ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons: http://cat.creativecommons.org/?page_id=184

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons: http://es.creativecommons.org/blog/licencias/

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license: https://creativecommons.org/licenses/?lang=en

Entomopathogenic nematodes and *Drosophila suzukii*: from biological control to immunology perspective

PhD Thesis

Anna Garriga Oliveras

Bellaterra, 2021

Facultat de Biociències
Departament de Biologia Animal, Biologia Vegetal i Ecologia
Unitat de Zoologia

PhD Thesis

Entomopathogenic nematodes and *Drosophila suzukii*: from biological control to immunology perspective.

Dissertation presented by

Anna Garriga Oliveras

for the degree of Doctor in Biodiversity under the supervision of
Dr. Fernando García del Pino and Dra. Ana Morton Juaneda

Supervisor	Supervisor	PhD candidate
Fernando García del Pino	Ana Morton Juaneda	Anna Garriga Oliveras

Bellaterra, 2021

Per tu mare,
per ensenyar-me a ser feliç

"*All that is gold does not glitter,
not all those who wander are lost;
the old that is strong does not wither,
deep roots are not reached by the frost.*"

J.R.R. Tolkien

Agraïments

He acabat aquesta tesis igual com la vaig començar, escrivint mentre fora la finestra les orenetes sobrevolen els camps de blat. I és complicat mirar aquests anys enrere i recordar tants bons moments i aprenentatges, així que intentaré no deixar-me ningú dels que m'heu acompanyat.

Primer de tot, voldria agrair al suport institucional rebut ja que aquesta tesi ha estat realitzada gràcies al finançament del projecte de *Drosophila suzukii* de FEDER / Ministerio de Ciencia, Innovación y Universidades—Agencia Estatal de Investigación / Proyecto: AGL2017-86770-R. D'altra banda, a la UAB que des de 2010 quan vaig començar el Grau de Biologia ha sigut un referent d'il·lusió per la ciència i font aprenentatge. I actualment he gaudit de la beca contractual de Personal Investigador en Formació (PIF). A més per la realització de les dues estades internacionals vaig poder comptar amb el programa Erasmus+ doctorat i l'ajut per estades breus PIF-UAB.

Un agraïment molt gran als meus directors, Ana i Fernando, per la confiança que vau dipositar en mi des del principi i donar-me la oportunitat de fer la tesis amb vosaltres. El fet de sempre voler anar un pas més enllà ha suposat un gran repte però també una satisfacció i creixement com a investigadora. Gràcies per aguantar-me durant aquests anys, que no soc fàcil de portar. I per tants moments que hem compartit al laboratori, en la docència i en congressos, i per tot el que m'heu ensenyat tan professional com personalment.

M'agradaria transmetre un fort agraïment a tota la Unitat de Zoologia, que m'ha acollit des del primer dia com una més i he aprés moltíssim des de la recerca fins a la docència. Ha sigut per mi una gran experiència poder transmetre als alumnes la il·lusió per la Zoologia a través de les pràctiques. Gràcies companys de la unitat pels molts moments viscuts: Francesc, Anna, Xavi, Ona, Maite, Maria i Emili. A tu Manolo per estar sempre al peu del canó, disposat a ajudar, saber on és tot i sinó solucionar-ho. Voldria agrair també a la comissió de seguiment de doctorat; Maite, Anselm i Anna, per aconsellar-me i guiar-me durant la tesis.

Un agraïment molt especialment als "*Pifs*" de la unitat: Ana Filipa, Ester i Oriol que hem compartit aquest camí i molts cafès per animar-nos i desentrellar la burocràcia molts cops. Al Dani, que vas ser el meu referent quan vaig entrar en el món dels nematodes i em vas ensenyar a criar-los amb tots els secrets del laboratori. I als companys amb qui he compartit laboratori al llarg d'aquests anys: Maira, Laura, Albert i Andrea. Finalment, una menció especial per tu Ivan, que et quedes com qui diu amb les claus del laboratori i et desitjo molta sort i ànims en aquesta etapa. I sense deixar la universitat ja que compartim la càrrega de doctorands, també vull agrair els infinits cafès amb la Lídia, la Cris i l'Adam que m'heu aguantat des d'abans de començar la tesis i espero poder seguir compartint amb vosaltres molts anys més.

Un agraïment pel suport tècnic rebut a l'inici de la tesis de la Unitat de Genètica i IRTA per la cria de Drosophiles. També més recentment al Servei de Microscopia UAB per l'ajuda rebuda durant l'assaig amb nematodes fluorescents.

In my stage at Varese, I had the opportunity to meet an amazing team that works like a clock-mechanism. Grazie mille Maurizio and Maristella, you made an outstanding job teaching me every detail of comparative immunology. Besides, Maristella thanks for opening your home to me, you known that you are a referent for me of kindness and excellent laboratory habits.

I have not enough words to thank Duarte and Prof. Nelson. Because even in a crazy year 2020 with all the odds against it, we manage to reschedule and carry on the stage at the Universidade. I learned a lot in your lab from genetics to all multidisciplinary projects you are involved in. Besides, I am really grateful to all the amazing people I met in Ponta, because with few months you made me feel like home and showed me the amazing Açorian life. Muito obrigada!

A la meva família, per acompanyar-me durant tot aquest camí, des de petita que em vau ensenyar a estimar la terra, tot el que hi conviu i hi creix. Per encoratjar-me a perseguir els meus somnis i això si, sempre treballant molt. Sense vosaltres al meu costat no hauria arribat a ser la científica de la família. Gràcies per ser-hi sempre avis, mare, pare i Jordi. Y también a mi familia en València, que en pocos años os habéis convertido en una pieza tan importante para mí, muchas gracias por vuestro apoyo.

Finalment vull agrair a Mike, que has sigut un pilar per mi durant tota aquesta aventura. Per estar al meu costat cada dia, animant-me a seguir endavant, donant valor a qualsevol pas que feia per insignificant que fos. I sobretot per escoltar sempre les meves peripècies diàries, poder comptar amb la teva opinió i coneixement com a referència. Gràcies pel dia a dia, per fer tan fàcil compaginar un projecte com aquesta tesi amb una qualitat de vida excepcional. Ara acabo aquesta aventura però segur que aviat n'emprendrem una de nova.

Anna

Table of contents

Abstract 9

Abbreviations 11

1. General introduction 13

    1.1 Biology and expansion of *Drosophila suzukii* 15

    1.2 Control measures for *Drosophila suzukii* 17

    1.3 Biology of entomopathogenic nematodes 20

    1.4 Application of entomopathogenic nematodes 22

    1.5 Immune system ahead of an infection process 23

2. Objectives 27

3. Is *Drosophila suzukii* as susceptible to entomopathogenic nematodes as *Drosophila melanogaster*? 31

4. Soil emergence of *Drosophila suzukii* adults: a susceptible period for entomopathogenic nematodes infection 33

5. Compatibility of entomopathogenic nematodes with natural enemies for horticultural pest control 35

6. Immune response of *Drosophila suzukii* larvae to infection with the nematobacterial complex *Steinernema carpocapsae–Xenorhabdus nematophila* 37

7. The modulation effect of *Steinernema carpocapsae* - *Xenorhabdus nematophila* on immune-related genes of *Drosophila suzukii* larvae 61

8. General discussion 85

    8.1 Biological control of *Drosophila suzukii* 87

    8.2 Immune response to infective process 89

9. Conclusions 93

10. References 97

Abstract

Drosophila suzukii is the major invasive pest of soft-skinned fruits involving crops such as strawberries, cherries and berries. This fly infests ripen fruits before harvest affecting fruit production that entail great economical losses. The present thesis intends to settle the foundations for biological control using entomopathogenic nematodes (EPNs) and to outline the insect immune response to the infection.

The susceptibility of *D. suzukii* was evaluated under laboratory conditions with four nematode species: *Steinernema feltiae* (Ext4), *Steinernema carpocapsae* (B14), *Heterorhabditis bacteriophora* (DG46 and Larvanem®) and *Steinernema* sp. (D122). Larvae resulted highly susceptible to EPNs infection with mortality rates of 60 – 94% with all nematode treatments. Besides, the most virulent nematodes, *S. feltiae* and *S. carpocapsae* were able to reproduce using larvae. In contrast, non EPNs infected *D. suzukii* pupae during the assay. The adult stage of the fly presented diverse susceptibility due to *S. carpocapsae,* being the only nematode to reach 65% of infection in mature adults. Meanwhile, teneral adults were more vulnerable to this nematode which reached 89% of infection.

From these results, two preliminary assays were designed to explore EPNs application under controlled laboratory conditions. To target the larval stage, infested strawberries were sprayed with *S. feltiae* and *S. carpocapsae* solution using two different doses (25 and 50 IJs cm^{-2}). After 13 days, adult emergence from fruit was reduced with all treatments with a peak efficacy of 35% with *S. feltiae*. Besides, a soil application of *S. carpocapsae* was evaluated to infect teneral adults during dispersal period. A column arena with three levels was used to evaluate the dispersion from nematode contact and the flight capability while infected. Total infection rate reached 84% of adults and from these, 17% flew carrying IJs. The number of nematodes inside adults limited their capacity to fly being 4 IJs the maximum load. These results entailed optimistic prospects to use EPNs to control *D. suzukii* even more applied assays should be carried out.

Considering the differential susceptibility of *D. suzukii* stages, compatibility of tested EPNs was evaluated with natural enemies of the pest used also as biological control agents. In Petri dish assay, treatments with *H. bacteriophora* and *S. feltiae* caused no mortality to neither predators nor parasitoids. Thus, these EPNs were considered harmless to the natural enemies. Only adult predators showed a decrease of survival with the treatment of *S. carpocapsae*. In consequence, a plant pot assay was designed to study the combined application under more natural conditions. Foliar treatment of *S. carpocapsae* caused no infection of any insects during the experiment as nematode persistence on leaves' surface was short. These results encourage a combined application with EPNs to target all *D. suzukii* stages.

The response of *D. suzukii* immune system to EPNs infection was determined through larva infection with the complex *S. carpocapsae* – *Xenorhabdus nematophila* as well as to detect the

infection strategy of this nematobacterial complex. In order to elucidate this topic physiological and molecular methods were employed. These analyses exposed an evasion strategy of *S. carpocapsae* IJs to the immune system of the fly in both signaling and effective levels. Before the bacterial release, IJs avoid triggering any defense response as immune-related genes were practically unresponsive to infection. Only after *X. nematophila* presence, hemolymph receptors detected the pathogen and activated the immune responses. Mainly, antimicrobial peptides (AMPs) were upregulated together with the interleukin Upd3 which promotes cellular mechanisms. However, the latter was unable to activate the effector gene Turandot C. Meanwhile, genes in charge of the melanization response (PO) remained essentially constant along the infection. These variations of the genetic expression were accompanied with the evaluation of the effector processes. Concerning the cellular response, larvae of *D. suzukii* were unable to encapsulate IJs or to phagocyte bacteria showing a lack of recognition and activation of these mechanisms. Crystal cells which are responsible for melanization response remained inactive even with bacterial presence. Besides, *X. nematophila* also inhibited the enzymatic activity of PO present in larvae hemolymph. In contrast, as seen in the genetic expression results, AMPs were activated under bacterial pressure although these peptides presented a reduced antimicrobial activity in presence of *X. nematophila*. These results revealed the strategies employed by *S. carpocapsae* – *X. nematophila* to assure the infection success through an evasion of recognition and active inhibition of enzymatic and peptide compounds.

Abbreviations

AMPs – Antimicrobial peptides

BCA – Biological control agents

EPNs – Entomopathogenic nematodes

GNBP – Gram-negative binding proteins

Jak/STAT – Janus kinase / signal transducer and activator of transcription

JNK – c-Jun N-terminal kinase

IJs – Infective juveniles

Imd – Immune deficiency

IPM – Integrated pest management

NE – Natural enemies

PGRP – Peptidoglycan recognition proteins

PI – Post-infection

PO – Phenoloxidase

PRR – Pattern recognition receptor

ROS – Reactive oxygen species

SWD – Spotted wing Drosophila

TGF – Transforming growth factor

1. GENERAL INTRODUCTION

“*Join the harvest of hundred fields, hearty and tame*
All going back to one single grain
Offer light to the coming day, inspire a child
Water the field, surrender to the earth”

Nightwish - Harvest

Insect pests are a major threat to agricultural production with 18% in world estimated crop losses (Oerke 2006). Meanwhile, agriculture is facing the challenge of reducing pesticide use and migrating to a biological control approach (Hossard et al. 2014). The European Committee established through the Directive 2009/128/CE a framework for the sustainable use of pesticides which regulate the overuse of broad-spectrum products that carry serious health risks for agrarian workers and consumers (European Parliament 2009). In consequence, the development of biological control strategies was encouraged to achieve a more sustainable management of insect pests (Chandler et al. 2011). These strategies include from augmentative biological control in which Biological Control Agents (BCA) are released to the agrarian system to conservation biological control that promote indigenous natural enemies already present in the area (Bale et al. 2008).

The increase of international fruit trade had contributed to the introduction of exotic pest species (Chapman et al. 2017). Rising temperatures due to climate change may cause an increased ecological impact of insect pests and facilitate the colonization of new environments mainly in temperate regions (Parmesan 2006; Chakraborty 2013; Lehmann et al. 2020). Insect pests already benefited from global temperature change to be established in new introduced areas, such as *Bactericera cockerelli* (Šulc)(Hemiptera: Triozidae), *Bemisia tabaci* (Gennadius)(Hemiptera: Aleyrodidae) or *Tuta absoluta* (Meyrick)(Lepidoptera: Gelechiidae) (Lamichhane et al. 2015). In addition, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) is considered another example of this effect (Little et al. 2020).

1.1 Biology and expansion of *Drosophila suzukii*

Drosophila suzukii became a global threat in 2008 and its invading range is still expanding. The Asian vinegar fly is widely known as spotted wing Drosophila (SWD) and has been considered the major risk of small-stone and soft-skinned fruits (Asplen et al. 2015). This fly belongs to the Melanogaster group in the Sophopora subgenus (Yang et al. 2012) and two morphological traits are commonly used to identify SWD (Fig. 1). Male adults have a dark spot in the leading edge of each wing and females possess a large and serrated ovipositor (Walsh et al. 2011).

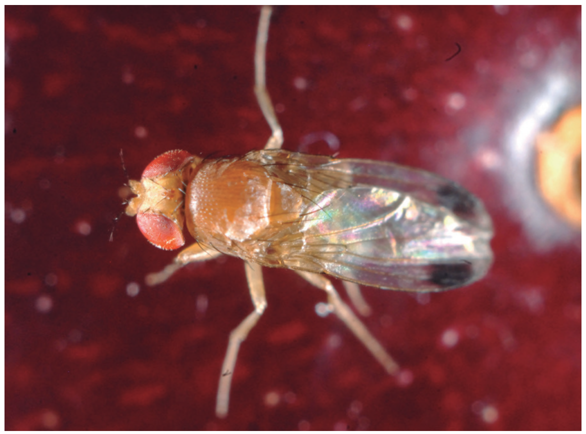


Fig 1. Adults of *D. suzukii*; male (left) and female (right).

This fly was described in Japan in 1916 and reported as common in all central Asia including Korea, China and India (Kanzawa 1939). In 1980, Kaneshiro (1983) reported the establishment of *D. suzukii* in Hawaii. This record implied the start of expansion out of its native range. First, the fly reached the Pacific coast of North America in 2008 and quickly spread to eastern coast and north central regions (Hauser 2011). Moreover, in late 2008 appeared the first reports of *D. suzukii* in Europe specifically in Spain and Italy (Calabria et al. 2012). The spread of the pest affected mainly the Mediterranean areas. SWD reached northern and eastern countries of Europe in 2014 with a slower rate of expansion (Asplen et al. 2015). In 2013, it was reported the presence of the fly in South America for the first time in Brazil and Argentina (Deprá et al. 2014; de la Vega et al. 2020). In 2015, *D. suzukii* presence was detected for the first time in Iran and Middle East (Parchami-Araghi et al. 2015). The last reports registered the fly presence in Chile, Morocco, Israel, and European Russia (Bienkowski and Orlova-Bienkowskaja 2020; Boughdad et al. 2020; EPPO 2021). This fly has a temperature tolerance of 0 to 32ºC being able to live and reproduce in temperate and subtropical climates (Walsh et al. 2011). This temperature range together with an easy dispersal permitted the quick invasion and establishment of SWD. Despite the active dispersal of *D. suzukii*, passive spread due to global trade is probably the main cause of expansion (Westphal et al. 2008). This was likely because fruit appearance is healthy until the last stage of larvae when intense feeding can be detected. There are three main traits to define invasion success of SWD discussed below: high reproductive potential, wide temperature tolerance and being a polyphagous species.

Females of *D. suzukii* are mature after two - four days of emergence and can oviposit an average of 380 eggs during their lifetime (Walsh et al. 2011). In contrast to other drosophilid flies, the serrated ovipositor allows *D. suzukii* females to pierce the skin and oviposit in ripening or healthy fruit. Eggs hatch during the first 2 days inside the fruit where the three larval instars mature in 5 – 10 days (Fig. 2). Third instar larvae wander to the outside of fruit and pupation was described to occur in the fruit surface (Walsh et al. 2011). However, Woltz and Lee (2017) reported with field assays that 82-93% pupation occurs in the soil since larvae fall from the fruit. This phase takes 6 to 10 days and pupae are hard without openings (Fig. 2). From adult emergence, *D. suzukii* have a lifespan of 20 to 60 days, but overwintering adults can reach 200 days (Kanzawa 1939). Commonly with other insects, the development period is temperature dependent (Wiman et al. 2016). The optimal thermal range for *D. suzukii* reproduction is from 18 to 27ºC with a development time of 10 – 17 days from egg to adult (Kanzawa 1939; Tochen et al. 2014). In favorable weather conditions, SWD could have 13 generations per year having short development time with high reproductive potential. However, adult activity decreases below 15ºC. Then, adults of *D. suzukii* enter in a reproductive diapause to overcome low winter temperatures, especially females (Wiman et al. 2014). In contrast, larvae and pupae are unable to survive strong winter periods. Asplen et al. (2015) described in *D. suzukii* a winter adult morphology; a larger and darker phenotype more cold-tolerant. These adults enter in a partial quiescence that prevents them from freezing. Females are more abundant during these winter periods and their number is a predictor of summer population in the area (Rossi-Stacconi et al. 2016).

Fig 2. Developmental stages of *D. suzukii* with three instar larvae: L1, L3 and L2 (left) and pupation process at day 1, 4 and 7 (right).

A key point of SWD invasion is its polyphagous characteristic. *Drosophila suzukii* infest soft-skinned and small stone fruits, such as strawberries, all kinds of berries, cherries, grapes and peaches (Cini et al. 2012). Besides, more than 80 fruit plants are considered to be susceptible hosts in Europe, including cultivated, ornamental and wild species (Arnó et al. 2016; Kenis et al. 2016). This diversity of hosts provides fruits all over the year and acts as a reservoir when crop-fruits are unavailable (Diepenbrock et al. 2016; Santoiemma et al. 2019). Besides, *D. suzukii* lack host fidelity and can use suboptimal fruits when high-quality fruits are unable, despite fitness cost.

Drosophila suzukii affects preferentially ripening fruits, thus damaging the crops before harvest. The main harm is done by larvae feeding from inside. Nevertheless, the serrated ovipositor puncture produces a physical deterioration in the skin fruit that can cause secondary infection of bacteria and fungi (Lee et al. 2011a). In the native region of SWD, Japan and China reported economic losses in blueberries and cherries with field infestations between 21 to 80% (Kawase et al. 2008; Zhang et al. 2011). But major losses were registered in North America and southern Europe. Goodhue et al. (2011) reported annual losses of $500 million in Western US production of berries and cherries. In Italy and France, some locations communicated losses by 3 million € per year on damaged fields of strawberries, cherries and cranberries (De Ros et al. 2013). Every year, more reports of economic losses caused by SWD appeared in the affected countries.

1.2 Control measures for *Drosophila suzukii*

The first measures applied to control SWD were based on chemical and cultural methods. Most used insecticides are conventional broad-spectrum products such as pyrethroids and organophosphates (Bruck et al. 2011). In addition, spinosad and chlorantraniliprole were also confirmed as highly efficient (Cuthbertson et al. 2014). However, strategies based on chemical products resulted in several inconveniences. The intense use of these products led SWD to develop insecticide resistance (Gress and Zalom 2019) that is a process widely described in *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) (Perry et al. 2008). Moreover, both growers and researchers observed

secondary pests in the same crops infested by *D. suzukii* forcing a more frequent application of insecticide (Woltz et al. 2017; Lee et al. 2019). Therefore, these applications must accomplish the safety terms to avoid hazardous wastes that can affect the consumers, because in a short period fruits are harvested. In consequence, these broad-spectrum products are not compatible with Integrated Pest Management (IPM) programs (Van Timmeren and Isaacs 2013). The IPM methods include a wide range of tools and techniques including cultural methods, organic compounds and BCA (Schetelig et al. 2018; Dam et al. 2019). Some cultural measures for SWD are sanitation of infested fruits (Lee et al. 2011b), usage of nets to cover trees or shrubs (Kawase et al. 2008), strict harvest schedules (Leach et al. 2018) or mass trapping to control the adults (Walsh et al. 2011). In addition, novel biotechnology-based techniques as sterile males and RNA interference were prospected to control *D. suzukii* (Taning et al. 2016; Nikolouli et al. 2020).

Among IPM methods, there is an increasing interest in BCA to control the pest and achieve environmentally sustainable fields (Haye et al. 2016). Biological control offers a potential alternative to insecticides by using natural enemies (NE) of the pest such as predators, parasitoids or entomopathogenic fungi and nematodes. A list of insect species tested for the control of *D. suzukii* is provided in Table 1 (Fig. 3). Gabarra et al. (2015) reported the predatory behavior of *Orius laevigatus* (Fieber) (Hemiptera: Anthochoridae) against larvae, pupae and adults of SWD although, this predation was not enough to control significantly the fly population. Other predator species as *Dalotia coriaria* Kraatz (Coleoptera: Staphylinidae) and *Forficula auricularia* Linnaeus (Dermaptera: Forficulidae) presented up to 50% of reduction of *D. suzukii* under laboratory conditions (Renkema et al. 2015; Bourne et al. 2019). Despite the lower predation registered on semi field assays, the combined release of *Orius insidiosus* (Say) (Hemiptera: Anthochoridae) and *D. coriaria* resulted in 60-70% of *D. suzukii* reduction (Renkema and Cuthbertson 2018). These authors reported that combined use of BCA achieved a better control of the pest than application of one predator species alone.

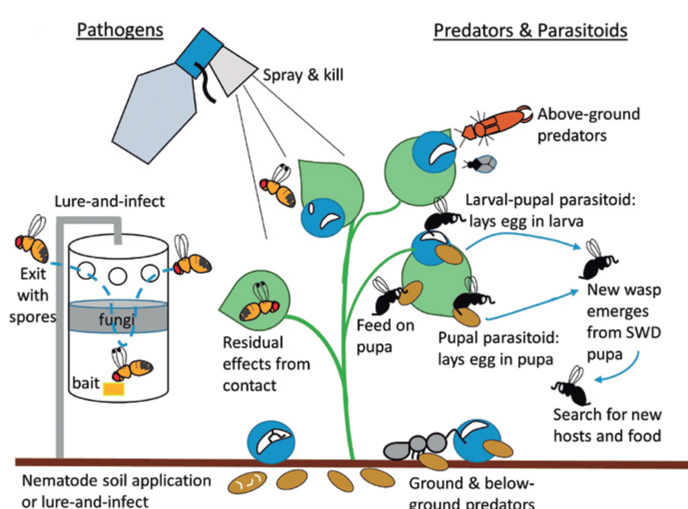


Fig 3. Biological control strategies (Lee et al. 2019)

Parasitoid species regulate *Drosophila* populations in natural conditions and consequently are used as agents for biological control (Fleury et al. 2009). In their native region, populations of *D. suzukii* are widely regulated by parasitoids such as *Asobara japonica* (Förster) (Hymenoptera: Braconidae), *Ganaspis* spp. (Hymenoptera: Figitidae) or *Leptopilina* spp. (Hymenoptera: Figitidae) (Asplen et al. 2015). Two pupal parasitoids were found naturally attacking *D. suzukii* in invaded areas and were effective under laboratory assays (Rossi Stacconi et al. 2015; Wang et al. 2016).

Table 1. Prospected natural enemies of *D. suzukii* for biological control

Insect species	**Type**	**Reference**
Orius laevigatus (Fieber) (Hemiptera: Anthochoridae)	Predator	(Gabarra et al. 2015)
Orius insidiosus (Say) (Hemiptera: Anthochoridae)	Predator	(Renkema and Cuthbertson 2018)
Dalotia coriaria Kraatz (Coleoptera: Staphylinidae)	Predator	(Woltz et al. 2015)
Forficula auricularia Linnaeus (Dermaptera: Forficulidae)	Predator	(Gabarra et al. 2015)
Chrysoperla carnea (Stephens) (Neuroptera: Chrysopidae)	Predator	(Bonneau et al. 2019)
Podisus maculiventris (Say) (Hemiptera: Pentatomidae)	Predator	(Bonneau et al. 2019)
Pachycrepoideus vindemmiae (Rondani) (Hymenoptera: Pteromalidae)	Pupal parasitoid	(Chabert et al. 2012)
Trichopria drosophilae (Perkins) (Hymenoptera: Diapriidae)	Pupal parasitoid	(Rossi Stacconi et al. 2019)
Leptopilina heterotoma (Thomson) (Hymenoptera: Figitidae)	Larval parasitoid	(Poyet et al. 2013)
Leptopilina japonica Novkovic & Kimura (Hymenoptera: Figitidae)	Asian larval parasitoid	(Daane et al. 2016)
Asobara japonica (Förster) (Hymenoptera: Braconidae)	Asian larval parasitoid	(Kacsoh and Schlenke 2012)
Ganaspis brasiliensis (Ihering) (Hymenoptera: Figitidae)	Asian larval parasitoid	(Daane et al. 2016)

Besides, Rossi Stacconi et al. (2019) presented field release of *Trichopria drosophilae* (Perkins) (Hymenoptera: Diapriidae) strategy compared to an unmanaged area and reported a decreased emergence of *D. suzukii* within 10 m radius from release point. However, this strategy involved several parasitoid releases and strict supervision to assure parasitoid efficacy (Bezerra Da Silva et al. 2019; Lee et al. 2019). In contrast, European species of larval parasitoids were unsuccessful in fly parasitization. Kacsoh and Schlenke (2012) reported a strong immunologic response that ended with the encapsulation of *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae) eggs inside larvae. However, Asian parasitoids as *A. japonica* are successful and have been suggested for classical biological control (Daane et al. 2016; Haye et al. 2016).

Considering the entomopathogenic species, fungi *Beauveria bassiana* and *Metarhizium anisopliae* were tested to control *D. suzukii* through different applied techniques (Woltz et al. 2015; Cuthbertson and Audsley 2016). These authors reported non-effect on fly emergence after dipping infested fruits on fungi solution. Besides, direct spray with *B. bassiana* caused 44% of adult mortality but could not prevent the new generation of SWD. Nevertheless, subsequent studies developed traps with fungi inoculum to lure and infect *D. suzukii* adults which achieved 62% of mortality with *M. brunneum* (Yousef et al. 2018).

Few studies evaluated entomopathogenic nematodes (EPNs) as BCA of *D. suzukii* before the beginning of this thesis. The first ones reported the fly as non-susceptible to nematodes with an

infection rate of 1% (Cuthbertson et al. 2014; Woltz et al. 2015). However, a contradictory study reported 80% of larval mortality with high dose of EPNs (Cuthbertson and Audsley 2016). The ambiguity and absence of solid results were the motivation to prospect more deeply into the relation between EPNs and *D. suzukii*.

1.3 Biology of entomopathogenic nematodes

In the Nematoda phylum, 23 families contain species related to insect parasitism. Among them, mainly species in Steinernematidae and Heterorhabditidae families are considered for biological control (Alatorre-Rosas and Kaya 1990; Peters 1996). Nematodes of these families are obligated parasites of a wide range of insect species associated with endosymbiotic bacteria of the genus *Xenorhabdus* for Steinernematidae and *Photorhabdus* for Heterorhabditidae (Poinar 1979; Goodrich-Blair and Clarke 2007). These bacteria are also called entomopathogenic bacteria and in fact EPNs commonly refers to the nematode-bacterial complex. *Xenorhabdus* spp. and *Photorhabdus* spp. belong to the Enterobacteria family being gram-negative bacillus with two different phases, although only phase I is associated with nematodes (Boemare 2002). Bacterial amount held for a nematode vary from 50 to 200 and are carried inside a bacterial vesicle (Steinernematidae) or in the intestine (Heterorhabditidae) (Bird and Akhurst 1983; Boemare et al. 1996; Forst and Clarke 2002; Goetsch et al. 2006). Nematodes only harbor the bacteria during the infective form of third larval stage which is called infective juvenile (IJ) or dauer larvae and is the only one that lives outside the host. Besides, IJs present double cuticula with mouth and anus closed since are only responsible to search and infect new host insects (Kaya and Gaugler 1993). EPNs penetrate the host through natural openings (mouth, anus or spiracles) avoiding the intense grooming of insects (Gaugler et al. 1994). Exceptionally, some Heterorhabditidae species have a cuticular tooth in the anterior area that permits penetration through insect cuticle (Bedding and Molyneux 1982). When IJs reach the hemocoel cavity, they get rid of the second cuticle and continue their development into larval stage four (L4). Meanwhile, symbiont bacteria are released and proliferate quickly into the host (Ciche and Ensign 2003; Martens et al. 2003). Both nematode and bacteria kill insect host within 24-72h through septicemia or toxemia (Dowds and Peters 2002). Symbiotic bacteria prevent other bacteria and fungi from colonizing the host while providing nutrients to nematodes (Koppenhöfer 2007). Adult nematodes reproduce in two or more generations depending on the insect size (Fig. 4). When resources are depleted, new IJs carrying bacteria emerge from the insect cadaver to find another host (Griffin et al. 2005). EPN families present different reproductive mechanisms. First generation of heterorhabitids are all hermaphrodite with auto-fertilization while in next generations separate sexes are mainly present (Dix et al. 1992). In contrast, all generations of steinernematid species are amphimictic except from *Steinernema hermaphroditum* (Stock, Griffin and Chaerani) (Stock et al. 2004). Both families are oviparous and lay the eggs in the host cavity, although old females or hermaphrodites can hatch eggs inside the uterus where juveniles develop from mother's tissue. This process is known as endotokia matricida and represents an ecological adaptation to ensure the maturity of IJs (Johnigk and Ehlers 1999).

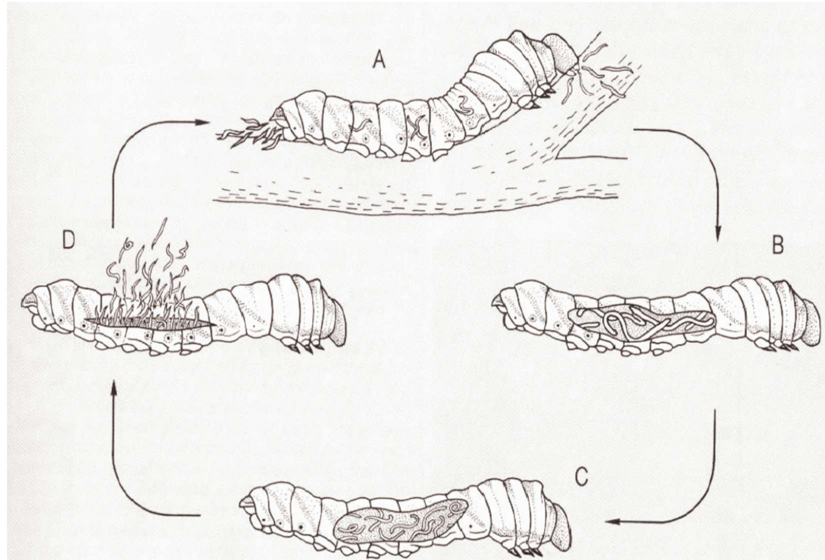


Fig. 4 Reprodutive cycle of EPNs: A. IJs locate and penetrate into the host; B. First generation of adults reproduce; C. Succesive generations of nematodes develop in the insect; D. Exit of the new IJs (Altalba et al. 1991).

