroform (Analysis grade, Merck, Germany), samples were incubated for 3 min at RT and centrifuged at 12000 ×g

for 15 min at 4 ºC (Mikro 200R, Hettich, North America). The aqueous phase was transferred to a new microtube and DNase I was applied to all samples according to manufacturer's instructions (NZYtech, Lisbon, Portugal), to eliminate the presence of potential genomic DNA. After DNAase treatment, RNA was precipitated with 400 µL of cold isopropyl alcohol (Fischer Scientific) for 1 h at -20 ºC, and then samples were centrifuged at 12000 ×g for 10 min at 4º C. The supernatant was discarded, the pellet was washed with 800 µL of ethanol 70% and samples were centrifuged at 12000 ×g for 5 min at 4 ºC. After discarding the supernatant, the pellet was air-dried, and resuspended in 80 µL of RNase-free water. RNA was stored at -80 ºC until further processing.

The cDNA synthesis was processed using an NZY First-strand cDNA synthesis Kit (NZYTech, Lisbon, Portugal) following the manufacturer's protocol: 100 ng of total RNA was used in a 20 µL total reaction volume. The reaction samples were gently mixed and incubated for 10 min at 25 ºC, 30 min at 50 ºC, and 5 min at 85 ºC, and chilled on ice. At this point, 1 µL of NZY RNase H was added to remove the RNA bonded to cDNA and incubated for 20 min at 37 ºC. The cDNA product was diluted in sterile distilled water in a 1:10 ratio.

The absorbances at 230, 260, and 280 nm and the ratios 260/280 and 260/230 were determined to assess the purity of RNA and cDNA, with a FLUOstar Omega Microplate Reader (BMG LABTECH, Ortenberg, Germany). The RNA and cDNA concentrations were calculated following the equations 1 and 2, respectively. Working solutions of 60 ng/µL cDNA were made and stored at -20 ºC until further use.

$$\text{RNA concentration} = 40\ \mu g/mL \times OD_{260} \times \text{dilution factor} \quad (1)$$

$$\text{cDNA concentration} = 33\ \mu g/mL \times OD_{260} \times \text{dilution factor} \quad (2)$$

2.3.2. Design of primers

The PCR primer sequences used in this study are listed in Table 4. *D. suzukii* genes' sequences were obtained from the Spotted Wing Fly Base (http://spottedwingflybase.org/). Unless referenced, primers were designed using Primer3Plus (version: 3.2.6)[161] using the following settings: melting temperatures between 55 and 62 ºC, GC content between 40-60%, amplicon size range between 70-120 bps, primer size between 18 and 24 bps, and the maximum self-complementarity of the primers was set at 5 and the maximum 3' complementarity at 2. As housekeeping reference genes, the elongation factor 1 beta (*ef1a48D*), TATA binding protein (*tbp*) and arginine kinase (a*rgK*) were selected based on Zhai *et al.*[162].

Table 4 - *Drosophila suzukii* primers used for Real-Time quantitative PCR analysis.

Gene symbol	Gene ID[a]	Primer sequences (5' to 3')[b]
Housekeeping		
ef1a48D[c]	DS10_00002426	F: TGGGCAAGGAAAAGATTCAC
		R: CGGCCTTCAACTTATCCAAA
tbp[c]	DS10_00003466	F: CCACGGTGAATCTGTGCT
		R: GGAGTCGTCCTCGCTCTT
argK[c]	DS10_00003811	F: CTACCACAACGATGCCAAGA
		R: AAGGTCAGGAAGCCGAGA
Heat shock proteins		
hsc70-4[d]	DS10_00009978	F: TGCTGATCCAGGTGTACGAG
		R: CGTTGGTGATGGTGATCTTG
hsp83[e]	DS10_00007878	F: CCGAGGATGAGAACGAGAAG
		R: TGGACTTCATCAGCTTGCAC
Insulin/Tor pathway		
tor	DS10_00007688	F: AACTGGCTAACATGCCGACT
		R: CGGCGGTACTCTTGTCTCTC
foxo[d]	DS10_00012524	F: CTCCCTGAACACGTACAGCA
		R: CTTCGACATTGCACTCCAGA
gadd45α	DS10_00005644	F: GCGACATGGATATGGAGATG
		R: AGAAGGGCCGATTTCACC
sod2	DS10_00003278	F: TGGGAGCACGCCTACTATCT
		R: GTCGTCCCAGTTAGCGATGT
vg	DS10_00005777	F: ACCACCAGGTTAGCGACTTG
		R: CGTGGGCATAGGAACCATAG
chico	DS10_00000455	F: TTATTTGGCGATGTCAACCA
		R: GCTCTGGAAAGTCGAAATGC
thor[d]	DS10_00000228	F: ATCATCTCGGATCCAATCCA
		R: ATTTGCGGAAGGGAGTACG
P450 pathway		
ac13E	DS10_00006210	F: TCACCTCGTTGAGCATGAAG
		R: GGATGGATAATGCCACGTTC
mpk2	DS10_00008948	F: CCCATCCGTATCTGGAGAAG
		R: GGCAGATCCATATCCTCGAA
cyp12d1-d	DS10_00002643	F: GACGGTCTGGATTCGATTGT
		R: TCGTCTTGTGAAGCAACCAG
Apoptotic stimuli		
dcp-1	DS10_0002956	F: ACACGCCGACTTTCTGTTCT
		R: CCAGGAGCCGTTGTTAATGT
th	DS10_00011088	F: CGGGCATGTTCTACACACAC
		R: GGGCAGATCCTCCTGCTC

[a] In Spotted Wing Fly Base (http://spottedwingflybase.org/); [b] F and R refer to forward and reverse primers, respectively; [c] Primers from Zhai *et al*.[162]; [d] Primers from Toxopeus *et al*.[163].

2.3.3. Gene expression evaluation by Real-time quantitative PCR

For the quantification of gene expression in *D. suzukii*, real-time quantitative PCR (RT-qPCR) amplifications were performed with a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, California, United States), using the primers listed in Table 4. The reaction mixture consisted of 10 µL of NZYSupreme qPCR Green Master Mix (2x), ROX plus (NZYTech, Lisbon, Portugal), 400 nM of each primer, 2 µL of cDNA template (120 ng), and 6.4 µL of sterile Milli-Q water, for a reaction volume of 20 µL. No-template controls were included in each set of reactions. The amplification program consisted of an initial polymerase activation at 95 ºC for 2 min, followed by 45 cycles, each one consisting of denaturation for 5 sec at 95 ºC and 30 sec at 60 ºC for the annealing and extension. The product fluorescence was detected at the end of each cycle. At the end of the qPCR run, melting curves were obtained by 0.5 ºC increments in 5 s/cycle from 65 to 95 ºC, with continuous fluorescence detection, to detect the presence of contaminations or off-target amplifications. All PCR experiments were carried out in triplicate. RDML-LinRegPCR tool was used to calculate primers efficiency. Relative quantification of gene expression was calculated using the standard ΔΔCq method as described by Taylor *et al.*[164]

2.4. Statistical analysis

GraphPad Prism (Version 9.0.0) was used for statistical analysis. Survival curves were calculated and plotted as Kaplan-Meier curves. The Log-rank (Mantel-Cox) test was used to compare survival probabilities among control and treatments, and interpretation of individual *p* values was performed using the Bonferroni method. Flies that escaped during the assay were censored. Comparisons between treatments and the control for gene expression levels were made with the One-way ANOVA test, corrected with Dunnett's T3 multiple comparisons test. Multivariate analyses for data correlation used Principal Component Analysis (PCA). Significance was defined as $p < 0.05$.

3. Results

3.1. Synthesis and oxidative folding of J-ACTX-Hv1c and µ-TRTX-Hhn2b

The J-ACTX-Hv1c and µ-TRTX-Hhn2b synthesis was performed with 2-CTC resin by automatic SPPS using the Fmoc/^{t}Bu chemistry. Figure 15 shows the chromatograms obtained for the synthesis crude products, where it is possible to observe impure products with major peaks (highlighted with the symbol *). As such, these peaks were analysed by LC-MS and confirmed to match the target peptides' molecular weights (Figure 15). The HPLC chromatogram of crude J-ACTX-Hv1c reveals a major impurity on the left side of the major peak (Figure 15A), which was analysed by LC-MS and MALDI-TOF (data not shown), revealing to have a molecular weight of 3665.3 Da, so it was determined it corresponded to the peptide J-ACTX-Hv1c less one cysteine coupled.

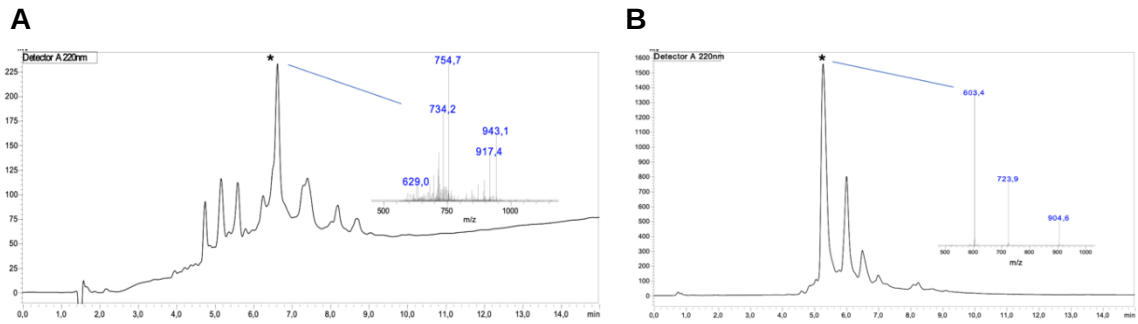


Figure 15 - HPLC chromatogram and LC-MS spectra of crude products from solid-phase synthesis of peptides A. J-ACTX-Hv1c (Rt = 6.64 min) and B. µ-TRTX-Hhn2b (Rt = 5.28 min). The symbol * highlights the major peak of each synthesis, which were confirmed by LC-MS analysis to be the desired peptides. The elution gradient used was 5-50% (for J-ACTX-Hv1c) and 15-50% (for µ-TRTX-Hhn2b) of solvent B into A (A: 0.1% TFA in water; B: 0.1% TFA in ACN), for 15 min, with a running flow of 1 mL/min and detection at λ = 220 nm.