Once IJs exit the insect, they disperse on soil in vertical and horizontal distribution to search for new hosts (Griffin et al. 2005; Lewis et al. 2006). EPNs perceive chemical, thermic, and mechanical signals from the environment (Lei et al. 1992; Rasmann et al. 2005). These signals can be from either insects or plants under herbivorous attack (Ennis et al. 2010; Hallem et al. 2011). IJs present foraging behavior that vary from ambushing to cruise (Lewis et al. 2006). With ambush strategy IJs are close to the soil surface and nictate to attack insects, while in cruise strategy IJs move actively through soil to find hosts (Campbell and Gaugler 1997). Generally, bigger nematode species such as *Steinernema glaseri* (Filipjev) (1130 µm) or *Steinernema kraussei* (Steiner) are considered cruisers while smaller species as *Steinernema carpocapsae* (Weiser) (558 µm) are considered ambushers. However, these strategies are not strictly bonded to species despite having a preferred behavior. The best example is *Steinernema feltiae* (Filipjev) that adopts an intermediate strategy (Campbell et al. 2003). Nevertheless, Wilson et al. (2012) reported *S. carpocapsae* to be able to switch over cruiser strategy depending on the environment. Thus, it was further assured that nematodes vary foraging behavior depending on target host, soil texture, environmental conditions, age of IJs or symbiont bacteria instead of adapting a unique foraging strategy (Griffin 2012). In consequence, the main factors that impact on nematode distribution are soil texture, vegetation, and host availability. EPNs can be found from grasslands, forests or sandy soil as most species are widely cosmopolitan (Griffin et al. 2005). Nevertheless, soil type and texture will determine infectivity success of IJs and their ability to disperse and persist. Nematode survival is also limited by extreme temperatures, desiccation and ultraviolet light revealing their soil-dwelling ecology (Glazer 2002). Similarly, IJs can be attacked by other microbial and invertebrates that inhabit the soil, such as nematophagous fungi, predatory mites or collembolans (Shapiro-Ilan et al. 2017).

EPNs are distributed in all continents except from Antarctica in either cultivated or wild soils (Hominick 2002). Among the global sampling, *S. feltiae* and *S. carpocapsae* were identified as widely cosmopolitan in temperate regions and *Heterorhabditis bacteriophora* (Poinar) is common in continental and Mediterranean climate. Although, other EPNs have a restricted distribution such as *Steinernema cubanum* (Mrácek, Hernandez and Boemare) or *Steinernema riojaense* (Griffin et al. 2005; Půža et al. 2020).

1.4 Application of entomopathogenic nematodes

In 1996, the EU-COST and OECD (European Cooperation in Science and Technology, and Organization for Economic Cooperation and Development) approved the safety use of EPNs for biological control (Ehlers and Hokkanen 1996). Nonetheless, the implementation and success of EPNs control strategies depends on the efficacy and adaptability of nematode species to the pest and crop system. In addition, application technique and user training to manipulate EPNs play a major role to achieve positive control (Georgis et al. 2006). The use of a specific nematode species is determined by the natural host preferences as some species are generalists able to infect a wide range of insects such as *S. feltiae* while other nematodes are more specialized (Peters 1996). However, the EPNs selection of the fittest species considering the efficiency rate is limited by the commercialized EPNs that can be accessed by users. The classical rearing of nematodes was carried out *In vivo* processes through *Galleria mellonella* L. (Lepidoptera: Pyralidae) infection. Nevertheless, techniques of mass production were developed based in *In vitro* culture in solid and liquid fermentation (Ehlers 2001). Liquid fermentation is the most efficient regarding production and nematode cost despite higher technology involvement (Koppenhöfer et al. 2020). In consequence, production efficiency and reduction of EPNs cost facilitated a wider usage. This fact prompted the commercialization of at least 13 different species over the world (Lacey et al. 2015).

The application of commercial formulates of EPNs to crops is done with aqueous solution through sprayer equipment or standard irrigation system (Georgis 1990). The firsts applications of EPNs were made into soil to control *Popillia japonica* Newman (Coleoptera: Scarabaeidae) in the United States (Glaser 1932). Due to their ecological niche, soil applications are the most efficient against pest attacking roots as *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae) or insects with soil-dwelling stages such as *Cydia latiferreana* (Walsingham) (Leptidoptera: Tortricidae) or *Curculio nucum* L. (Coleoptera: Curculionidae) (Morton and García del Pino 2008; Chambers et al. 2010; Batalla-Carrera et al. 2013). Whereas, researchers had pointed to the efficacy of nematodes against insects on cryptic habitats as the interior of branches, trunks, or fruits or on leaves (Kaya 1985; Tomalak et al. 2005). In foliar applications, *S. carpocapsae* and *S. feltiae* are the most used EPNs against pests such as *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), *B. tabaci* or the Dipteran *Liriomyza* spp. (Diptera: Agromyzidae) and *Bradysia* spp. (Diptera: Sciaridae) (Williams and Walters 2000; Head et al. 2003; Jagdale et al. 2004; Schroer and Ehlers 2005). In these applications, desiccation and UV light are critical for IJs survival before reaching a host.

Therefore, new formulations were developed to accompany the nematode application and ensure IJs survival and efficacy in foliar applications (Shapiro-Ilan et al. 2016, 2017).

IPM measures involve combined application strategies to control insect pests. Thus, compatibility of EPNs with other BCA or chemical treatments must be established in advance. Nematodes can tolerate short exposition to agrochemical products, insecticides and fertilizers that favors their use (Garcia-del-Pino et al. 2013; Koppenhöfer et al. 2020). However, few studies have been carried to determine the compatibility of EPNs and NE of different pest insects (Jandricic et al. 2006; Lordan et al. 2014).

1.5 Immune system ahead of an infection process

The understanding of infection process and interaction of host and pathogen are crucial while working with parasites beyond the mortality rate. The most known immune mechanism against EPNs infection is the encapsulation of IJs (Peters and Ehlers 1997; Li et al. 2007). However, a complex net of signaling and effector mechanisms are triggered once a parasite enters the insect organism. Insects enjoy a well-developed innate immune system able to recognize microbial and parasite infections (Castillo et al. 2011). This system is composed of the signaling or transcriptomic level and the effector one, which consists of humoral and cellular defense processes. Insect immune response was well studied in model insects such as *D. melanogaster* or *G. mellonella* (Lemaitre and Hoffmann 2007; Strand 2008) (Fig. 5). These studies reported substantial changes among insect orders in effector mechanisms as hemocytes and antimicrobial peptides (AMPs), while other signaling pathways are highly conserved even with mammals.

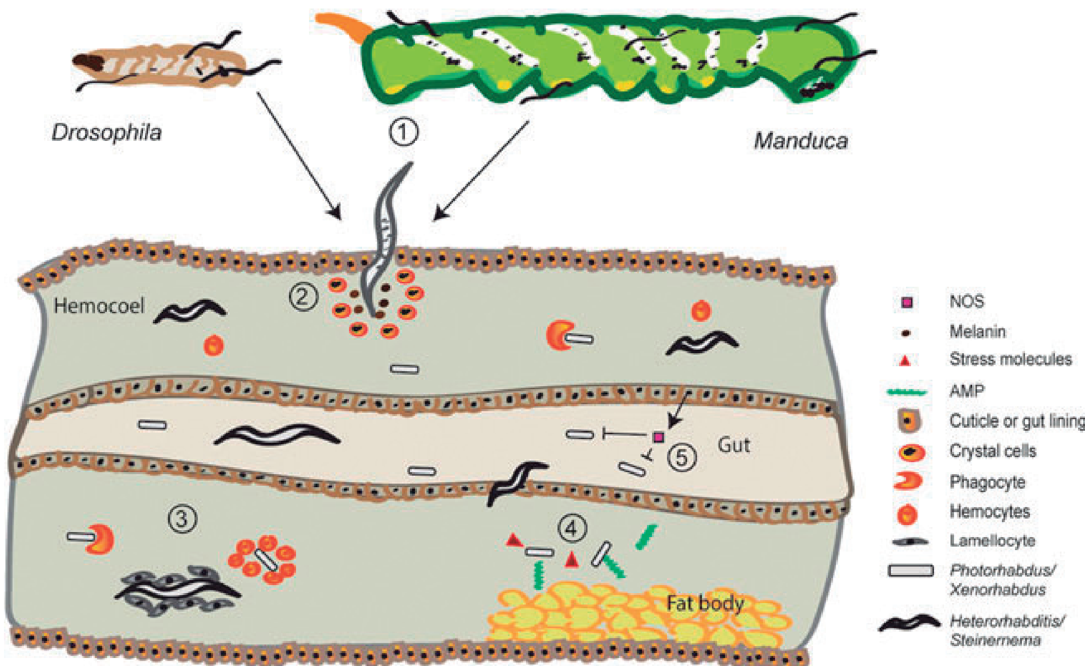


Fig. 5 Insect immune response to nematode-bacterial infection (Eleftherianos et al. 2016)

The first step of any response is the recognition of non-self through pattern-recognition receptors (PRR). These receptors are free in hemolymph or attached in the cell membrane and bind to pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway 2002). These molecules are secreted or derived from the surface of parasites, bacteria, fungi, or viruses such as lipopolysaccharide, peptidoglycans, β-glucans, lipoteichoic acid or nucleic acid. PRR detect the presence of non-self and trigger the signaling cascades to activate the effector mechanisms. At the transcriptomic level of *D. melanogaster*, six pathways are involved in this activation. These pathways are not immune exclusive and some of them are involved too in regulatory functions of embryology, development, or homeostasis (Eleftherianos et al. 2016). On one hand, Toll and Immune deficiency (Imd) represent the main exponentials of the immune system with their PRR: peptidoglycan recognition proteins (PGRP) and gram-negative binding proteins (GNBP). These receptors transmit the signal through a cascade until a nuclear factor (NF-κβ) activates the transcription of AMPs. Besides, c-Jun N-terminal kinase (JNK) and transforming growth factor-β (TGF-β) pathways receive input signals from Imd pathway to mediate stress response and global activation. On the other hand, PRR in hemocytes trigger the activation of pro-phenoloxidase (pro-PO) and Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathways to mediate cellular defense responses. At the effector level, active hemocytes promote phagocytosis, encapsulation, and nodulation of non-self (Lavine and Strand 2002; Strand 2008; Stanley et al. 2012). In *Drosophila*, mainly three hemocyte types are identified as plasmatocytes, lamellocytes and crystal cells which are involved in phagocytosis, encapsulation and melanization response, respectively (Wood and Jacinto 2007). In healthy larvae, lamellocytes are barely noticeable in circulant hemolymph and only after infection plasmatocytes induce the lamellocyte differentiation. AMPs carry the main humoral response in hemolymph all together with Lysozyme enzymes (Hultmark 1996; Bulet and Stöcklin 2005). Besides, two effector defenses combine cellular with humoral factors. One is to develop the hemolymph clotting and avoid dissemination of pathogens. The other is the melanization response in which crystal cells release PO enzyme that trigger an enzymatic cascade of hemolymph factors ending with melanin formation (Schmidt et al. 2001; Nappi et al. 2004). Besides the global response, specialized epithelial cells can promote local immune responses based on the localized release of AMPs and production of reactive oxygen species (ROS) (Lemaitre and Hoffmann 2007).

In regard to parasite perspective, EPNs interaction with the insect's immune system was mainly investigated also in model species such as *D. melanogaster*, *G. mellonella* and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Peña et al. 2015; Eleftherianos et al. 2016; Yadav et al. 2017). The infection process is characterized by three stages after entering the host (Binda-Rossetti et al. 2016) (Fig. 6). In the short or early phase (1 – 2 h Post-Infection (PI)) only nematodes are present until the intermediate phase (2 – 24 h PI) when IJs release the symbiotic bacteria into hemocoel. The host dies in the final or late phase (24 – 48 h PI) while nematode and bacteria reproduce. EPNs had coevolved to overcome insect immune defenses through different strategies from mimicry processes, immunomodulation and toxic compounds (Brivio and Mastore 2018). In early phase,

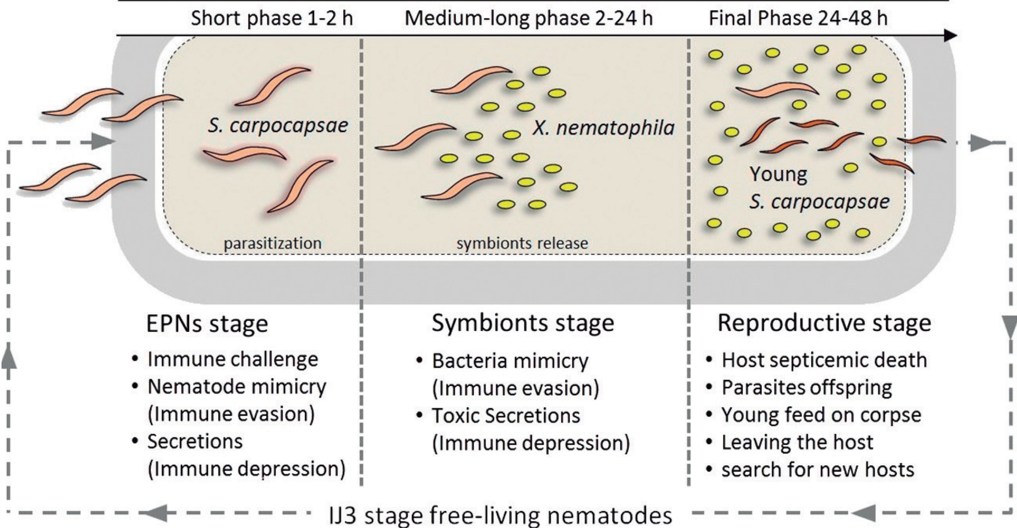


Fig. 6 Infection stages of EPNs into the host (Binda-Rossetti et al. 2016)

nematodes deploy evasive or depressive strategies to avoid being detected and prepare the bacterial release. For instance, *S. feltiae* modify its cuticular composition to disguise inside insects and avoid being recognized (Mastore and Brivio 2008). In contrast, *S. carpocapsae* secret molecules with proteolytic and toxic activity to immune suppress the system before the bacterial release (Laumond et al. 1989; Toubarro et al. 2010). In the intermediate and late phase, nematode and bacterial strategies act merged to ensure the infection's success. Symbiont bacteria *Xenorhabdus* spp. lead the main depressive and lethal role during the infection with toxic compound release and direct damage of hemocytes (Chattopadhyay et al. 2004; Herbert and Goodrich-Blair 2007). Bacteria rearrange the host's environment to favor its proliferation and nematode reproduction. Despite these strategies, immune response of some insects can avoid the nematode success and close phylogenetic insects even present different susceptibility. Thus, the relations established among host and parasite are species specific and should be studied by this nature.

2. OBJECTIVES

"*Nothing in life is to be feared, it is only to be understood.*
Now is the time to understand more, so that we may fear less."

Marie Curie

Drosophila suzukii is considered a major threat as a global invasive pest and the absence of a potential biological control has entailed important economic losses to producers and an overuse of chemical products. Meanwhile, EPNs were not properly tested despite the successful infection rate in similar dipteran species. Besides, while EPNs interaction with insect immune system is well-known in model species, this interplay is undetermined for pest insects as SWD. Thus, the aim of the thesis is to study the relation of *D. suzukii* and EPNs as BCA. To describe it, two different approaches were considered: one based on the basic parameters needed to develop a pest control strategy and the other based on the immunological relationship between host and parasite. Therefore, this goal serves to integrate biological and applied knowledge into the framework of biological pest control.

In order to accomplish this aim, specific objectives were designed:

1. To evaluate the susceptibility of *D. suzukii* stages (larvae, pupae and adults) to EPNs under laboratory conditions and which nematode species perform better results.
2. To perform preliminary assays with nematode applications under laboratory conditions.
3. To study the compatibility of EPNs with NE of *D. suzukii* that permits the building of combined applications of BCA.
4. To describe the effector immune responses of *D. suzukii* larvae infected with *S. carpocapsae* and symbiotic bacteria.
5. To characterize immune regulatory genes of *D. suzukii* and identify variations in the expression after *S. carpocapsae* and bacterial infection.

The fulfilment of these objectives became in the development of the following five chapters:

Chapter 3: Is *Drosophila suzukii* as susceptible to entomopathogenic nematodes as *Drosophila melanogaster*? Published in Journal Pest Science 2018, 91:789-798. [Objectives 1 and 2]

Chapter 4: Soil emergence of *Drosophila suzukii* adults (Matsumura) (Diptera: Drosophilidae): a susceptible period for entomopathogenic nematodes infection. Published in Journal Pest Science 2020, 93:639-646. [Objectives 1 and 2]

Chapter 5: Compatibility of entomopathogenic nematodes with natural enemies for horticultural pest control. Published in Biological Control 2019, 138. [Objective 3]

Chapter 6 Immune response of *Drosophila suzukii* larvae to infection with the nemato-bacterial complex *Steinenerma carpocapase – Xenorhabdus nematophila*. Published in Insects 2020, 11:210. [Objective 4]

Chapter 7: The modulation effect of *Steinernema carpocapsae* on immune-related genes of *Drosophila suzukii* larvae – In preparation 2021. [Objective 5]

CHAPTER 3

Is *Drosophila suzukii* as susceptible to entomopathogenic nematodes as *Drosophila melanogaster*?

Journal Pest Science 2018, 91:789-798

DOI: 10.1007/s10340-017-0920-6

CHAPTER 4

Soil emergence of *Drosophila suzukii* adults: a susceptible period for entomopathogenic nematodes infection

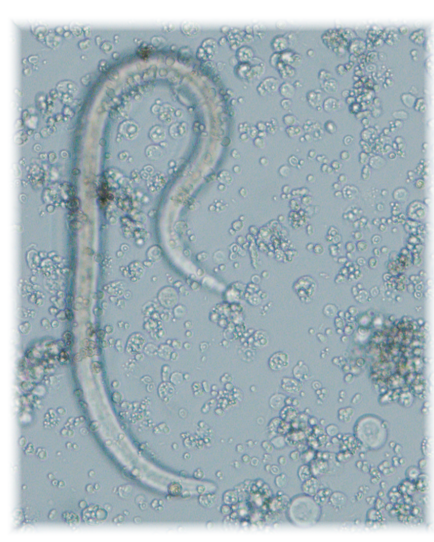
Journal Pest Science 2020, 93:639–646
DOI: 10.1007/s10340-019-01182-w

CHAPTER 5

Compatibility of entomopathogenic nematodes with natural enemies for horticultural pest control

Biological Control 2019, 138
DOI: 10.1016/j.biocontrol.2019.104050

CHAPTER 6



Immune response of *Drosophila suzukii* larvae to infection with the nematobacterial complex *Steinernema carpocapsae–Xenorhabdus nematophila*

Insects 2020, 11:210

DOI:10.3390/insects11040210

Article

Immune Response of *Drosophila suzukii* Larvae to Infection with the Nematobacterial Complex *Steinernema carpocapsae–Xenorhabdus nematophila*

Anna Garriga [1], Maristella Mastore [2], Ana Morton [1], Fernando Garcia del Pino [1,*] and Maurizio Francesco Brivio [2,*]

1 Departament de Biologia Animal, Biologia Vegetal i Ecologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain; anna.garriga.oliveras@uab.cat (A.G.); ana.morton@uab.cat (A.M.)

2 Laboratory of Comparative Immunology and Parasitology, Department of Theoretical and Applied Sciences, University of Insubria, 21100 Varese, Italy; maristella.mastore@uninsubria.it

* Correspondence: fernando.garcia@uab.cat (F.G.d.P.); maurizio.brivio@uninsubria.it (M.F.B.); Tel.: +39-0332-421404 (M.F.B.)

Received: 14 February 2020; Accepted: 26 March 2020; Published: 28 March 2020

Abstract: Entomopathogenic nematodes have been proposed as biological agents for the control of *Drosophila suzukii*, an invasive pest of small-stone and soft-skinned fruits. Larvae of the fly are susceptible to *Steinernema carpocapsae* infection but the reaction of immune defenses of the host are unknown. To determine the immune response, larvae were infected with *S. carpocapsae* and *Xenorhabdus nematophila* to evaluate the effector mechanisms of both humoral and cellular processes. The symbiont bacteria presented an inhibitory effect on the phenoloxidase cascade with a low level of melanization. Besides, *X. nematophila* activated the synthesis of putative antimicrobial peptides on the hemolymph of infected larvae. However, those peptides presented a lower antimicrobial activity compared to hemolymph from larvae infected with non-symbiont bacteria. *Xenorhabdus nematophila* avoided also the phagocytosis response of hemocytes. During in vitro and in vivo assays, *S. carpocapsae* was not encapsulated by cells, unless the cuticle was damaged with a lipase-treatment. Hemocyte counts confirmed differentiation of lamellocytes in the early phase of infection despite the unrecognition of the nematodes. Both *X. nematophila* and *S. carpocapsae* avoided the cellular defenses of *D. suzukii* larvae and depressed the humoral response. These results confirmed the potential of entomopathogenic nematodes to control *D. suzukii.*

Keywords: *Drosophila suzukii*; immunity; entomopathogenic nematodes; *Steinernema carpocapsae*; *Xenorhabdus nematophila*; humoral defenses; cellular defenses

1. Introduction

Entomopathogenic nematodes (EPNs) belonging to families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) are obligate parasites of a wide range of insects [1]. These nematodes have a mutualistic relationship with a bacteria of the genera *Xenorhabdus* and *Photorhabdus* respectively, that helps to kill the insect [2]. The infective juveniles (IJs) enter the host through natural body openings or by penetrating the cuticle and release the bacteria [3]. The nematode-bacteria complex kills the host within 24 to 48 h through septicemia or toxemia [4]. Thus, nowadays, EPNs are used as biological control agents in the management of agricultural pests [5]. An important factor that affects the efficacy of EPNs is the immune response of the insect host [6]. The cuticle of the insects is the first defense against nematodes together with an intense grooming behavior [7]. When IJs penetrate through the cuticle into the hemocoel, physiological and immune defenses are

activated in response to nematode presence [8,9]. Recognition of non-self, mainly based on the interaction between pathogen-associated molecular patterns and pattern-recognition receptors (PAMPs and PRRs), is crucial for the proper occurrence of cellular and humoral immune responses [10,11]. In insects, PAMPs and PRRs mediate the discriminatory step before triggering humoral responses, such as proPO system or antimicrobial peptide synthesis (AMPs). The proPO system is a complex enzymatic cascade responsible for the melanization reaction. This process leads to the production of melanin that can encapsulate invaders and opsonic factors enhancing immune reactions; moreover, drosophila phenoloxidases (PO) seem to play a role also in hemolymph clotting as a further defensive mechanism aimed to prevent the entry of nematodes and microorganisms [12–14]. Unlike the proPO system, which is rather well preserved and homogeneous among arthropod species, AMPs show different structural conformations among insects and various mechanisms to kill microorganisms [15].

PRRs also activate cellular responses like phagocytosis and encapsulation; phagocytosis is a conserved process mediated by hemocytes against various small targets including bacteria and yeast [16,17]. Instead, encapsulation is the main defense against the presence of multicellular targets, such as nematodes or endo-parasitoids. In the *Drosophila* family, three main types of hemocytes or immunocompetent cells (plasmatocytes, lamellocytes, and crystal cells) are found in the hemolymph and are responsible for the immune functions described [18]. Plasmatocytes represent the most abundant hemocytes and play a crucial role in target recognition, phagocytosis activity, and as promoters of encapsulation. These cells recall and differentiate to lamellocytes [19], which are involved in the formation of multi-layered capsules. The third cell population consists of crystal cells, which contain the enzymes of the proPO cascade and quickly degranulate in the presence of non-self [20].

Nevertheless, EPNs have developed strategies to evade and suppress the insect immune defenses during all stages of infection [6]. During a nematobacterial infection, three steps can be identified: in the early phase, IJs must evade and/or depress the host immune system just after entry. Afterward, in the midterm phase, symbiont bacteria are released and secret toxic compounds that contribute to killing the host. Finally, the long phase is the reproductive stage of nematodes [21]. Nemato-bacterial strategies are based on mimicry processes [22] or active suppression of host defenses [9]. *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae) has been reported using mimic insect recognition proteins expressed in the epicuticle of IJs that evade detection [23,24]. This nematode can also damage immune defenses with proteolytic secretions, modulate proPO activity, and avoid encapsulation in different insect species [25–27]. In addition, its symbiont bacteria *Xenorhabdus nematophila* can cause general immunodeficiency using toxins that jointly with nematode defenses overcome the insect's immune response [21]. Besides, Park and Kim [28] reported the ability of *X. nematophila* to avoid the activation of proPO cascade.

Our work is focused on *Drosophila suzukii* (Matsumura) (Diptera: Drosophilae) or spotted wing drosophila, the most important pest that attacks soft-skinned and small stone fruits causing significant losses to crops [29,30]. Despite chemical and culture methods are widely used, biological control of this fly has been attempted using natural enemies and entomopathogenic agents [31]. Studies with larvae of *D. suzukii* showed a strong immune response of encapsulation to parasitoid eggs of *Leptopilina heterotoma* Thompson (Hymenoptera: Figitidae) that discourages their use for controlling the pest [32,33]. Instead, pupal parasitoids, entomopathogenic fungi, and EPNs achieved better results controlling the fly under laboratory conditions [34–37]. Susceptibility of *D. suzukii* larvae was evaluated against different EPN species, as *S. carpocapsae, Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae), and *Heterorhabditis bacteriophora* (Poinar) (Rhabditida: Heterorhabditidae) [36]. This study reported a high susceptibility of the fly after nematodes killed the larvae and reproduced inside them. Nevertheless, the immune response of *D. suzukii* to EPNs infection has not yet been studied.

Therefore, this work aimed to study the relationships between *D. suzukii* larvae and the nematobacterial complex *S. carpocapsae/X. nematophila*, from an immunological point of view. We evaluated humoral defenses, as the proPO system and lysozyme activity, the presence of antimicrobial peptides (AMPs) pool and its activity against bacteria. We analyzed the cellular response of *D.*

suzukii larvae determining the phagocytosis and encapsulation ability of hemocytes and describing the immunoevasion strategies of *S. carpocapsae*.

2. Materials and Methods

2.1. Chemicals and Instruments

All reagents used in the assays were supplied by Sigma Chemicals (St. Louis, MO, USA), ICN (ICN Biomedicals, GmbH), Merck Millipore Ltd. (Tullagreen, Cork, Ireland), Bio-Rad Laboratories (Detroit, MI, USA). The equipment was supplied by Bio-Rad Laboratories (Detroit, MI, USA) and Celbio Spa (Milan, Italy, EU). Centrifugations were carried out with a SIGMA 1-14 (SciQuip Ltd., Newtown, Wem, Shropshire, UK) and an Eppendorf 5804R (Eppendorf, AG, Hamburg, Germany). Spectrophotometric assays were performed with a Jasco V-560 spectrophotometer (Easton, MD, USA). All materials, buffers, and solutions were autoclaved or filtered with 0.22 µm Minisart filters (Sartorius, Goettingen, Germany). For microscopy observations, a microscope Olympus IX-51 epifluorescence connected to a Nikon digital camera was used.

2.2. Insects and Nematodes

The third stage of *D. suzukii* larvae used for all assays was obtained from a laboratory culture of specimens collected in Catalonia (NE Spain) in 2012. These insects were reared on a *Drosophila* diet [37] and maintained in a climate chamber at 25 °C, 45% RH, and a 12:12 h photoperiod.

The EPN species used in this study was *S. carpocapsae* (B14) isolated from urban garden soil in Barcelona (Catalonia, NE Spain). Nematodes were reared at 25 °C in the last instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) according to the method described by Woodring and Kaya [38]. The IJs emerging from insect cadavers were collected with modified White traps [39] and stored in sterile tap water in culture flasks at 9 °C for a maximum of two weeks. Before use, IJs were acclimatized at room temperature for 3 h and their viability was checked by observation of movement under a stereomicroscope. IJs were selected, washed several times with sterile phosphate buffer (PBS) (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per liter, pH 7.4), and centrifuged at 100× g for 2 min at 20 °C. Assays were performed using alive, dead and lipase-treated dead nematodes, to evaluate the role of the body surface of *S. carpocapsae* in the immune-evasive processes.

To kill the nematodes, they were frozen at −20 °C for at least 5–6 h in PBS plus 20% of glycerol. To modify the cuticular lipid layer, killed nematodes were treated with 50 µl of lipase (10 U/µL in 30 mM of Tris-HCl, pH 8), at 37 °C, for 90 min; after the enzymatic digestion, nematodes were washed several times in sterile buffer.

2.3. Bacteria Cultures and Infection Protocol

To culture the symbiotic bacteria, *X. nematophila* were isolated from *S. carpocapsae* according to the method of Park and Kim [40]. *X. nematophila* Green Fluorescent Protein (GFP-labeled) was also kindly provided by the laboratory of Prof. Givaudan (University of Montpellier, France). Bacteria were inoculated into liquid broth (30g/L Tryptic Soy Broth) and incubated at 30 °C in agitation, overnight under dark conditions. The culture was grown to an optical density (OD) of 0.6; the growth curve was measured spectrophotometrically at 600 nm. Bacteria were centrifuged at 1700× g for 20 min and bacterial pellets were washed several times with PBS. Aliquots at different concentrations were prepared for corresponding assays.

Cultures of *Escherichia coli* (C1a), *Bacillus subtilis* (ATCC N° 6633), and *Micrococcus luteus* (ATCC N° 4698) were prepared for positive stimulation of *D. suzukii* larvae. Bacteria were inoculated and grown in Luria broth (20 gr/L) at 37 °C and 30 °C for *M. luteus* in agitation, overnight under dark conditions. Bacteria were grown up to 0.6 OD (10^9 CFU/mL) and were centrifuged at 1500× g for 15 min at 20 °C. After centrifugation, the pellets were washed several times in PBS. Final bacterial concentrations and species mixtures were reported in each assay.

2.4. Hemolymph Collection

To extract the hemolymph, third stage *D. suzukii* larvae were washed in PBS and 70% ethanol solution and anesthetized at 4 °C. Depending on the assay, 20 to 40 larvae were cut in the dorsal region with a microsurgical scissor and transferred in PCR tubes properly prepared for the procedure. The bottom of the tube was holed several times with a needle and inserted into a 0.5 mL Eppendorf so that during centrifugation, the hemolymph was collected in the large tube. Samples were centrifuged at 250× g for 5 min at 4 °C to collect whole hemolymph containing hemocytes. For humoral assays (Section 2.6, Section 2.7), the supernatant, corresponding to a cell-free fraction (CFF), was recovered, centrifuged at 720× g and a few phenylthiourea (PTU) crystals were added; all the humoral immunity assays were carried out according to the methods described in Mastore and Brivio [41]. For cellular assays (Section 2.8, Section 2.9), 10 µL of PTU (from a saturated stock solution) were added to the whole hemolymph to prevent unwanted activation of phenoloxidase.

2.5. proPO System Relative Activity in the Host Hemolymph

To test the proPO activity of *D. suzukii* larvae against bacterial infection, phenoloxidase relative activity was analyzed in the hemolymph by spectrophotometric analysis with L-Dopa as a substrate.

Larvae of *D. suzukii* were washed with PBS and anesthetized by exposure to cold; then, they were infected with bacteria using a pricking method. Pricking consisted of a puncture with a wolfram needle soaked in bacteria pellet obtained from a suspension of 10^9 CFU/mL. Four different treatments were evaluated: naive larvae, control pricked larvae, *X. nematophila* infected larvae, and *E. coli*/*B. subtilis* infected larvae. A mixture of *E. coli* and *B. subtilis* 1:1 (v/v) was prepared. Larvae were kept at rearing conditions until hemolymph was collected 30 min after any treatment. After the extraction, the total protein content was determined and the reaction volumes were normalized according to concentration, 2.5 µL of hemolymph was added in 1 mL of L-Dopa buffer (8 mM L-Dopa in 10 mM Tris-HCl, pH 7.2). The increase of absorbance was recorded at 490 nm (ΔA_{490} 5 min^{-1}) at 25 °C, by a double-beam Jasco V-560 spectrophotometer (Easton, MD, USA) using L-Dopa buffer as blank. For each treatment, 20 larvae were used, and the experiment was repeated 5 times.

2.6. Lysozyme Activity after Bacterial Infection

To evaluate the activity of lysozyme in naïve and infected larvae of *D. suzukii*, the turbidimetric method was used with *Micrococcus lysodeikticus* as a substrate. This method is based on the decrease of absorbance due to the lysozyme-induced cell lysis determined as a unit of lysozyme produced in one minute a variation of OD 450 nm of 0.001. Larvae of *D. suzukii* were infected with *X. nematophila* and *E. coli*/*M. luteus* mixture 1:1 (v/v) at a concentration of 10^9 CFU/mL, using the pricking method. After infection, larvae were kept with diet at 25 °C for 24 h. Then, hemolymph samples were extracted from larvae, cells were removed and CFF samples, added with PTU crystals, were diluted with PBS (1:10). For the analysis, 0.45mg/mL of *M. lysodeikticus* lyophilizate were resuspended in 0.3 M PBS, pH 6.8 and mixed for 1 min at room temperature. For each treatment, 60 µL of diluted CFF were added to 90 µL of *M. Iysodeikticus* suspension into a well of 96-MicroWell™ plate. As a control, a suspension of *M. lysodeikticus* (90 µL) plus PBS (60 µL) was used, and a PBS (90 µL) plus hemolymph sample (60 µL) was used as a blank for each treatment. The activity of lysozyme was assessed immediately using a microplate-Reader (Bio-Rad, Hercules, CA, USA) and absorbance variations were recorded every min for 10 min at 450 nm. For each treatment, 40 larvae were used, and the experiment was repeated three times.