The purification of the desired peptides from crude products was possible and successfully performed by preparative HPLC (data not shown). An attempt to synthesize µ-TRTX-Hhn2b using a Fmoc-L-Leu-Wang resin revealed to produce a product of very low quality (Figure I. 1), significantly hindering its purification, so it was no further used.

For the oxidative folding step, the HPLC-purified linear peptides were used. The *in vitro* folding was carried out in an 0.1 M NH_4OAc buffer containing both GSH and GSSG under anaerobic conditions. This process was monitored by HPLC (Figure 16) and LC-MS (data not shown) over time. For both peptides, the plateau was almost reached after 5 h from the beginning of the oxidative process, with the major peaks corresponding to 62.7% and 65.5% of the final products (respectively, J-ACTX-Hv1c and µ-TRTX-Hhn2b)

(Figure 16C and D). The oxidation yield was the maximum after 22 h for peptide J-ACTX-Hv1c (69.2%) and after 23h for peptide μ-TRTX-Hhn2b (67.6%) (Figure 16C and D). The impurities present in these chromatograms represent peptides that have acquired different conformations, i.e., with different disulphide connectivity. So, the oxidation products were also purified to isolate the main products, which by LC-MS (Figure 17) and MALDI-TOF (Figure I. 2 and Figure I. 3) analysis were confirmed to have experimental masses corresponding to the theoretical ones (Table 5).

As it was necessary to repeat the complete synthesis for both peptides to obtain a higher amount of the desired product, it was possible to verify the reproducibility in the profile of the peptides from different synthesis batches (data not shown). From three synthesis of J-ACTX-Hv1c it was obtained 17.4 mg, and 46.1 mg of μ-TRTX-Hhn2b was obtained from two independent synthesis. J-ACTX-Hv1c and μ-TRTX-Hhn2b final products were obtained in high purity degrees of 97.3% and 99%, respectively (Table 5). The high similarity of chemical shifts (data not shown) compared to those previously reported in the literatures,[81, 87] supported that each of the synthetic peptides have the same disulphide connectivity and adopt the same backbone structure in solution, similarly to

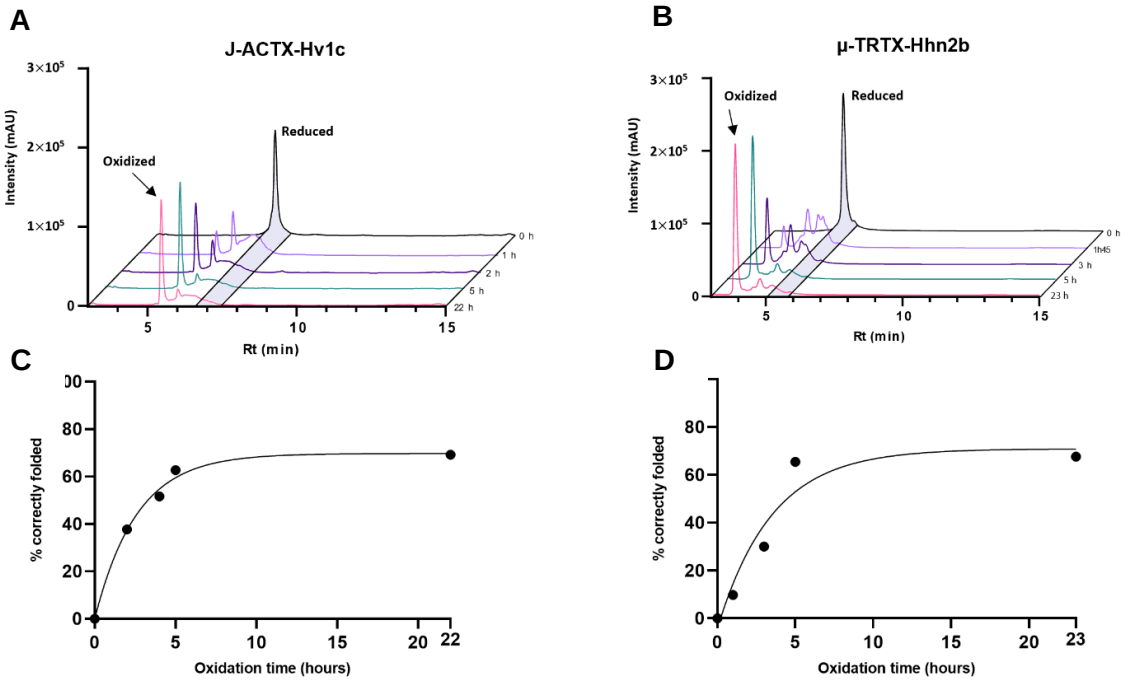


Figure 16 - HPLC chromatograms of A. J-ACTX-Hv1c and B. μ-TRTX-Hhn2b during oxidative folding. Peptides' oxidations were promoted at RT in an anaerobic environment, with 0.1 M NH_4OAc and 1 M Gd·HCl in the presence of GSH/GSSG, pH 7.8. The folding was monitored at multiple time points by HPLC and confirmed by LC-MS (data not shown). Arrows indicate the major peaks after oxidative folding, corresponding to the peptides' native conformation (later confirmed by NMR analysis); progress of C. J-ACTX-Hv1c and D. μ-TRTX-Hhn2b folding reaction as monitored by integration of the major peak at each time point.

the corresponding wildtype, which confirms the success of the established synthesis process.

Table 5 – Analytical characterization of the final oxidized peptides. Purity and retention time (Rt) were obtained by HPLC, and m/z detected by LC-MS. In both analyses, elution was performed with a gradient of 5-50 % ACN (0.1% TFA) in water (0.1% TFA), 1 mL/min, for 15 min.

Peptide	Theoretical MW (Da)	Purity (%)	Rt (min)	m/z
J-ACTX-Hv1c	3760.24	97.3	5.43	$[P+2H]^{2+}$ = 1881.15; $[P+3H]^{3+}$ = 1253.85; $[P+4H]^{4+}$ = 941.05; $[P+5H]^{5+}$ = 753.05
µ-TRTX-Hhn2b	3608.17	99	7.07	$[P+3H]^{3+}$ = 1203.35; $[P+4H]^{4+}$ = 903.00; $[P+5H]^{5+}$ = 722.60; $[P+6H]^{6+}$ = 602.35

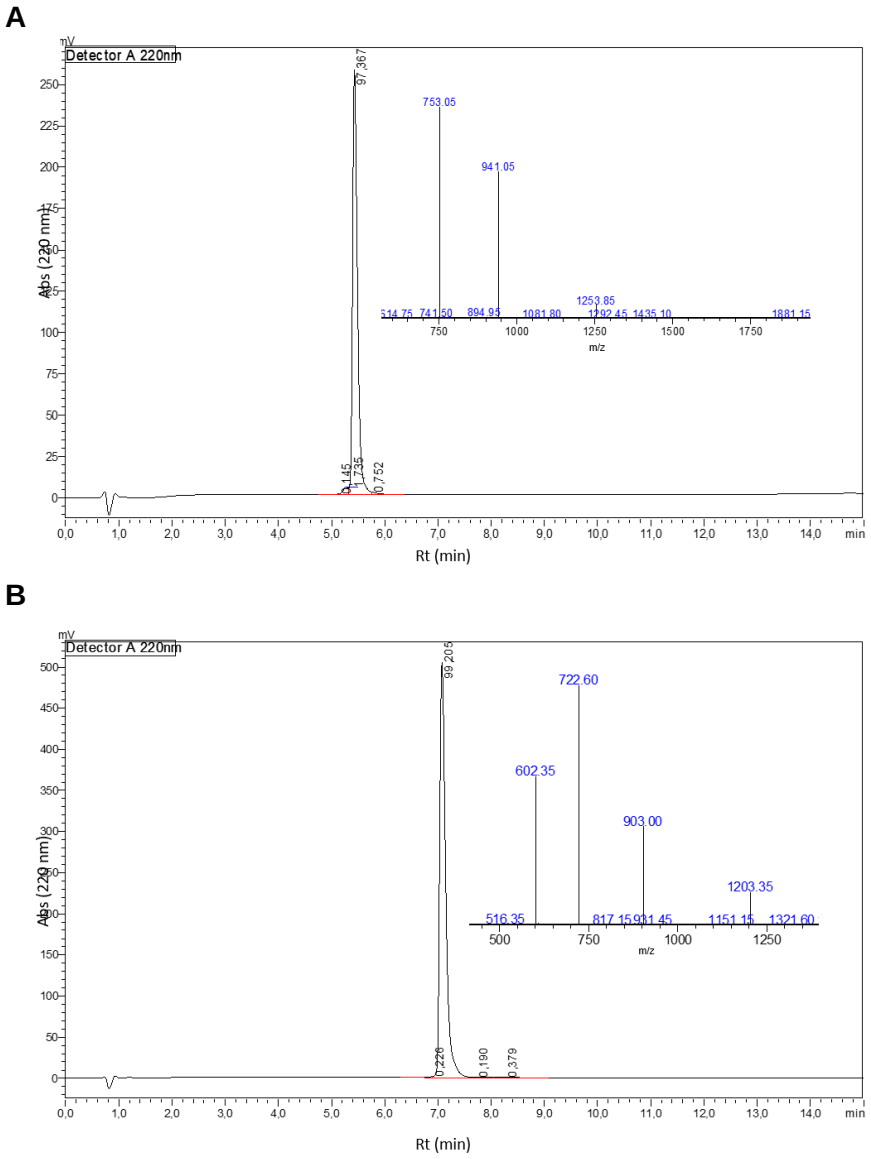


Figure 17 - A. chromatogram of purified J-ACTX-Hv1c after oxidation (inserted is corresponding LC-MS spectrum: $[P+2H]^{2+}$ = 1881.15; $[P+3H]^{3+}$ = 1253.85; $[P+4H]^{4+}$ = 941.05; $[P+5H]^{5+}$ = 753.05); B. chromatogram of purified µ-TRTX-Hhn2b after oxidation (inserted is the corresponding LC-MS spectrum: $[P+3H]^{3+}$ = 1203.35; $[P+4H]^{4+}$ = 903.00; $[P+5H]^{5+}$ = 722.60; $[P+6H]^{6+}$ = 602.35).