2.7. Analysis by Tricine-PAGE and Activity of AMPs

We analyzed changes in proteins and peptides patterns in hemolymph samples (especially in CFF) of *D. suzukii* larvae using the electrophoretic separation by Tricine-SDS-PAGE methodology [42]. Moreover, the antimicrobial activity of these CFF samples was evaluated with bacteria growth tests.

Larvae of *D. suzukii* were infected using the pricking method with a bacterial suspension of *X. nematophila* and *E. coli/M. luteus* mixture 1:1 (v/v) at a concentration of 10^9 CFU/mL. Larvae were kept in rearing conditions for 24 h. Then, the hemolymph from naïve infected *X. nematophila* and *E. coli/M. luteus* infected larvae was extracted. From these CFF samples, we carried out the analysis for the presence of putative AMPs molecules and the evaluation of the antimicrobial activity.

For the electrophoretic separations, hemolymph was fractioned by Amicon® Ultrafilters (Millipore, Burlington, MA, USA) cut-off 30 KDa and precipitated with trichloroacetic acid (20% V/V). Then, samples were resuspended in 1× Tricine-PAGE sample buffer [42] and denatured for 5 min at 100 °C. Electrophoresis was carried by a vertical PROTEAN® II xi Cell (Bio-Rad) at 50 V (constant voltage) overnight. Protein patterns were detected by Silver Staining.

For the antimicrobial activity, CFF samples were centrifuged at 1700× g for 15 min and fractioned <30 kDa; *E. coli*, *M. luteus*, and *X. nematophila* cultures were diluted to a final concentration of 10^6 CFU/mL with culture broth. For each treatment, 20 µL of CFF sample were added to 180 µL of bacteria culture. To evaluate the expected bacterial growth, 20 µL of PBS were added to the bacteria culture (180 µL). All samples were incubated for 3 h under shaking at the optimal growth temperature of the tested bacterium. After incubation, 100 µL of each sample was placed in a well of a 96-MicroWell™ plate and samples were serially diluted with phosphate buffer (61.4 mM K_2HPO_4, 38.4 mM H_2PO_4). Each dilution was plated on solid agar and incubated for 24 h more. Finally, bacteria colonies were counted. The antibacterial activity in hemolymph samples was intended as a percentage of bacterial survival compared with the control (bacterial suspension incubated without *D. suzukii* hemolymph). The final concentration of hemolymph total proteins used in the antimicrobial activity tests was 3.3 µg/µL. For each analysis and treatment, hemolymph of 40 larvae was extracted and the experiment was done three times.

2.8. Phagocytic Activity Assay

Phagocytic activity of *D. suzukii* hemocytes was evaluated both in vivo and in vitro in the presence of *X. nematophila*. For the in vivo assay, 60 larvae of *D. suzukii* were infected with *X. nematophila*-GFP (10^4 CFU/50nL). Microinjections were performed by a Drummond Nanoject II nanoliter injector (Drummond Scientific Company, PA, USA). As a positive control, 60 larvae were injected with 50 nl (1 mg/mL) of a suspension of pHrodo® Red *Staphylococcus aureus* Bioparticles®-Conjugate (Life Technologies, Carlsbad, CA, USA). Larvae were kept in rearing conditions and hemolymph was collected after 2 h. Extracted hemolymph with hemocytes was added to Schnëider medium in 96-MicroWell™ (Thermofisher Scientific, Waltham, MA, USA) plates (final concentration 2×10^5 cells/mL) and incubated for 30 min at 25 °C in the dark. To evaluate the phagocytic activity of hemocytes, after adhesion, cells were observed under a fluorescence microscope.

For in vitro assay, hemolymph of 40 naïve larvae was extracted and hemocytes were plated in 96-MicroWell™ plates at a concentration of 2×10^5 cells/mL in Schnëider medium. To allow cells to adhere to the substrate, plates were incubated for 1 h at 25 °C in the dark. Then, 5 µL (10^3 CFU) of *X. nematophila*-GFP or *S. aureus*-pHrodo® were added to cells and incubated for 4 h at 25 °C. After incubation, cells were observed under a fluorescence microscope to assess the phagocytic activity of hemocytes. Both experiments were performed three times.

2.9. In Vitro Encapsulation Assay

We assessed the ability of the hemocytes of *D. suzukii* larvae to recognize and encapsulate *S. carpocapsae* with an in vitro assay performed with three treatments. Alive IJs were used to test the physiological response of the host immune cells to the presence of the nematode. To exclude that unrecognition could be caused by active secretions, cold-killed nematodes were incubated with hemocytes. Besides, to investigate a possible mimetic function of the body-surface of *S. carpocapsae*, we modified the nematode cuticle using lipase enzyme treatment.

Hemocytes from naïve larvae were extracted and plated with Schnëider medium in a 96-MicroWell™ plate (2×10^5 cells/mL). Then, 5–10 nematodes per treatment (alive, cold-killed, or lipase-treated dead) were added to the microwells and incubated in a climate chamber at 25 °C. As positive control to evaluate encapsulation capability, we added 10–15 agarose beads (DEAE Sepharose®) into wells. Encapsulation processes were monitored at different times along 24 h with an inverted fluorescence microscope. Each treatment was performed three times.

2.10. Hemocytes Populations Count after Natural Infection

To evaluate the in vivo ability of *D. suzukii* hemocytes to recognize and isolate *S. carpocapsae*, we carried out encapsulation assays by a natural infection. Concurrently, total and differential cell counts (plasmatocytes, lamellocytes, and crystal cells) were performed to assess any possible variation of cell populations after nematode infection.

Ten larvae were placed in a Petri dish (3 cm diameter) filled with filter paper and exposed to *S. carpocapsae* (50 IJs cm^{-2}) for 20 h. The control treatment was carried out with sterile tap water. To detach any adherent cells from the hemocoel cavity, larvae were gently brushed, and then washed with PBS [43]. An incision was done behind the mandibles and all hemolymph (approximately 2 µL) was bled in 48 µL of buffered PTU (saturated PTU diluted 1:4 in PBS). After bleeding, hemolymph content of each separated larvae was observed under the microscope, to detect the presence and number of nematodes inside the larvae, cell encapsulation processes, and the possible presence of symbiotic bacteria released in the hemolymph. Depending on the phase of infection, larvae were divided into two groups: early infection (only nematodes were present) or midterm infection (bacteria were released). Total hemocyte count was made applying the diluted hemolymph into the hemocytometer (Neubauer chamber, Brand®). The cell counts were performed immediately by determining the total number of cell populations and the number of different types of hemocytes identified. For each treatment, ten larvae were evaluated, and the experiment was done twice.

2.11. Statistical Analysis

To analyze the differences in enzymatic activity of proPO and lysozyme, a General Linear Model (GLM) analysis has been used to ascertain differences among the tested samples. GLM was also used to elucidate differences among CFF samples of their antimicrobial activity against different bacterial cultures. Hemocyte counts were measured as the number of cells/µL of hemolymph and differences among treatments were analyzed with GLMs. For all experiments, when the GLM was significant, differences were evaluated by Tukey test and means without transformation (±SD) are presented. All statistical analyses were performed with the R studio software (version 1.0.153) [44], and any comparison was considered significant if the p-value was < 0.05.

3. Results

3.1. proPO System Relative Activity in the Host Hemolymph

The relative activity of phenoloxidase enzyme in *D. suzukii* samples was evaluated by recording spectrophotometrically the formation of dopachrome (Figure 1, Figure S1, and Table S1).

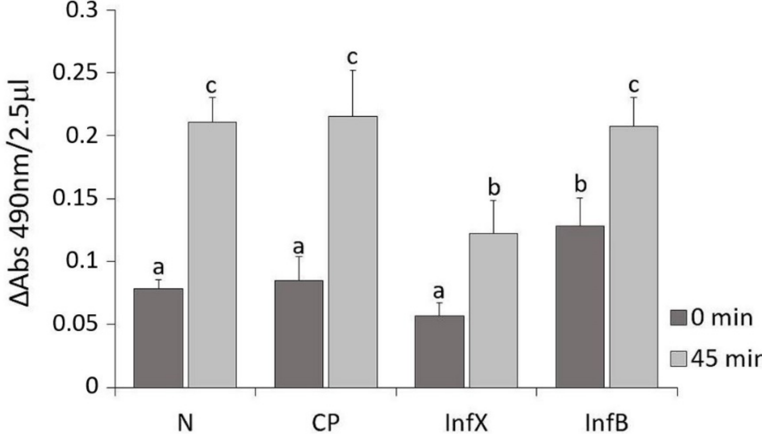


Figure 1. Phenoloxidase relative activity in the hemolymph of *D. suzukii* shown as the mean ± SD increase of absorbance, recorded at 0 and 45 min (= 490 nm), in hemolymph samples from naïve (N), control pricked (CP), *X. nematophila*-infected (InfX) and *E. coli/B. subtilis*-infected (InfB) larvae. Different letters indicate statistical significance differences between hemolymph samples ($p < 0.05$).

Samples from naïve larvae (N), control pricked larvae (CP), infected larvae with *X. nematophila* (InfX) and infected larvae with *E. coli/B. subtilis* (InfB) were analyzed. The absorbance values recorded showed no significant difference between naïve and control pricked larvae. These values represent the physiological activity of the enzyme until the formation of all dopachrome (time 45 min). Larvae infected with *X. nematophila* presented similar values to both naïve and control larvae at 0 min, but the presence of symbiotic bacteria produced an inhibition of phenoloxidase activity 45 min after the start of reaction. The enzyme showed a significantly minor activity compared to the other hemolymph samples (GLM: $F = 42$, $df = 7$, $p < 0.001$). In contrast, the proPO system of *D. suzukii* larvae resulted in *E. coli* and *B. subtilis* infection showing a significantly high increase of absorbance at 0 min after hemolymph extraction.

3.2. Lysozyme Activity after Bacterial Infection

The assays of lysozyme activity in the hemolymph of naïve *D. suzukii* larvae showed extremely low activity (1×10^{-1} Units mL^{-1}). Moreover, those infected with *X. nematophila* also presented a low level of activity (1.1×10^{-1} Units mL^{-1}). Only after infection with *E. coli* and *M. luteus*, a slight increase in the enzyme activity was recorded (1.3×10^{-2} Units mL^{-1}). Nevertheless, lysozyme activity in the hemolymph of *D. suzukii* larvae was not significantly stimulated by the infection of *X. nematophila* or the bacterial mixture of *E. coli* and *M. luteus* (GLM: $F = 0.12$, $df = 2$, $p = 0.81$).

3.3. Analysis by Tricine-PAGE and Activity of AMPs

Electrophoretic separation of fractioned CFF (<30 kDa) showed proteins and peptide patterns of hemolymph samples from naïve and infected larvae (Figure 2).

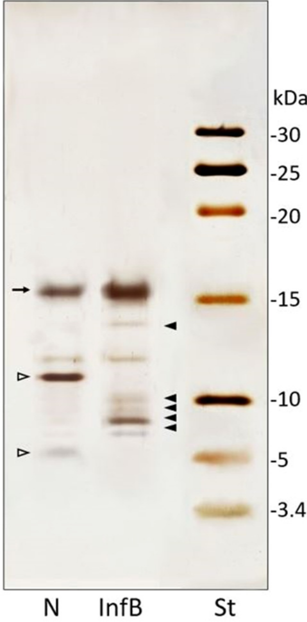


Figure 2. Tricine-SDS-PAGE (16%) of hemolymph samples. Patterns of low molecular weight proteins (<30 kDa) of hemolymph from naïve (N) and *E. coli/M. luteus*-infected (InfB) larvae; standard molecular weights marker (St). Full arrowheads indicate newly synthesized bands and the arrow an increased band, observed after bacterial infection. Empty arrowheads indicated disappeared peptides after bacterial infection.

In naïve larvae CFF, four main bands were present and two of them disappear in the infected larvae sample (Figure 2, empty arrowhead). Moreover, peptides pattern from *D. suzukii* larvae infected with *X. nematophila* (Figure S2) and infected with the mixture of *E. coli/M. luteus* showed newly synthesized or quantitatively increased peptides of low molecular weight (Figure 2, full arrowheads). Five main bands, ranging from 5 to 16 kDa, absent in CFF from naïve larvae (Figure 2, N), were observable in samples from treated larvae (Figure 2, InfB). Moreover, a band of about 16 kDa, increased in samples from infected larvae (Figure 2, InfB, arrow). The infection with *X. nematophila* (Figure S2) and *E. coli/M. luteus* resulted in the disappearance of two peptides (Figure 2, N, empty arrowheads).

After identification of the peptides pattern, the antimicrobial activity in the hemolymph was analyzed by co-incubation of CFF samples with bacterial cultures of *E. coli*, *M. luteus*, and *X. nematophila*. Bacterial growth (CFU/mL) represented a negative correlation of AMPs activity present in larvae hemolymph of naïve, infected with *X. nematophila* and infected with *E. coli/M. luteus* (Figure 3, Table S2).

As there was significant higher proliferation of all bacteria incubated with naive hemolymph compared to buffer incubation (PBS), we evaluated the bacterial mortality percentage assuming as 100% the data obtained with naive hemolymph samples (Table S3). CFF samples from larvae infected with *X. nematophila* reduced significantly the bacterial growth of *E. coli* to 6.11×10^7 CFU/mL and *M. luteus* to 3.86×10^7 CFU/mL. These results showed a degree of antimicrobial activity in hemolymph against both bacteria strains with 55.0% and 41.5% of mortality respectively (Table S3). However, CFF samples of *E. coli/M. luteus*-infected larvae presented a drastic antimicrobial effect against *E. coli* (1.06×10^7 CFU/mL) causing mortality of 92.2% and 76.1% against *M. luteus* (1.58×10^7 CFU/mL). In contrast, when tested on *X. nematophila* the recorded mortality was markedly lower (17.2%) (Table S3). Thus, CFF from larvae infected with *X. nematophila* showed antimicrobial activity against the three bacterial cultures tested, although lower than the obtained after *E. coli/M. luteus* infection.

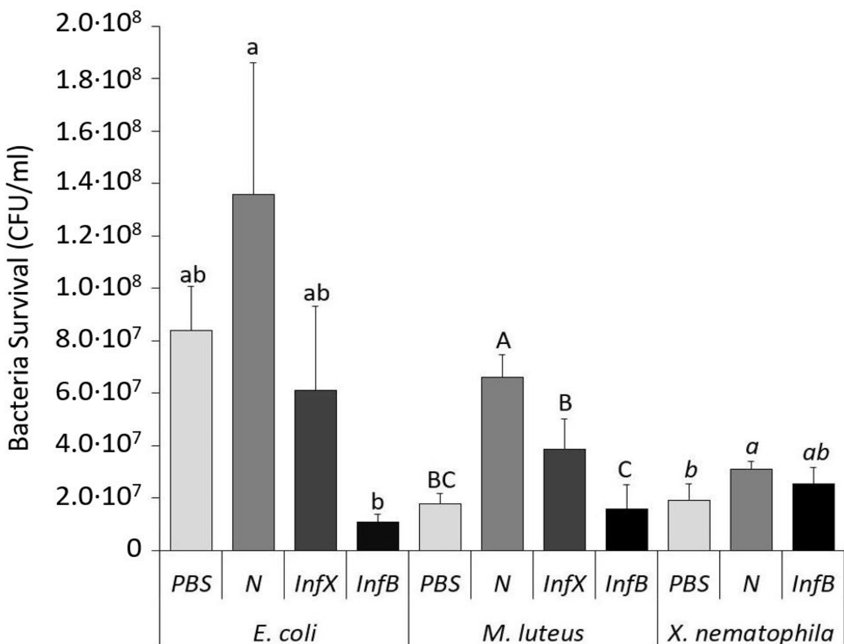


Figure 3. Antimicrobial activity of *D. suzukii* hemolymph after bacterial infection evaluated through co-incubation with *E. coli*, *M. luteus*, and *X. nematophila*. Host hemolymph samples were from PBS-control (PBS), naïve larvae (N), infected with *X. nematophila* (InfX), and with *E. coli/M. luteus* (InfB). Survival of *E. coli, M. luteus*, and *X. nematophila* are shown as the mean ± SD of CFU/ml. Different letters on the bars represent statistically significant differences among each antimicrobial sample. GLM of *E. coli:* $F = 8.39$, $df = 3$, $p = 0.007$; GLM of *M. luteus:* $F = 21.73$, $df = 3$, $p < 0.001$; GLM of *X. nematophila*: $F = 3.66$, $df = 2$, $p = 0.091$.

3.4. Phagocytic Activity Assay

During in vivo phagocytosis assay, hemolymph of *D. suzukii* larvae injected with *X. nematophila*-GFP was extracted and monitored by fluorescence microscopy.

To evaluate the phagocytosis capability of hemocytes, we carried out in vivo assays using *S. aureus* pHrodo®-conjugated which fluorescence was activated only at acidic pH (inside phagolysosomes). *Staphylococcus aureus*-pHrodo was effectively engulfed by host cells as confirmed by the intense fluorescence of the probe (pHrodo) (Figure 4, A1, left). Besides, in the bright field, hemocytes of *D. suzukii* were properly adhered to the substrate and are viable and showing a morphology typical of cells that have engulfed (Figure 4, A1, right). Otherwise, hemocytes seem to be unable to phagocytize *X. nematophila*-GFP (Figure 4, A2, left); both the elongated rod shape and swipes of the symbiont bacteria, indicate their extracellular localization. The entomopathogenic bacteria, in addition to not being phagocytized, seem to have cytotoxic effects on the host hemocytes, affecting the morphology of the cells (Figure S3). Besides, Figure 4 (A2, right) shows the micrograph obtained by combining fluorescence and phase contrast, in which the extracellular location of the symbiont bacteria can be detected.

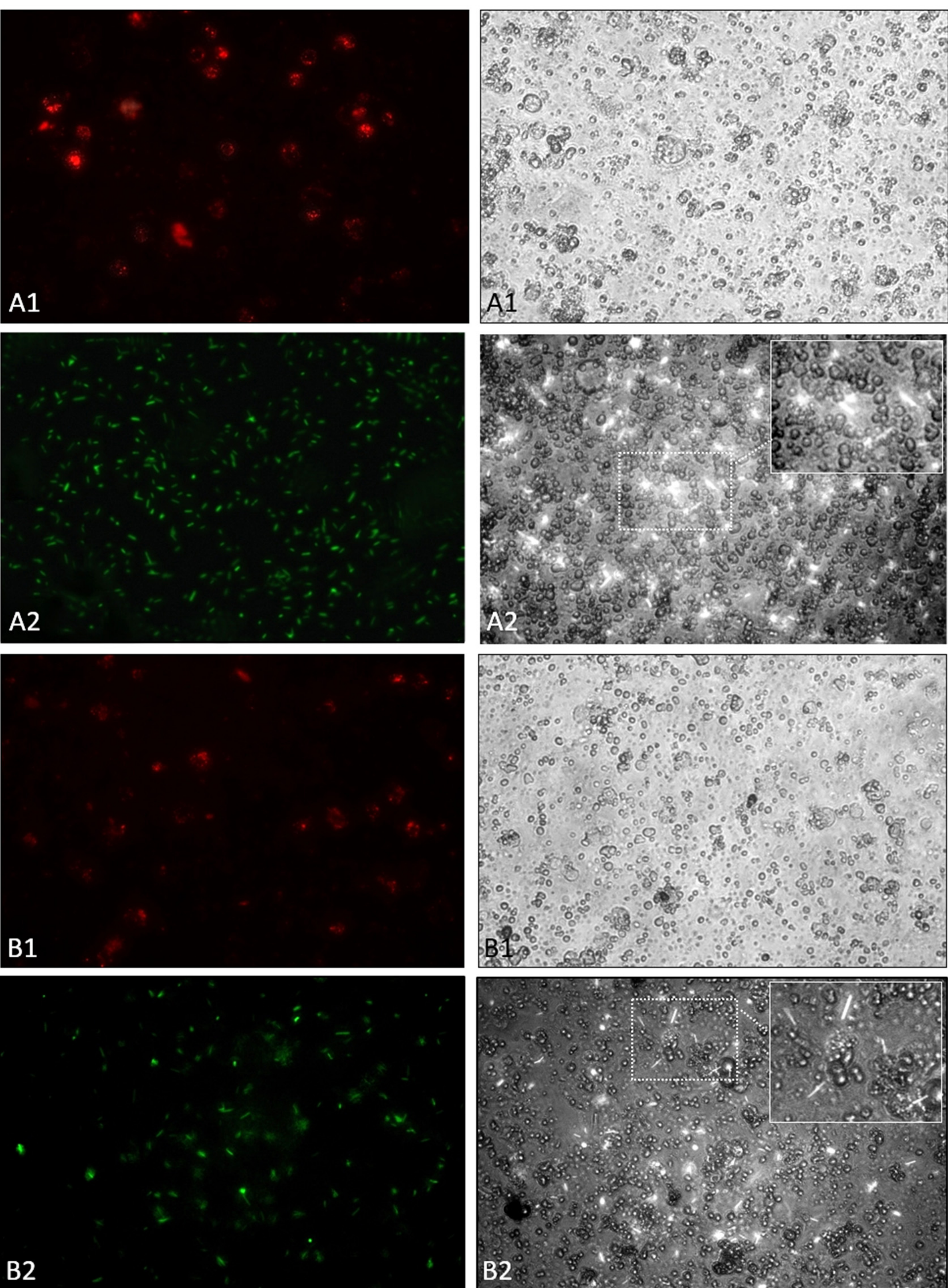


Figure 4. In vivo (**A**) and in vitro (**B**) phagocytic activity of *D. suzukii* larvae hemocytes against bacteria. (**A1**) in vivo assay at 2 h with *S. aureus*-pHrodo™ BioParticles®; (**A2**) in vivo assay at 2 h with *X. nematophila*-GFP, inside A2 right an enlargement of the central area of the image. (**B1**) in vitro assay at 4 h with *S. aureus*-pHrodo™ BioParticles®; (**B2**) in vitro assay at 4 h with *X. nematophila*-GFP, inside B2 right an enlargement of the central area of the image. All images on the right are bright field of the respective fluorescence micrographs on the left. Images magnification is 400×.

Hemocytes of *D. suzukii* larvae established in the in vitro cultures showed comparable results to those obtained in vivo; *S. aureus*-pHrodo was efficiently phagocytized (Figure 4, B1, left) and hemocytes

show the morphology of cells that are engulfing (Figure 4, B1, right). When *X. nematophila*-GFP was added to the culture, hemocytes were not able to phagocyte the symbiont bacteria as confirmed by the observation of Figure 4, B2, left and right.

3.5. In Vitro Encapsulation of S. carpocapsae

Cellular encapsulation of nematocomplexes was assessed using in vitro long-term co-incubation. Hemocyte response against *S. carpocapsae* alive, dead (cold-killed), or surface lipase-treated was monitored under an inverted microscope for 24 h. We observed that alive nematodes were not recognized nor encapsulated by hemocytes even after 24 h (Figure 5A,B: A1,A2,A3,A3i).

Moreover, no cellular processes directed against *S. carpocapsae* were evident. We performed the same experiment with cold-killed nematodes to exclude the influence of active secretions. As seen before, hemocytes were unable to recognize dead IJs of *S. carpocapsae* and no encapsulation processes were observed also at long times (Figure 5A,B: B1,B2,B3,B3i). When lipase-treated nematodes were used, it was possible to observe a reactivity of hemocytes adhering to the body surface of the treated nematodes (Figure 5C,D: C1 and C1i, C2 and C2i). After 2 h (Figure 5, C1), numerous layers of cells were attached to the cuticle and would contribute to the building of the cellular capsule. The abiotic targets (Sepharose DEAE microbeads) used as a control presented cellular reactivity with hemocytes adhered to beads and the presence of melanin clots at 12 and 24 h after incubation (Figure 5C,D: D1 and D2, respectively).

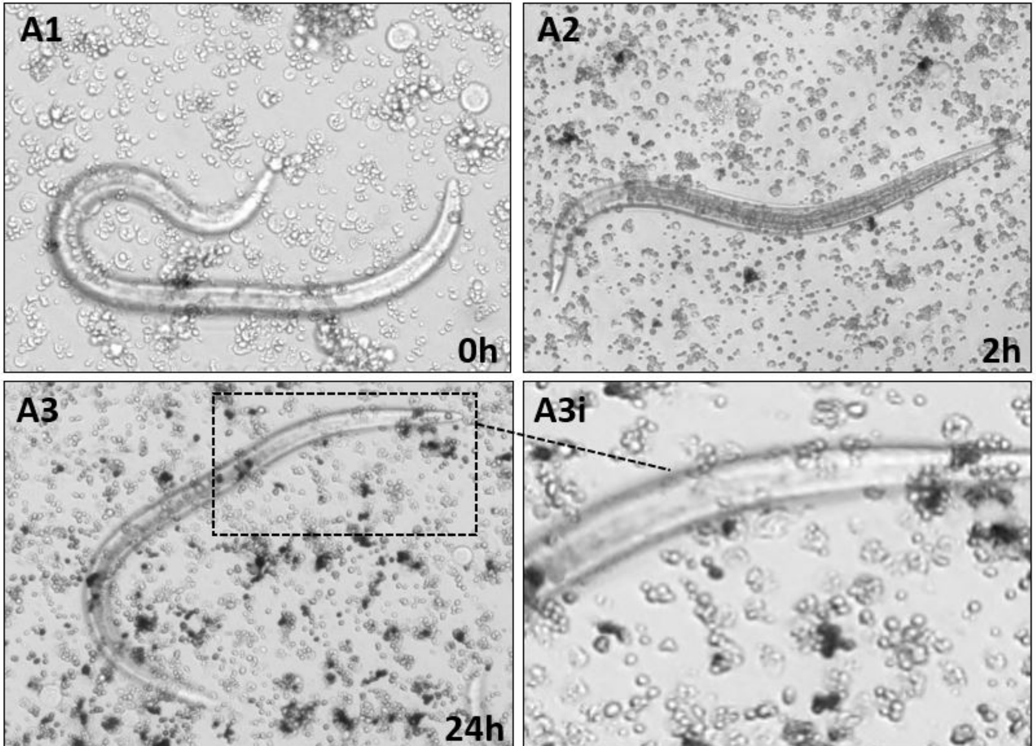


Figure 5. *Cont.*

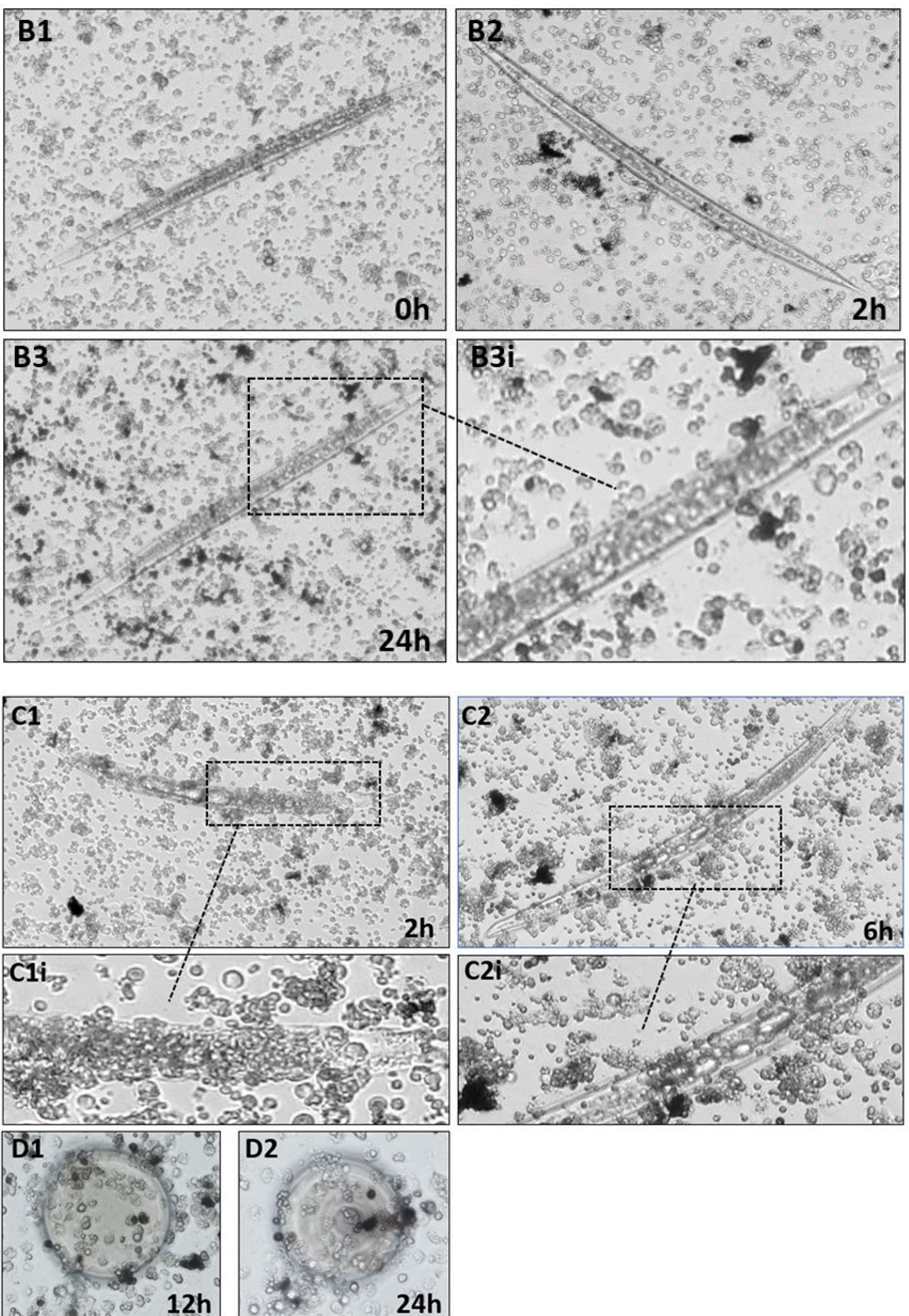


Figure 5. (**A**,**B**) In vitro encapsulation assays of S. carpocapsae by hemocytes of D. suzukii larvae. Nematodes were co-incubated with hemocytes. From top to bottom: alive nematodes at 0 h (**A1**), 2 h (**A2**) and 24 h (**A3**), A3i inset is an enlargement of A3. Cold-killed nematodes at 0 h (**B1**), 2 h (**B2**), and 24 h (**B3**), B3i inset is an enlargement of B3. A1 (200×); A2, A3, B1, B2, B3 (100×). (**C**,**D**). In vitro encapsulation assay of lipase treated dead nematodes at 2 h (**C1**) and 6 h (**C2**), C1i and C2i are enlargements of the respective images. In vitro encapsulation of agarose beads, at 12 h (**D1**) and 24 h (**D2**). C1 and C2 (100×); D1 and D2 (200×).

3.6. Hemocytes Populations Count after Natural Infection

Natural infection of *S. carpocapsae* in *D. suzukii* larvae showed no cellular encapsulation processes of nematodes, as we observed in the previous in vitro assay. IJs were found free without attached cells on the cuticle, even if *X. nematophila* was released in the hemolymph. Hemocyte counts were performed when the mean number of nematodes inside larvae was 5.9 ± 4.3 IJs. Counts were avoided when the nematode number was over 15 IJs because the massive entry of nematodes into small larvae like *D. suzukii* generate tissue damage quickly. Infected larvae were separated in the early phase or midterm phase of infection if bacteria were released. Larvae in the early phase were mostly still alive (95%), while 30% of larvae were alive in the midterm phase.

Total hemocytes count showed a high number of immunocompetent cells (2.63×10^4 cells/µl) in naive *D. suzukii* larvae hemolymph (Table 1).

Table 1. * Evaluation of hemocytes population (mean ± SD) of total and differential count of three *D. suzukii* larvae treatments: naïve larvae (no infected), early phase of infection (with only nematode presence) and midterm phase of infection (nematode and bacterial presence). Different letters indicate statistically significant differences among treatments for each cell type ($p < 0.05$).