3.2. Bioassays with SVPs

3.2.1. Orally administered SVPs may disrupt *D. suzukii* survival

To evaluate the insecticidal potency of selected SVPs against SWD, flies were first fed with Carolina® medium prepared with a solution of μ-TRTX-Hhn2b for a final concentration of 2.23 μM. There was no statistically significant effect on *D. suzukii* mortality when treated for 2 and 4 h ($p > 0.05$) (Figure 18A), so the time of exposure was increased for 72 h and the peptide concentration to 10- and 100-fold higher. Despite the increased concentrations and exposure period, the flies' survival was not significantly affected by the ingestion of this peptide ($p > 0.05$) (Figure 18B). It is important to note that, at this stage, only one replicate was made for the concentration 223 μM, as this involved a highly significant amount of peptide, and the protocol was being optimized. The Kaplan-Meier survival graphics of this study only present the results of the first 40 days of the experiments, since from this stage onwards mortality showed a general decline, and from the point of view of the biological question of this study, there was no interest in evaluating such long-term effects.

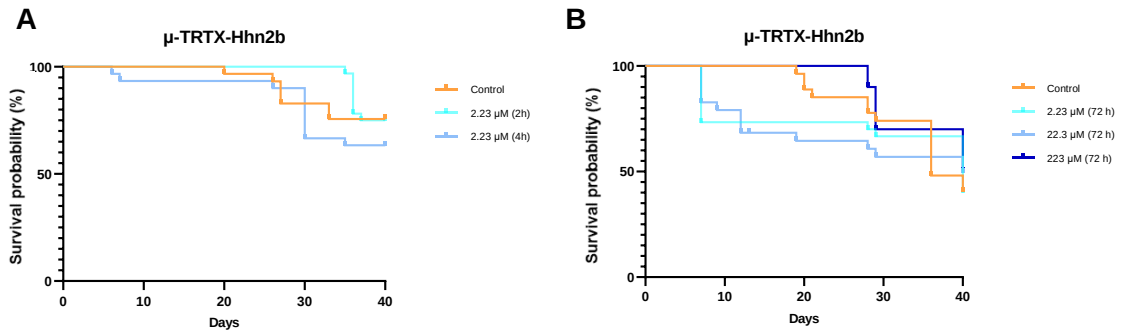


Figure 18 - Kaplan-Meier survival curves of *D. suzukii* flies treated with peptide μ-TRTX-Hhn2b incorporated in Carolina® medium at concentrations of A. 2.23 μM (for 2 and 4 h) and B. 2.23, 22.3 and 223 μM for 72 h. *Y*-axis indicates the probability of survival over time. Censoring is indicated as vertical marks. Only the results of the first 40 days after exposure to the peptide are shown.

Assessment of *D. suzukii* flies' mortality fed with peptide-soaked filter paper was first performed with the lowest concentration of 2.23 μM for short periods of time (2 h for J-ACTX-Hv1c; 2 and 4 h for μ-TRTX-Hnn2b), which showed no statistically significant variation in mortality when comparing flies treated with μ-TRTX-Hnn2b with untreated control flies ($p > 0.05$) (Figure 19A). The median survival time (days) of flies for control, 2.23 μM J-ACTX-Hv1c for 4 h, and 2.23 μM μ-TRTX-Hhn2b for 2 and 4 h was 20, 33, 20 and 26, respectively (data not shown). Flies treated with 2.23 μM of J-ACTX-H1vc were shown to have increased viability in the first days after treatment, but by day 34 all flies of this condition were dead, thus showing a decreased longevity when comparing with

control and μ-TRTX-Hnn2b-treated flies (Figure 19A). Kaplan-Meier survival curves revealed significant differences between all groups (p = 0.0295) but comparing the control survival curve with each treatment curve, results were not statistically significant using the family-wise significance level of 5% (p values > 0.0167).

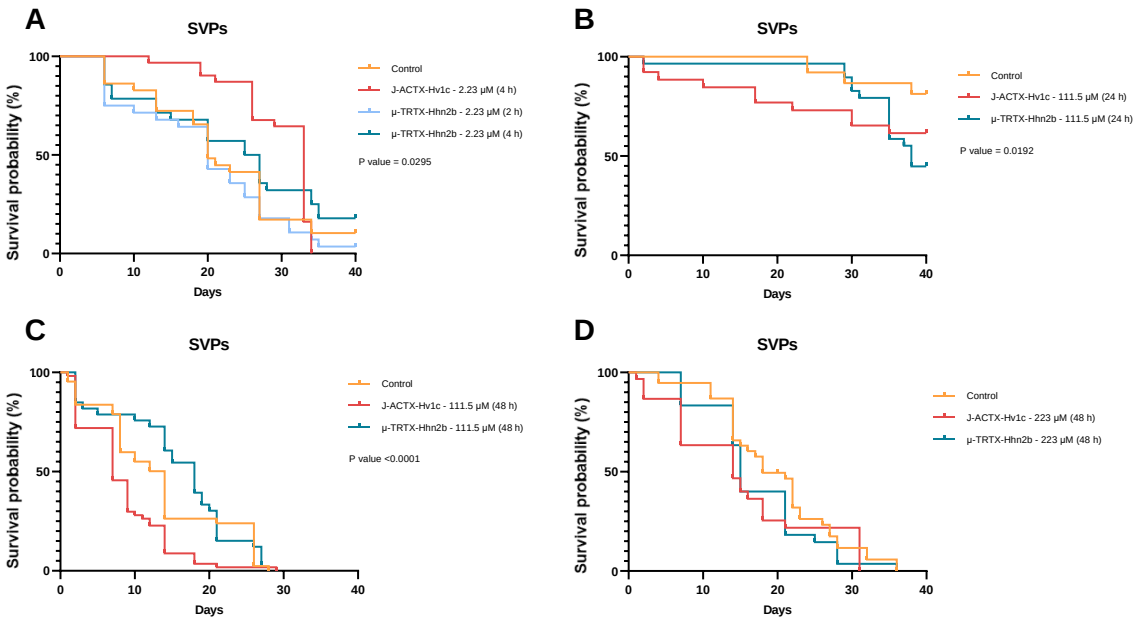


Figure 19 - Kaplan-Meier survival curves of *D. suzukii* flies orally exposed to SVPs at different concentrations and treatment times: A. 2.23 μM of peptides J-ACTX-Hv1c (4 h) and μ-TRTX-Hhn2b (2 and 4 h); B. SVPs at 111.5 μM for 24 h; C. SVPs at 111.5 μM for 48 h; D. SVPs at 223 μM for 48 h. *Y*-axis indicates the probability of survival over time. Censoring is indicated as vertical marks. Only the results of the first 40 days after exposure to the peptide are shown.

Taking into account the results obtained with the first series of concentrations, it was next increased the peptides' concentrations to 111.5 μM (24 and 48 h) and 223 μM (48 h). The Figure 19B reveals a significant difference between the control and treatment groups (p = 0.0192), although the correction for multiple comparisons only considers statistically significant the treatment with μ-TRTX-Hhn2b (p = 0.0036). In this case, flies treated with J-ACTX-Hv1c (111.5 μM, 24 h) show a slight increased mortality compared to the other two conditions, however this difference revealed to be not statistically significant.

The Kaplan-Meier survival curves of Figure 19C showed that the median lifespans of flies fed with J-ACTX-Hv1c at 111.5 μM for 48 h was significantly decreased to 7 days compared to the median lifespan of 14 days for the control group (p = 0.0017). It was also observed that, as already pointed out for low concentrations of J-ACTX-Hv1c, in this case flies treated with μ-TRTX-Hnn2b at 111.5 μM for 48 h had a higher survival, with a median lifespan of 18 days, although this increase was not statistically significant (p > 0.05) (Figure 19C). Doubling the peptides' concentration to 223 μM had no visible effects on the percentage of fly survival, as there were no significant differences amongst control and treatments across the whole experiment (Figure 19D). Although the susceptibility to

different conditions did vary, the different dose-response assays were developed at different times, consequently no statistical comparison was made between assays.

The control with 5 mL/100 L of Spintor® led to a 100% mortality of flies within 30 min when administered by both incorporation in the Carolina® medium and through filter paper (data not shown).

3.2.2. Gene expression in response to SVPs

Considering the previous results, in order to understand whether the peptides were having non-visible effects or if stress response mechanisms were being activated at the molecular level, it was further evaluated the transcript expression of some stress-related genes by RT-qPCR.

The expression patterns of genes *ac13E*, *mpk2*, *cyp12d1-d*, *hsp83*, *hsc70-4*, *chico*, *foxo*, *tor*, *vg*, *sod2*, *gaad45α*, *thor*, *th*, *dcp-1* for control and treated flies with J-ACTX-Hv1c and μ-TRTX-Hhn2b at 111.5 μM for 48 h are presented in Figure 20. Analysis of the expression of genes revealed that *D. suzukii* flies did not show significant differences in the expression of the 14 genes evaluated, when comparing treated and control flies. Although no differential expression was observed, some genes displayed very modest fold changes. The transcript level of *cyp12d1-d* was up-regulated by 1.56-fold after treatment with μ-TRTX-Hhn2b (Figure 20C). In flies treated with J-ACTX-Hv1c, the transcript level of *hsc70-4* and *dcp-1* was also up-regulated by 0.73-fold (Figure 20E) and 0.84-fold (Figure 20N), respectively. The transcript level of *chico* was 0.45-fold down-regulated for flies fed with μ-TRTX-Hhn2b when compared to the control (Figure 20F).

3.2.3. Multivariate analysis reveals a correlation of increased gene expression in response to treatment with SVPs

Principal Component Analysis revealed a clear separation between control and treatment groups (Figure 21). The first axes (PC1) where most of the variance is explained (62.34%) separates the control from the treatments. The second axis (PC2) explains 37.66% of the variance and separates treatment J-ACTX-Hv1c from μ-TRTX-Hhn2b. Control is located at the upper right quadrant and correlates with gene *thor*. The J-ACTX-Hv1c is located on the upper left quadrant and is the treatment scoring the

highest for genes *hsc70-4*, *mpk2*, *sod2* and *th*. Treatment µ-TRTX-Hhn2b is located on the lower left quadrant and highly correlates with genes *ac13E*, *tor* and *cyp12d1-d*.

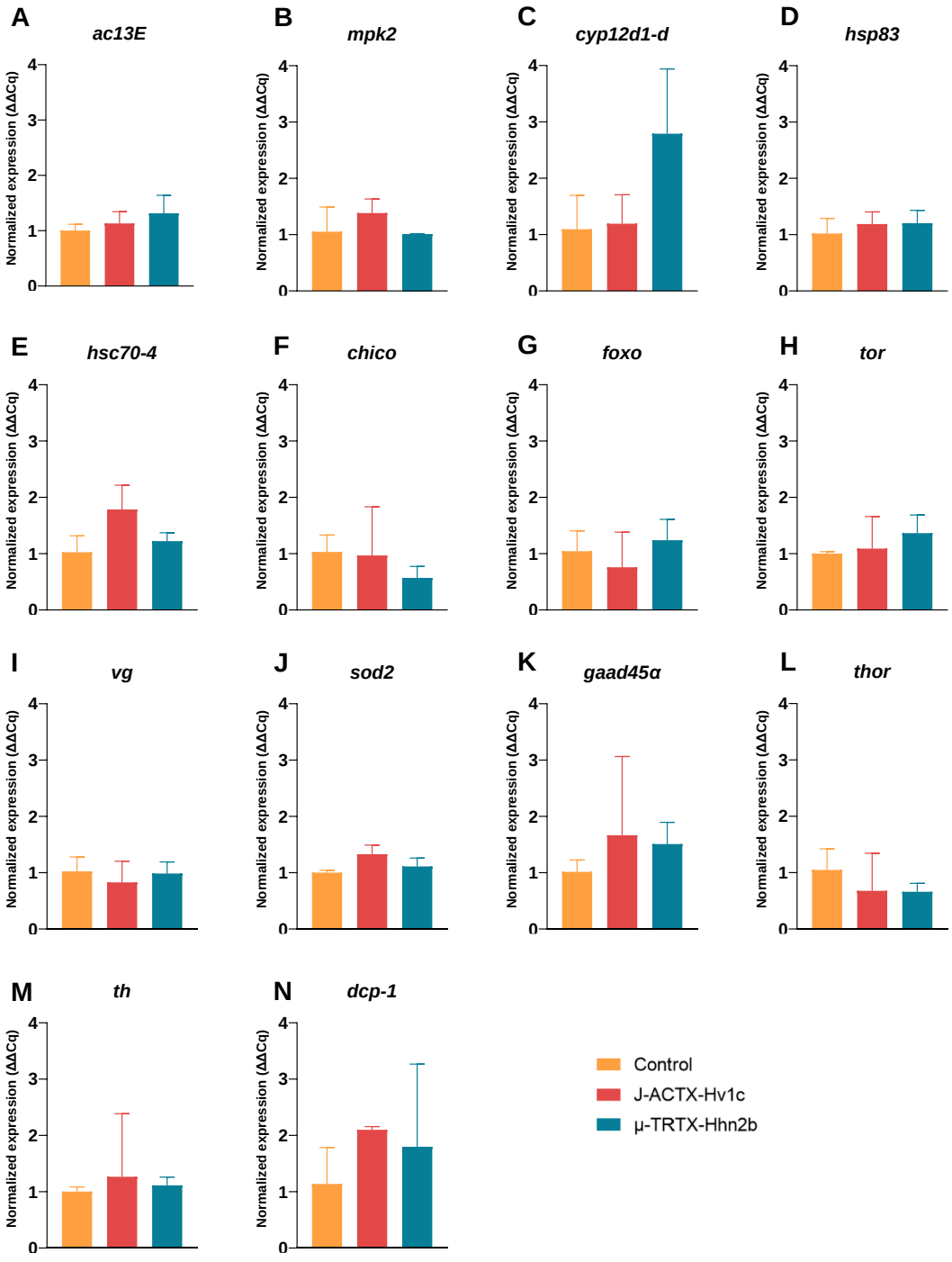


Figure 20 - Normalized expression levels of different genes in response to oral ingestion of J-ACTX-Hv1c and µ-TRTX-Hhn2b (111.5 µM for 48 h) by *D. suzukii* flies. Transcripts related with cytochrome P450 pathway: A. adenylyl cyclase 13E (*ac13E*), B. p38a MAP kinase (*mpk2*), C. cytochrome P450 12d1 proximal (*cyp12d1-d*). Transcripts related with HSPs expression: D. heat shock protein 83 (*hsp83*), E. heat shock protein 70 cognate 4 (*hsc70-4*). Transcripts related with insulin-like receptor/TOR pathway: F. *chico*, G. forkhead box, sub-group O (*foxo*), H. target of rapamycin (*tor*), I. vestigial (*vg*), J. superoxide dismutase 2 (*sod2*), K. growth arrest and DNA damage-inducible 45 (*gaad45α*), L. *thor*. Transcripts related with apoptotic stimuli: M. death-associated inhibitor of apoptosis 1 (*th*), N. death caspase-1 (*dcp-1*). Data are presented as means ± SE.

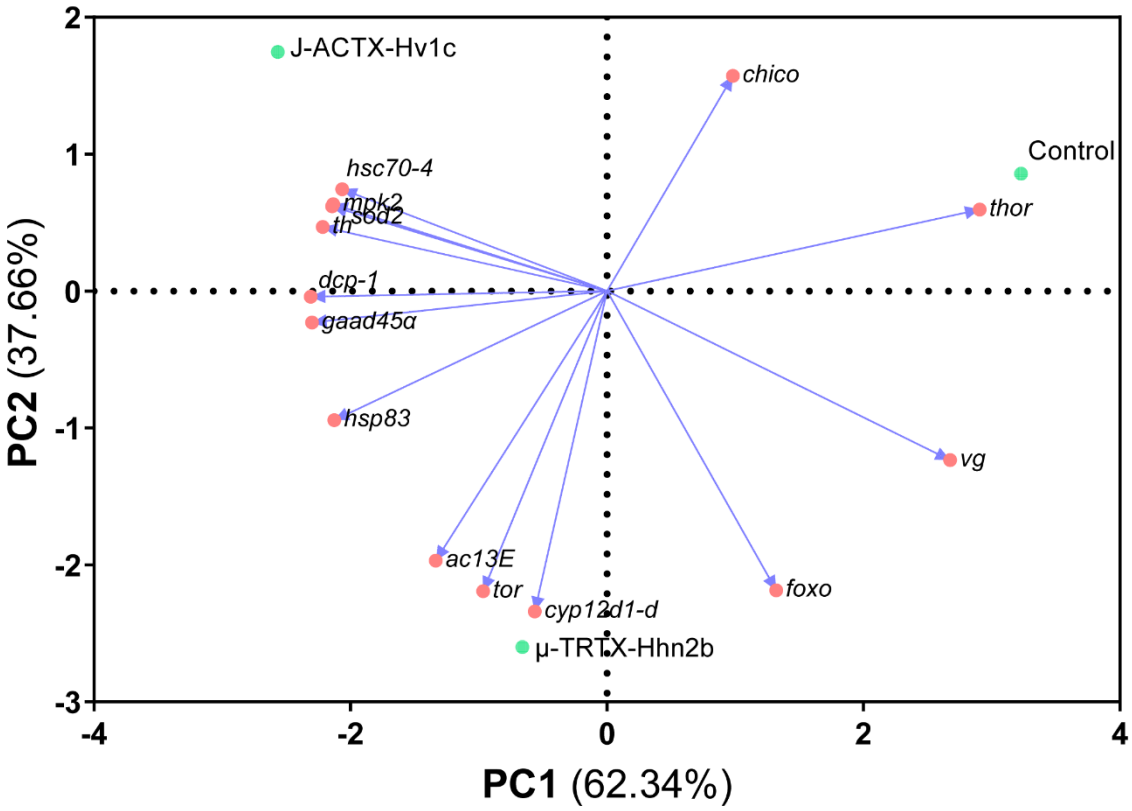


Figure 21 - Principal components analysis (PCA) biplot of the effects of peptides J-ACTX-Jv1c and μ-TRTX-Hhn2b at 111.5 μM (48 h of exposure) on *D. suzukii* genes expression. Abbreviations: *ac13E* - adenylyl cyclase 13E, *tor* – target of rapamycin, *foxo* - forkhead box, sub-group O, *gaad45α* - growth arrest and DNA damage-inducible 45, *sod2* - superoxide dismutase 2, *vg* – vestigial, *mpk2* – p38a MAP kinase, *cyp12d1-d* - Cytochrome P450 12d1 proximal, dcp-1 – death caspase-1, *th* - death-associated inhibitor of apoptosis 1, *hsp83* - heat shock protein 83, *hsc70-4* - heat shock protein 70 cognate 4.

3.3. Manipulation of *D. suzukii* microbiome with AMPs

3.3.1. Cationic AMPs display inhibitory activity against gut bacteria

To explore the potential to control the SWD by manipulating its microbiome, it was first tested *in vitro* the antibacterial activity of the AMPs 3.1, CA-M and RW-BP100 against six bacterial strains relevant in the *D. suzukii* microbiome. These peptides were already reported to have antimicrobial activity against Gram-negative and Gram-positive bacteria.[165-168]

Although the results were not very distinct between the three peptides tested, the antibiogram assay revealed the RW-BP100 as the preferred peptide for handling *D. suzukii* colonizing bacteria, as this AMP inhibited the growth of four bacterial strains at a concentration of 200 μM, while strain LCP21-3 (*Tatumella* sp.) was only susceptible at 400 μM, and the strain FCP21F-3 (*Acetobacter* sp.) was not susceptible to any of the AMPs in the range of concentrations tested (Figure 22). Interestingly, these two strains (LCP21-3 and FCP21F-3) were the only strains not susceptible to any of the antibiotics

(chlortetracycline, ampicillin, and streptomycin sulphate) used in this assay as positive controls (data not shown).