Hemocyte Number/µL of Hemolymph *				
Treatment	**Plasmatocytes**	**Lamellocytes**	**Crystal Cells**	**Total Hemocytes**
Naïve	25446.88 ± 4358.13 a	195.63 ± 49.88 a	677.50 ± 296.88 a	26320.00 ± 4311.47 a
Early phase	18676.88 ± 3676.56 b	564.38 ± 156.24 b	745.00 ± 184.04 a	19986.25 ± 3733.23 b
Midterm phase	20293.75 ± 3790.60 b	183.75 ± 54.73 a	841.88 ± 341.27 a	21319.38 ± 3791.21 b

Nevertheless, infected larvae showed a significant decrease in total hemocytes in the early and midterm phase of infection (GLM: $F = 14.26$, $df = 2$, $p = 0.000$). Plasmatocytes constituted the major fraction of hemocytes and presented a decrease in both phases of infection, as total hemocytes (GLM: $F = 16$, $df = 2$, $p = 0.000$). In naïve larvae, lamellocytes represent a very small fraction of immune cells but during the early phase of infection, the amount of lamellocytes increased significantly (GLM: $F = 93.99$, $df = 2$, $p = 0.000$). During the midterm phase, the bacteria were released, and we observed the attachment of lamellocytes and plasmatocytes with themselves, becoming big aggregations (Figure 6A,B). Due to the difficulty of counting only free cells were considered. In consequence, lamellocytes level decreased showing no significant difference with naïve value.

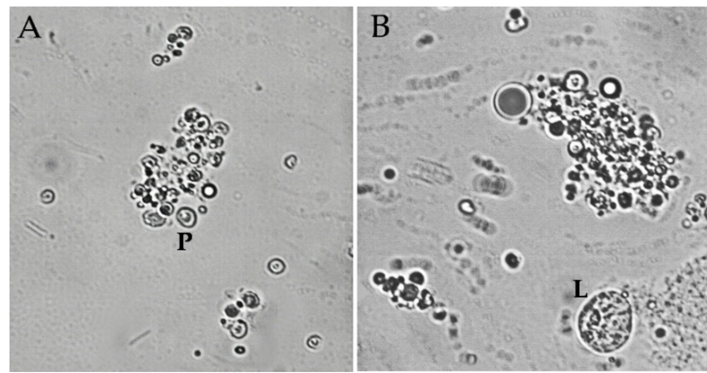


Figure 6. (**A**) Plasmatocytes (P) aggregation in in vivo encapsulation assay during the midterm phase of infection. (**B**) Aggregation of hemocytes next to a circulant lamellocyte (L). Image magnification is 400×.

Nevertheless, IJs were not found close to these cell aggregations. The only hemocytes population that seemed unaffected by nematode infection were the crystal cells. Neither nematode nor bacterial infection altered the number of crystal cells that remained constant in all assays (GLM: $F = 1.71$, $df = 2$, $p = 0.188$).

4. Discussion

D. suzukii represents a major threat to berry, cherry, and strawberry production as a globally invasive pest. This fly belongs to the *melanogaster* subgroup, as well as *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), which immune response to EPNs is widely studied as an insect model [18,43]. In contrast, few immunological studies have been conducted with *D. suzukii* and only focused on the parasitoid response. Poyet et al. [33] described a strong immune reaction against the parasitoid *L. heterotoma*, which leads to the encapsulation of wasp eggs. Even if *D. suzukii* larvae and adults are susceptible to nematodes [36], no immunological studies on the relationships between these pests and EPNs have been made before; consequently, we evaluated humoral and cellular responses upon infection of *S. carpocapsae* and *X. nematophila*.

The fastest defense of insect larvae is the activation of the phenoloxidase cascade. The symbiont bacteria *X. nematophila* showed an inhibitory effect on the host proPO system, with levels of phenoloxidase activity lower than naïve larvae. In contrast, *E. coli* and *B. subtilis* infection registered higher activity. These results agree with those obtained comparing the melanization rate of *D. melanogaster* infected with symbiont and axenic *S. carpocapsae* [45]. These authors reported that in the presence of *X. nematophila*, levels of melanization were significantly lower than using the axenic nematode. The inhibitory role of this symbiont bacteria and involvement of the eicosanoid pathway has been also reported in some lepidopteran species such as *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) and *Plutella xylostella* L. (Lepidoptera: Plutellidae) [28,46]. These authors related the secretion of a phospholipase A_2 (PLA_2) inhibitor with the suppression of the proPO activation in hemolymph and the alteration of cellular response. Despite no homologous genes were identified in *D. melanogaster*, Scarpati et al. [47] found other genes involved in eicosanoid pathway that could be functional equivalents.

The assay of lysozyme activity in naïve larvae of *D. suzukii* showed an extremely low activity compared to other insect species such as *Galleria mellonella* (Lepidoptera) 2.28×10^3 Units mL^{-1} or *Sarcophaga africa* (Diptera) 1.04×10^2 Units mL^{-1} [48]. There was no significant difference when larvae were stimulated by bacteria. Thus, lysozyme activity was not triggered by the infection *X. nematophila* nor *E. coli/M. luteus*. In agreement with our results, the *Drosophila* genus showed to have mainly digestive lysozyme and an insignificant amount of enzyme in the hemolymph for immune defense [49].

Along with phenoloxidase and lysozyme enzymes, AMPs perform a key role in humoral defense against bacterial infection. When AMPs presence was assessed by electrophoretic separations, both bacterial infections showed comparable band patterns with newly synthesized bands compared to naïve larvae. However, the antimicrobial activity test revealed a higher activity of the bacterial mixture infected larvae as opposed to larvae infected with *X. nematophila*. These results suggested that there was a synthesis of putative AMPs after symbiont infection. Nevertheless, larvae infected with *X. nematophila* showed lower activity with respect to that from larvae infected with non-entomopathogenic bacteria. With the conducted assay, we are unable to determine the causes, although these results could suggest an active mechanism of *X. nematophila* to disable the activity of those peptides. Some authors attributed to symbionts bacteria the ability to down-regulate AMPs genes in *S. exigua* or *Rynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) [50–52]. However, Peña et al. [45] reported in *D. melanogaster* an increased gene expression of AMPs in response to *X. nematophila* infection, over an infection of *S. carpocapsae*. Indeed, genes could be up-regulated in *D. suzukii*, as we detected the presence in the hemolymph of some peptides in the molecular mass range of AMPs. Although, the symbiont bacteria are known to release cytotoxic proteins and antimicrobial inhibitors to block the function of the peptides [53].

In addition to the analysis of humoral responses, we have also observed interference of the nematobacterial complex with the host cellular responses. As observed, either in in vivo or in vitro assays, *X. nematophila* was able to avoid phagocytosis response by the host immunocompetent cells; the phagocytic capability of the hemocytes was ascertained by the assays with *S. aureus*. Shrestha and Kim [54] reported in *S. exigua* larvae that the disruption of phagocytosis and avoidance of cell reaction to bacteria was caused by the synthesis of PLA_2 inhibitors by *X. nematophila*. Similar results were also obtained with *Manduca sexta* L. (Lepidoptera: Sphingidae) larvae, where *E. coli* was engulfed more than *Photorhabdus luminescens* (the symbiont bacteria of *H. bacteriophora*) [55]. Both symbionts bacteria use inhibitor of the PLA_2; likewise, in *D. suzukii* larvae, *X. nematophila* could implement the same strategy to avoid the host phagocytosis. Our data showed the ability of *S. carpocapsae* to avoid encapsulation of *D. suzukii* hemocytes in both in vitro and in vivo assays. Besides, nematode secretions do not seem to play a central role in the lack of encapsulation as we confirmed using dead IJs with unaltered cuticle. Encapsulation was only observed when the cuticle of nematodes was damaged after lipase treatment, suggesting an involvement of the body surface to avoid cellular recognition. Mastore et al. [26] reported similar results demonstrating a lack of encapsulation of alive and dead *S. carpocapsae* IJs in *R. ferrugineus*, although damaged cuticles of nematodes were strongly encapsulated. Furthermore, *S. carpocapsae* avoided the recognition by *G. mellonella* hemocytes while *H. bacteriophora* was recognized [56]. It has been described that *S. carpocapsae* have specific proteins in the epicuticle of IJs that provide a mimetic function to the nematode [23]; besides, in *S. feltiae*, disguise properties could be ascribed to lipids of the epicuticular layer, as suggested by Dunphy and Webster [57]. According to Brivio et al. [22,58], differences observed between *S. carpocapsae* and *S. feltiae* in the immunological relationships with their hosts supported the assumption that EPNs have developed peculiar immunoevasive strategies among different species.

During the in vitro encapsulation assay, a lack of lamellocyte differentiation was observed and resulted in the achievement only of the first steps of an encapsulation process with the attachment of plasmatocytes to lipase-treated nematodes and agarose beads. In contrast, in vivo assays evidenced the lamellocytes differentiation process after nematode infection, causing a decrease of plasmatocytes and an increase of lamellocytes from the constitutive level. The divergence of the differentiation process between assays could be expected due to a lack of natural physiological factors during in vitro assays. Moreover, the cell counts obtained during the in vivo assay showed a high amount of hemocyte populations, in agreement with Kacsoh and Schlenke [59] who suggested that *D. suzukii* larvae had five times more immunocompetent cells than *D. melanogaster*. The results of this assay also confirmed the lack of encapsulation of *S. carpocapsae* despite the differentiation of lamellocytes in the early phase of infection. During the midterm phase of infection, lamellocytes–plasmatocytes aggregation prevented counting hemocytes due to large cellular aggregates whose composition was not identifiable. An important role in the strategy of *S. carpocapsae* is attributable to its secretions of proteases and cytotoxic compounds which induce immunosuppression to the host. Some of these secretions have been identified as serine, cysteine, metallo, and aspartic proteases involved in processes of cell aggregation, clotting response, and cellular apoptosis [25,60]. These authors reported that when *S. carpocapsae* infects *D. melanogaster*, avoids clot enlargement by means of its inhibitor sc-spn6. Unlike plasmatocytes and lamellocytes, our assay crystal cell population remained unaffected even after the nematode released the bacteria. The regular count corroborated the low levels of melanization response observed whereas those cells produced and stored the components of proPO cascade. These results confirmed the different responses of *D. suzukii* larvae to parasitoids as their eggs activate the proPO reaction and cause a strong loss of crystal cells [33]. Moreover, the cellular response of the fly larvae to *L. heterotoma* or *L. boulardi* eggs presented the same pattern with a significant increase of plasmatocytes and lamellocytes [33,59]. In contrast, *D. suzukii* larvae showed a cellular response to *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae) eggs more similar to nematode's reaction observed in this work, with a slight decrease of plasmatocytes and similar constitutive levels of lamellocytes after long-term parasitization [33].

Our immunological approach aimed to investigate the relationship between an insect pest, such as *D. suzukii* and EPNs to provide an essential understanding of the strategies by which nematobacterial complexes overwhelm the host defenses. The results reported the inhibitory properties of both *S. carpocapsae* and *X. nematophila* to larvae's immune defenses. Symbiont bacteria affected the humoral response of proPO resulting in lower levels of phenoloxidase activity. In addition, *X. nematophila* infection activated the synthesis of putative AMPs molecules, although their antimicrobial activity was lower than peptides produced from infections with non-entomopathogenic bacteria. Besides, the cell populations of *D. suzukii* were unable to phagocyte the symbiont bacteria or encapsulate *S. carpocapsae* IJs. The data obtained from the encapsulation assays confirmed the elusive properties of the body surface of *S. carpocapsae*. These results attribute to the cuticle a synergistic role with its secretions to prepare an immunologically favorable environment before the release of the symbiont in the hemocoel cavity.

5. Conclusions

Along this work, *S. carpocapsae* and *X. nematophila* showed the ability to overtake the immune defenses of *D. suzukii*, therefore confirming the potentiality of this nematode as a biological control agent for this pest. This is the first report that addresses the physiological relationship between EPNs and *D. suzukii* from an immunological aspect; thus, providing a useful starting point to understand the parasite-host relationship between these organisms and help to improve the biological control of *D. suzukii*.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/4/210/s1. Figure S1. Time course of phenoloxidase relative activity in the hemolymph of *D. suzukii*, shown as mean ± SD increase of absorbance recorded every 5 min for 45 min total. Hemolymph samples were from naïve (N), control pricked (CP), *X. nematophila*-infected (InfX), and *E. coli/B. subtilis*-infected (InfB) larvae. Figure S2. The tricine-PAGE pattern of fractioned hemolymph (<30 KDa) extracted from larvae after *X. nematophila* infection. Even though the pattern showed some bands comparable to that observed in *E. coli/M. luteus*-infected larvae, this sample, tested for antimicrobial capability, revealed lower activity compared with that of larvae infected with non-entomopathogenic bacteria. Figure S3. Plated *D. suzukii* hemocytes from naive larvae (**A**) and *X. nematophila* infected-larvae (**B**). Micrographs below (B1, B2, and B3) show the altered morphology of the hemocytes with blebs (arrowheads) protruding from the cell surface. In the micrographs of healthy cells (A) lamellipodia (arrowheads) are visible. Table S1. Statistic comparison of phenoloxidase relative activity in the hemolymph of *D. suzukii* of Figure 1. *P*-value obtained in the Tukey comparison of the hemolymph samples from naïve (N), control pricked (CP), *X. nematophila*-infected (InfX) and *E. coli/B. subtilis*-infected (InfB) larvae, at 0 min and 45 min. Any comparison was considered significant if the p-value was < 0.05. Table S2. Statistic comparison of antimicrobial activity in *D. suzukii* hemolymph of Figure 3. p-value obtained in the Tukey comparison of the hemolymph samples from naïve (N), control (PBS), *X. nematophila*-infected (InfX), and *E. coli/B. subtilis*-infected (InfB) larvae, in coincubation with *E. coli*, *M. luteus*, or *X. nematophila*. Any comparison was considered significant if the p-value was < 0.05. Table S3. Mortality rate (%) of *E. coli*, *M. luteus*, and *X. nematophila*, when treated with *D. suzukii* hemolymph from larvae infected with InfB or InfX.

Author Contributions: Conceived idea, A.M. and F.G.d.P.; conceptualization, M.F.B., M.M., and A.G.; methodology, M.M., A.G., and M.F.B.; investigation, A.G. and M.M.; data curation, A.G., M.M., and M.F.B.; writing—original draft preparation, A.G.; writing—review and editing, M.F.B., M.M., A.G., F.G.d.P., and A.M.; project supervision and administration, F.G.d.P. and M.F.B.; funding acquisition, F.G.d.P. and M.F.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by FEDER/Ministerio de Ciencia, Innovación y Universidades—Agencia Estatal de Investigación/Project (AGL2017-86770-R) and University of Insubria, FAR grant/2018.

Acknowledgments: We are very grateful to Alain Givaudan for kindly providing us with the GFP-transformed *Xenorhabdus* strains.

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

References

1. Kaya, H.K.; Gaugler, R. Entomopathogenic nematodes. *Annu. Rev. Entomol.* **1993**, *38*, 181–206. [CrossRef]
2. Boemare, N. Biology, taxonomy, and systematics of *Photorhabdus* and *Xenorhabdus*. In *Entomopathogenic Nematology*; Gaugler, R., Ed.; CABI International: Wallingford, UK, 2002; pp. 35–56.

3. Snyder, H.; Stock, S.P.; Kim, S.K.; Flores-Lara, Y.; Forst, S. New insights into the colonization and release processes of *Xenorhabdus nematophila* and the morphology and ultrastructure of the bacterial receptacle of its nematode host, *Steinernema carpocapsae*. *Appl. Environ. Microbiol.* **2007**, *73*, 5338–5346. [CrossRef] [PubMed]
4. Dowds, B.C.A.; Peters, A. Virulence mechanisms. In *Entomopathogenic Nematology*; Gaugler, R., Ed.; CABI: New York, NY, USA, 2002; pp. 79–98.
5. Lacey, L.A.; Grzywacz, D.; Shapiro-Ilan, D.I.; Frutos, R.; Brownbridge, M.; Goettel, M.S. Insect pathogens as biological control agents: Back to the future. *J. Invertebr. Pathol.* **2015**, *132*, 1–41. [CrossRef] [PubMed]
6. Brivio, M.F.; Mastore, M. Nematobacterial complexes and insect hosts: Different weapons for the same war. *Insects* **2018**, *9*, 117. [CrossRef] [PubMed]
7. Gaugler, R.; Wang, Y.; Campbell, J.F. Aggressive and evasive behaviors in *Popillia japonica* (Coleoptera, Scarabaeidae) larvae—defenses against entomopathogenic nematode attack. *J. Invertebr. Pathol.* **1994**, *64*, 193–199. [CrossRef]
8. Castillo, J.C.; Reynolds, S.E.; Eleftherianos, I. Insect immune responses to nematode parasites. *Trends Parasitol.* **2011**, *27*, 537–547. [CrossRef]
9. Eleftherianos, I.; Shokal, U.; Yadav, S.; Kenney, E.; Maldonado, T. Insect immunity to entomopathogenic nematodes and their mutualistic bacteria. *Curr. Top. Microbiol. Immunol.* **2017**, *402*, 123–156.
10. Gillespie, J.P.; Kanost, M.R.; Trenczek, T. Biological mediators of insect immunity. *Annu. Rev. Entomol.* **1997**, *42*, 611–643. [CrossRef]
11. Kim, C.H.; Park, J.W.; Ha, N.C.; Kang, H.J.; Lee, B.L. Innate immune response in insects: Recognition of bacterial peptidoglycan and amplification of its recognition signal. *BMB Rep.* **2008**, *41*, 93–101. [CrossRef]
12. Nappi, A.J.; Kohler, L.; Mastore, M. Signaling pathways implicated in the cellular innate immune responses of *Drosophila*. *Invertebr. Surv. J.* **2004**, *1*, 5–33.
13. Scherfer, C.; Karlsson, C.; Loseva, O.; Bidla, G.; Goto, A.; Havemann, J.; Dushay, M.S.; Theopold, U. Isolation and Characterization of Hemolymph Clotting Factors in *Drosophila melanogaster* by a Pullout Method. *Curr. Biol.* **2004**, *14*, 625–629. [CrossRef] [PubMed]
14. Dziedzeich, A.; Shivankar, S.; Theopold, U. *Drosophila melanogaster* Responses against Entomopathogenic Nematodes: Focus on Hemolymph Clots. *Insects* **2020**, *11*, 62. [CrossRef] [PubMed]
15. Bulet, P.; Stöcklin, R. Insect antimicrobial peptides: Structures, properties and gene regulation. *Protein Pept. Lett.* **2005**, *12*, 3–11. [CrossRef] [PubMed]
16. Strand, M.R. The insect cellular immune response. *Insect Sci.* **2008**, *15*, 1–14. [CrossRef]
17. De Lerma Barbaro, A.; Gariboldi, M.B.; Mastore, M.; Brivio, M.F.; Giovannardi, S. In Vivo Effects of A Pro-PO System Inhibitor on the Phagocytosis of *Xenorhabdus nematophila* in *Galleria mellonella* Larvae. *Insects* **2019**, *10*, 263. [CrossRef]
18. Lemaitre, B.; Hoffmann, J. The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* **2007**, *25*, 697–743. [CrossRef]
19. Letourneau, M.; Lapraz, F.; Sharma, A.; Vanzo, N.; Waltzer, L.; Crozatier, M. *Drosophila* hematopoiesis under normal conditions and in response to immune stress. *FEBS Lett.* **2016**, *590*, 4034–4051. [CrossRef]
20. Rizki, T.; Rizki, R.; Grell, E. A mutant affecting the crystal cells in *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **1980**, *188*, 91–99. [CrossRef]
21. Binda-Rossetti, S.; Mastore, M.; Protasoni, M.; Brivio, M.F. Effects of an entomopathogen nematode on the immune response of the insect pest red palm weevil: Focus on the host antimicrobial response. *J. Invertebr. Pathol.* **2016**, *133*, 110–119. [CrossRef]
22. Brivio, M.F.; Mastore, M.; Nappi, A.J. A pathogenic parasite interferes with phagocytosis of insect immunocompetent cells. *Dev. Comp. Immunol.* **2010**, *34*, 991–998. [CrossRef]
23. Wang, Y.; Gaugler, R. *Steinernema glaseri* surface coat protein suppresses the immune response of *Popillia japonica* (Coleoptera: Scarabaeidae) larvae. *Biol. Control* **1999**, *14*, 45–50. [CrossRef]
24. Mastore, M.; Brivio, M.F. Cuticular surface lipids are responsible for disguise properties of an entomoparasite against host cellular responses. *Dev. Comp. Immunol.* **2008**, *32*, 1050–1062. [CrossRef] [PubMed]
25. Toubarro, D.; Avila, M.M.; Hao, Y.; Balasubramanian, N.; Jing, Y.; Montiel, R.; Faria, T.Q.; Brito, R.M.; Simões, N. A serpin released by an entomopathogen impairs clot formation in insect defense system. *PLoS ONE* **2013**, *8*, e69161. [CrossRef] [PubMed]

26. Mastore, M.; Arizza, V.; Manachini, B.; Brivio, M.F. Modulation of immune responses of *Rhynchophorus ferrugineus* (Insecta: Coleoptera) induced by the entomopathogenic nematode *Steinernema carpocapsae* (Nematoda: Rhabditida). *Insect Sci.* **2015**, *22*, 748–760. [CrossRef] [PubMed]
27. Brivio, M.F.; Toscano, A.; Pasquale, S.M.; Barbaro, A.D.; Giovannardi, S.; Finzi, G.; Mastore, M. Surface protein components from entomopathogenic nematodes and their symbiotic bacteria: Effects on immune responses of the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae). *Pest Manag. Sci.* **2018**, *74*, 2089–2099. [CrossRef]
28. Park, Y.; Kim, Y. *Xenorhabdus nematophilus* inhibits p-bromophenacyl bromide (BPB)-sensitive PLA2 of *Spodoptera exigua*. *Arch. Insect Biochem. Physiol.* **2003**, *54*, 134–142. [CrossRef]
29. Walsh, D.B.; Bolda, M.P.; Goodhue, R.E.; Dreves, A.J.; Lee, J.; Bruck, D.J.; Walton, V.M.; O'Neal, S.D.; Zalom, F.G. *Drosophila suzukii* (Diptera: Drosophilidae): Invasive Pest of Ripening Soft Fruit Expanding its Geographic Range and Damage Potential. *J. Integr. Pest Manag.* **2011**, *2*, 1–7. [CrossRef]
30. Haye, T.; Girod, P.; Cuthbertson, A.G.S.; Wang, X.G.; Daane, K.M.; Hoelmer, K.A.; Baroffio, C.; Zhang, J.P.; Desneux, N. Current SWD IPM tactics and their practical implementation in fruit crops across different regions around the world. *J. Pest Sci.* **2016**, *89*, 643–651. [CrossRef]
31. Lee, J.C.; Wang, X.; Daane, K.M.; Hoelmer, K.A.; Isaacs, R.; Sial, A.A.; Walton, V.M. Biological control of spotted-wing drosophila—current and pending tactics. *J. Integr. Pest Manag.* **2019**, *10*, 13. [CrossRef]
32. Chabert, S.; Allemand, R.; Poyet, M.; Eslin, P.; Gibert, P. Ability of European parasitoids (Hymenoptera) to control a new invasive Asiatic pest, *Drosophila suzukii*. *Biol. Control* **2012**, *63*, 40–47. [CrossRef]
33. Poyet, M.; Havard, S.; Prevost, G.; Chabrerie, O.; Doury, G.; Gibert, P.; Eslin, P. Resistance of *Drosophila suzukii* to the larval parasitoids *Leptopilina heterotoma* and *Asobara japonica* is related to haemocyte load. *Physiol. Entomol.* **2013**, *38*, 45–53. [CrossRef]
34. Stacconi, M.V.; Amiresmaeili, N.; Biondi, A.; Carli, C.; Caruso, S.; Dindo, M.L.; Francati, S.; Gottardello, A.; Grassi, A.; Lupi, D.; et al. Host location and dispersal ability of the cosmopolitan parasitoid *Trichopria drosophilae* released to control the invasive spotted wing *Drosophila*. *Biol. Control* **2018**, *117*, 188–196. [CrossRef]
35. Yousef, M.; Aranda-Valera, E.; Quesada-Moraga, E. Lure-and-infect and lure-and-kill devices based on *Metarhizium brunneum* for spotted wing *Drosophila* control. *J. Pest Sci.* **2018**, *91*, 227–235. [CrossRef]
36. Garriga, A.; Morton, A.; Garcia-del-Pino, F. Is *Drosophila suzukii* as susceptible to entomopathogenic nematodes as *Drosophila melanogaster*? *J. Pest Sci.* **2018**, *91*, 789–798. [CrossRef]
37. Garriga, A.; Morton, A.; Ribes, A.; Garcia-del-Pino, F. Soil emergence of *Drosophila suzukii* adults: A susceptible period for entomopathogenic nematodes infection. *J. Pest Sci.* **2020**, *93*, 639–646. [CrossRef]
38. Woodring, J.L.; Kaya, H.K. Steinernematid and heterorhabditid nematodes: A handbook of techniques. *South. Coop. Bull.* **1998**, *331*, 1–30.
39. White, G.F. A method for obtaining infective nematode larvae from cultures. *Science* **1927**, *66*, 302–303. [CrossRef]
40. Park, Y.; Kim, Y. Eicosanoids rescue *Spodoptera exigua* infected with *Xenorhabdus nematophilus*, the symbiotic bacteria to the entomopathogenic nematode *Steinernema carpocapsae*. *J. Insect Physiol.* **2000**, *46*, 1469–1476. [CrossRef]
41. Mastore, M.; Brivio, M.F. Basic Methods to Evaluate Humoral Immunity Processes in Lepidoptera Larvae. In *Immunity in Insects. Springer Protocols Handbooks*; Sandrelli, F., Tettamanti, G., Eds.; Humana: New York, NY, USA, 2020; pp. 127–144. [CrossRef]
42. Schägger, H.; Von Jagow, H. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **1987**, *166*, 368–379. [CrossRef]
43. Arefin, B.; Kucerova, L.; Krautz, R.; Kranenburg, H.; Parvin, F.; Theopold, U. Apoptosis in hemocytes induces a shift in effector mechanisms in the *Drosophila* immune system and leads to a pro-inflammatory state. *PLoS ONE* **2015**, *10*, e0136593. [CrossRef]
44. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2017; Available online: http://www.R-project.org/ (accessed on 26 September 2019).
45. Peña, J.M.; Carrillo, M.A.; Hallem, E.A. Variation in the susceptibility of *Drosophila* to different entomopathogenic nematodes. *Infect. Immun.* **2015**, *83*, 1130–1138. [CrossRef] [PubMed]
46. Kim, Y.G.; Shrestha, S.; Song, C.J.; Seo, S.Y. Bacterial metabolites of an entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibit a catalytic activity of phenoloxidase of the diamondback moth, *Plutella xylostella*. *J. Microbiol. Biotechnol.* **2011**, *21*, 317–322.

47. Scarpati, M.; Qi, Y.; Govind, S.; Singh, S. A combined computational strategy of sequence and structural analysis predicts the existence of a functional eicosanoid pathway in *Drosophila melanogaster*. *PLoS ONE* **2019**, *14*, e0211897. [CrossRef] [PubMed]
48. Mastore, M.; Quadroni, S.; Toscano, A.; Mottadelli, N.; Brivio, M.F. Susceptibility to entomopathogens and modulation of basal immunity in two insect models at different temperatures. *J. Therm. Biol.* **2019**, *79*, 15–23. [CrossRef]
49. Daffre, S.; Kylsten, P.; Samakovlis, C.; Hultmark, D. The lysozyme locus in *Drosophila melanogaster*: An expanded gene family adapted for expression in the digestive tract. *Mol. Gen. Genet.* **1994**, *242*, 152–162. [CrossRef]
50. Ji, D.; Kim, Y. An entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibits the expression of an antibacterial peptide, cecropin, of the beet armyworm, *Spodoptera exigua*. *J. Insect Physiol.* **2004**, *50*, 489–496. [CrossRef]
51. Duvic, B.; Jouan, V.; Essa, N.; Girard, P.A.; Pages, S.; Khattar, Z.A.; Volkoff, N.A.; Givaudan, A.; Destoumieux-Garzón, D.; Escoubas, J.M. Cecropins as a marker of *Spodoptera frugiperda* immunosuppression during entomopathogenic bacterial challenge. *J. Insect Physiol.* **2012**, *58*, 881. [CrossRef]
52. Mastore, M.; Binda Rossetti, S.; Giovannardi, S.; Scarì, G.; Brivio, M.F. Inducible factors with antimicrobial activity after immune challenge in the haemolymph of Red Palm Weevil (Insecta). *Innate Immun.* **2015**, *21*, 392–405. [CrossRef]
53. McQuade, R.; Stock, S.P. Secretion systems and secreted proteins in gram-negative entomopathogenic bacteria: Their roles in insect virulence and beyond. *Insects* **2018**, *9*, 68. [CrossRef]
54. Shrestha, S.; Kim, Y. An entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibits hemocyte phagocytosis of *Spodoptera exigua* by inhibiting phospholipase A_2. *J. Invertebr. Pathol.* **2007**, *96*, 64–70. [CrossRef]
55. Au, C.; Dean, P.; Reynolds, S.E.; Ffrench-Constant, R.H. Effect of the insect pathogenic bacterium *Photorhabdus* on insect phagocytes. *Cell. Microbiol.* **2004**, *6*, 89–95. [CrossRef] [PubMed]
56. Ebrahimi, L.; Niknam, G.; Dunphy, G.B. Hemocyte responses of the Colorado potato beetle, *Leptinotarsa decemlineata*, and the greater wax moth, *Galleria mellonella*, to the entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*. *J. Insect Sci.* **2011**, *11*, 75. [CrossRef] [PubMed]
57. Dunphy, G.; Webster, J. Partially characterized components of the epicuticle of dauer juvenile *Steinernema feltiae* and their influence on the hemocyte activity in *Galleria mellonella*. *J. Parasitol.* **1987**, *73*, 584–588. [CrossRef]
58. Brivio, M.F.; Mastore, M.; Moro, M. The role of *Steinernema feltiae* body-surface lipids in host–parasite immunological interactions. *Mol. Biochem. Parasitol.* **2004**, *135*, 111–121. [CrossRef]
59. Kacsoh, B.Z.; Schlenke, T.A. High hemocyte load is associated with increased resistance against parasitoids in *Drosophila suzukii*, a relative of *D. melanogaster*. *PLoS ONE* **2012**, *7*, e34721. [CrossRef]
60. Balasubramanian, N.; Hao, Y.J.; Toubarro, D.; Nascimento, G.; Simões, N. Purification, biochemical and molecular analysis of a chymotrypsin protease with prophenoloxidase suppression activity from the entomopathogenic nematode *Steinernema carpocapsae*. *Int. J. Parasitol.* **2009**, *39*, 975–984. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Supplementary Materials:

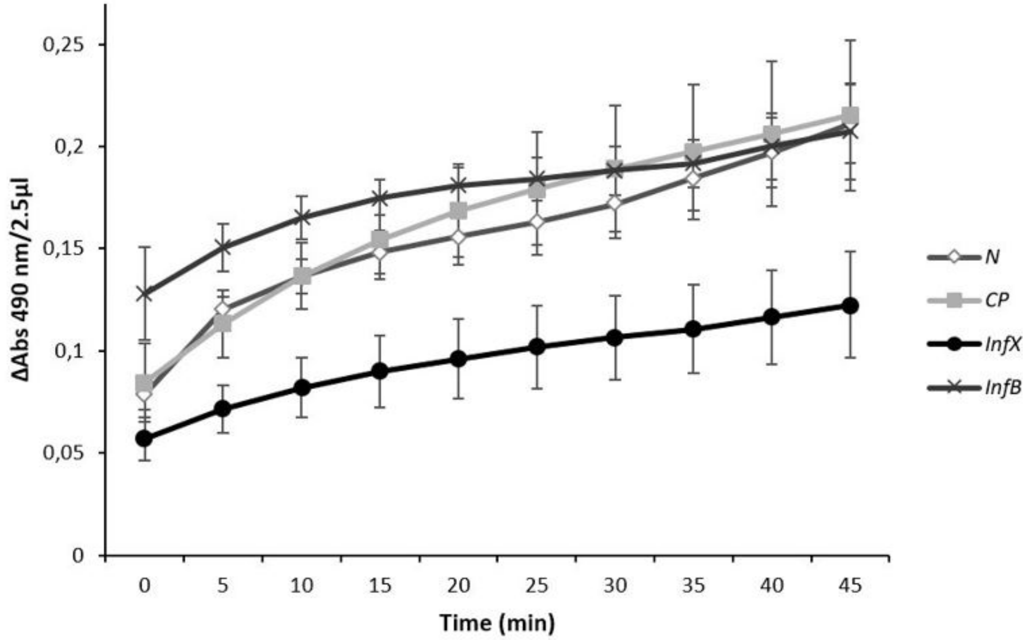


Fig. S1. Time course of phenoloxidase relative activity in hemolymph of *D. suzukii*, shown as mean ± SD increase of absorbance recorded every 5 min for 45 min total. Hemolymph samples were from naïve (N), control pricked (CP), *X. nematophila*-infected (InfX) and *E. coli/B. subtilis*-infected (InfB) larvae.