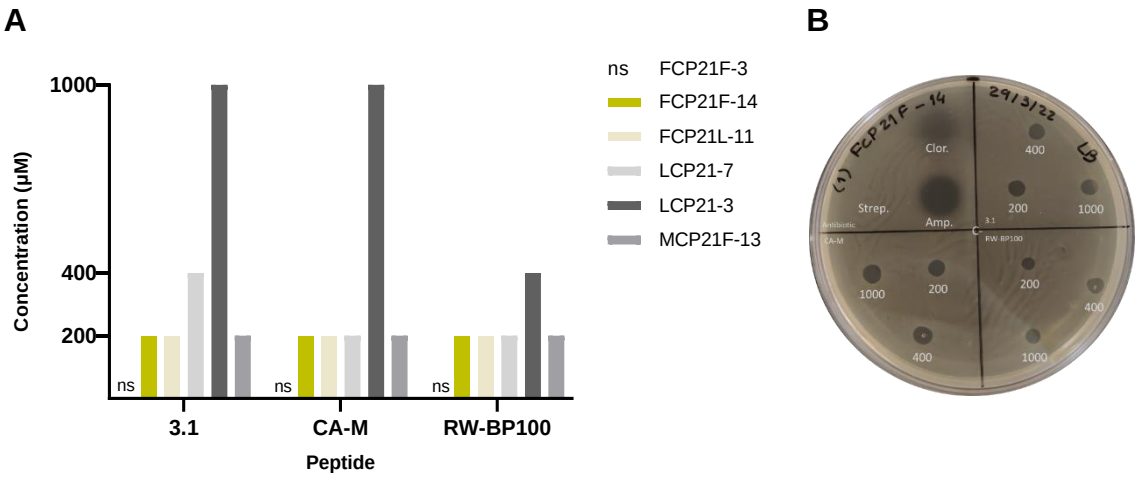


Figure 22 - A. Antibiogram results of peptides 3.1, CA-M and RW-BP100 against bacteria isolated from *D. suzukii*'s gut; B. Representation of the antibiogram assay in a plate with bacteria carpet. Abbreviations: Amp. – ampicillin, Clor. – chlortetracycline, C- - negative control, ns – non susceptible, Strep. - streptomycin sulphate).

3.3.2. Combination of RW-BP100 with SVPs may affect *D. suzukii* survival

Following the previous results, it was proceeded with RW-BP100 to determine the susceptibility of *D. suzukii* to the oral administration of this peptide. The Kaplan-Meier survival curve shows that there was no statistically significant difference in the survival of the flies after feeding with medium containing 400 µM RW-BP100 for 24 h, compared to the control group ($p > 0.05$) (Figure 23A). Although no differences were observed, it was hypothesised that a combination of RW-BP100 with SVPs could hinder the survival of *D. suzukii*. The mixture of 400 µM RW-BP100 with 111.5 µM J-ACTX-Hv1c (24 h treatment) led to an initial decrease in the survival probability, but this probability

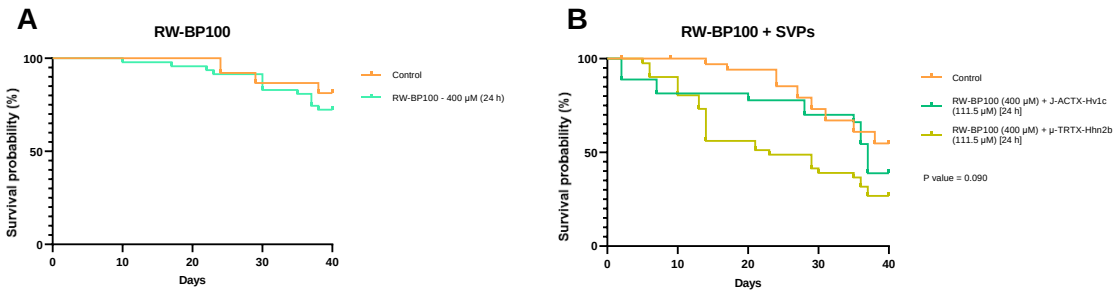


Figure 23 - Kaplan-Meier survival curves of *D. suzukii* flies orally exposed to A. RW-BP100 at 400 µM for 24 h and B. RW-BP100 (400 µM) conjugated with SVPs at 111.5 µM (for 24 h). *Y*-axis indicates the probability of survival over time. Censoring is indicated as vertical marks. Only the results of the first 40 days after exposure to the peptide are shown.

remained stable, with no further evidence of the action of the mixture on *D. suzukii* survival (Figure 23B). On the contrary, flies treated with 400 µM RW-BP100 and 111.5 µM µ-TRTX-Hv1c for 24 h presented much higher mortality compared to the control in the first 14 days after treatment (Figure 23B), with 40% less probability of survival. By day 40 this difference was equal to 27%, and the overall treatment showed to have a statistically significant difference with the control (p = 0.0022 after Bonferroni correction).

3.3.3. Genotypic identification results of 16s rDNA gene sequencing

To assess if there was a disturbance in the flies' microbiome after oral exposure to 400 µM of RW-BP100 for 24 h, the microbiome extraction and culture was performed in different media. After 48 h of incubation, colonies from control and treated flies showing morphological differences regarding colour, size, and brightness were selected (Figure II. 1) and their DNA was extracted for sequencing of 16s rDNA.

Table 6 - Identification of bacteria isolates from 16s rDNA partial sequencing. Bacteria were isolated from *D. suzukii* microbiome after 24 h of treatment with RW-BP100 at 400 µM through oral ingestion. Sequencing results were checked with BLASTn toll by NCBI to compare with published sequences. The top blast hit (Sequence ID) was identified as the isolated microbe.

Isolate code	Result	Sequence ID	Similarity	E-value
C3_F	*Acetobacter thailandicus*	MK049961.1	100%	0
C3_R	*Acetobacter thailandicus*	MG279703.1	100%	0
RW1_F	*Acetobacter thailandicus*	MK049961.1	99%	0
RW1_R	*Acetobacter thailandicus*	MG279703.1	100%	0
RW3_F	*Acetobacter thailandicus*	MK049961.1	100%	8.00E-131
RW3_R	*Acetobacter thailandicus*	MG279703.1	100%	0
RW5_F	*Acetobacter thailandicus*	MK049961.1	99%	1.00E-174
RW5_R	*Acetobacter thailandicus*	MG279703.1	100%	0
RW6_F	*Acetobacter thailandicus*	MK049961.1	100%	5.00E-97
RW6_R	*Acetobacter thailandicus*	MG279703.1	100%	0.00E+00

By visual observation of the cultivated media plates, there were no significant differences in the number of bacteria grown (data not shown). In terms of morphology, plates with bacteria from the treatment group had higher variety of bacteria than plates from the control group (untreated flies). However, partial sequencing of the 16s rDNA region revealed that all the isolates corresponded to the same bacterial species, i.e., *Acetobacter thailandicus* (Table 6).

4. Discussion

D. suzukii is an insect pest increasingly threatening crops around the world. Given the inefficiency of the control methos currently in use, and their toxicity effects to non-target species and to the environment, there is an urgent need for new classes of insecticides, that combine efficiency and safety to be used to control this pest. Within this context, this project aimed to explore new sustainable and efficient tools, based on natural peptides, to manage this pest.

The first step towards these objectives was the validation of a chemical synthesis method for the complex venom peptides J-ACTX-Hv1c and μ-TRTX-Hhn2b. The 2-CTC resin was selected for the synthesis of SVPs, as this resin offers several advantages, namely, it minimizes racemization during the coupling of the first AA, and allows the peptide to be cleaved from the resin at mild acidic conditions (e.g., 2-4% TFA), so the protected peptide can be obtained for further hybridization in solution or solid phase, in order to prepare larger peptides.[169] Furthermore, this resin offers the possibility to be re-used after complete peptide removal with TFA treatment, and subsequent reactivation with $SOCl_2$, which makes it a greener approach.[146, 169]

Despite the successful automated synthesis, J-ACTX-Hv1c had a substantially low synthesis yield, as it is a peptide with a longer sequence, which may hamper the correct coupling of some more challenging AAs.[101] As evidenced in Figure 15A, the major peak has a left-hand impurity (shorter retention time) in a sizeable amount, which significantly decreased the synthesis yield. Additionally, it hinders the purification of the target peptide, thus also contributing to a lower yield.

The need to perform two purifications along the synthesis, one before and one after the oxidative folding step, also contributes to lower the yield. However, a pre-oxidative folding purification is required to limit the number of unwanted interactions during oxidation, thus improving the overall efficiency of the *in vitro* folding process, and providing cleaner products.

The oxidative folding was validated as a time-efficient method to obtain the peptide's native structure. This approach aims to mimic physiological conditions that naturally-promotes the desired structures. It was observed that in the anaerobic redox environment, both peptides within a short period acquired its natural conformation, which is thermodynamically more stable.[154] The high dilution of peptides promoted the intramolecular reaction to provide monomeric disulphides as major products (> 67%) (Figure 18), however dimers and oligomers are also formed.[156] The success of the

synthesis and oxidative folding processes was subsequently confirmed by determining the NMR spectra (data not shown). Having 8 cysteines, the Janus-faced peptide can be structured in 105 different ways, whereas µ-TRTX-Hhn2b, with 6 cysteines, can acquire 15 different conformations,[156] thus emphasizing the importance of NMR-based structural determination. The oxidative folding of J-ACTX-Hv1c was already described by Wang *et al.*[87], having as starting point a purchased J-ACTX-Hv1c in its reduced conformation. In that study, the yield of the oxidative folding was much higher (100%) compared to the obtained in this work (69.2%) (Figure 18), which can be due to several factors, namely using a reduced peptide with a higher purity degree as starting point helps preventing the production of unwanted by-products. In that experiment, the GSH:GSSG ratio (2:1) was significantly different compared to 1:100 used in this study. In fact, GSH performs a critical role helping to disrupt non-native disulphide connectivities and allowing the reversibility of the reaction, which is crucial to ensure that a natural and stable product is formed.[170] The use of different experimental conditions, such as pH, co-solvents, temperature, buffer and GSH:GSSG ratio, may also affect the yield of cysteines' oxidation.[171] A study conducted with α-Conotoxin TxIB, a small SS-rich peptide with 16 AAs, showed that for this peptide the buffer pH clearly affected the folding yield, influencing more than the choice of the buffer.[171] An increase in pH from 6 to 8 led to an increase in yield of around 40-70% depending on temperature, although temperature revealed to be a minor factor.[171]

Overall, this study demonstrates that this approach is a transposable method for the synthesis of complex multi-disulphide peptides with similar structures. Solid-phase peptide synthesis is a fast and versatile way to obtain peptides, allowing to produce a great variety of compounds on a research scale.[101] Considering the growing interest of small biomolecules like peptides as compounds to agricultural purposes, such as bioinsecticides,[69, 70] or also to explore their therapeutic potential as pain modulators,[172, 173] anti-cancer drugs and ion-channels' modulators, as examples, it is of utmost importance to explore and endorse chemical synthesis as a way to obtain these compounds.