Table S1. Statistic comparison of phenoloxidase relative activity in hemolymph of *D. suzukii* of Fig. 1. P-value obtained in Tukey comparison of the hemolymph samples from naïve (N), control pricked (CP), *X. nematophila*-infected (InfX) and *E. coli/B. subtilis*-infected (InfB) larvae, at 0 min and 45 min. Any comparison was considered significant if p-value was < 0.05.

Tukey comparison	p-value at 0 min	p-value at 45 min
CP – InfB	0.003	0.963
CP – N	0.932	0.994
CP – InfX	0.067	0.0003
InfB – N	0.001	0.995
InfB – InfX	0.0001	0.0008
N – InfX	0.193	0.0005

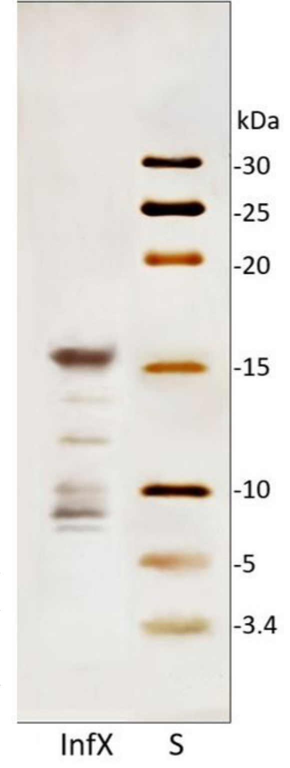


Fig. S2. Tricine-PAGE pattern of fractioned hemolymph (<30 KDa) extracted from larvae after *X. nematophila* infection. Even though the pattern showed some bands comparable to that observed in *E. coli/M. luteus*-infected larvae, this sample, tested for antimicrobial capability, revealed lower activity compared with that of larvae infected with non-entomopathogenic bacteria.

Table S2. Statistic comparison of antimicrobial activity in *D. suzukii* hemolymph of Fig. 3. P-value obtained in Tukey comparison of the hemolymph samples from naïve (N), control (PBS), *X. nematophila*-infected (InfX) and *E. coli/B. subtilis*-infected (InfB) larvae, in coincubation with *E. coli, M. luteus* or *X. nematophila*. Any comparison was considered significant if p-value was < 0.05.

Tukey comparison	p-value for *E. coli*	p-value for *M. luteus*	p-value for *X. nematophila*
PBS – InfB	0.077	0.991	0.188
PBS – N	0.250	0.0006	0.035
PBS – InfX	0.804	0.072	-
InfB – N	0.005	0.0005	0.269
InfB – InfX	0.265	0.048	-
N – InfX	0.072	0.020	-

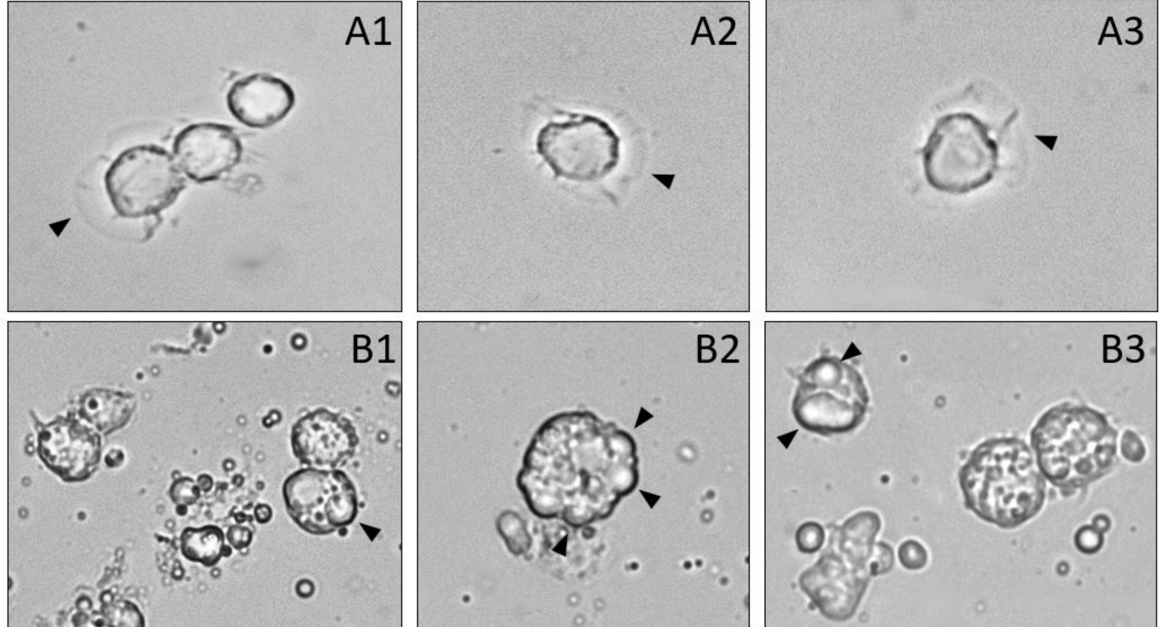
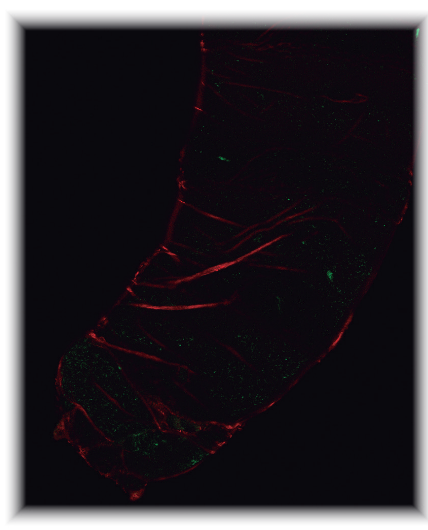


Fig. S3. Plated *D. suzukii* hemocytes from naive larvae (A) and from *X. nematophila* infected-larvae (B). Micrographs below (B1, B2 and B3) show the altered morphology of the hemocytes with blebs (arrowheads) protruding from the cell surface. In the micrographs of healthy cells (A) lamellipodia (arrowheads) are visible.

Table S3. Mortality rate (%) of *E. coli, M. luteus* and *X. nematophila*, when treated with *D. suzukii* hemolymph from larvae infected with InfB or InfX.

Bacteria mortality (%)		
	InfX	InfB
E. coli	55.0%	92.2%
M. luteus	41.5%	76,1%
X. nematophila	n.d.	17.2%

CHAPTER 7



The modulation effect of *Steinernema carpocapsae* - *Xenorhabdus nematophila* on immune-related genes of *Drosophila suzukii* larvae

In preparation, 2021

The modulation effect of *Steinernema carpocapsae - Xenorhabdus nematophila* complex on immune-related genes of *Drosophila suzukii* larvae

A. Garriga[1], D. Toubarro[2], N. Simões[2], A. Morton[1], F. García-del-Pino[1]

[1]Departament de Biologia Animal, Biologia Vegetal i Ecologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain. [2]CBA, Departamento de Biologia, Universidade dos Açores, Ponta Delgada, Portugal

Abstract

Larvae of the invasive pest *Drosophila suzukii* are susceptible to *Steinernema carpocapsae – Xenorhabdus nematophila* complex, even though the activation of immune-regulatory system was unknown. Thus, expression of 14 immune-related genes of different pathways was analyzed through qRT-PCR to determine variations after infection in three time-points. At 90 min and 4 h only nematodes were present in hemocoel and at 14 h of infection, bacteria were already released and established. Results revealed that before the bacterial release, infective juveniles (IJs) were not recognized by the immune system of larvae as practically none of the analyzed pathways presented variations compared to non-infected larvae. In contrast, with *X. nematophila* release, PGRP-LC was activated in response to the pathogen presence leading to the gene upregulation of antimicrobial peptides of both Toll and Imd pathways. These pathways presented a positive correlation with bacterial presence. Interestingly, cellular response remained inactive along the infection course with Jak/STAT and pro-phenoloxidase genes unresponsive to both nematode and bacteria presence. These results provided a better comprehension of nematode ability to modulate the defense response of *D. suzukii* and ensure the infection success.

Keywords: immune response, entomopathogenic nematodes, gene expression, drosophila.

Introduction

Entomopathogenic nematodes (EPNs) are obligate parasitic nematodes of insects that belong to the families Steinernematidae and Heterorhabditidae [1,2]. These nematodes have a symbiotic relation with entomopathogenic bacteria that help to kill the insect. In the case of the nematode *Steinernema carpocapsae*, their bacteria *Xenorhabdus nematophila* inhabit in a vesicle of infective juveniles (IJs) and when nematodes infect an insect, bacteria are released in few hours [3]. EPNs have been studied for its use as biological control agents against agricultural pests. Besides, these nematodes also serve as biological model to study parasitology for medical and veterinary purposes [4]. From the insect point of view, a detailed knowledge of *Drosophila melanogaster* immune genes and the pathways implicated in the insect defense has been studied [5]. More than 100 genes among several signaling pathways are known to be involved in the immune response against bacterial and parasitic infections. The main defense is based on two signaling pillars that are Toll

and Immune deficiency (Imd) pathways. These complementary cascades regulate immunologic defense besides other developmental process through members of nuclear factor κB family (NF – κB) which among others activate the encoding genes of the antimicrobial peptides (AMPs) that form the humoral response [6]. Imd is activated by peptidoglycan receptors (PGRP) that recognize the cellular peptidoglycan wall of gram-negative bacteria. In contrast, GNBP are free receptors in hemolymph that bind gram-positive and fungi to activate Toll pathway [5].

Apart from the humoral components, the cellular response is mediated by two pathways: Janus kinase / signal transducer and activator of transcription (Jak/STAT) and c-Jun N-terminal kinase (JNK). Jak/STAT is a pathway involved in multiple functions in *Drosophila* like embryonic segmentation and developmental processes but also has a role in cellular immune response activated under stress, tissue damage or infection [7]. The receptor Domeless interacts with three different cytokines (Unpaired1, Upd2 and Upd3) and activates the transcription factor Stat92E that induces effector genes such as *Turandot* family for immune purposes [8]. Activation of Upd2 and Upd3 due to bacterial or pathogen infection activate Jak/STAT pathway for a specific regulation of hemocytes and encapsulation process [9]. The melanization response is a component of cellular and humoral defense of *Drosophila* that is regulated by pro-phenoloxydase (PPO) pathway and ends with the trigger of phenoloxydase enzyme. This cascade is mediated by activation of three genes in different situations: PPO1, PPO2 and PPO3 [10]. Crystal cells are closely related with melanization defense and release PPO1 in early response to infection or injury in tissues. Furthermore, these cells activate PPO2 in the later phase of melanization, while PPO3 is only produced by lamellocytes [11].

JNK is involved in many developmental processes, metabolic and tissue homeostasis, cell differentiation, stress response and global signaling that are tightly involved with immune defense [12]. This pathway consists of a main kinase Basket which is activated through a wide range of inputs and its response is context-dependent on the signal received. JNK can receive Imd activation and as well can interfere upon activation of Upd3 of Jak-STAT pathway [13]. To limit its own activity the phosphatase Puckered acts as negative feedback inside the cascade [14]. Lastly, TGF-β is another well-conserved signaling pathway that receives signals from Toll, Imd and JNK activation [15]. The pathway is involved in wounding and inflammation after a bacterial infection and is activated through NF – κB that regulates two branches: bone morphogenic protein (BMP) and activin signaling. BMP ligand decapentaplegic (Dpp) is activated by wounding and suppress the production of AMPs, while activin ligand dawdle (Daw) represses the melanization induced by infection [16]. Thus, among other functions TGF-β genes limit an overexpression of humoral effector processes.

Effects of EPNs infection in *D. melanogaster* immune system and gene expression have been evaluated in different studies [17,18]. However, few assays have been performed with an applied focus using pest insects of interest [19], despite this wide knowledge in *D. melanogaster*. As it is the case of *Drosophila suzukii*, which is a global pest that affects soft-skinned fruits like berries,

cherries, and strawberries [20]. The pressure to reduce the use of chemical pesticides to control the fly lead to the development of strategies using biological control agents such as parasitoids or EPNs [21,22]. Asiatic parasitoids, from the native *D. suzukii* area, are effective in both larvae and pupae [23]. Nevertheless, parasitoid species from invaded areas showed mixed results being only pupal parasitoids able to develop. European larval parasitoids were unable to develop as larvae presented a strong immune reaction ending with the encapsulation of the egg [24,25]. In contrast, laboratory assays with EPNs proved that larvae of *D. suzukii* were susceptible to nematodes with mortality rates of 84.3% with *S. carpocapsae* treatment [26]. Moreover, the response of immune effector mechanisms of *D. suzukii* larvae were analyzed against an infection with this nemato-bacterial complex [27]. This study evidenced that nematodes and bacteria avoided cell encapsulation and lamellocytes differentiation. Another affected process was the phenoloxidase cascade as *X. nematophila* managed to reduce their activation on hemolymph. Moreover, bacteria modulated antimicrobial activity on hemolymph showing an inhibitory effect on those peptides.

Nevertheless, to better understand the role of nematodes and bacteria in the infection it should be considered not only alterations on the effector mechanisms but upon transcriptomic level too. The genome of *D. suzukii* was sequenced and available in NCBI GenBank database (Access Code: AWUT01000000) [28]. Yet, no study of genetic expression of immune genes has been carried out. As consequence, the objectives of this work were i) to identify *D. suzukii* immune genes belonging to different pathways in homology to *D. melanogaster*. ii) Evaluate the variation of immune gene expression of *D. suzukii* larvae infected with the complex *S. carpocapsae – X. nematophila* in three time points.

Results

1. Retrieve of immune-related genes in *D. suzukii*

To study the variation of gene expression upon nematode-bacterial infection, we selected 14 genes to provide a wide response of larval immunological status after infection with monoxenic nematodes including pattern recognition receptors (PGRP-LC and PGRP-LF) and 6 immune related pathways: Imd (Cecropin and Diptericin), Toll (Defensin and Drosomycin), Jak/STAT (Upd3 and Turandot C), pro-phenoloxidase (PPO1 and PPO2), JNK (Basket and Puckered) and TGF-β (Dawdle and Decapentaplegic) (Table 1). This gene selection was based on a pool of 40 immune-related genes described in *D. melanogaster* (Table S1). Thus, we carried out an identification of immune related genes in *D. suzukii* through alignment of *D. melanogaster* homolog genes. Peptidoglycan receptors (PGRP) are highly conserved among *Drosophila* species. In *D. suzukii* the alignment of those genes presented over 80% of identity with *D. melanogaster* genes PGRP-LC and PGRP-LF. Differently, the AMPs of both Imd and Toll pathways have high rates of variance among *Drosophila* species. Besides, for some encoding genes the homologs were only matched successfully using the protein sequence with tBlastn. In Imd, the Diptericin-A of *D. suzukii* showed 83.88% identity with *D. melanogaster* annotated Diptericin-B. Besides, Cecropin-A2 of

D. melanogaster aligned only through tBlastn to Cecropin-C-line of *D. suzukii* with 74.6% of identity. In Toll pathway, Drosomycin presented high homology between the flies with 87.68% of identity. In contrast, Defensin was aligned with tBlastn with 76.39% of identity in *D. suzukii* gene. Regarding, Jak/STAT pathway, Upd3 interleukin was not annotated in *D. suzukii*, in our analysis an ORF with 39% of cover and an identity of 75.25% was identified. This ORF harbors a well-preserved region with high homology among *Drosophila* and therefore, was used to analyze gene expression. The effector gene Turandot C was identified with 68,22% of identity. In the phenoloxidase pathway, PPO1 and PPO2 of *D. melanogaster* presented an identity of 85.75% and 89.84% with *D. suzukii* genes annotated as Phenoloxidase 2 LOC108009339 and Phenoloxidase 2 LOC108009476, respectively. The genes belonging to JNK pathway and TGF-β presented high rates of homology between both *Drosophila* with alignments over 83%. There are some discrepancies in the annotation of different genes of *D. melanogaster* and *D. suzukii*. Therefore, we used the gene identification of *D. melanogaster*.

Table 1. Selected genes of *D. suzukii* for quantitative expression of immune genes of different pathways and homolog correspondence to *D. melanogaster.* Alignment parameters are shown as e-value and percentage of identity (% id.) for Blastn algorithm and *tBlastn algorithm.

Pathway	*D. suzukii*	Access code	*D. melanogaster*	e-value	% id.
Imd	PGRP-LC	XM_017079226	PGRP-LC	0	82.59%
Imd	PGRP-LF	XM_017078922	PGRP-LF	0	80.20%
Imd	Cecropin-C-like	XM_017083110	Cecropin-A2	2E-27	74.60%*
Imd	Diptericin A	XM_017085064	Diptericin B	2E-101	83.88%
Toll	Drosomycin	XM_017070727	Drosomycin	1E-88	87.68%
Toll	Defensin	XM_017074017	Defensin	2E-35	76.39%*
Jak/STAT	Turandot C-like	XM_017086062	Turandot C	5E-66	68.22%*
Jak/STAT	Uncharacterized LOC108010411	XM_017075270	Upd-3	1E-103	75.25%
proPO	Phenoloxidase 2 (LOC108009339)	XM_017073614	PPO1	0	85.75%
proPO	Phenoloxidase 2 (LOC108009476)	XM_017073856	PPO2	0	89.84%
JNK	Stress-activated protein kinase	XM_017067534	Basket	0	91.59%
JNK	Tyrosine-protein phosphatase vhp1	XM_017086030	Puckered	0	87.52%
TGF-β	Growth/differentiation factor 8	XM_017090335	Dawdle	0	83.62%
TGF-β	Decapentaplegic X3	XM_017089893	Dpp	0	87.19%
-	60S RpL32	XM_017087128	RpL32	0	96.19%

2. Infection process of nematode-bacterial complex

IJs of *S. carpocapsae* were able to infect *D. suzukii* larvae after a short time of exposure. Infection rates reached 62.83 ± 14.94% and 75.14 ± 6.77% of exposed larvae after 1 h and 2 h, respectively. The mean number of nematodes found inside larvae was 3.44 ± 2.39 and 6.68 ± 4.45 IJs after 1 h and 2 h, respectively. A maximum of 18 IJs was found in infected larvae after 2 h of exposition although inappropriate for assay parameters.

GFP-marked *X. nematophila* (strain F1D3) permitted to follow infection progression in the fluorescent stereomicroscope observation. Symbiont bacteria localized in a receptacle vesicle of *S. carpocapsae* (Fig. 1) allowed us to observe IJs in hemocoel cavity within the first 90 min of exposition. Interesting, the bacteria started to be observed free in insect hemocoel 8 h post exposition and at 12 h bacteria spread all over the larvae (Fig. 2). The qRT-PCR analysis of bacterial amount (based on 16S) amplified in 90 min and 4 h post-infection was trivial while after 14 h it was more than 10 times higher (Table 2) indicating the bacterial proliferation. Amplification of nematode 18S confirmed *S. carpocapsae* presence on larvae.

Based on these observations, we selected 90 min, 4 h and 14 h as time-points for the analysis of expression patterns of immune - related genes. The time point 90 min corresponded to nematode invasion into haemocoel (early infection); 4 h corresponded to nematode establishment in haemocoel before the release of bacteria (mid infection); and 14 h corresponded to the proliferation of symbiotic *X. nematophila* in insect haemocoel (late infection).

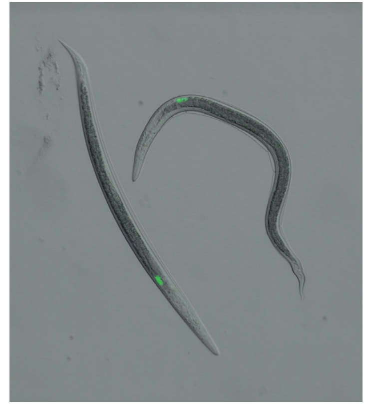


Fig. 1 *S. carpocapsae* IJs with GFP-marked *X. nematophila* used for the assay.

Table 2. Mean ± SD of ΔΔCT values corresponding to 18S gene of *S. carpocapsae* and 16S gene of *X. nematophila* inside infected larvae of *D. suzukii*.

Sample	ΔΔCT of *S. carpocapsae*	ΔΔCT of *X. nematophila*
Early Inf.	-2.26 ± 1.95	1.44 ± 1.19
Mid-Inf.	-4.33 ± 2.46	1.16 ± 0.39
Late Inf.	-8.21 ± 1.28	-9.22 ± 0.51

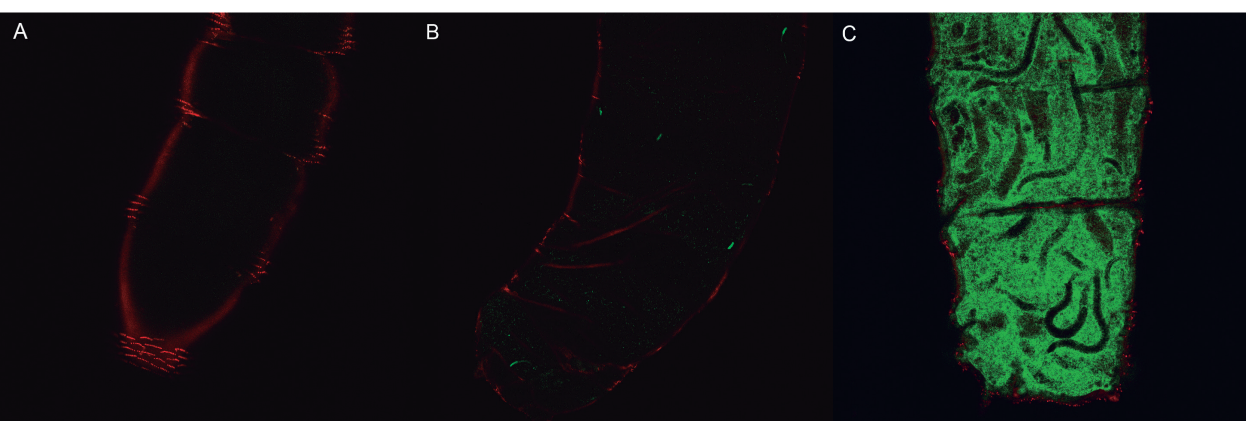


Fig. 2 Florescent images of *D. suzukii* larvae to evaluate nematode and bacterial infection progress. A) Untreated larvae only presented cuticular fluorescence (in red false color) B) Infected larvae at 8h of incubation, some nematodes still had bacteria inside the vesicle, while some were already released in hemocoel (in green). C) Infected larvae at 14h of incubation, bacteria spread through all larvae and proliferated with intensity. Images were obtained with confocal microscope Leica TCS SP5 with differential detection of marked GFP bacteria exited at 488 nm (detection between 500 – 580 nm) and autofluorescence of larva cuticle excited at 405 nm (detection between 440-485 nm).

3. Expression pattern of immune related genes before bacterial release

In order to investigate the response of the insect to the presence of the nematode, we compared the expression rate of pathogen-associated molecular patterns in infected and non-infected insects. In the untreated larvae, the level of expression of PGRP-LC (12.24 ΔCT) was lower than that of PGRP-LF (4.82 ΔCT) (Fig. 3A - B). After infection, the expression rate was not significantly different, PGRP-LC (Log2FC = 0.88) and PGRP-LF (Log2FC = -0.50), which suggests that the insect did not detect nematode invasion. In what concerns Imd pathway, both genes analyzed have similar patterns of expression, which are not significantly different from the untreated larvae. Either in the expression rate of Cecropin or Diptericin we observed a slight decrease in the expression at 90 min of infection (Log2FC = -1.26, Log2FC = -1.80, respectively) whereas at 4 h the rate of expression was closer to the control (Log2FC = 0.60, Log2FC = 1.29, respectively) (Fig. 3C - D). Particularly, Diptericin presented a significant upregulation between 90 min and 4 of infection. Regarding Toll pathway, the expression of Defensin remained constant between infected and healthy larvae (Log2FC = -1.10). The expression rate of Drosomycin did not change with the infection at 90 min (Log2FC = -1.05) but had a slight increase at 4 h (Log2FC = 1.65) (Fig. 3E - F).

Paralleling the humoral response, the Upd3 expression rate in cellular defenses was not modified in the presence of the nematode at 90 min and 4 h (Log2FC = 0.49) (Fig. 4A), thus suggesting the absence of recognition. Consistent with non-induction of Upd3 expression, the expression of Turandot C remained constant at 90 min and 4 h of infection (Log2FC = -1.21 and -0.37), indicating the lack of JAK/STAT pathway activation (Fig. 4B). Besides, PPO1 and PPO2 showed no significant variation after nematode infection (Log2FC = 0.52, Log2FC = 0.68, respectively), although a non-significant increase was observed at 90 min of infection in both genes (Fig. 4C - D).

In infected insects, the expression rates of the activator and negative feedback genes of JNK stress response pathway, Basket and Puckered remained stable at 90 min (Log2FC = 0.04, Log2FC = -0.14, respectively) and 4 h (Log2FC = 0.52, Log2FC = 0.09) post-infection (Fig. 5A - B). The last pathway analyzed was TGF-β with Dawdle (Daw) and Decapentaplegic (Dpp) genes (Fig. 5C - D). The expression rate of TGF-β genes presented non-significant variation during the penetration and nematode infection (Log2FC = -0.59, Log2FC = -0.11, respectively).

4. Expression pattern of immune related genes after bacterial release

We analyze the expression of defense genes at 14 h post infection when the symbiotic bacteria spread in the entire insect hemocoel. Receptors PGRP-LC and PGRP-LF, showed a noteworthy increase in the expression rate at this time point (Log2FC = 1.97, Log2FC = 2.02, respectively) compared to 4 h, despite being not significant (Fig. 3A – B). The increased expression of PGRP-LC was followed by an upregulation of AMPs of Imd pathway probably in response to bacterial challenge (Fig. 3C - D). In fact, Diptericin and Cecropin showed a significant increase of expression reaching a 2.85 and 4.80 (Log2FC), respectively. Similarly, the presence of bacteria

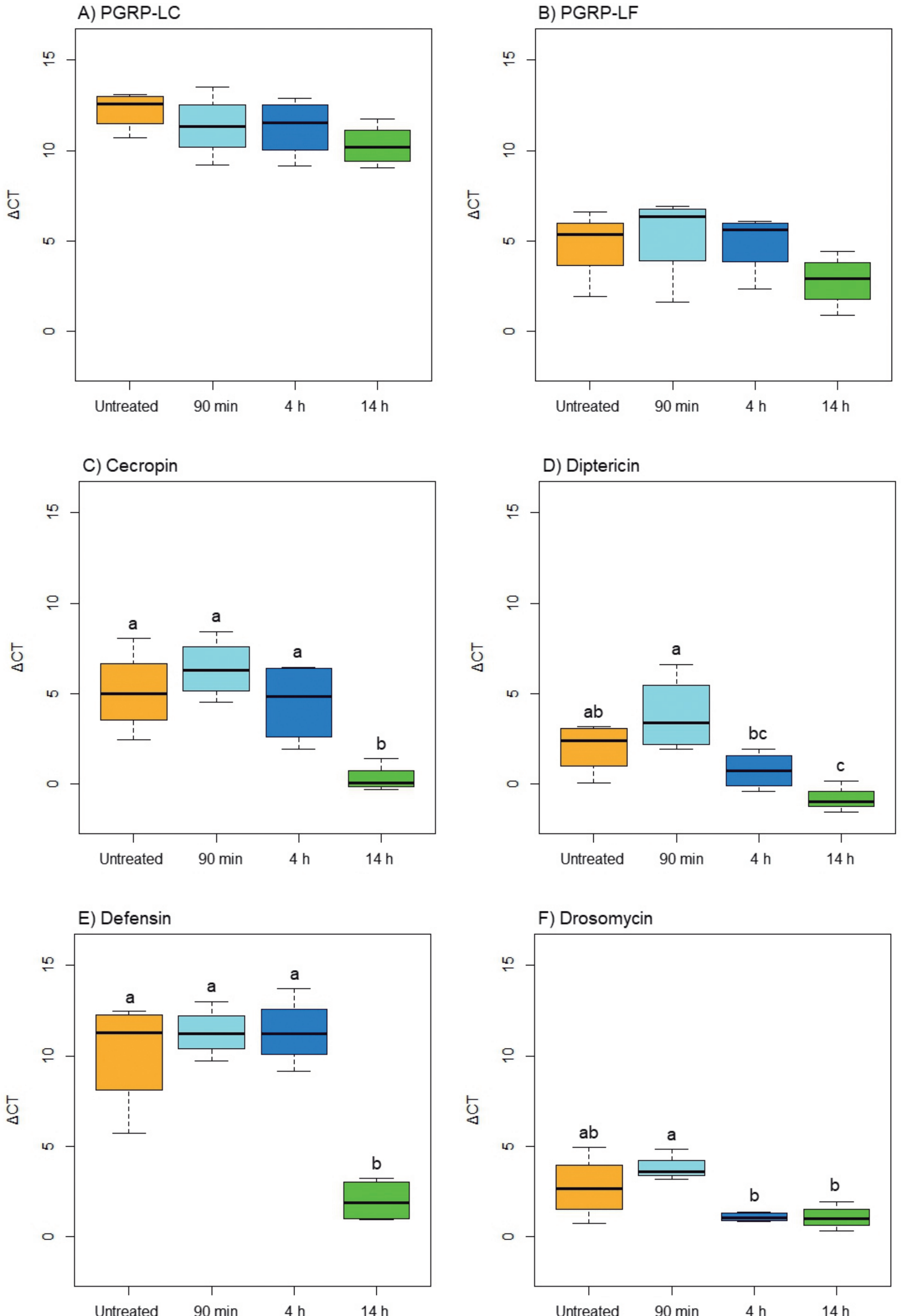


Fig. 3 Gene expression of *D. suzukii* larvae untreated and infected with *S. carpocapsae* at 90 min, 4 h and 14 h of infection expressed as ΔCT, for Imd and Toll genes: A) PGRP-LC (GLM: F= 1.25, df= 3, p= 0.3335), B) PGRP-LF (GLM: F= 1.34, df= 3, p= 0.3061), C) Cecropin (GLM: F= 8.04, df= 3, p= 0.0033), D) Diptericin (GLM: F= 7.67, df= 3, p= 0.0039), E) Defensin (GLM: F= 20.04, df= 3, p< 0.0001) and F) Drosomycin (GLM: F= 7.053, df= 3, p= 0.0054). Different letters indicate statistical significance among infection time-points ($p < 0.05$).