To elucidate the insecticidal activity of the synthesized SVPs against *D. suzukii*, a series of bioassays to orally-administer these compounds were developed. Every treatment with µ-TRTX-Hhn2b incorporated within Carolina® medium, as well as treatments with both SVPs at 2.23 µM incorporated within filter paper had no effect on the survival and lifespan of *D. suzukii*, except for treatment with J-ACTX-Hv1c. In this case, the results suggest that this treatment promoted an initial stimulation of *D. suzukii* survival. However,

increasing the peptides concentration to 111.5 µM, the flies proved to be more susceptible to J-ACTX-Hv1c. Although treatment with µ-TRTX-Hhn2b for 24 h showed statistically significant differences, in the first 30 days after treatment there was no visible effect on the survival of *D. suzukii* flies. From a practical point of view, it is of most interest to have a higher initial mortality, as observed for the equivalent treatment with J-ACTX-Hv1c. An earlier reduction in the survival will result in fewer flies reproducing and laying their eggs on the fruit, thus decreasing the impact in fruit crops. In the case of J-ACTX-Hv1c, the results are more in line with the objective of developing a tool to help reducing the impacts of *D. suzukii* on crops. Despite this trend, and contrary to our initial hypothesis, these results showed that doubling the concentration of tested SVPs (223 µM) had no visible impact on the survival and lifespan of *D. suzukii* flies.

The fact that lower concentrations of SVPs (2.23 µM in the case of J-ACTX-hv1c, 111.5 µM for µ-TRTX-Hhn2b) lead to an apparent increase in survival of *D. suzukii* flies, and increasing concentrations reduced this survival probability, may also be explained by the concept of hormesis often observed in dose-effect studies. This phenomenon consists of a biphasic response characterised by low-dose stimulation and high-dose inhibition.[174] This differential response favours insects exposed to sublethal concentrations of compounds, which result in a stimulation of protective mechanisms, such as change in behaviour and stimulation of reproduction.[175, 176] A study by Krüger *et al.*[177] conduced on *D. suzukii* showed that a neurotoxic insecticide directly acting on insect coordination, when applied at sublethal doses, was however able to positively affect mating, contrary to expected. Similarly, a study developed by Deans and Hutchison[178] on spotted-wing *Drosophila* described evidence of hormesis response to sublethal doses of the insecticides zeta-cypermethrin, spinetoram, and pyrethrin. This study also described survival as being sex-dependent, since males were more susceptible to the studied compounds than females.[178] In fact, differential responses according to the insect sex have been described for xenobiotics. Treatment with phenobarbital and atrazine, a medicinal product for human use and an herbicide, respectively, promoted differential sex-related expression of genes encoding for P450 and GST expression in *Drosophila melanogaster*.[179] In males, gene expression was in general up-regulated in response to atrazine, while females had a higher response to treatment with phenobarbital. Moreover, the study described a selectively expression of P450-encoding genes in response to the different xenobiotics to adaptatively respond to chemically adverse environments.[179]

The evaluation of the expression of some stress-response related genes, conducted for flies treated with the SVPs at 111.5 µM for 48 h, demonstrated a clear peptide-dependent response (Figure 24). These results showed that *cyp12d1-d* was up-regulated only on µ-TRTX-Hhn2b-treated flies. Although this change was not statistically significant, it is hypothesized that this higher expression may be linked to peptide detoxification in *D. suzukii*, thus promoting the higher survival observed for flies of this treatment (Figure 19C). As a matter of fact, some studies relate the overexpression of detoxification genes with insecticide tolerance, metabolic resistance, and also stimulation of longevity and fertility.[128, 180, 181] Civolani *et al*.[182] described that *D. suzukii* flies treated with increasing concentrations of cyantraniliprole had a dose-dependent response in the expression of P450 *cyp12d1*. They also performed an insecticide resistance selection assay starting with a susceptible colony and by the 8th generation the LC_{50} value increased 2.2 fold compared to the control population, suggesting that this adaptive response relies on the expression of detoxifying agents such as P450.[182] According to the PCA (Figure 21), the increased expression of *cyp12d1-1* gene seems to be related to the adenylyl cyclase pathway rather than the MAPK pathway, since this analysis clearly correlates the expression of the *ac13E* with the peptide µ-TRTX-Hhn2b (Figure 24A).

Gene expression levels suggest that stress-induced expression of *hsc70-4* mRNA in flies may be associated with some tolerance to the treatment with J-ACTX-Hv1c at 111.5 µM for 48 h. Furthermore, the PCA correlates this treatment with the insulin-like receptor/TOR and apoptosis pathways. Concerning the insulin-like receptor/TOR pathway, J-ACTX-Hv1c seems to correlate more closely with *sod2* and *gaad45α* expression. Although there was no differential expression of the up-stream genes of this pathway (i.e., *chico* and *foxo*), in unfavourable conditions (such as insecticide-induced stress)[128] it is expected that the expression of *foxo* increases, leading to increased expression of genes associated with oxidative stress response and DNA repair, like *sod2* and *gaad45α*.[183] The expression of these proteins has already been associated with increased lifespan in *Drosophila* spp.[131, 183] Furthermore, *foxo* influences reproduction, inhibiting the expression of reproduction-associated genes (e.g. vitellogenin, encoded by the gene *vg*, an egg yolk protein responsible for ovarian maturation) leading to reproductive arrest.[128] Effects on fertility and reproduction of SWD were not evaluated in this study. The results presented here refer to gene expression analysis from a pool of male and female flies. The effects of these SVPs in the fertility and fecundity of *D. suzukii* are unknown, so the expression of genes associated to reproduction should be further studied only in females.

J-ACTX-Hv1c may have triggered the apoptosis pathway due to a slight increase in the expression of *dcp-1*, which encodes for an effector apoptosis caspase. Although not assessed in this study, it would be of interest to understand whether this peptide triggered the production of reactive oxygen species (ROS) that may have activated the apoptosis pathway, through signalling of the apoptosis regulator p53.[184] Treated *D. melanogaster* flies with sublethal doses of ciprofloxacin showed shorter lifespan, delayed development and smaller body size, and these observations were linked with elevated oxidative stress markers and down-regulation of *diap1* (here, *th*), which is also a target gene of Yorkie.[185]

Altogether, the activation by J-ACTX-Hv1c of these stress response pathways may have hampered the peptides' activity, leading to a lower-than-expected mortality for the concentration of 111.5 µM. Furthermore, the same mechanisms may have led to a lack of response to both peptides for treatment at 223 µM. Therefore, it is of most importance to evaluate the expression of genes for this group of treatments. Answering these questions is paramount to improve the design of new control tools.

One of the major challenges of SVPs is their bioavailability via oral delivery. To reach their target (i.e., the nerve cells) they often require an appropriate delivery system to overcome some biological barriers.[186] Examples of these barriers are the insect cuticle, an apolar lipid matrix lining the fore- and hindgut, representing a major impediment to the oral delivery of peptides;[186] the peritrophic membrane (composed of chitin and proteins) covering the midgut of insects, which also poses an obstacle to peptides transport;[186] the stability of peptides to the diverse pHs and proteases from the gut and haemolymph.[66] Numerous proteins, namely bovine serum albumin (BSA) and immunoglobulins, were already described as effective in crossing from the gut to the haemolymph in insects from the Diptera order (*D. suzukii* order).[186] Nonetheless, some of these molecules can also transpose across mammalian epithelial cells. Fitches *et al*.[73] demonstrated that the fusion of the peptide Hv1a with the snowdrop lectin GNA (*Galanthus nivalis* agglutinin) highly increased the insecticide activity of the Hv1a/GNA (compared to Hv1a and GNA alone) when orally administered to *Mamestra brassicae* (cabbage moth) larvae, leading to around 80% larval mortality in 10 days. They also evaluated the presence of Hv1a/GNA in the insect haemolymph and nerve chords, by Western blotting and immunoblotting analysis, respectively.[73] This plant lectin has the ability to play systemic effects via binding to glycoproteins in the gut membrane, and cross this membrane into the haemolymph following oral ingestion.[187] In summary, the

experiments developed here demonstrate that synthetized natural SVPs show bioactivity and may act against SWD.

A second strategy studied here to the control of *D. suzukii* was the manipulation of its microbiome with AMPs, with the hypothesis that by interfering with the flies' gut microbiome, SVPs could perform a better insecticidal activity. The efficacy of AMPs in inhibiting the growth of *D. suzukii* microbiome-related bacteria was assessed by antibiogram for peptides 3.1, CA-M and RW-BP100. Although little difference was observed between peptides, RW-BP100 had the highest bacteriostatic activity. However, it is noteworthy that RW-BP100 did not affect the growth of an *Acetobacter* species at concentrations up to 1000 µM. This result was unexpected, considering the high concentration tested and the fact that this peptide has already proved efficient against Gram-positive, but mainly Gram-negative bacteria,[165] as is the case of the *Acetobacter* sp.. This result may be explained by the fact that bacteria produce proteases with broad-spectrum activity, thus reducing the susceptibility of bacteria to these antimicrobial molecules.[188]

To elucidate the potential of RW-BP100 as a microbiome-manipulation tool, in this study it was developed an oral administration assay of this AMP at 400 µM for 24 h, which revealed to have no effect on the survival and lifespan of *D. suzukii* flies. The partial sequencing of the 16s rDNA region of bacteria from control and treated flies revealed no difference between groups, with only the species *Acetobacter thailandicus* being identified. This may help to explain the *in vivo* lack of activity in the survival of AMP-treated flies, since RW-BP100 failed to inhibit the *in vitro* growth of *Acetobacter* sp. at concentrations 2.5 times (1000 µM) higher than the tested *in vivo.* The concentration of 400 µM for the *in vivo* assays was chosen considering the limited amount of peptide available, and given that 400 µM was enough to inhibit the growth of 5 out of the 6 bacteria tested in the antibiogram assay, only not inhibiting the growth of an *Acetobacter* sp., as already pointed out. However, from the results of 16s rDNA sequencing, where only *A. thailandicus* was identified, and in light of the antibiogram screening, it would be expected that an increase in the concentration of orally administered RW-BP100 would have no effect on the survival of *D. suzukii*. To better understand what is hampering the peptides' activity, further molecular studies (e.g., with a fluorescence-labelled peptide) should be performed.

Furthermore, it was observed that flies from our laboratory colony only have in their microbiome a culturable bacterial species, although flies may be colonized by others that, due to their symbiotic character, may not survive out of the relationship with the

host. This was not unexpected because, as already pointed out in the introduction, nutrition and environment have direct impact on the microbiome of *D. suzukii*, and in the laboratory, where these conditions are controlled, flies are exposed to less diversity of nutrients as well as to microorganisms.[93] Interestingly, some studies distinguish *A. thailandicus* as a species that can maintain and proliferate in the gut of *D. melanogaster* (a non-pest insect species closely related to *D. suzukii*) and its presence is linked with a faster host development and increased fertility of offsprings.[100, 189] Obata *et al*.[190] described *Acetobacter* as a "live fast, die young"-promoting factor in *Drosophila*, and concluded that depletion of these bacteria in early-life stages by low dose of oxidants could extend longevity, but the same treatment during adult life stages leads to gut dysfunction and limit the insect lifespan. Altogether, these results emphasise the potential of controlling this pest by manipulating the gut microbiome, through impairing the bacteria-host interaction, or by supressing the proliferation of bacteria essential for the correct functioning of the flies' physiological processes. Thus, it will be of most relevance to develop new ways of reaching this target, i.e., the symbiotic bacteria *A. thailandicus*. This may be accomplished with the design of peptides with target specificity, and further evaluation of their viability as insecticidal agents to apply in crops, as sprays or in attract-and-kill traps, for example.

The mixture of RW-BP100 and μ-TRTX-Hhn2b showed to negatively affect the survival of *D. suzukii*, contrary to the expected from the results from treatments with RW-BP100 and μ-TRTX-Hhn2b applied separately. Although the results from this assay with those obtained for the treatment with SVPs alone should not be compared, it is interesting to note that RW-BP100 seems to have decreased the activity of J-ACTX-hv1c while boosting μ-TRTX-Hhn2b. To better understand these results, it would be important to perform a metagenomic analysis to assess the identity and quantify the bacteria colonizing the flies after each treatment. This way, it would be possible to evaluate the presence of non-culturable bacteria, and better understand whether peptides may be leading to a reduction in the amount rather than in the diversity of colonising bacteria.

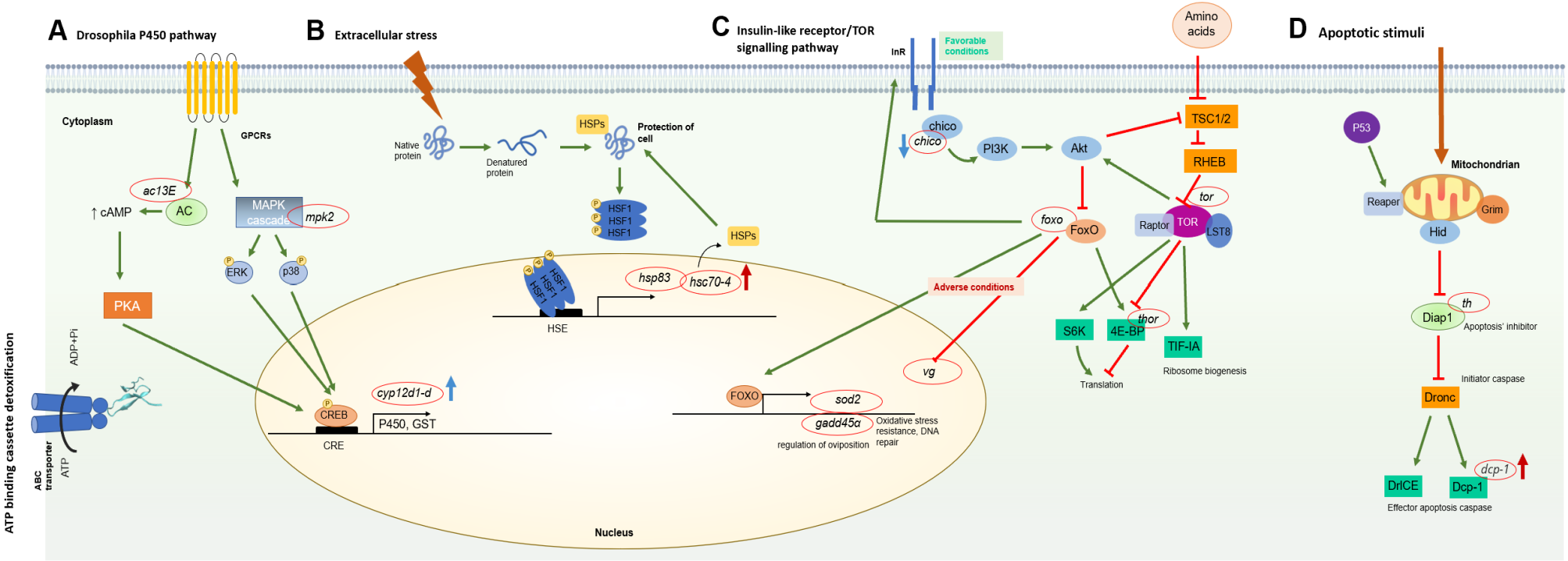


Figure 24 - Proposed general scheme of gene expression pathways triggered in the response to xenobiotics in *D. suzukii*. A. upon exposure of cells to xenobiotics, GPCRs may activate the adenylyl cyclase (AC) pathway, leading to an increased cAMP in the cell, and activate the phosphorylation cascade through MAPK, both pathways signalling to the expression of P450 enzymes and glutathione S-transferase (GST). B. heat shock proteins (HSTs) are expressed to protect cell components under stressful conditions. C. the insulin-like receptor/TOR signalling pathway interprets growth factors, oxygenation state and nutrition, promoting appropriate changes in cell growth and proliferation, survival, and fecundity. D. under apoptotic stimuli (e.g., DNA damage), apoptosis may be promoted by the regulation of pro-apoptotic proteins, such as Reaper, Hid and Grim, complex which will promote the apoptosis inhibitor Diap1. This inhibition promotes de activation of initiation caspase Dronc and, consequently, leading to the activation of effector caspases DrICE and Dcp-1. Blue arrows indicate up- (*cyp12d1-d*) or down-regulated (*chico*) expression of the corresponding genes for flies treated with µ-TRTX-Hhn2b (111.5 µM, 48 h), and dark red arrows indicate up-regulated expression of the corresponding genes (*hsc70-4* and *dcp-1*) for flies treated with J-ACTX-Hv1c (111.5 µM, 48 h).

5. Conclusion and Future Perspectives

The present work illustrates a simple method to chemically synthesize the complex peptides J-ACTX-Hv1c and μ-TRTX-Hhn2b, which is predicted to be easily transposable to other disulphide-rich peptides. Along with, this work aims to sow the importance of chemical synthesis for an academic context, as it allows to quickly acquire the desired peptides in sufficient quantities to perform biological assays, while enabling to easily make deletions or additions as a way to improve the sequence according to the target.

Following the *in vivo* data, the oral activity of J-ACTX-Hv1c and gene expression data show that this peptide is a suitable tool for controlling *D. suzukii*, by shortening the insect pest lifespan. As future work it would be important to identify the specific target of these peptides in *D. suzukii*, to place them as feasible tools to control insecticide resistance. Furthermore, it would also be worth understanding the factors hindering the peptides' mode of action. Future work should address areas such as studying the oral bioavailability of peptides by controlled delivery through the insect gut and haemolymph towards the nervous system, to overcome possible barriers to peptides' metabolization. In any case, much more research is needed to better understand the potential of peptides to impact the survival of the challenging pest SWD. It would also be interesting to analyse the whole flies' transcriptome with total RNA sequencing (RNA-seq) to help identify other possible peptide-triggered responses in addition to those hypothesised in this study.

Although our results do not yet prove the previous hypothesis of influencing the flies by fragilizing its microbiome with RW-BP100, it is also of great importance to focus on the insect microbiome as a target for pest control. Future studies should be pursued on the design of target-specific antimicrobial peptides, with improved chemical stability and resistance to proteases.

Finally, it is essential to assess the toxicity of peptides to mammals and non-target insects, such as the extremely important pollinators, and also study the effects on the whole ecosystem involved.

Overall, this work aims to outline the potential of peptides as sustainable and safe tolls to manage *D. suzukii* in a present where there is an urgent need to develop alternatives for the conventional insecticides.

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Appendices

Appendix II.