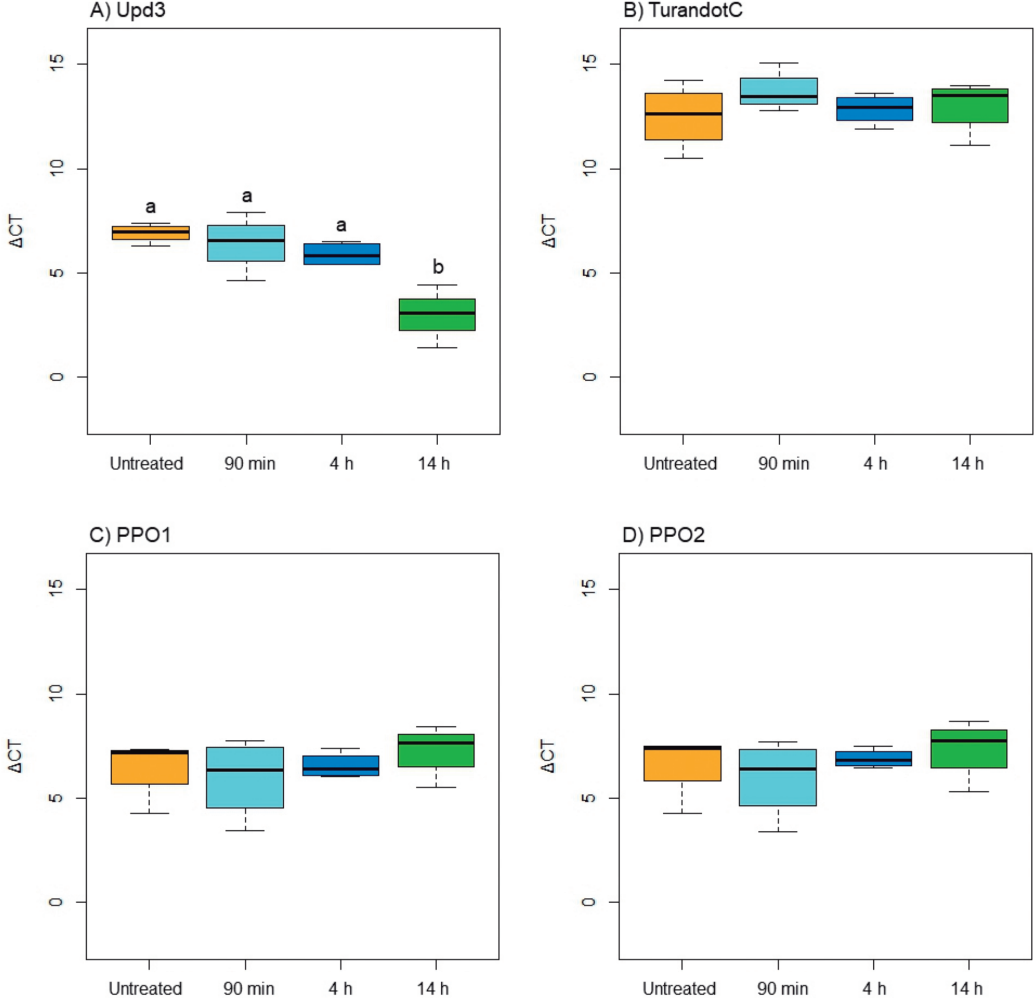


Fig. 4 Gene expression of *D. suzukii* larvae untreated and infected with *S. carpocapsae* at 90 min, 4 h and 14 h of infection expressed as ΔCT, for Jak/STAT and proPO genes: A) Upd3 (GLM: F= 12.84, df= 3, p= 0.0004), B) Turandot C (GLM: F= 0.73, df= 3, p= 0.5485), C) PPO1 (GLM: F= 0.60, df= 3, p= 0.6246) and D) PPO2 (GLM: F= 0.64, df= 3, p= 0.6004). Different letters indicate statistical significance among infection time-points ($p < 0.05$).

induced a huge upregulation of Defensin in Toll pathway (Log2FC = 8.19) (Fig. 3E). In contrast, the expression of Drosomycin did not change between 4 and 14 h of infection (Log2FC = 1.67) (Fig. 3F). Worth to be mentioned, the positive correlation between the expression rate of *D. suzukii* genes and the amount of bacteria in the insect measured by the amount of 16S RNA (Fig. S1). Defensin and Cecropin presented a high positive correlation to bacterial load (Corr = 0.96 and 0.85 respectively). While Diptericin and PGRP-LF showed a significant but weaker correlation (Corr = 0.67 and 0.59 respectively). These data are in accordance with postulated knowledge that gram-negative bacteria target the Imd pathway.

In the presence of bacteria, the expression of the interleukin Upd3 was also significantly upregulated (Log2FC = 3.90) (Fig. 4A). Despite the production of the pro-inflammatory Upd3, JAK/STAT pathway was not activated, as evidenced by the non-variation of the expression of the effector gene Turandot C (Log2FC = -0.53) (Fig. 4B). The expression of pro-phenoloxidase genes, PPO1 and PPO2, did not change after bacterial release (Fig. 4C – D). Moreover, we observed a

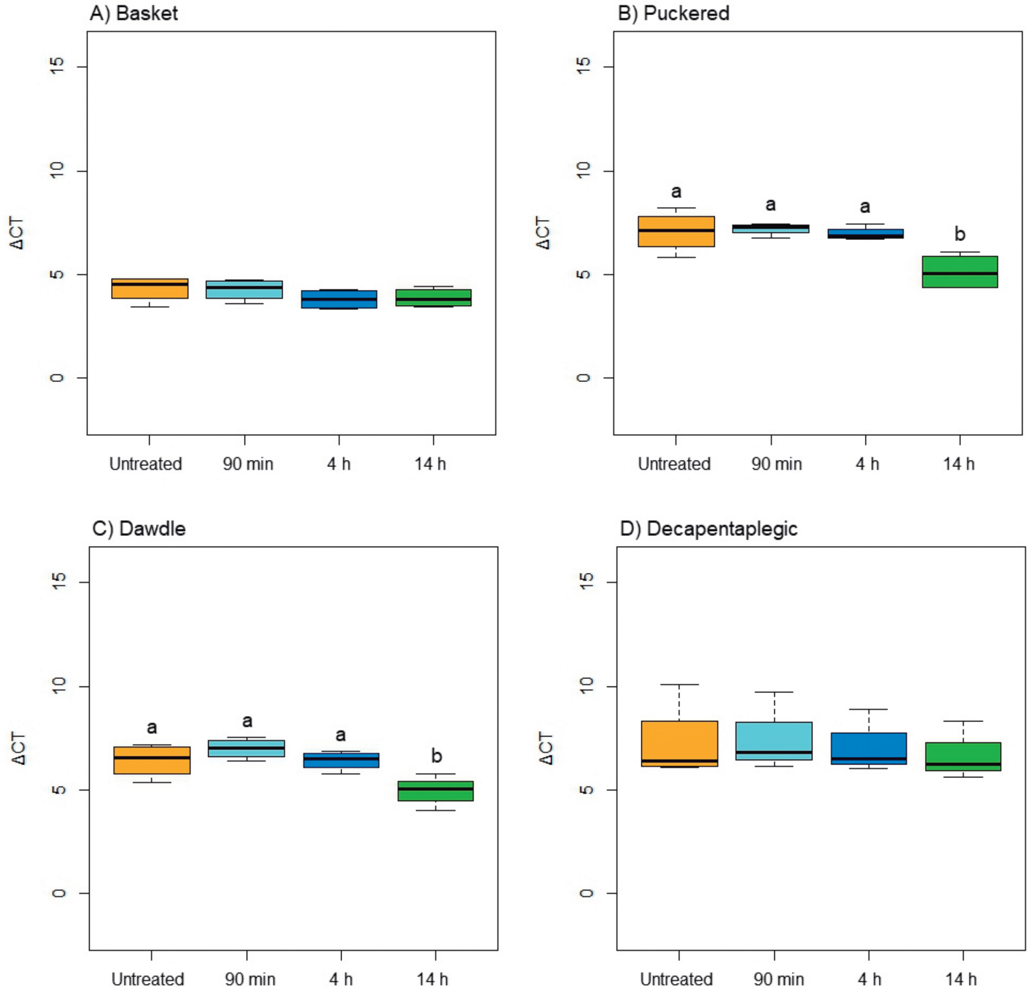


Fig. 5 Gene expression of *D. suzukii* larvae untreated and infected with *S. carpocapsae* at 90 min, 4 h and 14 h of infection expressed as ΔCT, for JNK and TGF-β genes: A) Basket (GLM: F= 1.05, df= 3, p= 0.4047), B) Puckered (GLM: F= 7.75, df= 3, p= 0.0038), C) Dawdle (GLM: F= 7.30, df= 3, p= 0.0047) and D) Dpp (GLM: F= 0.19, df= 3, p= 0.8958). Different letters indicate statistical significance among infection time-points ($p < 0.05$).

slight downregulation tendency of both genes after 14 h of infection (Log2FC = -0.81, Log2FC = -0.72, respectively), thus suggesting crystal cells were not activated. Concerning JNK pathway, the expression of Basket remained stable at 14 h compared to 4 h of infection (Log2FC = 0.44), whereas negative feedback gene Puckered was significantly upregulated (Log2FC = 1.92) (Fig. 5A – B), indicating that stress mechanisms were not induced even with the presence of the bacteria. In TGF-β pathway, Dawdle ligand presented a significant upregulation under bacterial pressure at 14 h of infection (Log2FC = 1.47) (Fig. 5C). In contrast, Dpp ligand kept the expression rate constant along all nematode-bacterial infection (Log2FC = 0.65) (Fig. 5D).

Discussion

The high susceptibility of *D. suzukii* larvae to EPNs was reported with 84.3% of *S. carpocapsae* infection although the involvement of immune defenses was unknown [26]. Thus, to ensure an

efficient biological control with EPNs was crucial to comprehend the interaction between nematodes and *D. suzukii*. The immune system operates as a complex net of signaling in constant variation and communication among hemocytes, humoral receptors, fat body and effector mechanisms. For this reason, mRNA analysis provides accurate information of switched-on genes, although no information about resulting product efficacy is given [29]. In consequence, transcriptomic results provided in this work will be compared to post-transcriptomic processes already reported [27] to better understand *D. suzukii* response.

The immune genes of this fly are barely unknown; thus, we characterized these genes involved in *D. suzukii* for the first time through *D.* melanogaster homology. Imd receptors, PGRP-LC and PGRP-LF are highly conserved in *D. suzukii* as well as genes in JNK and TGF-β pathways which are well preserved among insects and even mammals [5,12,30]. In contrast, AMPs are short sequences that present variations not only in the nucleotide sequence but also complete gene duplications and losses among species [31]. As seen in *D. suzukii* which presented a wide range of isoform variations of Diptericin and Cecropin genes compared to *D. melanogaster*. The pathway involved in cellular defense presented less percentage of identity in *D. suzukii* for Upd3 and Turandot genes. *Dome* and *mtm* genes which are involved in Jak/STAT and hemocyte regulation, presented in *D. suzukii* fast evolutionary rates that could facilitate defense adaptation in invaded regions [32]. Changes in those regulatory genes can be related with the increased number of hemocyte levels reported in this fly [25]. Interestingly, homolog sequences of *D. melanogaster* PPO1 and PPO2 were identified as PPO2, which are two different genes in *D. suzukii* using the same name.

Foreign organisms that invaded the insect body are recognized by the immune system when pathogen-associated molecular patterns bind host-derived pattern recognition receptors. However, in our assay, genes involved in cellular and humoral response were unresponsive to the presence of nematodes during the first 4 h of infection (Fig. 6). Only genes of humoral response changed their expression once bacteria were released into the hemocoel. For instance, Imd receptors PGRP-LC and PGRP-LF only presented a slight upregulation at 14 h which evidenced an absence of nematode recognition during early infection. In consequence, the expression of AMPs genes was mainly upregulated at 14 h of infection as seen in Cecropin and Diptericin pattern of expression. Genes regulated in Toll pathway were also activated after the release of *X. nematophila*. Defensin displayed the greatest upregulation at 14 h of infection (Fig. 6). Besides, Drosomycin was upregulated at 4 h and maintained its expression after. This gene presented a similar pattern of response as Diptericin due to the cross-activation signaling that Drosomycin receives from Imd pathway [5]. Despite being not significant, a downregulation tendency was observed in the expression of AMPs genes at 90 min of infection that suggested a modulatory effect of IJs to inhibit antimicrobial activity before bacterial release. In agreement with these results, AMPs were detected in hemolymph of *D. suzukii* infected with *X. nematophila* after 24 h, even though the antimicrobial activity of those peptides was reduced [27]. Interestingly, the expression of AMP genes displayed in *D. suzukii* a big variance in control conditions as already reported in *D. melanogaster* larvae [33]. In *D. melanogaster,*

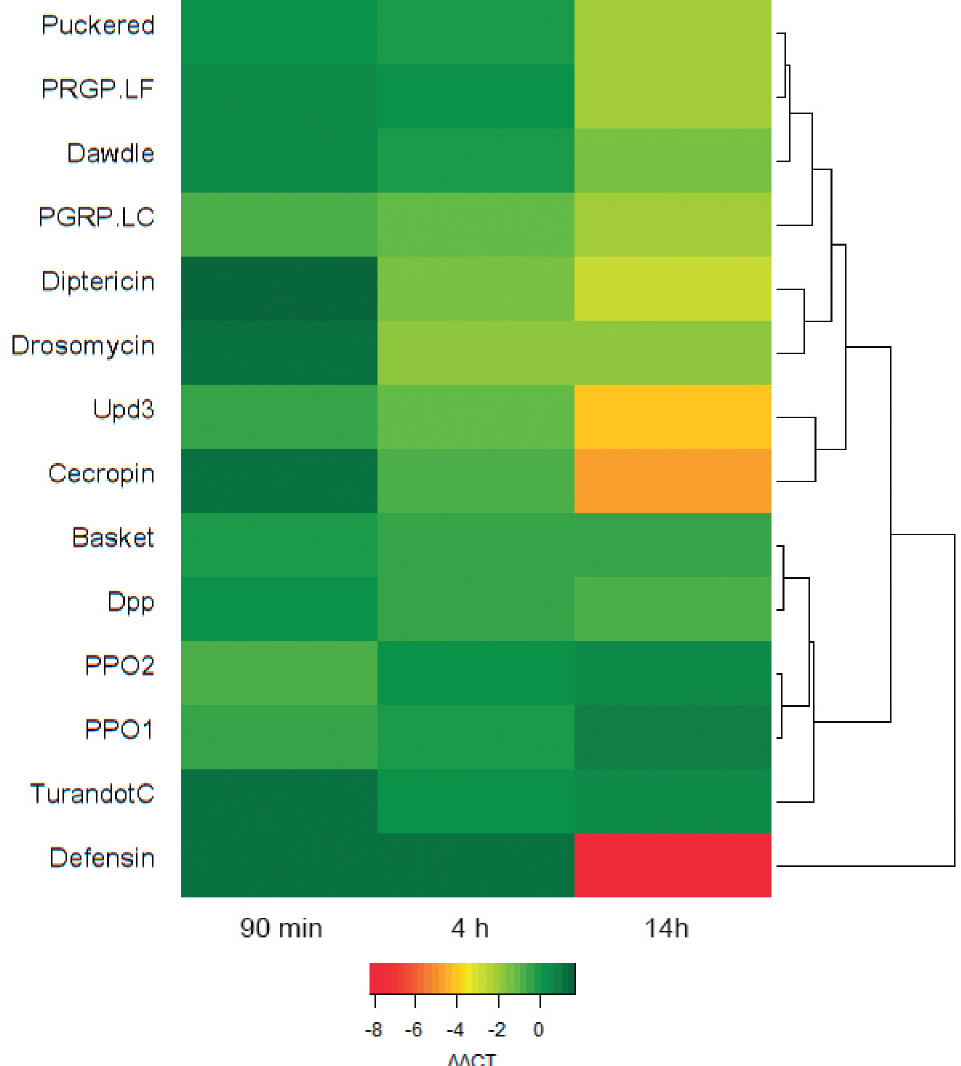


Fig. 6 Heatmap graph summarize the variation of ΔΔCT all treatments, in green are presented the downregulated genes and in red the upregulated ones. The dendrogram groups the genes with similar response pattern through the four infection stages.

AMPs expression after *S. carpocapsae* infection presented similar results with those observed in *D. suzukii* for Diptericin and Drosomycin expression, although Cecropin and Defensin were only upregulated after 24 h [34]. Moreover, in *D. melanogaster* was reported the upregulation of AMP genes without an activation of PGRP receptors suggesting the involvement of another signaling receptor [35].

Regarding the cellular response, Jak/STAT pathway should be quickly activated through hemocyte receptors. Nevertheless, an increase of expression was only registered by Upd3 gene after *X. nematophila* challenge (Fig. 6). Besides, the effector gene Turandot C remained constant in all measured time-points as control larvae. In *D. melanogaster* larvae infected by EPNs, an upregulation of Turandot genes was reported only after 18 h and 30 h of infection [36,37]. These results confirmed that under EPNs infection the quick cellular response was not developed. In agreement with the gene expression results, lamellocyte differentiation in *D. suzukii* larvae was observed in low levels under *S. carpocapsae* infection and hemocytes were unable to encapsulate

IJs [27]. The inactivation of Jak/STAT pathway entailed a lack of regulation upon cellular response which ended with the absence of effector mechanisms. Indeed, *S. carpocapsae* was described to use an evasive and immunosuppressive strategy as IJs and bacteria secrete proteolytic compounds to avoid hemocyte action, clot formation and melanization [38,39].

Regarding the melanization response, our results showed that PPO1 and PPO2 genes displayed a constant expression during infection and only an upregulation tendency was observed at 90 min (Fig. 6). Despite being a quick mechanism of response, phenoloxidase cascade was not activated upon IJs entrance as confirmed by gene expression. In contrast, infected *D. melanogaster* larvae presented an increased expression of PPO1 while PPO2 remained constant [10]. Therefore, this expression of PPO2 pointed to a lack of involvement of this gene to nematode infection. PPO2 involvement with stored pro-phenoloxidase in crystal cells may be the cause of inactivation as the response was not triggered in first place. The complex *S. carpocapsae* and *X. nematophila* has been previously described to avoid melanization response using post-transcriptional mechanisms in different insects [34,38,40,41]. Moreover, *D. suzukii* larvae infected with *X. nematophila* showed a lower level of phenoloxidase enzymatic activity in hemolymph compared to control larvae [27]. In the scope of these results, the inactivity of PPO1 and PPO2 confirmed a modulation of the *S. carpocapsae - X. nematophila* complex to avoid the melanization response in collaboration to already described post-transcriptional mechanisms.

In the JNK pathway, the gene expression of Basket presented in *D. suzukii* larvae a constant expression all along infection (Fig. 6). Only Puckered was upregulated after 14 h of infection although its role as negative feedback to avoid hyperactivation of the pathway. Thus, the pathway was unresponsive to nematode-bacterial infection and as a result, stress response in larvae was not triggered. This pattern of expression was also observed in *D. melanogaster* after EPNs infection [35,36]. In addition, larvae of *D. suzukii* showed practically no variation of TGF-β genes over the infection course. *Drosophila melanogaster* larvae infected with the same EPNs did not trigger Dawdle or Dpp activation [35]. However, in *D. melanogaster* adults, TGF-β was found upregulated, so activation of the pathway seems to be restricted to the adult stage [16,30]. In regard to those results, *D. suzukii* could behave like *D. melanogaster* with a higher involvement of TGF-β genes in adults rather than larvae in response to EPNs infection.

This study provides the first characterization of *D. suzukii* genes involved in the immune system. Moreover, the combined information provided by gene expression analysis and post-transcriptional mechanisms, permitted to comprehend the development of the immune response of *D. suzukii* to EPNs infection. Nematodes avoided being detected by humoral and cellular receptors and thus, trigger the fast responses of immune defense. Only after bacterial release the receptors increased its expression and AMPs were upregulated. Although, cellular response was inactive even in late infection due to steadiness of Turandot and PPO genes. Besides, JNK was unresponsive to infection indicating a lack of stress response in larvae. Nevertheless, the grade of involvement of nematodes or bacteria in each gene activation was beyond the scope of this work. Further

experiments should be done to elucidate the activation origin and enlarge the analyzed genes to provide a wider understanding of *D. suzukii* response to EPNs infection.

Material and Methods

Insects and nematodes

Third-instar larvae of *D. suzukii* used in the assay were from laboratory culture. Wild specimens were collected in 2012 in Maresme, Catalonia (NE Spain) and established under laboratory conditions since then. Insects were reared on a modified drosophila diet [26] at 25ºC with 12:12h photoperiod. All experiments were conducted under these environmental conditions.

The assay was performed with *S. carpocapsae* (B14) isolated from an urban garden soil in Barcelona, Catalonia (NE Spain). Symbiont bacteria *Xenorhabdus nematophila* was changed to *X. nematophila* Green Fluorescent Protein (GFP-labeled strain F1D3) (provided by the laboratory of Prof. Givaudan, University of Montpellier, France), according to the method of McMullen and Stock [42]. Nematodes were reared in late instar of *Galleria mellonella* L. (Lepidoptera: Pyralidae) at 25ºC, according to the method of Woodring and Kaya [43]. Once IJs emerged from insect, nematodes were collected with modified White traps. IJs were stored with sterile tap water (STW) in culture flasks at 9ºC for a maximum of 2 weeks. Before use, nematodes were acclimatized at room temperature for 3 h, to ensure maximum activation and infection during assay. IJs viability was assessed by movement observation under a stereomicroscope.

Retrieve of immune-related genes

Genome of *D. suzukii* was previously sequenced but only some of its genes were annotated and others presented discrepancies between the annotation in D. *suzukii* and homologous in *D. melanogaster*. Thus, a first identification of genes involved with the immune system was required. For this reason, we used *D. melanogaster* which immune system is well known as reference. From literature review, we identified 40 key genes involved in different levels of the immune pathways [5,10,17,29,35,36]. *Drosophila melanogaster* sequences were extracted from GenBank NCBI and compared to *D. suzukii* genome using BLAST [44]. Two methods, Blastn (MegaBlast algorithm) and tBlastn, were performed to find potential homolog sequences considering best score hit, Query coverage, E-value and Max identity. Transcript sequences of best hits were aligned back to *D. melanogaster* to confirm protein similarity. From the identified homolog sequences of *D. suzukii*, two genes of each main pathway (Imd receptors, Imd, Toll, Jak-STAT, ProPO, JNK, TGF-β) were selected for the genetic expression assay.

Infection methodology

Drosophila suzukii infections were carried out in 96-multiwell and using sterile filter paper as substrate. Larvae were placed individually with a nematode dose of 300 IJs per larvae in 15 µL and sealed with Parafilm® with an exposition time of 1 – 2h. For untreated larvae, only STW

was applied. After exposition, larvae were thoroughly rinsed with STW to remove any possible nematode on its surface and transferred to a Petri dish (3 cm diameter) with humid filter paper and diet. Then, infected larvae were incubated during different times to evaluate early (30 min), mid (2h) and late (12h) infection response in order to see gene expression variations. After incubation, all larvae were thoroughly rinsed again with STW and transferred to PCR tubes with 20 µL of RNAlater and frozen immediately at -80ºC. Infected larvae after 14 h were checked for positive fluorescent signal of GFP bacteria by fluorescent stereomicroscope Leica MzfIII before freeze (Fig. 2). The final time-points considering the exposure and incubation times were set at 90 min, 4 h and 14 h post-infection.

RNA extraction

Drosophila suzukii larvae were dissected individually in presence of RNAlater to verify nematode presence after 90 min, 4 h and 14 h of infection. For qRT-PCR assay, only larvae with nematodes inside were used after inspection. Larvae with more than 10 IJs were rejected to avoid outliers in expression due to massive nematode entry. Polls of five larvae per treatment were used for mRNA extraction and four biological replications were carried out per treatment. mRNA isolation was performed with TRIzol Reagent (Invitrogen) combined with Invitrogen PureLink RNA Mini Kit (ThermoFisher) following manufacture procedures. Samples were treated with ezDNase and cDNA was prepared with SuperScript™ IV VILO™ MasterMix (ThermoFisher) using PoliA hexamers. RNA of untreated larvae was extracted under the same conditions.

Analysis of immune – related gene expression

Primers were designed for *D. suzukii* genes with OligoPerfect software. A relation of primers used and access number for each gene could be found in Table S2 for *D. suzukii*. For those genes with isoforms (PGRP-LC, Cecropin, Diptericin, Drosomycin, Dawdle and Decapentaplegic) primers were designed to amplify a conserved region. The housekeeping ribosomal gene RpL32 was used as endogenous control for the analysis of genes expression levels. To elucidate load of *S. carpocapsae* and *X. nematophila* inside infected larvae was used the 18S and 16S genes, respectively (Table S3). Primer specificity was tested for all pairs by melt curve analysis (Fig. S2). qRT-PCR was done using *Power* SYBR Green MasterMix and conducted following the protocol: 95ºC for 10 min and 40 cycles of 95ºC for 15 s followed by 60ºC for 60 s. Each sample was run in triplicate and considered consistent if their threshold cycle (CT) were within 0.5 variance.

Data analysis

We performed the statistical analysis using ΔCT values (ΔCT = CT target gene – CT endogenous gene) and transformed fold change values (Log2FC) were also provided. Those values were extracted using DataAssist (Applied Biosystems). Variations of gene relative expression were statistically analyzed using General Lineal Model (GLM) and differences among treatments were elucidated with Tukey Test. Any comparison was considered significant if *p* value was less than

0.05. To analyze correlation and elucidate similar pattern responses of all genes though infection course, data was normalized to ΔΔCT (ΔΔCT = ΔCT target gene – mean ΔCT of untreated larvae). Pearson's correlation analysis was run to determine if variations in gene expression for each biological repetition was due to nematode and bacterial load, measured as ΔΔCT of *S. carpocapsae* 18S gene and *X. nematophila* 16S gene. Graphical representations were done with the R library "*corrplot"* function *"corrplot"* and library "*PerformanceAnalytics*" function "*chart.Correlation*". The heatmap was generated with R function "*heatmap*" and using a dendrogram per row. All statistical analysis were run in R Studio software (version 3.4) [45].

Acknowledgments

We would like to thank the Servei de Microscopia - Universitat Autònoma de Barcelona for the technical support.

Funds

The research presented in this paper was supported by FEDER/Ministerio de Ciencia, Innovación y Universidades—Agencia Estatal de Investigación/Project (AGL2017-86770-R) and the project PTDC/BAA/30609/2017 of FCT.

References

1. Poinar G. Biology and taxonomy of Steinernematidae and Heterorhabditidae. In: Gaugler R, Kaya HK, editors. Entomopathogenic nematodes in biological control. Florida: Boca Raton: CRC Press; 1990. p. 23–62.
2. Boemare NE. Biology, taxonomy, and systematics of *Photorhabdus* and *Xenorhabdus*. In: Gaugler R, editor. Entomopathogenic nematodology. Wallingford, UK: CABI International; 2002. p. 35–56.
3. Snyder H, Stock SP, Kim S-K, Flores-Lara Y, Forst S. New insights into the colonization and release processes of *Xenorhabdus nematophila* and the morphology and ultrastructure of the bacterial receptacle of its nematode host, *Steinernema carpocapsae*. Appl Environ Microbiol. 2007;73(16):5338–46.
4. Cooper D, Eleftherianos I. Memory and specificity in the insect immune system: Current perspectives and future challenges. Front Immunol. 2017;8:1–6.
5. Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. Annu Rev Immunol. 2007;25:697–743. doi: 10.1146/annurev.immunol.25.022106.141615
6. Eleftherianos I, Shokal U, Yadav S, Kenney E, Maldonado T. Insect immunity to entomopathogenic nematodes and their mutualistic bacteria. Life Sci J. 2016;6(4):23–7.
7. Zeidler MP, Bach EA, Perrimon N. The roles of the *Drosophila* JAK/STAT pathway. Oncogene. 2000;19(21):2598–606.
8. Yang H, Kronhamn J, Ekström J, Korkut GG, Hultmark D. JAK / STAT signaling in *Drosophila* muscles controls the cellular immune response against parasitoid infection. EMBO Rep. 2015;16(12):1664–72.
9. Agaisse H, Petersen UM, Boutros M, Mathey-Prevot B, Perrimon N. Signaling role of hemocytes in *Drosophila* JAK/STAT-dependent response to septic injury. Dev Cell. 2003;5(3):441–50.

10. Cooper D, Wuebbolt C, Heryanto C, Eleftherianos I. The prophenoloxidase system in *Drosophila* participates in the anti-nematode immune response. Mol Immunol. 2019;109:88–98. doi: 10.1016/j.molimm.2019.03.008

11. Dudzic JP, Kondo S, Ueda R, Bergman CM, Lemaitre B. *Drosophila* innate immunity: Regional and functional specialization of prophenoloxidases. BMC Biol. 2015;13(1):1–16. doi: 10.1186/s12915-015-0193-6

12. Delaney JR, Stöven S, Uvell H, Anderson K V., Engström Y, Mlodzik M. Cooperative control of *Drosophila* immune responses by the JNK and NF-κB signaling pathways. EMBO J. 2006;25(13):3068–77.

13. Biteau B, Karpac J, Hwangbo DS, Jasper H. Regulation of *Drosophila* lifespan by JNK signaling. Exp Gerontol. 2011;46(5):349–54.

14. Tafesh-Edwards G, Eleftherianos I. JNK signaling in *Drosophila* immunity and homeostasis. Immunol Lett. 2020;226:7–11. doi: 10.1016/j.imlet.2020.06.017

15. Clark RI, Woodcock KJ, Geissmann F, Trouillet C, Dionne MS. Multiple TGF-β superfamily signals modulate the adult drosophila immune response. Curr Biol. 2011;21(19):1672–7.

16. Patrnogic J, Heryanto C, Eleftherianos I. Transcriptional up-regulation of the TGF-β intracellular signaling transducer Mad of *Drosophila* larvae in response to parasitic nematode infection. Innate Immun. 2018;24(6):349–56.

17. Hallem EA, Rengarajan M, Ciche T, Sternberg PW. Nematodes, bacteria, and flies: A tripartite model for nematode parasitism. Curr Biol. 2007;17(10):898–904.

18. Castillo JC, Reynolds SE, Eleftherianos I. Insect immune responses to nematode parasites. Trends Parasitol. 2011;27(12):537–47. doi: 10.1016/j.pt.2011.09.001

19. Huot L, George S, Girard PA, Severac D, Nègre N, Duvic B. *Spodoptera frugiperda* transcriptional response to infestation by *Steinernema carpocapsae*. Sci Rep. 2019;9(1):1–13.

20. Walsh DB, Bolda MP, Goodhue RE, Dreves AJ, Lee J, Bruck DJ, et al. *Drosophila suzukii* (Diptera: Drosophilidae): invasive pest of ripening soft fruit expanding its geographic range and damage potential. J Integr Pest Manag. 2011;2(1):1–7.

21. Gabarra R, Riudavets J, Rodríguez GA, Pujade-Villar J, Arnó J. Prospects for the biological control of *Drosophila suzukii*. BioControl. 2015;60:331–9.

22. Woltz JM, Donahue KM, Bruck DJ, Lee JC. Efficacy of commercially available predators, nematodes and fungal entomopathogens for augmentative control of *Drosophila suzukii*. J Appl Entomol. 2015;1–12.

23. Daane KM, Wang X-G, Biondi A, Miller B, Miller JC, Helmut R, et al. First exploration of parasitoids of *Drosophila suzukii* in South Korea as potential classical biological agents. J Pest Sci (2004). 2016;89(3):823–35.

24. Poyet M, Havard S, Prevost G, Chabrerie O, Doury G, Gibert P, et al. Resistance of *Drosophila suzukii* to the larval parasitoids *Leptopilina heterotoma* and *Asobara japonica* is related to haemocyte load. Physiol Entomol. 2013;38(1):45–53.

25. Kacsoh BZ, Schlenke TA. High hemocyte load is associated with increased resistance against parasitoids in *Drosophila suzukii*, a relative of *D. melanogaster*. PLoS One. 2012;7(4).

26. Garriga A, Morton A, Garcia-del-Pino F. Is *Drosophila suzukii* as susceptible to entomopathogenic nematodes as *Drosophila melanogaster*? J Pest Sci (2004). 2018;91(2):789–98.

27. Garriga A, Mastore M, Morton A, Garcia-del-Pino F, Brivio MF. Immune response of *Drosophila suzukii* larvae to infection with the nematobacterial complex *Steinernema carpocapsae–Xenorhabdus nematophila*. Insects. 2020;11(4).

28. Chiu JC, Jiang X, Zhao L, Hamm CA, Cridland JM, Saelao P, et al. Genome of *Drosophila suzukii*, the spotted wing *Drosophila*. G3 (Bethesda). 2013;3(12):2257–71.

29. Neyen C, Bretscher AJ, Binggeli O, Lemaitre B. Methods to study *Drosophila* immunity. Methods. 2014;68(1):116–28. doi: 10.1016/j.ymeth.2014.02.023

30. Eleftherianos I, Castillo JC, Patrnogic J. TGF-β signaling regulates resistance to parasitic nematode infection in *Drosophila melanogaster*. Immunobiology. 2016;221(12):1362–8. doi: 10.1016/j.imbio.2016.07.011

31. Hanson MA, Lemaitre B, Unckless RL. Dynamic evolution of antimicrobial peptides underscores trade-offs between immunity and ecological fitness. Front Immunol. 2019;10.

32. Ometto L, Cestaro A, Ramasamy S, Grassi A, Revadi S, Siozios S, et al. Linking genomics and ecology to investigate the complex evolution of an invasive *Drosophila* pest. Genome Biol Evol. 2013;5(4):745–57.

33. Hanson MA, Hamilton PT, Perlman SJ. Immune genes and divergent antimicrobial peptides in flies of the subgenus *Drosophila*. BMC Evol Biol. 2016;16(1):228. doi: 10.1186/s12862-016-0805-y

34. Peña JM, Carrillo MA, Hallem EA. Variation in the susceptibility of *Drosophila* to different entomopathogenic nematodes. Infect Immun. 2015;83(3):1130–8.

35. Yadav S, Gupta S, Eleftherianos I. Differential regulation of immune signaling and survival response in *Drosophila melanogaster* larvae upon *Steinernema carpocapsae* nematode infection. Insects. 2018;9(1).

36. Castillo JC, Shokal U, Eleftherianos I. Immune gene transcription in *Drosophila* adult flies infected by entomopathogenic nematodes and their mutualistic bacteria. J Insect Physiol. 2013;59(2):179–85. doi: 10.1016/j.jinsphys.2012.08.003

37. Yadav S, Frazer J, Banga A, Pruitt K, Harsh S, Jaenike J, et al. Endosymbiont-based immunity in *Drosophila melanogaster* against parasitic nematode infection. PLoS One. 2018;13(2):1–20.