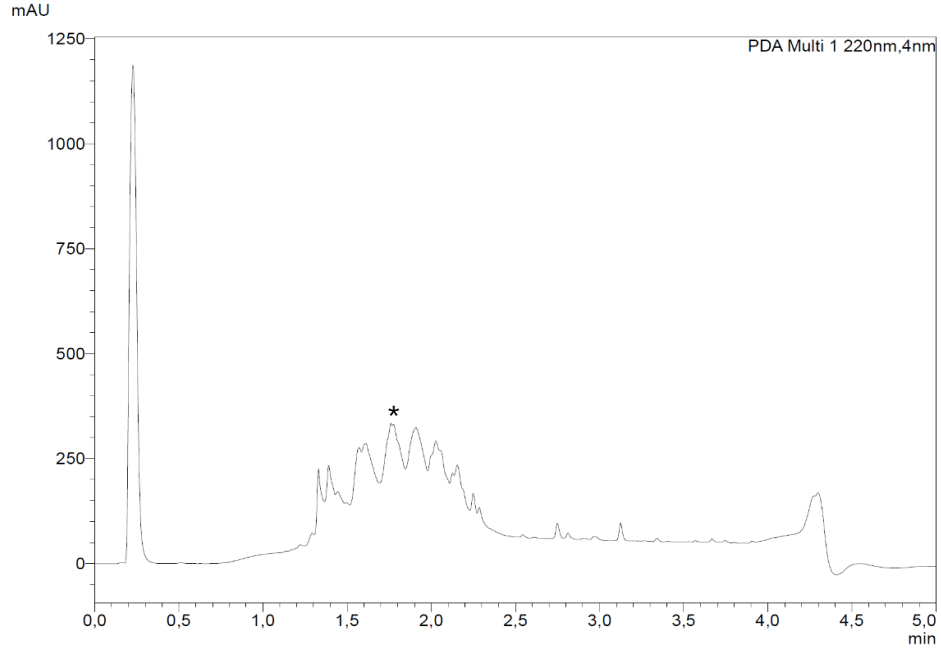


Figure I. 1 - HPLC chromatogram of the crude product from solid-phase synthesis of μ-TRTX-Hhn2b with Wang resin (Rt = 1.75 min). The symbol ***** highlights the major peak of the synthesis, which were found by LC-MS analysis to be the desired peptide. The elution gradient used was 5-95% of solvent B into A (A: 0.1% TFA in water; B: 0.1% TFA in ACN), for 3 min, with a running flow of 0.7 mL/min and detection at λ = 220 nm.

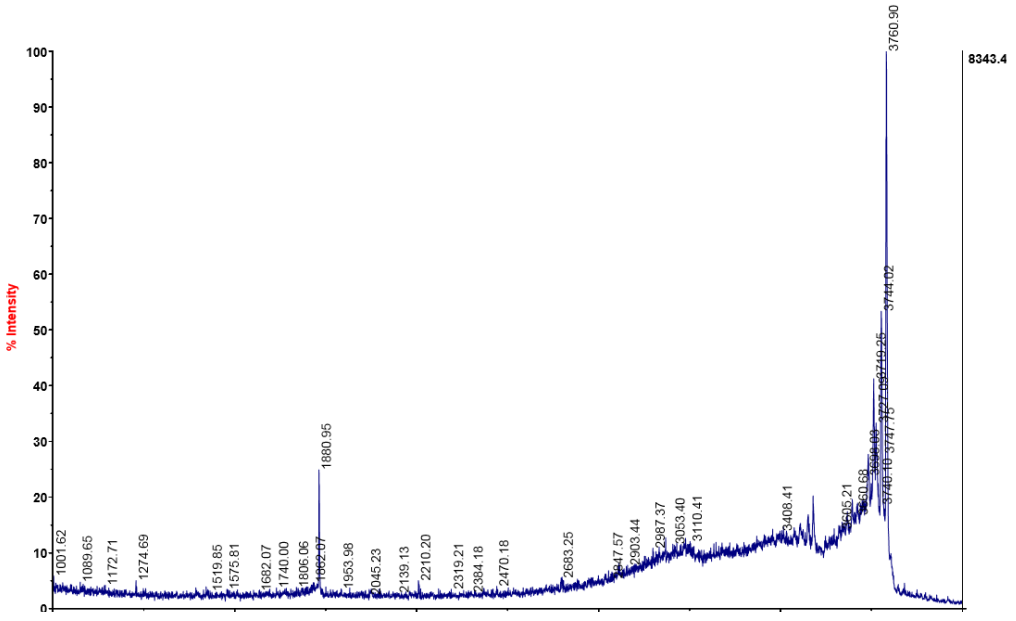


Figure I. 2 - MALDI-TOF mass spectrum of oxidized J-ACTX-Hv1c in positive ion mode. Mass spectrum was acquired from the mixture of equal volumes of the matrix solution (CHCA, 15 mg/mL in 50% MeCN in H_20) with peptide (1 mg/mL). The percentual intensities of the ions are shown on the y-axis and the ratio between mass and charge of the ions (m/z) are shown on the x-axis. m/z = 3760.90 corresponds to the peptide, and m/z = 1880.95 to peptide two times protonated (P+2H).

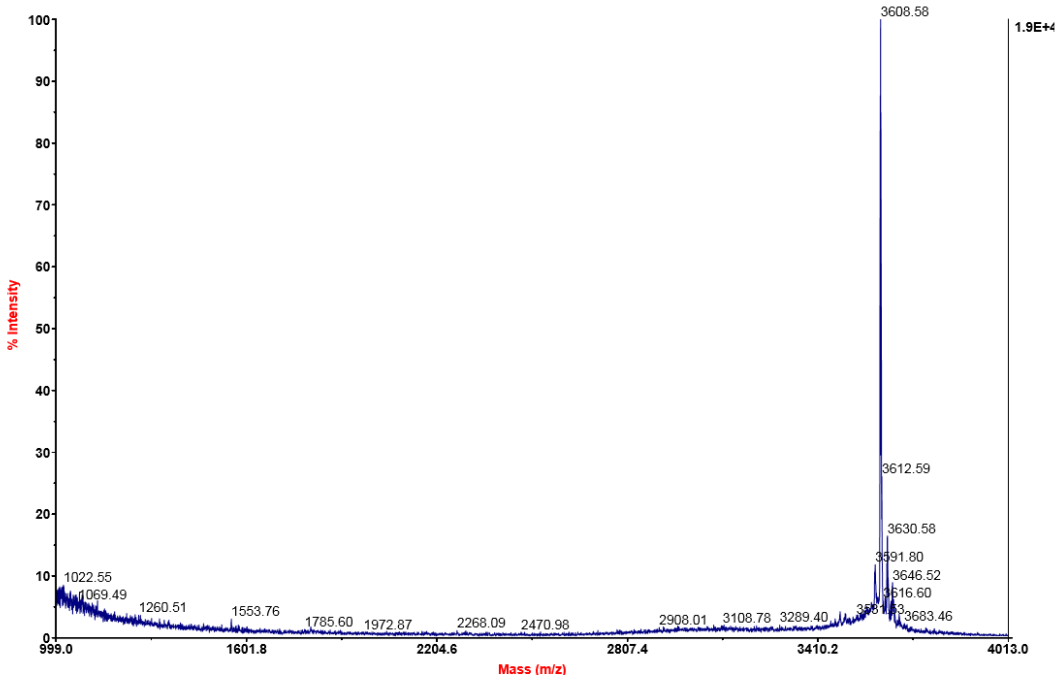


Figure I. 3 - MALDI-TOF mass spectrum of oxidized µ-TRTX-Hhn2b in positive ion mode**.** Mass spectrum was acquired from the mixture of equal volumes of the matrix solution (CHCA, 15 mg/mL in 50% MeCN in H_20) with peptide (1 mg/mL). The percentual intensities of the ions are shown on the y-axis and the ratio between mass and charge of the ions (m/z) are shown on the x-axis. m/z = 3608.58 corresponds to the oxidized peptide.

Appendix II.

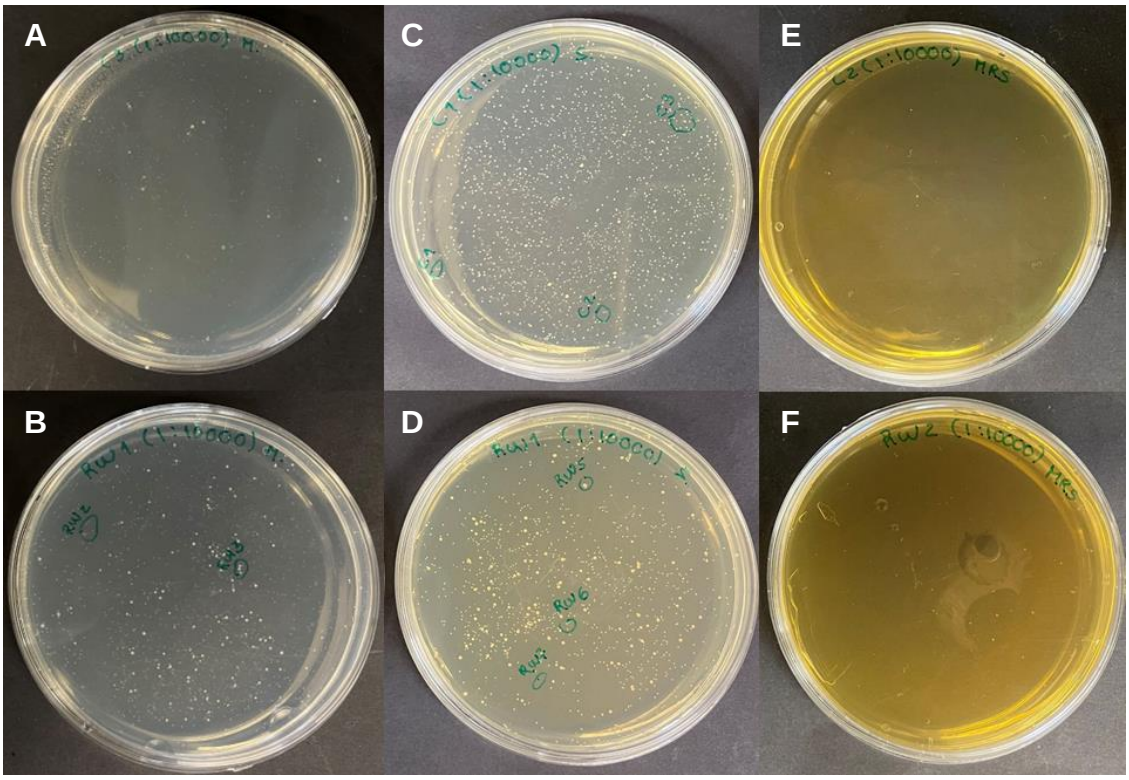


Figure II. 1 - Culture of bacteria from the microbiome of control *D. suzukii* flies (A. C. and E.) and treated flies with RW-BP100 at 400 µM for 24 h (B., D. and F.). Bacteria were inoculated in mannitol medium (A. and B.), Sabouraud Dextrose Agar medium (C. and D.) and, MRS medium (E. and F.), incubated for 48 h.