38. Brivio MF, Mastore M. Nematobacterial complexes and insect hosts: Different weapons for the same war. Insects. 2018;9(3).

39. Toubarro D, Avila MM, Montiel R, Simões N. A pathogenic nematode targets recognition proteins to avoid insect defenses. PLoS One. 2013;8(9):1–13.

40. Balasubramanian N, Toubarro D, Simões N. Biochemical study and in vitro insect immune suppression by a trypsin-like secreted protease from the nematode *Steinernema carpocapsae*. Parasite Immunol. 2010;32(3):165–75.

41. Eliáš S, Hurychová J, Toubarro D, Frias J, Kunc M, Dobeš P, et al. Bioactive excreted/secreted products of entomopathogenic nematode *Heterorhabditis bacteriophora* inhibit the phenoloxidase activity during the infection. Insects. 2020;11(6):1–19.

42. McMullen JG, Stock SP. In vivo and In vitro rearing of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae). J Vis Exp. 2014;91:e52096, doi: 10.3791/52096.

43. Woodring JL, Kaya HK. Steinernematid and heterorhabditid nematodes: A handbook of biology and techniques. Arkansas Agricultural Experiment Station, Fayetteville, AR: Southern Cooperative Series Bulletin 331; 1988. 30 p.

44. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403–10.

45. R Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2017.

Supplementary Materials:

Table S1

D. melanogaster		*D. suzukii*				
Gene name	Code number	Gene name	Code number	Cover	e-value	%Id.
PGRP receptors						
PGRP-LC	NM_140041	PGRP-LC	XM_017079226	97%	0	82.6%
PGRP-LE	CG8995	PGRP-LE	XM_017087015	92%	0	81.9%
PGRP-LF	NM_140042	PGRP-LF	XM_017078922	100%	0	80.2%
Imd pathway						
Attacin-A	CG10146	Attacin-A	XM_017073965	100%	8E-144	88.6%*
Attacin-B	NM_001169681	Attacin-A	XM_017073965	91%	3E-136	94.0%*
Attacin-C	NM_079005	Attacin-C	XM_017087554	86%	0	82.2%
Attacin-D	NM_079667	Attacin-A-like	XM_017084362	78%	1E-68	81.7%*
Cecropin-A1	CG1365	Cecropin-A2	XM_017083108	61%	5E-73	90.4%
Cecropin-A2	NM_079850	Cecropin-C-like	XM_017083110	100%	2E-27	74.6%*
Cecropin-B	NM_079851	Cecropin-B	XM_017083107	48%	4E-60	86.9%
Cecropin-C	NM_079852	Cecropin-A2	XM_017083108	52%	2E-53	85.6%
Diptericin-A	CG12763	Diptericin	XM_017085063	100%	3E-24	44.3%*
Diptericin-B	CG10794	Diptericin-A	XM_017085064	86%	2E-101	83.9%
Drosocin	NM_079020	Drosocin	XM_017074307	58%	3E-70	89.5%
Relish	NM_206467	Nuclear factor kappa-β p110	XM_017076711	61%	0	83.1%
Toll pathway						
Cactus	NM_057594	NF-kappa-B inhibitor cactus	XM_017087906	96%	0	87.6%
Defensin	CG1385	Defensin	XM_017074017	78%	2E-35	76.4%*
Drosomycin	CG10810	Drosomycin	XM_017070726	71%	1E-88	87.6%
GNBP3	NM_079262	GNB-BP3	XM_017078351	97%	0	85.0%
Metchnikowin	CG8175	Metchnikowin	XM_017085179	98%	3E-74	85.6%
Myd88	NM_001299317	Uncharacterized LOC	XM_017073595	95%	0	89.0%*
Spatzle	NM_170312	Spatzle-like protein	XM_017082745	57%	4E-175	76.7%
Jak/STAT						
Domeless	CG14226	Cytokine receptor	XM_017081527	87%	0	83.0%
Fondue	NM_165287	Fibroin heavy chain	XM_017089984	49%	5E-53	57.8%*
STAT 92E	NM_001275833	STAT	XM_017071568	90%	0	87.0%
Turandot-C	NM_080518	Turandot C-like	XM_017086062	100%	5E-66	68.2%*
Turandot-M	CG14027	Turandot M	XM_017076988	100%	6E-68	73.3%*
UPD-3	NM_001103544	Uncharacterized LOC108010411	XM_017075270	39%	1E-103	75.2%
Phenoloxidase						
PPO1	NM_057464	Phenoloxidase2 LOC108009339	XM_017073614	92%	0	85.7%
PPO2	NM_136599	Phenoloxidase2 LOC108009476	XM_017073856	96%	0	89.8%
PPO3	NM_080021	Phenoloxidase 3-like	XM_017072531	91%	0	79.9%
JNK						
Basket	CG5680	Stress-activated protein kinase	XM_017067534	99%	0	91.6%
dARK	NP_725638	Uncharacterized LOC108009795	XM_017074457	99%	0	82.9%
dRICE	NM_079827	Caspase (LOC108015517)	XM_017081980	96%	0	84.8%
Puckered	CG7850	Tyrosine-protein phosphatase	XM_017086030	83%	0	87.5%
Wengen	CG6531	Wengen	XM_017075346	81%	0	86.8%
TGF-β						
Dawdle	CG16987	Growth/differentiation factor 8	XM_017090335	91%	0	83.6%
Decapentaplegic	CG9885	Decapentaplegic	XM_017089893	95%	0	87.2%
Glass bottom boat	CG5562	Protein 60A	XM_017074586	99%	0	87.9%

Table S1. Relation of all *D. melanogaster* aligned genes involved in the immune pathways with the correspondent homolog in *D. suzukii*. Alignment parameters are shown as query cover, e-value and % of identity (%Id.). Those values marked (*) represent alignments only achieved using tBlastn algorithm while the other values were achieved using Blastn.

Table S2. Nucleotide sequence of primers used for q-PCR of *D. suzukii* selected genes.

Gene name	Access number		Primer
Cecropin	XM_017083110	Forward	TTTGTCGCCCTCATCTTGGC
		Reverse	GACATTGGCAGCTTGTTGGG
Diptericin	XM_017085064	Forward	TCTCCAGCTCCTTGGCCTAT
		Reverse	GACTCTGCCAAACTGGAGCA
PGRP-LC	XM_017079226	Forward	CGCCGACGGTTTCTATACGA
		Reverse	CCACCTGACGACTGATCACC
PGRP-LF	XM_017078922	Forward	ACCCACATCTTAAGCTGCCC
		Reverse	CGTCCCACGTAGACTTGACC
Defensin	XM_017074017	Forward	CTTTGGTGGCCTGTTTGGTG
		Reverse	CAGGCGGTATGGTTCCAGTT
Drosomycin	XM_017070727	Forward	CACCCAACAGGCTCTCTGAG
		Reverse	GACTCGTCCTTCCTCCTTGC
PPO1	XM_017073614	Forward	ACATGCACCACCAGATCCTG
		Reverse	GCATCTTCGCGGTTCACATC
PPO2	XM_017073856	Forward	TCCTTCCTGACCGATCGCTA
		Reverse	CGGAGCGCATGTTCATGAAG
Upd3	XM_017075270	Forward	GTACGCATCTCGACTGGGAG
		Reverse	TTGTCCACAGTGATCCTCGC
Turandot C	XM_017086062	Forward	TGCTTTGCACTGCTCCTGAT
		Reverse	CCCTTTCCTCAGGCGACAAA
Basket	XM_017067534	Forward	TTACAGCCAACCGTCCGAAA
		Reverse	CCTCGTCCACAGATATCCGC
Puckered	XM_017086030	Forward	CCGCACCTGAACAGTCCTAG
		Reverse	CCGCTGTCCACATCATCGTA
Daw	XM_017090335	Forward	GACCATAGCCATCCAGTCGG
		Reverse	TCATGATGAACGGCCGGTAG
Dpp	XM_017089893	Forward	CCCACCCATCTACCCAACAC
		Reverse	CGTGGCATGGCGGCTATATA
RpL32 60S	XM_017087128	Forward	GCCGCTTCAAGGGACAGTAT
		Reverse	GACGATCTCCTTGCGCTTCT

Table S3. Nucleotide sequence of primers used for q-PCR detection of *S. carpocapsae* and *X. nematophila* inside *D. suzukii* larvae.

Organism / Gene	Access number		Primer
S. carpocapsae 18S	LC157427	Forward	GAACGGCTCATTACAACGCC
		Reverse	AACGTATCGCCGGAACAAGT
X. nematophila 16S	DQ282116	Forward	GGGTGAGTAATGTCTGGGGATC
		Reverse	CCCACTTTACTCCCAAGAGGTC

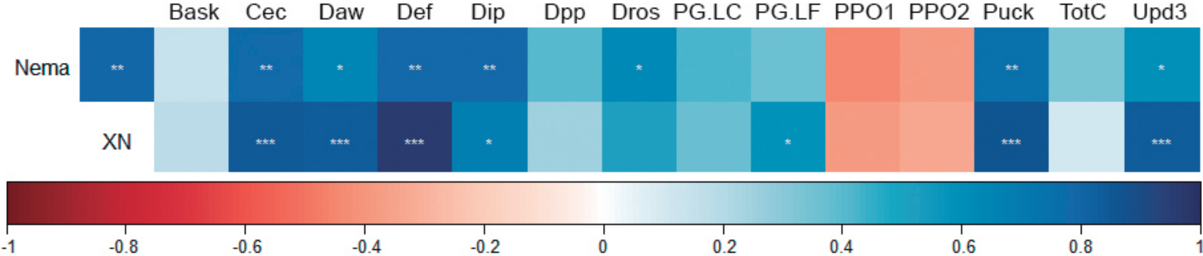


Fig. S1 Correlation matrix of ΔΔCT of Nema (18S *S. carpocapsae*), XN (16S *X. nematophila*) and *D. suzukii* genes: Bask (Basket), Cec (Cecropin), Daw (Dawdle), Def (Defensin), Dip (Diptericin), Dpp (Decapentaplegic), Dros (Drosomycin), PGLC (PGRP-LC), PGLF (PGRP-LF), PPO1, PPO2, TotC (Turandot C), Upd3. Correlation values are shown by color together with significance key ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

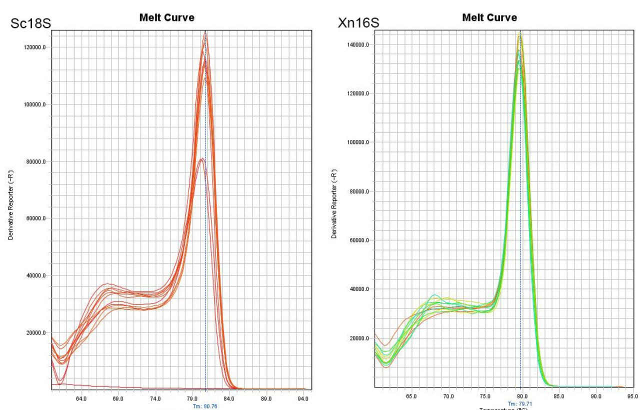
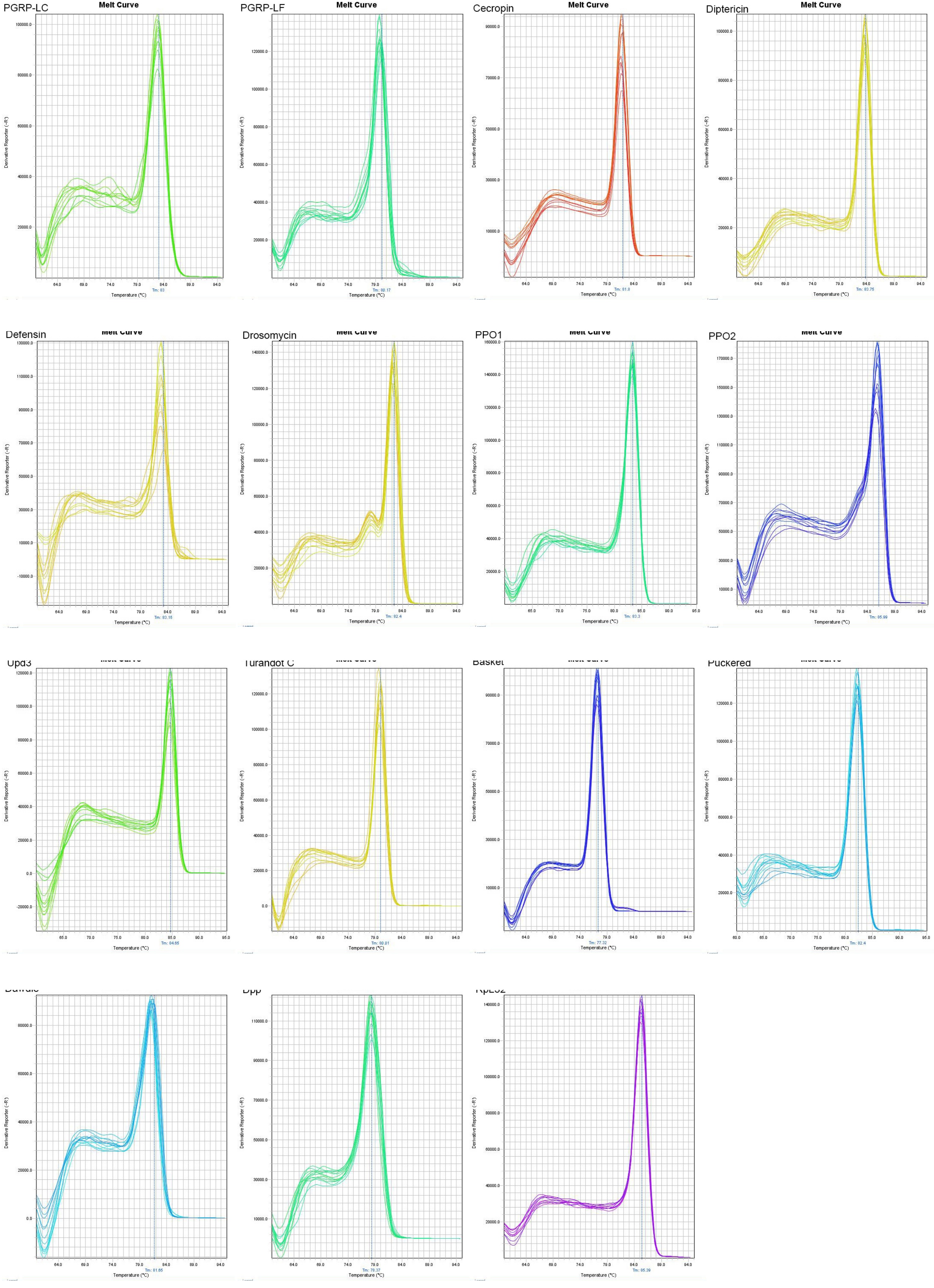


Fig. S2 Melt curve analysis of q-PCR reaction for each gene to test primer specificity: Sc18S (*S. carpocapsae*) and Xn16S (*X. nematophila*), continuing in the next page with *D. suzukii* genes: PGRP-LC, PGRP-LF, Cecropin, Diptericin, Defensin, Drosomycin, PPO1, PPO2, Upd3, Turandot C, Basket, Puckered, Dawdle, Dpp, RpL32).


PGRP-LC
Melt Curve
PGRP-LF
Melt Curve
Cecropin
Melt Curve
Diptericin
Melt Curve
Defensin
Drosomycin
PPO1
PPO2
Puckered

8. GENERAL DISCUSSION

“*An understanding of the natural world and what’s in it is a source of not only a great curiosity but great fulfillment.*”

David Attenborough

8.1 Biological control of *Drosophila suzukii*

For the development of a biological control strategy with EPNs is essential to determine the susceptible stages of the pest. For this reason, infectivity of *D. suzukii* was evaluated under laboratory conditions in the Chapter 3 and 4. Larvae and pupae assays were carried out in parallel with *D. melanogaster* to validate the methodology and compare the results with a well-known fly. The assessment of larvae was performed with second – third instar and the four EPN treatments achieved high infectivity rates which demonstrated the susceptibility of this stage. These results pointed that *D. suzukii* showed an EPNs sensitivity similar to other Drosophilid flies (Peña et al. 2015). Furthermore, *S. carpocapsae* exhibited the ability to infect *D. suzukii* larvae with short exposure time evidencing the infective potential of the nematode (Chapter 7). Aside from *S. carpocapsae*, *S. feltiae* is a tightly related nematode to dipteran parasitism and naturally isolated from larval stages (Peters 1996). Therefore, this nematode achieved the peak infection in larval assays, although IJs were unable to infect adult *D. suzukii*. During the course of this thesis, other researchers reached comparable findings when *D. suzukii* larvae were evaluated with EPNs under laboratory conditions (Cuthbertson and Audsley 2016; Hübner et al. 2017; Evans and Renkema 2020).

The first reports about pupae susceptibility of *D. suzukii* showed an infectivity with *H. bacteriophora* comparable to larval stage (Cuthbertson and Audsley 2016). However, in our assays, pupae remained uninfected under any nematode treatment despite the observed attraction of IJs described during the experiments. These results were corroborated also during the assays with teneral adults, as no pupae were found infected before adult emergence. In the development of *D. suzukii,* pupae became inaccessible for IJs to infect due to the cuticle hardening. The puparium presents only the spiracle holes which are too small to permit IJs entry along with the hard cuticle. In agreement, Hübner et al. (2017) also provided findings with no infection of *D. suzukii* pupae along their experiments. The lack of susceptibility of this stage could lead to a combined application of other BCA to target the pupae.

The adult stage of *D. suzukii* was less studied as a target for biological control, while larvae were tested several times. *Drosophila suzukii* executes the post-ecdysis process near the puparium in a resting status comparable to *D. melanogaster* development process (Peabody and White 2013). Along with the evidence gathered from the EPNs performance during adult's assay, only *S. carpocapsae* displayed the potentiality to infect teneral adults whose vulnerability resulted in a massive infection. Previous observations pointed to the high infectivity of *S. carpocapsae* during the ecdysis process of *Blattella germanica* L. (Blattodea: Blattellidae) (Garcia-del-Pino and Morton 2001). After ecdysis completion, mature adults were only infected by *S. carpocapsae* with lower rates than the teneral period. This nematode was already reported to display an increased infectivity to adult stages of *Rhagoletis indifferens* (Diptera: Tephritidae), *Ceratitis capitata* (Diptera: Tephritidae) or *T. absoluta* (Yee and Lacey 2003; Malan and Manrakhan 2009; Garcia-

del-Pino et al. 2013). Besides the infectivity rate, the reproductive potential exhibits great variations in micro-insects (less than 5 mm) due to resource scarcity to nourish nematode offspring (Bastidas et al. 2014). Indeed, *Drosophila suzukii* is considered a micro-insect (Walsh et al. 2011), although larvae and adults were sufficient to carry on nematode reproduction and develop offspring.

The determination of susceptible stages led to the development of two preliminary experiments based on potential field application of EPNs. The nematode treatment of fruits was already approached in experiments with *D. suzukii* with low infection results (Cuthbertson et al. 2014; Woltz et al. 2015). Thus, a strawberry assay was designed to target the larval stage before the pupation in Chapter 3. *Steinernema feltiae* and *S. carpocapsae* decreased adult emergence from fruit with a positive correlation of the IJs dose. Despite the encouraging results, the recorded efficacy requires more research to improve infection rate. In consideration, Hübner et al. (2017) suggested that acidic pH of these fruits could intervene in the nematode infective process and reduce the efficacy of fruit treatment. The other prospected approach was the soil application of EPNs to target *D. suzukii* teneral adults (Chapter 4). The design was inspired by previous experiments with *S. exigua* and *T. absoluta* in which *S. carpocapsae* achieved a high infection rate of emerged adults from soil (Kaya and Grieve 1982; Garcia-del-Pino et al. 2013). The addition of distinct levels in the columns permitted us to observe the effect of nematode infection along the adult dispersion from soil. The post-ecdysis process of *D. suzukii* barely took 2 h, even though *S. carpocapsae* managed to massively infect these teneral adults in the soil level. Interestingly, the number of IJs limited the adult mobility but a percentage of adults flew with enough IJs to permit *S. carpocapsae* reproduction. Through further research of this soil application, nematode efficacy in adult reduction could be ascertained and also explore the possible dispersion of nematodes though *D. suzukii* adults.

The environment of EPNs application involves a microhabitat composed by the affected crop, the pest but also other NE which could be implicated in the control of the pest too. Considering the variations of EPNs susceptibility through *D. suzukii* stages, a combined use of BCA could enrich a strategy to cover all the biological cycle of the fly. Nevertheless, few studies had deepened into these relations with EPNs, despite the importance of establishing compatibility relations among BCA (Jandricic et al. 2006; Lordan et al. 2014; Tourtois and Grieshop 2015). Nematode compatibility with *D. suzukii* predator and parasitoid species was prospected to evaluate their possible coexistence (Chapter 5). The plant pot assay simulated the conditions of foliar application of IJs that involve a high concentration of nematode on the plant surface in the subsequent hours after application. Although, when NE enjoyed space to avoid IJs, *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* were considered harmless as there were no infected insects. In consequence, combined applications of EPNs with the tested species could be implemented without negative impact to NE insects. To pursue more complex biological control strategies the knowledge of compatibility and synergic effects among BCA need to be determined in advance to exploit all assets.

In the light of the exposed results in this section, EPNs have the potential to control *D. suzukii* in larval and adult stages due to high infectivity rates. Besides, fruit and soil assays provided hints which should be expanded with further research as these trials serve as a baseline to design applied field experiments in the future. *Steinernema carpocapsae* presented the major versatility to infect either larvae and adults in fruit or soil applications. For the fruit treatment, it would be necessary to study with different crops affected by *D. suzukii* while in soil treatments, the application time is the key to mass infect teneral adults. Nematode doses should be adjusted depending on field efficacy. Besides, considering the results of pupal parasitoids and the compatibility shown in our assay, a combined release of these agents would provide a wide strategy to control the fly. The knowledge developed in this thesis provides a solid and consistent understanding of *D. suzukii* susceptibility to EPNs from which a biological control strategy could be designed after field experiments.

8.2 Immune response to infective process

In the infective process of any parasitic species, the immune response of the host embodies the main shield against pathogen development. The involvement of *D. suzukii* immune system and the nematode-bacterial complex remained unclear after susceptibility assays. Thus, due to observed differences in the immune system between *D. suzukii* and *D. melanogaster* (Kacsoh and Schlenke 2012), Chapters 6 and 7 of this thesis pursued to describe the immune response developed after EPNs infection which comprise effector mechanisms and regulatory system. For the development of the immune system is fundamental the recognition of non-self to activate the effector response through the signaling pathways (Lemaitre and Hoffmann 2007). Nevertheless, evidence exposed in both chapters supported the hypothesis that the success of nematode infection was achieved as a result of an evasive strategy to the recognition process, as discussed in detail below.

The melanization reaction is an immediate response to pathogen entry before the whole hemocyte activation. In *D. melanogaster*, infection with entomopathogenic bacteria *X. nematophila* and *P. luminiscens* was reported to inhibit the enzymatic activity of PO to prevent the melanization response (Brivio et al. 2002; Peña et al. 2015). This inhibition was demonstrated also in *D. suzukii* as larvae infected by *X. nematophila* showed low levels of PO activity in hemolymph. Moreover, the expression of both proPO genes make it difficult to consider that a melanization response was triggered due to *S. carpocapsae* infection since expression remained constant. Besides, the inhibition of proPO reaction was consistent with the recount of crystal cells which remained constant up to 20 h of infection. Indeed, *S. carpocapsae* and *X. nematophila* presented their own inhibitory mechanism to counter the melanization response from early to late infection.

Hemocytes accomplish a crucial role against nematode-bacterial infections with the encapsulation and phagocytosis of pathogens. However, the conducted specific assays confirmed the complete overcome of *S. carpocapsae* and *X. nematophila* to cellular response. The non-recognition of IJs was noticeable since Upd3 receptor was only upregulated after bacterial release while the effector

gene Turandot C remained constant in agreement with the lack of cellular activation registered during the effector experiments. After *S. carpocapsae* infection, lamellocytes differentiation was observed in early infection, although it represented an infimum increase compared to the amount reported in *D. suzukii* during parasitoid infection (Poyet et al. 2013). The absence of nematode recognition was manifested too through *In vitro* assays where hemocytes could not even encapsulate dead IJs. Therefore, there was a passive involvement of *S. carpocapsae* cuticle to avoid encapsulation as described in other insects (Mastore and Brivio 2008; Mastore et al. 2015). Cellular response against *X. nematophila* also failed in the phagocytosis and nodulation of bacteria in hemolymph. However, entomopathogenic bacteria took a direct correlation with the upregulation of humoral pathways and the subsequent AMPs increment. Before the bacterial release, these gene expressions barely changed with nematode presence. This peptide increase was detected through electrophoresis of larval hemolymph after 24 h of bacterial infection. However, *X. nematophila* demonstrated a modulatory process to disable these peptides and download their antimicrobial activity. This bacterial action is usually supported with the secreted products of *S. carpocapsae* which exhibit toxic activity although no assessment was made along our assays (Laumond et al. 1989).

The direct research with a pest insect instead of model species provides reliable results that have direct impact to field application. In the genetic background, high levels of homology with *D. melanogaster* were reported in the main base of the immune pathways of *D. suzukii* which reflect a strong conservation to avoid posterior mutations. In contrast, genes encoding the effector mechanisms revealed more changes among species that could hint to ecological adaptations following each *Drosophila* nature (Early and Clark 2017). *Drosophila suzukii* was reported to have genetic variations compared to close relative *Drosophila* that facilitate the adaptation to new regions and resist a wider number of NE compared to *D. melanogaster* (Ometto et al. 2013). In consequence, direct research with pest insects provides accurate results to assure a successful EPNs strategy for biological control.

In the evaluation of the immune system, genetic analysis brought light into some effective processes which could not be tested due to technical and biological incompatibilities. The integration of both studied levels permitted us to elaborate a global picture of the infection process, although there are complex processes modified by EPNs infection which are still far from understood. The combination of techniques yielded complementary approaches to comprehend the vastness of the infective process. Indeed, to continue the genetic prospect of the infection, a comparative analysis of the complete transcriptome is still underdevelopment from larval samples of Chapter 7. This analysis will provide a wider view of up and down regulation of genes after nematode-bacterial infection. These results could verify some variations detected through qRT-PCR or detect new modulations of other genes in the analyzed pathways or in complementary ones. With the complete transcriptome image, further research could deepen in the pathways that presented a higher involvement with EPNs such as Jak/STAT or pro-PO to comprehend the modulation mechanism

employed by *S. carpocapsae*. In addition, a subsequent analysis could also entail a detailed study of AMPs from receptor activation to hemolymph peptides in relation to *X. nematophila* up to 24 h of infection. A better understanding of EPNs strategies upon insect infection could help to design biological control strategies considering insect resistance and adequate the nematode species in consideration. The study of *S. feltiae, H. bacteriophora* and other EPNs may provide different strategies to deal with the immune system. Furthermore, with a complete knowledge of the insect – nematode interaction, researchers could explore the use of molecules, enzymes or concrete genes to enhance nematode infection and overcome insect resistance.

9. CONCLUSIONS

"*Conservation is a state of harmony between men and land.*"

Aldo Leopold

1. Susceptibility of *Drosophila suzukii* to entomopathogenic nematodes was evaluated by developmental stages to determine the suitable stage for biological control. Our assays demonstrated a high susceptibility of the larval stage to *Steinernema feltiae, Steinernema carpocapsae* and *Heterorhabditis bacteriophora* under laboratory conditions, while these EPNs were unable to infect *Drosophila suzukii* pupae.

2. Nematode infection in *Drosophila suzukii* mature adults was limited to *Steinernema carpocapsae*. Moreover, the highest infection rate was registered in teneral adults as the post-ecdysis process favored the nematode entry.

3. *Steinernema feltiae* and *Steinernema carpocapsae* reproduced on infected larvae providing a new generation of IJs, although only the later was able to reproduce in the adult stage of *Drosophila suzukii*.

4. The preliminary fruit assay focused on the larval stage showed that *Steinernema feltiae* and *Steinernema carpocapsae* decreased the emergence of the fly. The achieved efficacy was limited and thus, further research should be done to develop an efficient fruit application.

5. The preliminary soil application of *Steinernema carpocapsae* caused high rates of infectivity on teneral adults during the dispersion process under laboratory conditions. Even though nematode load limited the flight ability of *Drosophila suzukii,* 21% of the adults flew with infective juveniles promoting the nematode dispersion. The application time is predicted as a limiting factor for a strategy based on teneral adults due to the short time to infect that entails the stage.

6. The combined application of natural enemies and entomopathogenic nematodes evidenced a viable compatibility with *Steinernema feltiae* and *Heterorhabditis bacteriophora* even in constant contact in Petri dish experiments, while *Steinernema carpocapsae* infected adult predators in these conditions. Nonetheless, in plant conditions this nematode displayed an appropriate compatibility with non-infection of the beneficial insects.

7. Regarding the pathogen-host relation, the success of *Steinernema carpocapsae* infection was based on the avoidance and modulation of the immune system of *Drosophila suzukii*. In the early phase of infection, the immune system presented no upregulation of the signaling pathways which evidenced a lack of recognition of infective juveniles. The analysis of gene expression variations and evaluation of the effector mechanisms permitted to weave an integrative vision of the infection process.

8. The melanization response was not triggered by *Steinernema carpocapsae* infection as indicated by the absence of upregulation of proPO genes. In accordance, the number of crystal cells was constant after 20 h of infection. Besides, *Xenorhabdus nematophila* decreased the enzymatic activity of PO in hemolymph of *Drosophila suzukii.*

9. The cellular response of *Drosophila suzukii* was unable to phagocyte IJs which avoided the recognition of hemocytes through passive involvement of the cuticula. Lamellocyte differentiation was only observed in infimum numbers that corresponded to a constant expression of Turandot C. Only the interleukin Upd3 was upregulated at 14 h of infection but there was no activation of effector genes. In addition, plasmatocytes were unable to phagocyte *Xenorhabdus nematophila.*

10. The humoral response of *Drosophila suzukii* exhibited an acute correlation with bacterial pressure. After the bacterial release, Imd and Toll pathways were activated through peptidoglycan recognition and followed by an important upregulation of AMPs. However, *Xenorhabdus nematophila* displayed a mechanism to decrease the antimicrobial activity of these peptides once in hemolymph.

10. REFERENCES

Alatorre-Rosas R, Kaya HK (1990) Interspecific competition between entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* for an insect host in sand. J Invert Pathol 55:179–188

Altalba C, Alòs C, Alvà V, et al (1991) Invertebrats no artròpodes. In: Ros JD (ed) Història natural dels Països Catalans. Enciclopèdia Catalana, Barcelona, Spain.

Arnó J, Solà M, Riudavets J, Gabarra R (2016) Population dynamics, non-crop hosts, and fruit susceptibility of *Drosophila suzukii* in Northeast Spain. J Pest Sci (2004) 89:713–723. https://doi.org/10.1007/s10340-016-0774-3

Asplen MK, Anfora G, Biondi A, et al (2015) Invasion biology of spotted wing Drosophila (*Drosophila suzukii*): a global perspective and future priorities. J Pest Sci (2004) 88:469–494.

Bale JS, Van Lenteren JC, Bigler F (2008) Biological control and sustainable food production. Philos Trans R Soc B Biol Sci 363:761–776. https://doi.org/10.1098/rstb.2007.2182

Bastidas B, Portillo E, San-Blas E (2014) Size does matter: The life cycle of *Steinernema spp.* in micro-insect hosts. J Invertebr Pathol 121:46–55. https://doi.org/10.1016/j.jip.2014.06.010

Batalla-Carrera L, Morton A, Garcia-del-Pino F (2013) Field efficacy against the hazelnut weevil, *Curculio nucum* and short-term persistence of entomopathogenic nematodes. Spanish J Agric Res 11:1112–1119. https://doi.org/10.5424/sjar/2013114-4210

Bedding R., Molyneux A. (1982) Penetration of insect cuticle by infective juveniles of *Heterorhabditis spp.* (Heterorhabditidae: Nematoda). Nematologica 28:354–359

Bezerra Da Silva CS, Park KR, Blood RA, Walton VM (2019) Intraspecific competition affects the pupation behavior of spotted-wing drosophila (*Drosophila suzukii*). Sci Rep 9:1–9. https://doi.org/10.1038/s41598-019-44248-6

Bienkowski AO, Orlova-Bienkowskaja MJ (2020) Invasive agricultural pest *Drosophila suzukii* (Diptera, Drosophilidae) appeared in the Russian Caucasus. Insects 11:1–7. https://doi.org/10.3390/insects11110826

Binda-Rossetti S, Mastore M, Protasoni M, Brivio MF (2016) Effects of an entomopathogen nematode on the immune response of the insect pest red palm weevil: Focus on the host antimicrobial response. J Invertebr Pathol 133:110–119. https://doi.org/10.1016/j.jip.2015.11.001

Bird A, Akhurst R (1983) The nature of the intestinal vesicle in nematodes of the family Steinernematidae. Int J Parasitol 13:599–606

Boemare NE (2002) Biology, taxonomy, and systematics of *Photorhabdus* and *Xenorhabdus*. In: Gaugler R (ed) Entomopathogenic nematodology. CABI International Wallingford-UK, pp35–56

Boemare NE, Laumond C, Mauléon H (1996) The nematode-bacterium complexes: biology, life cycle, and vertebrate safety. Biocontrol Sci Techn 6:333–345

Bonneau P, Renkema JM, Fournier V, Firlej A (2019) Ability of *Muscidifurax raptorellus* and other parasitoids and predators to control *Drosophila suzukii* populations in raspberries in the laboratory. Insects 3:68

Boughdad A, Haddi K, El Bouazzati A, et al (2020) First record of the invasive spotted wing *Drosophila* infesting berry crops in Africa. J Pest Sci (2004). https://doi.org/10.1007/s10340-020-01280-0

Bourne A, Fountain MT, Wijnen H, Shaw B (2019) Potential of the European earwig (*Forficula auricularia*) as a biocontrol agent of the soft and stone fruit pest *Drosophila suzukii*. Pest Manag Sci 75:3340–3345. https://doi.org/10.1002/ps.5459

Brivio MF, Mastore M (2018) Nematobacterial complexes and insect hosts: Different weapons for the same war. Insects 9. https://doi.org/10.3390/insects9030117

Brivio MF, Pagani M, Restelli S (2002) Immune suppression of *Galleria mellonella* (Insecta, Lepidoptera) humoral defenses induced by *Steinernema feltiae* (Nematoda, Rhabditida): Involvement of the parasite cuticle. Exp Parasitol 101:149–156. https://doi.org/10.1016/S0014-4894(02)00111-X

Bruck DJ, Bolda M, Tanigoshi L, et al (2011) Laboratory and field comparisons of insecticides to reduce infestation of *Drosophila suzukii* in berry crops. Pest Manag Sci 67:1375–1385. https://doi.org/10.1002/ps.2242

Bulet P, Stöcklin R (2005) Insect antimicrobial peptides: Structures, properties and gene regulation. Protein Pept Lett 12:3–11

Calabria G, Máca J, Bächli G, et al (2012) First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. J Appl Entomol 136:139–147. https://doi.org/10.1111/j.1439-0418.2010.01583.x

Campbell JF, Gaugler R (1997) Inter-specific variation in entomopathogenic nematode foraging strategy: dichotomy or variation along a continuum? Fundam Appl Nematol 20:393–398

Campbell JF, Lewis EE, Stock SP, et al (2003) Evolution of host search strategies in entomopathogenic nematodes (Nematoda: Steinernematidae). J Nematol 35:142–145

Castillo JC, Reynolds SE, Eleftherianos I (2011) Insect immune responses to nematode parasites. Trends Parasitol 27:537–547. https://doi.org/10.1016/j.pt.2011.09.001

Chabert S, Allemand R, Poyet M, et al (2012) Ability of European parasitoids (Hymenoptera) to control a new invasive Asiatic pest, *Drosophila suzukii*. Biol Control 63:40–47. https://doi.org/10.1016/j.biocontrol.2012.05.005

Chakraborty S (2013) Migrate or evolve: options for plant pathogens underclimate change. Glob Chang Biol 19:1985–2000

Chambers U, Bruck DJ, Olsen J, Walton VM (2010) Control of overwintering filbertworm (Lepidoptera: Tortricidae) larvae with *Steinernema carpocapsae*. J Econ Entomol 103:416–422. https://doi.org/10.1603/EC09255

Chandler D, Bailey AS, Mark Tatchell G, et al (2011) The development, regulation and use of biopesticides for integrated pest management. Philos Trans R Soc B Biol Sci 366:1987–1998. https://doi.org/10.1098/rstb.2010.0390

Chapman D, Purse B V., Roy HE, Bullock JM (2017) Global trade networks determine the distribution of invasive non-native species. Glob Ecol Biogeogr 26:907–917. https://doi.org/10.1111/geb.12599

Chattopadhyay A, Bhatnagar N., Bhatnagar R (2004) Bacterial insecticidal toxins. Crit Rev Microbiol 30:33–54

Ciche TA, Ensign JC (2003) For the insect pathogen *Photorhabdus luminescens*, which end of a nematode is out? Appl Env Microbiol 69:1890–1897

Cini A, Ioriatti C, Anfora G (2012) A review of the invasion of *Drosophila suzukii* in Europe and a draft research agenda for integrated pest management. Bull Insectology 65:149–160

Cuthbertson AGS, Audsley N (2016) Further screening of entomopathogenic fungi and nematodes as control agents for *Drosophila suzukii*. Insects 7:24. https://doi.org/10.3390/insects7020024

Cuthbertson AGS, Collins DA, Blackburn LF, et al (2014) Preliminary screening of potential control products against *Drosophila suzukii*. Insects 5:488–498. https://doi.org/10.3390/insects5020488

Daane KM, Wang X-G, Biondi A, et al (2016) First exploration of parasitoids of *Drosophila suzukii* in South Korea as potential classical biological agents. J Pest Sci (2004) 89:823–835. https://doi.org/10.1007/s10340-016-0740-0

Dam D, Molitor D, Beyer M (2019) Natural compounds for controlling *Drosophila suzukii*. A review. Agron Sustain Dev 39:. https://doi.org/10.1007/s13593-019-0593-z

De la Vega GJ, Corley JC, Soliani C (2020) Genetic assessment of the invasion history of *Drosophila suzukii* in Argentina. J Pest Sci (2004) 93:63–75. https://doi.org/10.1007/s10340-019-01149-x

De Ros G, Anfora G, Grassi A, Ioriatti C (2013) The potential economic impact of *Drosophila suzukii* on small fruits production in Trentino (Italy). IOBC/WPRS Bull 91:317–321

Deprá M, Poppe JL, Schmitz HJ, et al (2014) The first records of the invasive pest *Drosophila suzukii* in the South American continent. J Pest Sci (2004) 87:379–383. https://doi.org/10.1007/s10340-014-0591-5

Diepenbrock LM, Swoboda-Bhattarai KA, Burrack HJ (2016) Ovipositional preference, fidelity, and fitness of *Drosophila suzukii* in a co-occurring crop and non-crop host system. J Pest Sci (2004) 89:761–769. https://doi.org/10.1007/s10340-016-0764-5

Dix I, Burnell A., Griffin CT, et al (1992) The identification of biological species in the genus *Heterorhabditis* (Nematoda: Heterorhabditidae) by cross breeding second-generation amphimictic adults. Parasitology 104:509–518

Dowds BCA, Peters A (2002) Virulence mechanisms. In: Gaugler R (ed) Entomopathogenic nematodology. CABI, New York, EE.UU., pp 79–98

Early AM, Clark AG (2017) Genomic signatures of local adaptation in the *Drosophila* immune response. Fly (Austin) 11:277–283. https://doi.org/10.1080/19336934.2017.1337612

Ehlers RU (2001) Mass production of entomopathogenic nematodes for plant protection. Appl Microbiol Biotechnol 56:623–633. https://doi.org/10.1007/s002530100711

Ehlers RU, Hokkanen HMT (1996) Insect biocontrol with non-endemic entomopathogenic nematodes *Steinernema* and *Heterorhabditis spp.*: conclusions and recommendations of a combined OECD and COST workshop on scientific and regulatory policy issues. Biocontrol Sci Techn 6:295–302

Eleftherianos I, Shokal U, Yadav S, et al (2016) Insect immunity to entomopathogenic nematodes and their mutualistic bacteria. Life Sci J 6:23–27. https://doi.org/10.1007/82

Ennis D, Dillon AB, Griffin CT (2010) Simulated roots and host feeding enhance infection of subterranean insects by the entomopathogenic nematode *Steinernema carpocapsae*. J Invert Pathol 103:140–143

EPPO (2021) *Drosophila suzukii* (DROSSU) reporting service articles. In: EPPO Glob. Database. https://gd.eppo.int/taxon/DROSSU/reporting. Accessed 22 Feb 2021

European Parliament (2009) Directive 2009/128/Ce. Off J Eur Union 71–86

Evans BG, Renkema JM (2020) Initial tests of *Steinernema riobrave* against spotted wing drosophila, *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) and comparison to other entomopathogenic nematode species in the laboratory. Biocontrol Sci Technol 0:1–7. https://doi.org/10.1080/09583157.2020.1846682

Fleury F, Gibert P, Ris N, Allemand R (2009) Ecology and life history evolution of frugivorous *Drosophila* parasitoids. Adv Parasitol 70:3–44. https://doi.org/10.1016/S0065-308X(09)70001-6

Forst S, Clarke DJ (2002) Bacteria-nematode symbiosis. In: Gaugler R (ed) Entomopathogenic nematodology. Entomopathogenic nematology. CABI, New York, EE.UU., pp 57–77

Gabarra R, Riudavets J, Rodríguez GA, et al (2015) Prospects for the biological control of *Drosophila suzukii*. BioControl 60:331–339. https://doi.org/10.1007/s10526-014-9646-z

Garcia-del-Pino F, Alabern X, Morton A (2013) Efficacy of soil treatments of entomopathogenic nematodes against the larvae, pupae and adults of *Tuta absoluta* and their interaction with the insecticides used against this insect. BioControl 58:723–731. https://doi.org/10.1007/s10526-013-9525-z

Garcia-del-Pino F, Morton A (2001) Los nematodos entomopatógenos para el control biológico de la cucaracha alemana, *Blattella germanica* (Dictyoptera: Blattellidae). Proc III Congr Nac Entomol Apl Pamplona Spain 122

Gaugler R, Wang Y, Campbell JF (1994) Aggressive and evasive behaviors in *Popillia japonica* (Coleoptera, Scarabaeidae) larvae—defenses against entomopathogenic nematode attack. J Invertebr Pathol 64:193–199

Georgis R (1990) Formulation and application technology. In: Gaugler R, Kaya HK (eds) Entomopathogenic Nematodes in Biological Control. RC Press, Boca Raton, Florida, EE.UU., pp 173–194

Georgis R, Koppenhöfer AM, Lacey LA, et al (2006) Successes and failures in the use of parasitic nematodes for pest control. Biol Control 38:103–123. https://doi.org/10.1016/j.biocontrol.2005.11.005

Glaser R (1932) Studies on *Neoaplectana glaseri*, a nematode parasite of the Japanese beetle (*Popilia japonica*). New Jersey Dep Agric Circ 211:34

Glazer I (2002) Survival biology. In: Gaugler R (ed) Entomopathogenic nematodology. CABI, New York, EE.UU., pp 169–187

Goetsch M, Owen H, Goldman B, Frost SDW (2006) Analysis of the PixA inclusion body protein of *Xenorhabdus nematophila*. J Bacteriol 188:2706–2710

Goodhue RE, Bolda M, Farnsworth D, et al (2011) Spotted wing drosophila infestation of California strawberries and raspberries: economic analysis of potential revenue losses and control costs. Pest Manag Sci 67:1396–1402. https://doi.org/10.1002/ps.2259

Goodrich-Blair H, Clarke DJ (2007) Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: Two roads to the same destination. Mol Microbiol 64:260–268. https://doi.org/10.1111/j.1365-2958.2007.05671.x

Gress BE, Zalom FG (2019) Identification and risk assessment of spinosad resistance in a California population of *Drosophila suzukii*. Pest Manag Sci 75:1270–1276. https://doi.org/10.1002/ps.5240

Griffin CT (2012) Perspectives on the behavior of entomopathogenic nematodes from dispersal to reproduction: traits contributing to nematode fitness and biocontrol efficacy. J Nematol 44:77–84

Griffin CT, Boemare NE, Lewis EE (2005) Biology and behaviour. In: Grewal PS, Ehlers RU, Shapiro-Ilan DI (eds) Nematodes as Biological Control. CABI International, pp 47–64

Hallem EA, Dillman AR, Hong A, et al (2011) A sensory code for host seeking in parasitic nematodes. Curr Biol 21:377–383

Hauser M (2011) A historic account of the invasion of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) in the continental United States, with remarks on their identification. Pest Manag Sci 67:1352–1357. https://doi.org/10.1002/ps.2265

Haye T, Girod P, Cuthbertson AGS, et al (2016) Current SWD IPM tactics and their practical implementation in fruit crops across different regions around the world. J Pest Sci (2004) 89:643–651. https://doi.org/10.1007/s10340-016-0737-8

Head J, Palmer LF, Walters KFA (2003) The compatibility of control agents used for the control of the South American leafminer, *Liriomyza huidobrensis*. Biocontrol Sci Technol 13:77–86. https://doi.org/10.1080/0958315021000054403

Herbert E, Goodrich-Blair H (2007) Friend and foe: The two faces of *Xenorhabdus nematophila*. Nat Rev Microbiol 5:634–646

Hominick WM (2002) Biogeography. In: Gaugler R (ed) Entomopathogenic nematodology. CABI Publishing, Wallingford, Gran Bretaña, pp 115–143

Hossard L, Philibert A, Bertrand M, et al (2014) Effects of halving pesticide use on wheat production. Sci Rep 4:1–7. https://doi.org/10.1038/srep04405

Hübner A, Englert C, Herz A (2017) Effect of entomopathogenic nematodes on different developmental stages of *Drosophila suzukii* in and outside fruits. BioControl 62:669–680. https://doi.org/10.1007/s10526-017-9832-x

Hultmark D (1996) Insect lysozymes. EXS 75:87–102

Jagdale GB, Casey ML, Grewal PS, Lindquist RK (2004) Application rate and timing, potting medium, and host plant effects on the efficacy of *Steinernema feltiae* against the fungus gnat, *Bradysia coprophila*, in floriculture. Biol Control 29:296–305. https://doi.org/10.1016/S1049-9644(03)00164-6

Jandricic S, Scott-Dupree CD, Broadbent AB, et al (2006) Compatibility of *Atheta coriaria* with other biological control agents and reduced-risk insecticides used in greenhouse floriculture integrated pest management programs for fungus gnats. Can Entomol 138:712–722

Johnigk S, Ehlers RU (1999) Endotokia matricida in hermaphrodites of *Heterorhabditis spp.* and the effect of the food supply. Nematology 1:717–726

Kacsoh BZ, Schlenke TA (2012) High hemocyte load is associated with increased resistance against parasitoids in *Drosophila suzukii*, a relative of *D. melanogaster*. PLoS One 7:. https://doi.org/10.1371/journal.pone.0034721

Kaneshiro K (1983) *Drosophila* (Sophophora) *suzukii* (Matsumura). Proc Hawaiian Entomol Soc 24:179

Kanzawa T (1939) Studies on *Drosophila suzukii* Matsumura. Yamanashi Agric Exp Station Kofu

Kawase S, Uchino K, Yasuda M, Motoori S (2008) Netting control of cherry drosophila *Drosophila suzukii* injurious to blueberry. Bull Chiba Prefect Agric Res Cent 7:9–15

Kaya HK, Grieve BJ (1982) The nematode *Neoaplectana carpocapsae* and the beet armyworm *Spodoptera exigua*: Infectivity of prepupae and pupae in soil and of adults during emergence from soil. J Invertebr Pathol 39:192–197. https://doi.org/10.1016/0022-2011(82)90009-X

Kaya HK (1985) Entomogenous nematodes for insect control in IPM systems. In: Hoy M., Herzog DC (eds) Biological Control in Agricultural IPM Systems. Academic Press, London, UK, pp 183–302

Kaya HK, Gaugler R (1993) Entomopathogenic nematodes. Ann Rew Entomol 38:181–206

Kenis M, Tonina L, Eschen R, et al (2016) Non-crop plants used as hosts by *Drosophila suzukii* in Europe. J Pest Sci (2004) 89:735–748. https://doi.org/10.1007/s10340-016-0755-6

Koppenhöfer AM (2007) Bacterial symbionts of *Steinernema* and *Heterorhabditis*. In: Nguyen K., Hunt D. (eds) Nematology Monographs and Perspectives. Brill, Leiden, Holanda, pp 735–808

Koppenhöfer AM, Shapiro-Ilan DI, Hiltpold I (2020) Entomopathogenic nematodes in sustainable food production. Front Sustain Food Syst 4:1–14. https://doi.org/10.3389/fsufs.2020.00125

Lacey LA, Grzywacz D, Shapiro-Ilan DI, et al (2015) Insect pathogens as biological control agents: Back to the future. J Invertebr Pathol 132:1–41. https://doi.org/10.1016/j.jip.2015.07.009

Lamichhane JR, Barzman M, Booij K, et al (2015) Robust cropping systems to tackle pests under climate change. A review. Agron Sustain Dev 35:443–459. https://doi.org/10.1007/s13593-014-0275-9

Laumond C, Simões N, Boemare NE (1989) Toxins of entomoparasitic nematodes. Pathogenicity of *Steinernema carpocapsae*–prospectives of genetic engineering. C R Acad Agric Fr 75:135–138

Lavine M., Strand MR (2002) Insect hemocytes and their role in immunity. Insect Biochem Mol Biol 32:1295–1309

Leach H, Moses J, Hanson E, et al (2018) Rapid harvest schedules and fruit removal as non-chemical approaches for managing spotted wing *Drosophila*. J Pest Sci (2004) 91:219–226. https://doi.org/10.1007/s10340-017-0873-9

Lee JC, Bruck DJ, Curry H, et al (2011a) The susceptibility of small fruits and cherries to the spotted-wing drosophila, *Drosophila suzukii*. Pest Manag Sci 67:1358–1367

Lee JC, Bruck DJ, Dreves AJ, et al (2011b) In Focus: Spotted wing drosophila, *Drosophila suzukii*, across perspectives. Pest Manag Sci 67:1349–1351. https://doi.org/10.1002/ps.2271

Lee JC, Wang X, Daane KM, et al (2019) Biological control of spotted-wing *Drosophila* (Diptera: Drosophilidae) - current and pending tactics. J Integr Pest Manag 10. https://doi.org/10.1093/jipm/pmz012

Lehmann P, Ammunét T, Barton M, et al (2020) Complex responses of global insect pests to climate warming. Front Ecol Environ 18:141–150. https://doi.org/10.1002/fee.2160

Lei Z, Rutherford T., Webster JM (1992) *Heterorhabditid* behavior in the presence of the cabbage maggot, *Delia radicum*, and its host plants. J Nematol 24:9–15

Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25:697–743. https://doi.org/10.1146/annurev.immunol.25.022106.141615

Lewis EE, Campbell J, Griffin C, et al (2006) Behavioral ecology of entomopathogenic nematodes. Biol Control 38:66–79. https://doi.org/10.1016/j.biocontrol.2005.11.007

Li XY, Cowles RS, Cowles EA, et al (2007) Relationship between the successful infection by entomopathogenic nematodes and the host immune response. Int J Parasitol 37:365–374. https://doi.org/10.1016/j.ijpara.2006.08.009

Little CM, Little CM, Chapman TW, Hillier NK (2020) Plasticity is key to success of *Drosophila suzukii* (Diptera: Drosophilidae) invasion. J Insect Sci 20:. https://doi.org/10.1093/jisesa/ieaa034

Lordan J, Alegre S, Alins G, et al (2014) Compatibility between *Forficula auricularia* and entomopathogenic nematodes to be used in pome fruit pest management. J Appl Entomol 138:635–643. https://doi.org/10.1111/jen.12118

Malan AP, Manrakhan A (2009) Susceptibility of the Mediterranean fruit fly (*Ceratitis capitata*) and the Natal fruit fly (*Ceratitis rosa*) to entomopathogenic nematodes. J Invertebr Pathol 100:47–49. https://doi.org/10.1016/j.jip.2008.09.007

Martens E., Heungens K, Goodrich-Blair H (2003) Early colonization events in the mutualistic association between *Steinernema carpocapsae* nematodes and *Xenorhabdus nematophila* bacteria. J Bacteriol 185:3147–3154

Mastore M, Arizza V, Manachini B, Brivio MF (2015) Modulation of immune responses of *Rhynchophorus ferrugineus* (Insecta: Coleoptera) induced by the entomopathogenic nematode *Steinernema carpocapsae* (Nematoda: Rhabditida). Insect Sci 22:748–760. https://doi.org/10.1111/1744-7917.12141

Mastore M, Brivio MF (2008) Cuticular surface lipids are responsible for disguise properties of an entomoparasite against host cellular responses. Dev Comp Immunol 32:1050–1062. https://doi.org/10.1016/j.dci.2008.02.003

Medzhitov R, Janeway CJ (2002) Decoding the patterns of self and nonself by the innate immune system. Science (80) 296:298–300

Morton A, García del Pino F (2008) Effectiveness of different species of entomopathogenic nematodes for biocontrol of the Mediterranean flatheaded rootborer, *Capnodis tenebrionis* (Linné) (Coleoptera: Buprestidae) in potted peach tree. J Invertebr Pathol 97:128–133. https://doi.org/10.1016/j.jip.2007.09.003

Nappi AJ, Kohler L, Mastore M (2004) Signaling pathways implicated in the cellular innate immune responses of *Drosophila*. Invertebr Surv J 1:5–33

Nikolouli K, Sassù F, Mouton L, et al (2020) Combining sterile and incompatible insect techniques for the population suppression of *Drosophila suzukii*. J Pest Sci (2004) 93:647–661. https://doi.org/10.1007/s10340-020-01199-6

Oerke E. (2006) Crop losses to pests. J Agr Sci 144:31–43

Ometto L, Cestaro A, Ramasamy S, et al (2013) Linking genomics and ecology to investigate the complex evolution of an invasive *Drosophila* pest. Genome Biol Evol 5:745–757. https://doi.org/10.1093/gbe/evt034

Parchami-Araghi M, Gilasian E, Keyhanian A (2015) Spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) an invasive fruit pest new to the Middle East and Iran. Drosoph Inf Serv 98:59–60

Parmesan C (2006) Ecological and evolutionary responses to recent climate change. Ann Rev Ecol Evol Syst 37:637–669

Peabody NC, White BH (2013) Eclosion gates progression of the adult ecdysis sequence of *Drosophila*. J Exp Biol 216:4395–4402. https://doi.org/10.1242/jeb.091595

Peña JM, Carrillo MA, Hallem EA (2015) Variation in the susceptibility of *Drosophila* to different entomopathogenic nematodes. Infect Immun 83:1130–1138. https://doi.org/10.1128/IAI.02740-14

Perry T, Heckel DG, McKenzie JA, Batterham P (2008) Mutations in Dα1 or Dβ2 nicotinic acetylcholine receptor subunits can confer resistance to neonicotinoids in *Drosophila melanogaster*. Insect Biochem Mol Biol 38:520–528. https://doi.org/10.1016/j.ibmb.2007.12.007

Peters A (1996) The natural host range of *Steinernema* and *Heterorhabditis spp.* and their impact on insect populations. Biocontrol Sci Technol 6:389–402.

Peters A, Ehlers RU (1997) Encapsulation of the entomopathogenic nematode *Steinernema feltiae* in *Tipula oleracea*. J Invertebr Pathol 69:218–222. https://doi.org/10.1006/jipa.1996.4648

Poinar G (1979) Nematodes for biological control of insects. CRC Press Boca Raton, FL, EEUU 143

Poyet M, Havard S, Prevost G, et al (2013) Resistance of *Drosophila suzukii* to the larval parasitoids *Leptopilina heterotoma* and *Asobara japonica* is related to haemocyte load. Physiol Entomol 38:45–53. https://doi.org/10.1111/phen.12002

Půža V, Campos-Herrera R, Blanco-Pérez R, et al (2020) *Steinernema riojaense n. sp.*, a new entomopathogenic nematode (Nematoda: Steinernematidae) from Spain. Nematology 22:825–841. https://doi.org/10.1163/15685411-00003343

Rasmann S, Köllner T., Degenhardt J, et al (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434:732–737

Renkema JM, Cuthbertson AGS (2018) Impact of multiple natural enemies on immature *Drosophila suzukii* in strawberries and blueberries. BioControl. https://doi.org/10.1007/s10526-018-9874-8

Renkema JM, Telfer Z, Gariepy T, Hallett RH (2015) *Dalotia coriaria* as a predator of *Drosophila suzukii*: Functional responses, reduced fruit infestation and molecular diagnostics. Biol Control 89:1–10. https://doi.org/10.1016/j.biocontrol.2015.04.024

Rossi-Stacconi MV, Kaur R, Mazzoni V, et al (2016) Multiple lines of evidence for reproductive winter diapause in the invasive pest *Drosophila suzukii*: useful clues for control strategies. J Pest Sci (2004) 89:689–700. https://doi.org/10.1007/s10340-016-0753-8

Rossi Stacconi MV, Buffington M, Daane KM, et al (2015) Host stage preference, efficacy and fecundity of parasitoids attacking *Drosophila suzukii* in newly invaded areas. Biol Control 84:28–35. https://doi.org/10.1016/j.biocontrol.2015.02.003

Rossi Stacconi MV, Grassi A, Ioriatti C, Anfora G (2019) Augmentative releases of *Trichopria drosophilae* for the suppression of early season *Drosophila suzukii* populations. BioControl 64:9–19. https://doi.org/10.1007/s10526-018-09914-0

Santoiemma G, Trivellato F, Caloi V, et al (2019) Habitat preference of *Drosophila suzukii* across heterogeneous landscapes. J Pest Sci (2004) 92:485–494. https://doi.org/10.1007/s10340-018-1052-3

Schetelig MF, Lee K-Z, Otto S, et al (2018) Environmentally sustainable pest control options for *Drosophila suzukii*. J Appl Entomol 3–17. https://doi.org/10.1111/jen.12469

Schmidt O, Theopold U, Strand MR (2001) Innate immunity and its evasion and suppression by hymenopteran endoparasitoids. Bioessays 23:344–351

Schroer S, Ehlers RU (2005) Foliar application of the entomopathogenic nematode *Steinernema carpocapsae* for biological control of diamondback moth larvae (*Plutella xylostella*). Biol Control 33:81–86. https://doi.org/10.1016/j.biocontrol.2004.12.009

Shapiro-Ilan D, Hazir S, Glazer I (2017) Basic and applied research: Entomopathogenic nematodes. Elsevier Inc.

Shapiro-Ilan DI, Cottrell TE, Mizell RF, Horton DL (2016) Efficacy of *Steinernema carpocapsae* plus fire gel applied as a single spray for control of the lesser peachtree borer, *Synanthedon pictipes*. Biol Control 94:33–36. https://doi.org/10.1016/j.biocontrol.2015.12.006

Stanley D, Haas E, Miller J (2012) Eicosanoids: exploiting insect immunity to improve biological control programs. Insects 3:492–510

Stock SP, Griffin CT, Chaerani R. (2004) Morphological and molecular characterisation of *Steinernema hermaphroditum n. sp.* (Nematoda: Steinernematidae), an entomopathogenic nematode from Indonesia, and its phylogenetic relationships with other member of the genus. Nematology 6:401–412

Strand MR (2008) The insect cellular immune response. Insect Sci 15:1–14. https://doi.org/10.1111/j.1744-7917.2008.00183.x

Taning CNT, Christiaens O, Berkvens N, et al (2016) Oral RNAi to control *Drosophila suzukii*: laboratory testing against larval and adult stages. J Pest Sci (2004) 89:803–814. https://doi.org/10.1007/s10340-016-0736-9

Tochen S, Dalton DT, Wiman N, et al (2014) Temperature-related development and population parameters for *Drosophila suzukii* (Diptera: Drosophilidae) on cherry and blueberry. Environ Entomol 43:501–510. https://doi.org/10.1603/EN13200

Tomalak M, Piggott S, Jagdale GB (2005) Glasshouse application. In: Grewal PS, Ehlers RU, Shapiro-Ilan DI (eds) Nematodes as Biological Control. CABI Publishing, Wallingford, UK, pp 147–166

Toubarro D, Lucena-Robles M, Nascimento G, et al (2010) Serine protease-mediated host invasion by the parasitic nematode *Steinernema carpocapsae*. J Biol Chem 285:30666–30675.

Tourtois J, Grieshop MJ (2015) Susceptibility of *Dalotia coriaria* (Kraatz) (Coleoptera: Staphylinidae) to entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae). Insects 6:224–235. https://doi.org/10.3390/insects6010224

Van Timmeren S, Isaacs R (2013) Control of spotted wing drosophila, *Drosophila suzukii*, by specific insecticides and by conventional and organic crop protection programs. Crop Prot 54:126–133. https://doi.org/10.1016/j.cropro.2013.08.003

Walsh DB, Bolda MP, Goodhue RE, et al (2011) *Drosophila suzukii* (Diptera: Drosophilidae): Invasive Pest of Ripening Soft Fruit Expanding its Geographic Range and Damage Potential. J Integr Pest Manag 2:1–7. https://doi.org/10.1603/IPM10010

Wang X, Kaçar G, Biondi A, Daane KM (2016) Foraging efficiency and outcomes of interactions of two pupal parasitoids attacking the invasive spotted wing drosophila. Biol Control 96:64–71. https://doi.org/10.1016/j.biocontrol.2016.02.004

Westphal MI, Browne M, MacKinnon K, Noble I (2008) The link between international trade and the global distribution of invasive alien species. Biol Invasions 10:391–398.

Williams EC, Walters KFA (2000) Foliar application of the entomopathogenic nematode *Steinernema feltiae* against leafminers on vegetables. Biocontrol Sci Technol 10:61–70. https://doi.org/10.1080/09583150029396

Wilson MJ, Ehlers RU, Glazer I (2012) Entomopathogenic nematode foraging strategies - Is *Steinernema carpocapsae* really an ambush forager? Nematology 14:389–394. https://doi.org/10.1163/156854111X617428

Wiman NG, Dalton DT, Anfora G, et al (2016) *Drosophila suzukii* population response to environment and management strategies. J Pest Sci (2004) 89:653–665. https://doi.org/10.1007/s10340-016-0757-4

Wiman NG, Walton VM, Dalton DT, et al (2014) Integrating temperature-dependent life table data into a matrix projection model for *Drosophila suzukii* population estimation. PLoS One 9. https://doi.org/10.1371/journal.pone.0106909

Woltz JM, Donahue KM, Bruck DJ, Lee JC (2015) Efficacy of commercially available predators, nematodes and fungal entomopathogens for augmentative control of *Drosophila suzukii*. J Appl Entomol 1–12. https://doi.org/10.1111/jen.12200

Woltz JM, Lee JC (2017) Pupation behavior and larval and pupal biocontrol of *Drosophila suzukii* in the field. Biol Control 110:62–69. https://doi.org/10.1016/j.biocontrol.2017.04.007

Woltz JM, Wiman NG, Lee JC (2017) Two pests overlap: *Drosophila suzukii* (Diptera: Drosophilidae) use of fruit exposed to *Halyomorpha halys* (Hemiptera: Pentatomidae). J Econ Entomol 110:1938–1941. https://doi.org/10.1093/jee/tox156

Wood W, Jacinto A (2007) *Drosophila melanogaster* embryonic haemocytes: Masters of multitasking. Nat Rev Mol Cell Biol 542–551

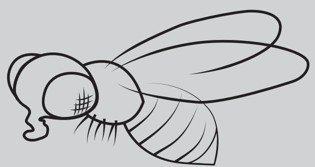
Yadav S, Daugherty S, Shetty AC, Eleftherianos I (2017) RNAseq analysis of the *Drosophila* response to the entomopathogenic nematode *Steinernema*. Genes|Genomes|Genetics 7:1955–1967. https://doi.org/10.1534/g3.117.041004

Yang Y, Hou ZC, Qian YH, et al (2012) Increasing the data size to accurately reconstruct the phylogenetic relationships between nine subgroups of the *Drosophila melanogaster* species group (Drosophilidae, Diptera). Mol Phylogenet Evol 62:214–223. https://doi.org/10.1016/j.ympev.2011.09.018

Yee WL, Lacey LA (2003) Stage-specific mortality of *Rhagoletis indifferens* (Diptera: Tephritidae) exposed to three species of *Steinernema* nematodes. Biol Control 27:349–356. https://doi.org/10.1016/S1049-9644(03)00029-X

Yousef M, Aranda-Valera E, Quesada-Moraga E (2018) Lure-and-infect and lure-and-kill devices based on *Metarhizium brunneum* for spotted wing *Drosophila* control. J Pest Sci (2004) 91:227–235. https://doi.org/10.1007/s10340-017-0874-8

Zhang CL, Zhang YH, He WT, et al (2011) Integrated control technology of cherry fruit flies and experimental demonstration in Aba, Sichuan province. China Plant Prot 31:26–28



Facultat de Biocièccncies
Departament de Biologia Animal, Biologia Vegetal i Ecologia
Unitat de Zoologia